



Classical Conditioning Alters Short Noncoding RNA Expression in *Drosophila*

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Classical Conditioning Alters Short Noncoding RNA Expression In *Drosophila*

A dissertation presented

by

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to

The Department of Molecular and Cellular Biology

in partial fulfillment of the requirements

for the degree of

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Biochemistry

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Classical Conditioning Alters Short Noncoding RNA Expression In *Drosophila***Abstract**

MicroRNAs (miRNAs) and other classes of short non-coding RNAs regulate essential processes in the development and function of the nervous system. Regulation of miRNAs by neural activity has also been reported. Recently, instances of piwi interacting RNA (piRNA) and endogenous short interfering RNA (esiRNA) mediated modulation of neural physiology have been reported. To better understand the role of miRNAs and other classes of short non-coding RNAs in long term memory (LTM) formation, we have conducted high throughput sequencing on 15-35nt RNAs isolated from heads of *Drosophila* that have been subjected to aversive olfactory conditioning. We developed genome wide profiles of miRNA, piRNA, and esiRNA, and tested for differential expression following conditioning. We find that 5 miRNAs exhibit significant regulation in the conditioned group. We identify several esiRNA generating loci within genes required for olfactory LTM formation. Our data reveal that an intron of the multiple wing hairs (mwh) gene forms secondary structures and generates esiRNAs following conditioning from regions that correspond to lysozyme family genes located within the mwh intron. We find that piRNAs are produced in fly heads, and that a small set of piRNA generating loci mapping to LTR retrotransposons are significantly down regulated following conditioning. In addition to the well characterized classes of short non coding RNAs, we describe a set of transcripts that produce large numbers of reads with a broad size distribution from the sense strand. We find that a subset of these are regulated following treatment and contain consensus elements that may be involved in their regulation. We investigate expression of one such gene with dramatically up-regulated reads following treatment, the *Drosophila* beta-site APP-cleaving enzyme (dBACE), and find that increased reads reflect increased mRNA levels. Further, we find that the target of dBACE protein, drosophila β amyloid protein precursor-like (APPL), is subjected to increased cleavage following conditioning, and that dBACE is required for LTM formation, but not for learning or STM.

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this while simultaneously pursuing her own career in architecture. She has achieved more than can be listed here, but her work in many ways reflects the person she is. Her decision to change the direction and pursue architecture in itself displays her bravery. Her elegance finds expression in many of her projects, her drive manifests in the volume and level of her work, and her toughness was in evidence when she learned to weld and to operate heavy machinery. Her accomplishments and motivation are my inspiration. I will spend the rest of my life making sure she understands how much I value all that she has done for me, how much I respect her, and this brief section of my dissertation cannot contain the years of gratitude I owe her.

I also have the great fortune of being born into a family for whom the philosophy of science is a guiding force. I am the son of a man once described to me by an accomplished professor of biology as “A scientist’s scientist in the same way that Ted Williams was a baseball player’s baseball player.” At the time, this sounded great, but I had not yet begun my graduate career, and was thus not well enough read in biology to appreciate the remark. Reflecting back upon the comment now, I realize how apt it was. Much as Williams’ book “The Science of Hitting” remains a foundational text for developing baseball players, “Molecular Cloning” has allowed generations of scientists working in diverse areas of biology to bring the power of molecular biology and biochemistry to their work, and I have had the pleasure of putting this text to use in my own work. My father’s course on gene regulation was required for all students in my program, and it was one of the most difficult I have taken. By relying solely on current publications for course materials, he and his co-

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My mother is also a scientist by training, having obtained a master's in geology before I was born. She put this career on hold for our family, yet fostered my intellectual curiosity and development every bit as much as my father has. Her caring turned ER visits for stitches into lessons in the biology of wounds, and her patience recognized the educational value in my near detonation of the bathroom following after school electrolysis of water. She even gave me my own plot in the garden, and let me plant something different and of my own choosing every year.

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In addition to my biological brother, I have acquired several brothers through friendship. Together, my brothers have taught me the things in life that one's parents and education can't. They have challenged me, disciplined me, held me up in triumph, and commiserated with me in defeat. They have expanded my intellectual horizons beyond academics, and honed my philosophy of life... But these things have always been a collaboration, and I know that we will continue to form like Voltron.

Without the help of those mentioned here, and others, I would not be who I am, nor would I have completed the work contained in this dissertation. My gratitude cannot be bound by words and is eternal... Thank you.

Chapter I
Introduction

Of the advantages gained by organisms possessing a nervous system, perhaps none is more remarkable than the ability to anticipate future events based upon past experience. Learning and lasting retention of acquired knowledge provide a means by which organisms can adapt to new environmental circumstances within the lifespan of an individual. In the human context, it is arguable that learning and memory are the core components that define who we are as individuals, as societies, and as a species. As such, understanding the basic mechanisms by which animals learn and remember have long been, and remain major areas of study. The underlying physiology of learning and memory is largely composed of the formation, destruction, and modulation of the efficacy of synaptic connections, a set of processes termed synaptic plasticity. Much as memories can be trivial and fleeting, or profound and long enduring, so can be the changes in synapses, and neural circuitry associated with learning and memory. Further, with repeated presentation of information, memories that might otherwise be short lived can be made to last. The underlying molecular mechanisms are known to reflect these experiential aspects of learning and memory as well, and are largely conserved from invertebrates to humans. For example, triggering of the cyclic adenosine monophosphate (cAMP) signaling pathway by synaptic activity relevant to learning results in the modification of existing synaptic proteins, and leads to the initial and least enduring changes in synaptic efficacy. Formation of lasting memories involves transcription and protein synthesis, and can lead to enduring changes in gene regulation, synaptic efficacy, and synaptic number. Modulation of the activity of genes under the control of the cAMP response promoter element

(CRE) via the transcription factor cAMP response promoter element binding protein (CREB) appears to be a central and conserved aspect of the conversion of an initial learning experience to a stable memory. The fact that these major mechanisms governing synaptic plasticity are evolutionarily conserved allows the study of gene regulation following long-term memory formation in invertebrates to yield insight into the most profound cognitive functions of higher organisms, including humans. Therefore, in the first part of this chapter, I will discuss the formation of aversive olfactory memory, its underpinnings in synaptic plasticity, and the role of *Drosophila* genetics in understanding the processes involved. Recently, studies of gene regulation in neural tissues have shown that a variety of short, noncoding RNAs modulate the production of factors required for neural development, synaptic plasticity, and learning and memory. Thus, in the second section of this chapter, I will discuss evidence that short noncoding RNAs regulate synaptic plasticity, and distinctions between the several classes of these RNAs. One of the most widely known neural disorders affecting memory is Alzheimer's disease (AD). A central feature of AD is the formation of synaptic plaques composed of β -amyloid precursor protein (APP) cleavage products. *Drosophila* possess a homologue of APP, the APP like protein (APPL), and recently *Drosophila* homologues of the proteases that cleave APP have been identified. Therefore, it is now possible to use the power of *Drosophila* behavioral genetics to study this disease. It has already been shown that disruptions of the regulated expression and cleavage of APPL result in synaptic abnormalities and long-term memory (LTM) defects in flies. Accordingly, in the

final section, I will provide an overview of APPL cleavage and its role in synaptic plasticity and memory.

Part I: Synaptic Plasticity In Learning And Memory, And Olfactory Memory In Drosophila

Synaptic plasticity is the physiological basis of learning and memory

For many organisms possessing a nervous system, the ability to form memories is crucial for survival. Learning the stimuli associated with a good place to find food can not only help an animal return to that location, but also to find new, similar locations where food is likely to be present. For animals spanning the evolutionary distance from mollusks to mammals, the distinctive odor of a predator can provide a crucial warning if the association of the odor with the predator can be learned and later remembered. Some birds, and many mammals are able to learn useful behaviors by observing others, and to retain the new behavior long enough to pass the behavior on to their own offspring. In this way, learning and memory are the foundation of culture. Memory in many ways defines who we are and how we react to life events, and as such, it was the focus of early work in psychology. To Freud, memories, conscious or repressed, were the root cause of psychopathologies. He and others exerted great effort toward an understanding of the formation, retention, and extinction of memories in the context of treating mental illness. Though Freud preferred empirical evidence, little was available in his time, and he

viewed observations by a neutral psychoanalyst as a valid method for understanding the inner workings of the mind. Accordingly, this early work dealt largely with the cognitive aspects of memory, and was in many ways as much philosophical as empirical. Ebbinghaus transformed the study of memory from philosophical reflection on one's own memories into a subject of quantitative scientific analysis. He quantified the relationship between repetitions of memorization, accuracy of recollection and durability of memory. Ebbinghaus described the capabilities of human memory in the first concrete terms by counting how many made up words a person can recall, and measuring the period of time during which they can be recalled. He showed that the trope "practice makes perfect" had a basis in fact, demonstrating that one could recall only 6 or 7 items after brief study, and that the ability to recall these items accurately degraded rapidly over the course of minutes, but with repeated study, one could dramatically increase both the number of items and the period over which they could be recalled. Further, by studying the rate at which recollection failed, he observed that memory degraded in two phases, one in the hours immediately after learning, and one that lasted for months. Thus, his work indicated that long-term memory (LTM) was an extension of short-term memory (STM), but that distinctions existed between the two. (1) The work of Ivan Pavlov provided seminal insights into the mechanistic aspects of learning and memory. His use of animal models and simple stimuli with easily quantifiable measures showed that techniques to empirically study the biology of the mind could be developed. His studies of reflex also provided a first hint to the physiological basis of learning. In this work, he developed what would

come to be termed classical conditioning, in which a subject can be taught to associate an arbitrary conditioned stimulus (CS), with a biologically significant, unconditioned stimulus (US) such as food or pain, which normally elicits an unconditioned response (UR) such as salivation or assuming a fear posture. Following conditioning, the CS will elicit a conditioned response (CR) as if the subject was presented with the US. Specifically, Pavlov showed that after repeated sessions in which a bell is rung just prior to giving a dog a taste of food, the dog will associate the ringing bell with the taste of food, and as a result, the dog will salivate in response to the ringing bell, even in the absence of food. Using this method, Pavlov was able to directly examine the formation and persistence of a newly created memory of an association of stimuli. Crucially, he found that conditioning was most effective when the CS was presented just prior to the US, thereby becoming predictive of the US. Another form of learning, in which animals are taught to associate a behavior with its consequences, was discovered by Konorski and studied extensively by Thorndike. In this paradigm, behaviors are punished or reinforced, resulting in the increase or decrease in the probability of the subject displaying the conditioned behavior. This form of learning came to be termed operant conditioning. Based on this work, Thorndike developed a theory of learning that included the idea that all animals learn the same way. Cajal, based upon his careful study of neuroanatomy, first proposed a physiological manifestation of memory in the form of synaptic connections between neurons. Later, Hebb would use behavioral studies as well as measurements of the electrical activity of the brain as the basis of what became known as Hebbian theory. A central component of this

theory is that the firing patterns of neurons affect the efficacy of their synapses. He suggested that when one neuron repeatedly participates in triggering the activity of another, physiological changes in one or both cells would occur, thereby enhancing the ability of the first cell to trigger activity in the second cell. This was shown to be true by Lomo and Bliss in studies of the rabbit hippocampus. In this work, they demonstrated that a single electrical stimulation of the perforant pathway to the dentate gyrus caused excitatory post-synaptic potentials (EPSP) in the neurons of the dentate gyrus. However, preceding such stimulation with a high-frequency train of electrical pulses would cause a change such that the post-synaptic cells of the dentate gyrus would produce stronger and longer lasting EPSPs. Further, this change could last 30 minutes to several hours. Lomo and Bliss termed this phenomenon long-term potentiation (LTP).(2) This observation strongly supported Hebbian theory, but the complexity of the mammalian brain hindered subsequent progress. Kandel and his colleagues made significant contributions in work on the cellular and molecular biology of the gill withdrawal reflex of the sea hare *Aplysia californica*. Having identified the gill withdrawal reflex as a behavior that could be easily observed and triggered, they mapped a simple circuit, composed of relatively large, and easily manipulated neurons, governing the reflex. In this circuit, a presynaptic glutaminergic sensory neuron forms direct synaptic connections with the motor neurons governing the gill withdrawal reflex, as well as synaptic connections with both excitatory and inhibitory interneurons that themselves form synapses with the sensory and motor neurons. Using this system, Kandel and his colleagues demonstrated that pairing stimulation of the siphon (the CS) with an

electric shock to the tail (the US) enhances the gill withdrawal reflex (the CR), and that this enhancement is correlated with changes in synaptic strength. A single Pavlovian pairing of US and CS transiently enhances the ability of the presynaptic sensory neuron to trigger an action potential in the postsynaptic motor neuron. This enhancement requires serotonergic modulatory input triggered by the US. 5 or more pairings leads to formation of a more persistent, intermediate-term memory (ITM) of the association, lasting several hours before the response returns to baseline. Previously quiescent synapses can become active, but the number of synaptic connections remains constant.(3) Repeated sessions of 5 or more pairings of siphon stimulation and tail shock, with periods of rest between sessions, leads to stable, long-term memory (LTM) formation, lasting for days or longer. This LTM forming paradigm results in strengthening of existing synapses as well as the formation of new synapses.(4) Thus, Kandel and his colleagues demonstrated that aspects of memory described by Ebbinghaus had invertebrate neural correlates akin to those predicted by Cajal and Hebb. They also showed that invertebrate neurobiology could provide insights that would be, at the time, precluded by the complexity of neural tissues in higher organisms and the obstacles to work in mammalian systems such as slower reproduction and ethical concerns. In this vein, Benzer and his colleagues used the power of *Drosophila* mutants to pioneer work in behavioral genetics, demonstrating that individual genes could govern complex behaviors. Subsequent work showed that many of these genes are broadly conserved, with similar contributions to behavior in other organisms. (5-7) The advent and dissemination of powerful biochemistry and molecular biology methods

since the 1970s has permitted study of learning and memory at the most fundamental level. It is now known that major features of learning and memory are evolutionarily conserved at molecular, cellular, and behavioral levels. Mollusks, insects, rodents and humans all possess phases of associative memory commonly categorized as STM, ITM, and LTM. At the cellular and molecular level, major mechanisms involved in the formation of each type of memory are also conserved. The initial, and least stable phase of memory formation involves modification of proteins already present at existing synapses via the cAMP pathway. Neural activity relevant to learning activates adenylyl cyclase (AC) and phospholipase-C (PLC). AC produces cyclic adenosine mono-phosphate (cAMP), and cAMP in turn activates the cAMP-dependent protein kinase (pKA). pKA phosphorylates K^+ channels, reducing the influx of K^+ , and prolonging the action potential. PLC cleaves phosphatidylinositol 1,4,5-bisphosphate (PIP_2) yielding inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG activates protein kinase C (pKC), leading to the opening of L-type Ca^{2+} channels, which have a prolonged response, and thus lead to greater calcium influx.(8) Consolidation of memories into LTM, involves the cAMP signaling pathway as well. During consolidation, pKA recruits the mitogen activated protein (MAP) kinase. Together, pKA and MAP translocate to the nucleus, where they phosphorylate the transcription factor cAMP response promoter element binding protein (CREB). This allows CREB to bind the cAMP response promoter element (CRE), activating transcription of genes under its control. These genes have numerous activities and trigger biochemical pathways that act in concert to stabilize the enhancement of synaptic efficacy. Key amongst these CREB regulated genes is

the transcription factor CCAAT-enhancer-binding protein (C/EBP), which itself activates a cascade that leads to the growth of new synaptic connections. (6-9)

Many Molecular and Genetic Tools Are Available For Studies Of Drosophila Learning And Memory

Drosophila was a well-developed model organism for use in genetic studies long before modern molecular biology became widely used. Researchers have been collecting and inbreeding *Drosophila* mutants since the early 1900s, and now have cultured strains carrying mutations in most genes. Furthermore, many methods with which to generate random and directed mutations in flies exist. The mobile DNA P-element found in *Drosophila* has been of particular utility for generating desirable mutants. Random insertion of P-elements within genes, or the excision of previously inserted P-elements within genes can produce mutations. Constructs carrying transgenes flanked by P-element sequences that permit insertion into the genome are a widely used method for delivering transgenes to the *Drosophila* genome. Several methods for tissue specific expression of transgenes in flies have been developed. Gene targeting has been developed in *Drosophila*, but its use remains infrequent. A widely used method for tissue specific expression is the binary GAL4 / UAS system, in which the yeast transcriptional activator GAL4 is placed under the control of a *Drosophila* regulatory element, and drives expression of a transgene at a separate genomic location under the control of the yeast upstream activation sequence (UAS). A large number of random P element

insertions carrying GAL4 enhancer trap constructs have been collected and cultured. Such insertions will drive expression of GAL4 according to regulatory elements in the genomic context surrounding the insertion. This can result in GAL4 expression closely mimicking the spatial and temporal expression patterns of genes near the enhancer trap insertion.(10) Recently, a consortium from the Howard Hughes Medical Institute's Janelia farm have cloned genomic fragments spanning much of the *Drosophila* genome, and placed these fragments immediately up-stream of a core promoter driving GAL4 expression. This promoter will become active only if the cloned fragment contains an enhancer. These constructs are then inserted into the *Drosophila* genome at a fixed location so as to avoid position effect variegation from random insertion. These constructs will drive GAL4 expression in patterns matching that of genes controlled by elements in the cloned fragment. Using this collection, the consortium is identifying neural expression patterns of genes, and mapping the circuitry of the brain. Much of the husbandry, imaging, and analysis in this work has been automated.(11) The binary nature of the GAL4/UAS and similar systems available in *Drosophila* makes such GAL4 collections enormously powerful tools, as experiments involving expression of different transgenes in defined tissues can be accomplished simply by crossing flies expressing GAL4 in the desired tissue to flies harboring the transgenes under UAS control. Similarly, a single transgene can be expressed in different tissues through simple crosses. Binary expression systems have been used to map circuitry relevant to learning and memory using GAL4 expression under the control of genes required for these processes to drive expression of UAS-GFP. Another approach uses

screening for learning or memory defects in flies carrying GAL4 enhancer traps driving expression of transgenes that silence neural activity under UAS control. One such transgene, the dominant negative, temperature sensitive dynamin mutant *Shiberet^{ts}* (*Shi^{ts}*), which reversibly blocks synaptic transmission when shifted to a temperature above 29°, has been used to map the behavioral contribution of various neural circuits. GAL4 enhancer trap lines from crosses exhibiting defects can then be crossed to flies harboring UAS-GFP or other reporter transgenes, thereby marking the neurons involved. Photoactivatable GFP variants (PA-GFP) permit tracing of individual neurons. Neurons expressing PA-GFP and innervating a neuropil of interest are illuminated such that PA-GFP is converted to its fluorescent state. This activated PA-GFP is then allowed to diffuse to the cell bodies of neurons innervating the neuropil. An individual soma is selected, and PA-GFP converting illumination is maintained at that soma while fluorescence in other neurons decays. In this way, the branching patterns of individual neurons, and wiring diagrams of circuits can be precisely mapped.(12) Neural activity in these circuits can be recorded during odor presentation by expressing genetically encoded fluorescent calcium sensors under the control of the same GAL4 lines, and using confocal or two-photon microscopy to collect images of the brain from intact heads as they are presented with odors.(13, 14) Neural activity in the same cells can also be directly controlled optogenetically by crossing the GAL4 line to flies expressing channelrhodopsin-2 or halorhodopsin under UAS control.(15) Further, a number of methods for conditional expression of transgenes in *Drosophila* are available. HSP70-GAL4 drives GAL4 in all tissues when flies are subjected to elevated

temperature. GAL80ts blocks transcription of genes under UAS control until flies expressing it are subjected to elevated temperature. Thus, by combining ubiquitously expressed GAL80ts with a GAL4 line of interest, expression of genes under UAS control can be manipulated spatially and temporally. For developmental studies, mosaic analysis with a repressible cell marker (MARCM) can be used to drive expression of transgenes in cells from specific lineages. These examples represent a subset of the genetic tools available in *Drosophila*, but underscore the modular design and combinatorial potential available in flies. This illustrates why *Drosophila* has become a uniquely well-suited model organism for experimental manipulation and study of learning and memory.

Paradigms For Behavioral Studies In *Drosophila*

Drosophila can form tactile, spatial, visual, gustatory, and olfactory memories in operant and/or classical conditioning paradigms. (7, 13, 16) A variety of behavioral methods has been developed to study learning and memory in *Drosophila*, with a broad range of complexity and scale. To study visual learning, flight simulators have been developed, in which individual tethered flies can learn to associate a shape or color with a noxious stimulus in the form of heat delivered by infrared (IR) laser light.(17) Others developed an arena, accommodating one or many larvae or adults, in which areas illuminated with light of a particular color contain sucrose. Large groups of flies can be trained and tested for their memory of the association of color and sucrose using this setup.(18) To study memory of place,

an analog of the Morris water maze has been developed in which an array of LEDs surround an arena in which a particular part of the floor is cooled, while the rest of the arena is heated. The ability of flies to find the cool location improves with repeated trials. By manipulating the visual scene presented on the LED array, flies can be tested for their ability to find the cool location when the visual cues are altered or disappear.(19) *Drosophila* can be conditioned to associate a taste with a noxious heat. *Drosophila* respond to presentation of sugars to gustatory sensory neurons in their tarsi by extending the proboscis. This proboscis extension reflex (PER) can be used to evaluate gustatory associative learning. By pairing presentation of a tastant to the tarsi with painful IR laser light to the antennae, flies can be conditioned to associate the tastant with the noxious heat. Following conditioning, PER is suppressed if the conditioned tastant is mixed into a sugar solution presented to the tarsi.(20) Male *Drosophila* readily attempt to mate with appropriate and inappropriate targets, including sexually immature males and females. However, after presentation with a previously mated adult female, the male will exhibit decreased courtship attempts with females, but not immature males for 2-3 hours. Repeated presentation can result in decreased courtship attempts lasting as long as 8 days. This learning is associative, with cuticle hydrocarbons being the CS, and the male deposited hormone cis-vinyl acetate (cVA) being the best characterized aversive US.(21) Recent advances in computational video analysis now permit simultaneous behavioral analysis and/or manipulation of individual flies within large freely behaving groups.(22) Exceedingly rich data sets can be rapidly collected with such methods, as they

permit the assessment of group dynamics, and/or high-throughput examination of individual behavior. However, aversive olfactory classical conditioning was one of the first paradigms developed for studies of *Drosophila* learning and memory, and remains one of the most widely used.(5) It is also the most developed and best understood paradigm in widespread use. In this paradigm, flies are presented with two odors, one of which is accompanied by inescapable electric shock. Conditioning occurs in the dark to ensure that the odors being presented are the most salient stimuli other than the US. Conditioned flies are then tested for odor choice in the dark, using a T-maze in which the flies begin at the bottom of the T, with one arm containing the conditioned odor, and the other arm containing the control odor or a novel odor. Odor concentrations are identical to those used during conditioning. Innate negative geotaxis leads the flies to the choice point. Following conditioning, the number of flies in the arm containing the conditioned odor is significantly reduced. Learning and memory can be evaluated by counting the number of flies in each arm, and then calculating a performance index (PI). PI is defined as the fraction of flies that avoid the conditioned odor, minus the fraction of flies that avoid the control odor. This means that a PI of 0 represents no learning, and a 50:50 distribution of flies in the two arms, while a PI of 1 represents perfect learning, with all flies avoiding the conditioned odor. This aversive olfactory conditioning method allows many flies to be simultaneously trained and tested, thereby vaulting over one of the major hurdles of behavioral work in other animal models. (7, 13, 23)

Recently, a semi-automated method for performing aversive olfactory conditioning of *Drosophila* has been developed, permitting multiple specimens to be conditioned

simultaneously, and in a reproducible manner.(24) This list is not exhaustive, and new paradigms with which to study *Drosophila* behavior continue to be developed. The diversity of methods with which to study and manipulate *Drosophila* sensation, learning, and memory demonstrates the surprising capabilities of the drosophila brain. This diversity also illustrates the exceptional suitability of *Drosophila* as a model organism with which to study learning and memory.

Olfactory Learning and Memory in Drosophila

Quinn and Tully used aversive olfactory conditioning to study the genetics of learning and memory, and were able to identify several genes that, when mutated, caused defects in specific aspects of these processes. (5, 6, 25-27) Subsequent work has identified many more mutations and P-element insertions that result in learning or memory defects. These have in turn been used to map the neural circuits involved in olfactory learning and memory in *Drosophila*. Identification of the genes and circuits involved has greatly contributed to understanding the molecular mechanisms underlying synaptic plasticity as well as learning and memory more broadly. Many of these molecular mechanisms are evolutionarily conserved, and analogous circuitry exists in many insect and non-insect species.(6) *Drosophila* aversive olfactory memory can be broken down into 4 distinct phases following learning: STM, middle-term memory (MTM), anesthesia resistant memory (ARM), and LTM. *Drosophila* STM is present immediately after a single session of aversive olfactory classical conditioning, and is subject to disruption by anesthesia. STM

persists for 30-60 minutes, and does not require transcription or protein synthesis. A single training session will also produce MTM lasting several hours, but by 24 hours after training, PIs return to baseline. MTM can be disrupted by anesthesia or reversal training, in which previously conditioned flies are exposed to the same odors used during conditioning, but the previously conditioned odor is now neutral and the previously neutral odor is now paired with shock. Following 5 -10 conditioning sessions with no intervening rest period (Massed training), STM, MTM, and a more durable form of memory are produced. This durable form of memory is not subject to disruption, and is therefore termed anesthesia resistant memory (ARM). ARM can last for as long as 4 days before PI diminishes completely, and does not require protein synthesis. 5-10 conditioning sessions with intervening periods of rest (Spaced training) produces STM, ARM, and LTM. LTM is distinguished from ARM by its requirement for protein synthesis, and CREB. LTM can persist for many days and is not subject to disruption by anesthesia. Spaced training also produces better PI immediately following conditioning than massed training, which in turn produces better PI than a single conditioning session. (6, 7, 16) However despite its crucial role in advancing the study of learning and memory, recent studies using other learning paradigms suggest that some aspects of *Drosophila* memory formation resulting from aversive olfactory classical conditioning, long thought to be generic to all modes of learning, may in fact differ between learning paradigms. For instance, appetitive olfactory conditioning, in which flies are taught to associate an odor with the reward of sugar, can produce LTM after a single training session. Though many such differences were reported long ago, they have only recently

begun to be investigated in detail, and no *Drosophila* training paradigm has been as widely used and well studied as aversive olfactory classical conditioning. (28) The automation of many newer learning paradigms may speed the identification of aspects of memory unique to different sensory modalities, unconditioned stimuli, or training methods, but significant work remains to categorize some features of learning and memory in *Drosophila* as truly general or more specific to a given paradigm. Nonetheless, aversive olfactory classical conditioning remains the standard to which other paradigms are compared.

Genetic Analysis of Drosophila Olfactory Memory

There are unique genetic requirements for aversive olfactory classical conditioning learning, and for each form of memory it produces. Indeed, much of what is known about the phases of *Drosophila* learning and memory comes from genetic analysis of mutants exhibiting behavioral defects when subjected to aversive olfactory classical conditioning. Learning itself requires components of the cAMP signaling pathway. Mutants of the drosophila PKA regulatory subunit (PKA-RI), the PKA catalytic subunit (Pka-C1), or the PKC inhibitor (14-3-3ζ) are unable to learn, but are sensitive to shock, and are able to perceive odors. (6, 16, 29) In addition to cAMP pathway components, fasciclin II (fasII) and latheo(lat) mutants show similar learning defects. Mutants for other genes in the cAMP signaling pathway exhibit both learning and STM defects. The cAMP phosphodiesterase dunce (dnc), and the Ca²⁺ / calmodulin dependent adenylate cyclase rutabaga (rut), are required for

learning and STM, but not MTM. Mutants of the integrin scab (scb) or neurofibromin 1 (NF1) also show learning and STM defects, but retain some MTM. MTM was itself confirmed as a distinct phase of memory based upon analysis of the amnesiac (amn) mutant. STM decays rapidly, and ARM appears gradually around 60 minutes after conditioning, but observations of memory retention showed a smooth decline following training. It had therefore been surmised that another disruptable phase of memory existed, and that the additive contributions of STM, ARM and this surmised phase produced the observed retention curve. Careful study of amn flies shows that they are able to learn, have near normal STM immediately following a single conditioning session, and exhibited memory retention 6-7 hours later. However, amn flies exhibit significantly reduced memory during the intervening period. Further, when reversal training is conducted at various time points following conditioning, the memory of normal flies is shown to be subject to disruption during the same period. Moreover, the retention curves of normal flies subjected to reversal training, and of amn flies are very similar, indicating that a disruptable phase of memory between STM and ARM exists and requires the amn gene product. Mechanistically, amn encodes a neuropeptide that binds a G-protein coupled receptor, thereby activating adenylate cyclase.(30) When flies harboring a temperature sensitive Pka-C1 (DC0^{ts}) are shifted to the restrictive temperature just before beginning behavioral experiments, they memory defects that are indistinguishable from amn. In this way, Pka-C1 and amn mutants demonstrate that MTM is an early form of memory that persists into the onset of ARM, that is distinct from STM, and that has unique genetic requirements. In many ways, ARM

resembles the other form of consolidated memory, LTM. However, While ARM decays within 4 days, LTM can persist for more than a week. The radish (*rsh*) gene is the only gene known to be specifically required for ARM induced by aversive olfactory classical conditioning and not for LTM. However, it has been reported that in other learning paradigms, *rsh* mutants display memory defects as early as 3 minutes after learning, suggesting that *rsh* may not be required generally for memory consolidation, and that the requirement for *rsh* in ARM is specific to olfactory aversion.(31) This view is counter to the prevailing model based upon results from aversive olfactory classical conditioning that implicate *rsh* in a serotonin mediated pathway for memory consolidation parallel to cAMP signaling. (6, 7, 32) In *Drosophila*, as in other species, administration of the protein synthesis inhibitor cycloheximide (CMX) blocks LTM formation. ARM does not require protein synthesis, and is thus unaffected by CMX administration. LTM also uniquely requires the transcription factors dCREB2, Adf1, and Notch. Additionally, flies require APPL expression specifically for LTM, and not for learning or other types of memory. Though APPL null mutants show defects in response to shock, likely due to developmental defects, conditional expression in the mushroom body of an RNAi inducing hairpin directed against APPL beginning 24 hours prior to conditioning significantly reduces LTM, but not STM or ARM. These flies are able to perceive odor and react to shock normally, indicating that APPL exerts its effect on LTM independent from its developmental role.(33) In addition to genes specifically required for distinct types of aversive olfactory memory, a large and growing list of genes have been implicated more broadly in various aspects of learning and

memory. Many of these were identified in screens for genes involved in LTM, but may act more broadly in memory formation. For instance, appetitive and aversive olfactory memory require G-protein coupled signaling triggered by the dopamine receptor (Dop1R1). (28, 34, 35) Dopaminergic signaling is thought to act through rut during memory formation, resulting in CREB mediated transcriptional changes.(34) Thus, flies with genetic disruptions at many downstream points in this pathway exhibit learning and memory defects. However, the critical role of Dop1R1 in olfactory memory is demonstrated by the complete ablation of aversive olfactory memory in Dop1R1 mutants, while mutants of other genes required for learning such as rut only exhibit decreased learning. The requirement for cAMP signaling pathway components at various stages of learning and memory illustrates the central role that this pathway plays in regulating the cellular processes involved in learning and memory. It also mirrors mechanisms found to be at work in classical conditioning of *Aplysia*. (8, 34) cAMP signaling is activated during learning, leading to rapid but temporary changes in synaptic efficacy. These initial changes can be stabilized through feedback loops such as CaMKII autophosphorylation and more permanently by altered gene expression resulting from CREB mediated transcription. Additionally, a number of genes involved in RNA localization and translational regulation are involved in memory formation. These include Staufén, Pumilio, Oskar, Fmr1, and components of the microRNA induced silencing complex (miRISC). Defects in RNA localization and translational control are an emerging area of study in several neurodegenerative diseases and cognitive disorders. In addition to a role in regulating gene expression generally, miRNA may play a crucial

role in regulating translation at the synapse of mRNAs involved in synaptic plasticity. Mounting evidence indicates that regulated RNA localization and synaptic translation is crucial in maintaining appropriate synaptic connectivity. This also suggests a mechanism by which plasticity could be modulated at specific synapses within a single neuron, though proof of such a mechanism is currently lacking.

The Neural Circuitry of Drosophila Olfactory Memory

The neural circuitry involved in aversive olfactory classical conditioning has been the subject of extensive study in *Drosophila*, and is therefore well understood. Much of what is known about this circuitry comes from genetic dissection, and recording from and manipulation of neural activity in selected brain regions using methods I have previously described. *Drosophila* olfactory sensation takes place in hair like structures called sensilla present on the third segment of the antennae, and on the maxillary palps. A total of approximately 1200 olfactory receptor neurons (ORN) innervate these structures. Each ORN expresses 1 of 59 olfactory receptor (DOR) genes and an invariant olfactory coreceptor (Orco). The DOR gene product complexes with Orco to form a functional DOR. All ORNs expressing the same DOR project to the same bilaterally symmetric glomeruli of both antennal lobes (AL). The antennal lobes are composed of 43 distinct glomeruli, each receiving input from ORNs expressing a fixed set of DORs. In this way, an odorant map is produced in the AL by the distinctive projection patterns of ORNs. Specific spatial patterns in the AL are activated by input from ORNs, and these patterns are determined by the unique

set of DORs triggered by a given odorant. (14, 36) In the AL, ORN axons form synapses with inhibitory and excitatory local interneurons (LN) that integrate information between glomeruli. ORNs and LNs form synapses with projection neurons (PN). Dendritic arbors of a given PN are typically confined to a single glomerulus. PNs relay olfactory information integrated in the AL to the calyx (CA) of the mushroom body (MB), and to the lateral horn (LH). (7, 28, 34) The set of PNs activated by a given odorant can be modified through conditioning, but this modification lasts only minutes.(37) The mushroom body has long been known to be involved in memory, as many of the genes required for memory formation are highly expressed in it, and disruptions of the MB's development or function result in memory defects. (6, 34) Whereas the MB is involved in behavioral response to learned olfactory information, the LH is thought to be involved in innate responses to olfactory information such as those triggered by pheromones. (28, 38) The MB is composed of several lobes, each innervated by 1 of 3 distinct Kenyon cell (KC) populations. The cell bodies of KCs are immediately adjacent to the CA, into which all KCs extend dendrites. In the CA, KCs receive olfactory input from PNs. While a stereotyped odorant map exists in the AL, odor representation in the MB differs between individuals, with KCs integrating input from PNs from apparently random AL glomeruli.(34) In the MB, olfactory information is represented by distinct patterns of sparse activity across all three 3 major classes of KCs. KCs of each major class extend two axon branches into specific MB lobes. α/β KCs extend one axon branch into the α lobe, and another into the β lobe. Similarly, each α'/β' cell extends one axon branch into the α' lobe and another into the β' lobe. γ KCs extend both

branches into the γ lobe. (28, 34) The sparse pattern of odor evoked KC activity is likely the result of two mechanisms. First, in the locust MB, KCs function as coincidence detectors, integrating input from multiple PNs. Examination of the dendritic arbors of *Drosophila* KCs shows that each KC typically has 7 dendritic claws, each receiving input from a single distinct PN, thereby indicating that the wiring for coincidence detection of PN activity is present in *Drosophila* KCs. Second, KCs also receive inhibitory GABA-ergic input from non-spiking neurons termed anterior paired lateral neurons (APL) throughout the MB, including in the CA. It has recently been shown that APL activity reduces odor evoked KC activity, further supporting the notion that GABA-ergic APL activity tunes KC activity.(39) In addition to olfactory information, the MB also receives input from separate aversive and appetitive reinforcement pathways. For many years, it was thought that dopamine only functioned in aversive signaling, with octopamine functioning in appetitive signalling. This idea is now known to be the result of an experimental artifact, and it has been shown that both aversive and appetitive input occurs via dopaminergic signaling through Dop1R1, albeit through distinct circuits. Dopaminergic protocerebral paired lateral 1 (PPL1) and protocerebral anterior lateral (PAM) neurons project to the α'/β' , α/β , and γ lobes of the MB, and provide aversive reinforcement input.(15, 40, 41) Electric shock of the sort used in aversive olfactory classical conditioning activates these neurons. (40, 42) However, restoring expression of Dop1R1 only in the γ lobe of Dop1R1 mutant flies is sufficient for aversive memory formation.(43) Dopaminergic input involved in appetitive reinforcement comes from a set of PAM neurons distinct from those involved in

aversive reinforcement. These appetitive dopaminergic PAM neurons project only to the β , β' , and γ lobes of the MB, and neurotransmission from these neurons during odor exposure leads to appetitive memory formation. Appetitive PAM neurons respond to octopamine, and octopaminergic reward signaling occurs through these PAM neurons. (44, 45) In addition to olfactory and reinforcement inputs, the MB also receives input from the dorsal paired medial (DPM) neurons. DPM neurons project throughout the MB, except to the CA. DPM neurons express the *amn* gene, and blocking neurotransmission in DPM neurons with *shi^{ts}* does not affect learning, but prevents consolidation of olfactory memories. Although they express the neuropeptide *amn*, DPM neurons are reported to be serotonergic. According to this report, serotonin signaling via the serotonin receptor (5-HT1A), expressed in α/β cells, is required for ARM.(32) Interestingly, DPM neurons form gap junctions with APL neurons, and blocking expression of innexins required for the formation of gap junctions in either APL or DPM neurons results in MTM defects.(46) However, blockade of APL neurotransmission using *shi^{ts}* inhibits STM but not LTM.(47) Taken together, data regarding DPM function implicate it in the process of consolidating memories.(28) Experiments in which *shi^{ts}* is used to block neurotransmission in each of the lobes of the MB at specific times during learning, memory consolidation, and behavioral testing, reveal that distinct MB regions are required for various aspects of learning, memory storage, and retrieval. Neurotransmission in α'/β' KCs is required during, and immediately after conditioning. However, blockade of neurotransmission in α'/β' KCs during testing has no effect. It therefore appears that α'/β' KCs are required for acquisition, and the earliest stage of memory

formation, but are not the ultimate locus of memory storage. Blockade of the neurotransmission in α/β and γ cells during aversive olfactory conditioning does not disrupt memory formation, but α/β neurotransmission is required during testing. Expression of *rut* in the γ lobes of *rut* null mutants rescues STM, but not LTM defects, whereas expression of *rut* in α/β and γ lobes rescues STM and LTM. Taken together, these results support a model for aversive olfactory memory storage in which olfactory memory is initially encoded in the α'/β' lobes and quickly transferred to the γ lobes. APL and DPM signaling help maintain the memory trace while it is consolidated, and transferred to the α/β lobes where it is stored as a stable LTM.(28, 34)

Though the circuitry of the drosophila brain is complex enough to produce a surprising array of behaviors, it is simple enough that one can identify the contributions of small groups of cells to these behaviors. Genetic dissection of olfactory memory has identified biochemical pathways and neural tissues involved in memory formation in drosophila. This work demonstrates that the MBs are the primary center for encoding and storing olfactory memory. The changes in behavior resulting from olfactory conditioning stem from altered patterns of neural activity in circuits residing within the MBs. Such insights demonstrate the unique suitability and capabilities of *Drosophila* as a model system for studying the physiology of learning and memory. Work in flies has shown that modification of existing synaptic proteins can produce the changes in neural activity underlying memory, but only transiently. LTM formation requires transcription and translation, and can

involve changes in synaptic number or patterns of synaptic connectivity. Thus, the importance of understanding the ways in which gene regulation is altered during LTM formation is clear. Again, the uniquely powerful combination of behavioral, genetic, and other tools available in *Drosophila* make it a premier model organism with which to study gene regulation in response to LTM formation.

Part II: Short Noncoding RNAs In *Drosophila* Memory Formation

A Variety Of Short, Non-Protein Coding RNAs Regulate Gene Expression In Animals

All somatic cells within a given organism share a common genome. Yet, an astounding diversity of sizes, shapes, and behaviors exists amongst the various cell types that organism possesses. The meter long motor neurons innervating muscles in the feet of mammals bear little resemblance in form or function to the fibroblasts of the same animal, but fibroblasts can be experimentally coaxed into becoming motor neurons, thereby demonstrating that they share the same genetic potential.(48) Moreover, a single cell type can exhibit diverse behaviors. For instance, upon infection, a relatively quiescent immune cell can become mobile, actively stalking pathogenic bacteria, engulfing the bacteria once it is caught, producing enzymes to destroy the bacteria, and secreting factors to recruit other immune cells to the site of infection. Both the diversity of cell types and the array of behaviors of a given cell type are largely achieved through dynamically regulated gene expression. As altered gene expression is required for LTM formation,

understanding the mechanisms by which this is achieved provides deep insight into the physiology of memory and cognitive disorders. Work in recent years has significantly broadened and deepened our knowledge of the regulatory potential of animal cells. Much of this new understanding stems from the continual identification of new and surprising activities of non-protein coding RNAs. The discovery of several families of short non-coding RNAs (sRNA) that regulate gene expression through related but distinct mechanisms is a major development in this vein. sRNAs are known to regulate gene expression via transcriptional gene silencing (TGS), post transcriptional gene silencing (PTGS), through DNA and histone modification, heterochromatin formation, and new roles continue to be discovered. (49-52) Three major classes of sRNAs have been identified: short interfering RNAs (siRNAs), micro-RNAs (miRNAs), and piwi-interacting RNAs (piRNAs). Though significant distinctions exist between species, all three classes are broadly conserved across animal phyla. The three major sRNA classes are distinguished by their biogenesis, size ranges, mechanisms of action, and protein complexes with which they associate. siRNAs and miRNAs are derived from double stranded RNA (dsRNA) precursors, while piRNAs are produced from single stranded RNAs transcribed from repetitive elements and transposons, or via a mechanism that amplifies previously produced piRNAs. All three sRNA classes act via direct binding to members of the Argonaute family of effector proteins. Argonaute proteins and their bound sRNAs are incorporated into multiprotein complexes termed RNA induced silencing complexes (RISCs). In *Drosophila*, each sRNA class is bound by distinct sets of Argonaute proteins, and these interactions are the major

determinants of the ultimate regulatory effect of sRNAs.(52) The Argonaute family of proteins is divided into the Ago and PIWI clades. *Drosophila* have two members of the Ago clade (Argonaute-1 (Ago1) and Argonaute-2 (Ago2)), and 3 PIWI clade members (P-element induced wimpy testes (piwi), Aubergine (Aub), and Argonaute-3 (Ago3)). The overwhelming majority of miRNAs bind Ago1 and negatively regulate mRNA expression via translational silencing.(52) siRNAs bind Ago2 and are involved in a variety of processes including post-transcriptional gene silencing (PTGS), heterochromatin regulation, PolII pausing, viral defense, and genome protection against selfish genetic elements. In *Drosophila*, both endogenously produced siRNAs (esiRNAs) and exogenously introduced siRNAs direct Ago2 mediated destruction of target RNAs. However, esiRNAs can also guide Ago2 in its other activities just mentioned.(51, 53-57) piRNAs can bind all three members of the PIWI clade and largely function in the germline to silence transposable elements (TEs).(52, 56) However, instances of piRNA activity in somatic cells, both in TE silencing and in gene regulation, have been reported. (58-62) The mechanism of sRNA biogenesis and sequence features of a given sRNA direct it into binding with a given Argonaute protein, and thereby largely dictate the ultimate function of the sRNA. (63-65) Some sRNAs are expressed at barely detectible levels, while others are highly expressed. Moreover, sRNA expression is dynamic, responding to and directing various processes from development to immunity.(51, 66, 67) The diversity and abundance of sRNAs adds significant complexity to the task of understanding gene regulation. However, it is now clear that sRNAs compose an ancient and vital component animal physiology. Recent

studies have identified both miRNAs and piRNAs as important factors in *Drosophila* neurobiology.(59, 68-70) The array of known esiRNA functions continues to expand, and recent work suggests that they are involved in olfactory sensation, learning, and other neural processes. (71-73) Though some aspects of sRNA biogenesis and function are well understood, these phenomena are relatively recent discoveries, and much work remains. Further, the relevance of individual sRNAs for particular biological processes have been characterized, but analysis of broader sets of sRNAs in most processes remain lacking.

Biogenesis And Function Of siRNAs

The first hints at the regulatory potential of sRNAs came in the 1980s from experiments in plants.(74) When two constructs carrying separate transgenes, but sharing regulatory sequences, were introduced into the same plant, expression of both transgenes was inhibited. However, when the transgenes were segregated in progeny, both were expressed. This was shown to be the result of both DNA methylation and PTGS. Both processes involve production of sRNAs that base pair with regulatory sequences and RNA products of the transgenes respectively. (75-77) Further, it was shown that transgenic expression of truncated viral coat proteins in plants lead to immunity from the corresponding virus through the destruction of both transgenic and viral RNAs encoding the coat protein.(78) These initial observations in plants were followed by the discovery of similar phenomena in virtually every animal examined. Though these processes in plants share features

with similar systems in animals, significant distinctions exist. I mention sRNA mediated silencing in plants here to underscore the ancient origin of these mechanisms, and to provide historical context. Although sRNAs remain the focus of active and vibrant work in plants, critical early advances in understanding sRNA mediated gene silencing came from *C. elegans* and *Drosophila*, and these species remain premier model organisms for studies of these phenomena. I will therefore restrict my subsequent discussion of sRNAs to animals unless otherwise explicitly stated. Early work in plants and animals indicated that genes could be silenced in an RNA dependent manner, but the relative ability of sense and antisense single stranded RNA (ssRNA), and double stranded RNA (dsRNA) was not yet clear.(79) In work designed to explore this issue, Fire et al showed that neither strand of ssRNA induced robust silencing when injected into *C. elegans*, but potent and sequence specific silencing was induced by introduction of dsRNA homologous to target RNA sequences.(80) This mechanism is known as RNA interference (RNAi). The potential utility of a means with which to experimentally silence genes of choice was clear, and thus others worked to duplicate this result in other animals. This work has shown the mechanism to be conserved from nematodes to humans. (49, 52, 64) Significant progress toward understanding the mechanism of RNAi soon came from work in *Drosophila*. Several papers published in rapid succession showed that introduction of perfectly matched dsRNA into *Drosophila* cell extracts leads to processing of the dsRNA into 21-23nt fragments from both strands, even in the absence of target mRNA. These short RNA fragments are termed short interfering RNA (siRNA). Further, cleavage of mRNAs matching the dsRNA in sequence occurs

only within the region corresponding to the dsRNA sequence, and at intervals of 21-23nt. This observation reflects the role siRNAs play as guides, base pairing with targeted RNAs, and thereby directing silencing machinery to the appropriate target. (81-84) This silencing machinery consists of the siRNA and a multi-protein complex termed the RNA induced silencing complex (RISC). siRNAs are produced from long dsRNA precursors via RNase III type processing by a Dicer protein. In *Drosophila*, two Dicer genes are present, Dicer-1 (Dcr1) and Dicer-2 (Dcr2). Cleavage of such long dsRNA with extensive complementarity by Dcr2 yields duplexes of 21-23nt with 2nt 3' OH overhangs, and 5' monophosphates. However, the vast majority of Dcr2 cleaved duplexes are composed of 21nt ssRNAs.(51) Following cleavage by Dcr2, the duplex is loaded into the effector protein Argonaute-2 (Ago2). One strand of the dsRNA, dubbed the passenger strand, is discarded and degraded. Ago2 retains the other strand such that the siRNA is able to base pair with target RNAs, thereby guiding Ago2 to the target.(52) The Ago2 retained strand is therefore termed the guide strand. The strand with a less stably base paired 5' end is preferentially retained as the guide strand. The details of siRNA excision from precursor RNAs, and subsequent loading into Argonaute proteins differ between species, though common themes exist. This phenomenon is perhaps best understood in flies. In *Drosophila*, the R2D2 protein functions in sensing the relative stability of the 5' ends of the two dsRNA strands. R2D2 thus plays an important role in strand selection and loading of Ago2.(52) R2D2 may function in localizing Dcr2, and R2D2 stability is Dcr2 dependent.(85) The D isoform of another dsRNA binding protein, Loquacious (loqs-PD) is also required for siRNA production via Dcr2. Its

function is less clear, but it is believed that loqs-PD facilitates Dcr2 binding of dsRNAs, and that this step is downstream of R2D2's activity.(86) The heat shock cognate protein 70kDa-heat shock protein 90 (Hsc70-Hsp90) chaperone complex holds Ago2 in a conformation able to accept the duplex. This Hsc70-Hsp90 mediated conformational change of Ago2 is ATP dependent, and results in the transfer of the Dcr2 cleaved duplex to Ago2. The passenger strand is subsequently cleaved and separated from the guide strand within Ago2 in an ATP independent manner. (87, 88) Following passenger strand cleavage by Ago2, a complex of the Translin and Trax proteins termed the Component 3 Promoter of RISC (C3PO) removes the passenger strand from Ago2. The interaction of C3PO and siRNA loaded Ago2 yields an active siRNA containing RISC (siRISC) that is able to cleave target mRNA.(89) The final step of in vivo maturation of siRISCs is 2' O-methylation of the 3' nucleotide of Ago2 bound siRNAs by the methyl transferase DmHen1.(90, 91) Uridines are added to the 3' ends of sRNAs with extensive complementarity to their targets by terminal uridyil transferases, and thereby marked for destruction. 2' O-methylation of the 3' nucleotide protects Ago2 bound siRNAs from nucleotide addition and exonucleolytic shortening, and thus stabilizes siRNAs.(91) Ago2 cleaves target RNAs with perfect, or nearly perfect complementarity to the siRNA with which it is loaded via an endonuclease activity, leading to the destruction of the target RNA. siRNA directed cleavage of mRNAs by Ago2 is a multi-turnover process, thus siRNAs are potent post-transcriptional silencers of gene expression.(52) As such, increased siRNA levels resulting from stabilization by 2' O-methylation of the 3' nucleotide enhances silencing. During the early 2000's siRNA induced RNAi was

developed into one of the most important and widely used methods for manipulating gene expression in animal cells. Researchers are now able to experimentally inactivate any mRNA for which they have sequence information by transfecting corresponding siRNAs, longer dsRNAs, or by generating animals that harbor transgenes producing dsRNA matching the target mRNA sequence. As this system must have evolved with a natural function involving exogenous RNAs in *Drosophila*, the siRNA pathway was long thought to function in viral defense, and indeed it does.(92, 93) However, the siRNA pathway does not act solely via exogenous RNA. With the spread of massively parallel DNA sequencing technology in the late 2000's and its use in sequencing sRNAs, researchers identified endogenous sRNAs bound to Ago2 that matched genomic sequences in *Drosophila*. Equivalent endogenous sRNAs were also found in mice, worms, humans, and other animals. (50, 94-97) As these sRNAs bind the siRNA effector protein Ago2 in *Drosophila*, and share the 21-22nt size range of siRNAs, these sRNAs are termed endogenous siRNAs (esiRNA). esiRNAs arise from a number of sources, and appear to have functions in addition to guiding Ago2 mediated destruction of target mRNAs. As is the case with siRNAs, esiRNAs are produced from long dsRNAs exhibiting extensive complementarity between strands. Such dsRNAs can be generated by annealing of RNAs transcribed from distant genomic locations, or from bi-directional transcription at loci where genes, pseudogenes, or ncRNAs reside on both strands. Long inverted repeats can form hairpin secondary structures with extensive complementarity and also produce esiRNAs. Lastly, heterochromatin, transposons, and other repetitive elements generate and are targeted by

esiRNAs.(94, 96, 97) esiRNAs can act in PTGS much as exogenous siRNAs. However, such instances appear to be rare. Czech et al reported that esiRNAs generated by a locus, dubbed esi-2, harboring 20 palindromic repeats of ~260nt produces abundant esiRNAs. Some of the most abundant esi-2 esiRNAs have extensive, though not perfect complementarity with a sequence in the coding region of the mus308 gene. Analysis of mus308 cleavage products indicated that they correspond to siRNA type cleavage directed by esi-2 esiRNAs. Further, Ago2 and Dcr2 mutants both exhibited elevated mus308 expression.(94) However, despite the large number of esiRNAs cloned thus far, this remains the only well documented case in which esiRNAs regulate endogenous gene expression through PTGS in *Drosophila*.(98) The esiRNA pathway also appears regulate gene expression transcriptionally through Pol II pausing. The negative elongating factor E (Nelf-E), causes transcriptional pausing via interactions with Pol II. Nelf-E co-immunoprecipitates with Ago2 and Dcr2 as well as Pol II. Nelf-E and Ago2 association with Pol II is disrupted in cells depleted of Dcr2. Depletion of Dcr2 disrupts transcriptional control of genes that have paused Pol II. Functioning Dcr2 and Ago2 are required for proper transcriptional silencing of heat shock loci under normal conditions. Single amino acid mutations that disrupt the helicase or dicing activities of Dcr2, or the slicing activity of Ago2, result in increased levels of heat shock protein transcripts under normal conditions. The role of esiRNAs themselves in Pol II pausing remains unclear. However, the presence of Ago2 bound antisense esiRNAs mapping to heat shock promoters suggests that they are involved in this process.(54) However, the major function of *Drosophila* esiRNAs appears to be in

silencing selfish genetic elements in somatic cells. The bulk of Ago2 bound esiRNAs correspond to transposon sequences. Loss of Ago2 or Dcr2 corresponds with elevated transcript levels of some transposons. (94, 95, 99, 100) Heterochromatin is largely composed of transposons and repetitive elements in *Drosophila*. Blocking biogenesis or function of esiRNAs with viral proteins alters patterns of H3K9 methylation and disrupts heterochromatin formation. These results are also seen in Dcr2, R2D2, and Ago2 mutants.(53) Though exogenously introduced siRNAs have been a widely used experimental tool for over a decade, much remains to be understood about the origin and functions of esiRNAs. Further, as components of the siRNA mediated silencing pathway are known to interact with many other proteins, both within and outside of RISCs, it is not surprising to find them implicated in a variety of biological processes. Recent work has shown that Ago2 unexpectedly functions in processes from alternative splicing to chromosomal looping. However, it appears that esiRNAs and the slicer function of Ago2 are dispensable for these activities.(55, 101) Further study is required to clearly delineate which processes are siRNA dependent, and in which RISC components merely serve as structural components of protein complexes unrelated to their silencing function.

Biogenesis And Function Of miRNAs

Mature miRNAs are ~21-24nt single stranded RNAs, derived from a variety of longer precursors that form duplexes with themselves via hairpin secondary

structures. miRNAs bind Ago family Argonaute proteins, and are thereby incorporated into protein complexes dubbed microRNA induced silencing complexes (miRISC). Animal miRNAs base pair imperfectly with sequences in mRNAs, thereby targeting these mRNAs for post-transcriptional silencing via miRISC activity. Base pairing with the target in the region of miRNA nucleotides 2-8 is sufficient to induce silencing, and the sequence of this region is therefore the major determinant of miRNA target specificity. This region of miRNAs is termed the seed sequence. Sequences outside of the seed seem to play little role in determining which mRNAs a given animal miRNA targets. While *Drosophila* have only 258 miRNA genes, the brevity of the seed sequence ensures that each miRNA targets many mRNAs. Indeed, most animals have a small number of miRNA genes, yet half or more of mRNAs in most animals are subject to silencing by miRNAs. Plant miRNAs usually exhibit extensive complementarity with their targets, and thereby guide post-transcriptional silencing via endonucleolytic cleavage and destruction akin to the activity of siRNAs. Though plant and animals both possess miRNAs, major distinctions exist in miRNA silencing mechanisms between the kingdoms. I will therefore restrict my discussion to animal miRNAs. miRNAs were first identified in *C. elegans*, but were subsequently found in plants, fungi, and all metazoans. Indeed, many miRNAs are extensively conserved in animals, as are many of the regulatory pairings of miRNAs and target genes. The conservation of sequence and functions in developmental gene regulation exhibited by many miRNAs is indicative of their ancient and vital role in controlling gene expression. (102-105) For this reason, miRNAs have been a major topic of study since their

discovery. Several mechanisms for miRNA biogenesis have been identified in *Drosophila*. Canonically, miRNAs are transcribed by RNA Polymerase II (PolII) from non-protein coding genes, yielding a hairpin primary miRNA precursor (pri-miRNA). In many cases, multiple pri-miRNAs exist within a single such transcript. In the nucleus, a heterodimer of the Pasha and Drosha proteins, termed the microprocessor, recognizes such hairpin structures and excises a shorter, ~50-70nt miRNA precursor from the pri-miRNA. This shorter precursor is known as a pre-miRNA. Cleavage by Drosha/Pasha results in a ~2nt 3' overhang on the pre-miRNA hairpin, allowing it to be recognized and actively exported to the cytoplasm via Exportin-5 (Exp-5) and Ran-GTP. In the cytoplasm, the RNase III protein Dicer-1 (Dcr1) cleaves the pre-miRNA ~2 helical turns into the hairpin. This second cleavage event yields a ~22nt dsRNA with 2nt 3' overhangs. (52, 63, 106, 107)

Specific isoforms of the Loquacious (loqs) protein bind Dcr1, positioning the pre-miRNA properly in Dcr1, and facilitating cleavage. Loss of loqs-PA and loqs-PB is embryonic lethal in *Drosophila*, but loss of loqs-PD is not. loqs-PA or loqs-PB transgene expression can rescue lethality in homozygotic loqs mutants. However, distinct sets of miRNAs are preferentially produced by loqs-PA or loqs-PB transgene expression in homozygotic loqs mutants. Further, the Dcr1 cleavage sites of some miRNAs differ when homozygotic loqs mutants are rescued with loqs-PA vs. loqs-PB transgenes. The distinct sets of Dcr1 cleavage products preferentially generated by loqs-PA and loqs-PB suggest a model in which relative abundance of different miRNAs, or Dcr1 cleavage products from the same pre-miRNA can be modulated through loqs splice site selection.(108) Such miRNA variants from the same pre-

miRNA are termed isomiRs. Following cleavage by Dcr1, the duplex is loaded into the effector protein Ago1. As is the case for Ago2, the relative stability of the 5' ends of each strand of the duplex is a major determinant of which strand will be loaded into Ago1 as the guide strand.(63, 107) Ago1 bound miRNAs usually exhibit a 5' uridine, whereas siRNAs and miRNA strands loaded onto Ago2 prefer a 5' cytidine. The strand not retained as the Ago1 guide is termed the miRNA* strand. Unlike Ago2, Ago1 does not cleave the miRNA* strand. However, after it is discarded, the miRNA* strand is still degraded. Nonetheless, a significant fraction of miRNA* species are incorporated into Ago2. This phenomena reveals the role that features other than 5' end stability play in determining strand selection in Ago1 loading. As pre-miRNAs feature bulges at positions of mismatched nucleotides, the dsRNA produced by Dcr1 cleavage will present different structures to Ago1, depending on in which orientation the dsRNA encounters Ago1. Ago1 is preferentially loaded with duplexes featuring an unpaired 5' end, and unpaired bases at nucleotides 8-11. Duplexes presenting perfect matches at nucleotides 8-11 are preferentially loaded onto Ago2(109) In this way, the strands of sRNA duplexes are sorted into different RISCs depending on the base pairing between precursor strands. This sorting is further refined by the absence of 2' O-methylation of the 3' nucleotide of Ago1 bound sRNAs. While this modification protects Ago2 bound sRNAs from nucleotide addition, trimming, and destabilization, Ago1 bound sRNAs are not modified, and remain vulnerable to destabilization by this mechanism. Ago1 bound sRNAs exhibiting extensive complementarity with their targets are subjected to 3' nucleotide addition and trimming. In flies lacking the Hen1, the enzyme responsible

for 2' O-methylation of the 3' nucleotide of sRNAs, Ago2 bound sRNAs with extensive complementarity to their targets are also tailed, trimmed, and destabilized. Similarly, artificial introduction of RNA that is perfectly complementary to a given miRNA leads to trimming, tailing, and reduced levels of that miRNA.(110) However, regardless of the methylation status of the 3' nucleotide of sRNAs, sRNA – target duplexes without perfectly complementary are bulged at positions of mismatched nucleotides. Such bulging prevents target dependent 3' nucleotide addition and trimming of sRNAs. In this way, target interactions help to purify the sRNA complement of Ago1 and Ago2, ensuring that Ago1 selectively retains only miRNA strands that have bulged base pairing with their targets.(91, 109, 110)

While pri-miRNA transcripts are the major source of miRNAs, other RNA precursors that form hairpin secondary structures also produce miRNAs. Deep sequencing of sRNAs typically yields large numbers of reads mapping to snoRNAs, rRNAs, and tRNAs. While few examples of regulatory functions for these ncRNA derived reads have been found, some snoRNAs appear to produce genuine miRNAs. snoRNAs form secondary structures and primarily act as antisense guides for enzymes that chemically modify rRNAs, tRNAs, and snRNAs. Recently, analysis of deep sequencing data from plants and evolutionarily distant metazoan species has shown that a conserved mechanism generates sRNAs with distinct and characteristic size ranges from snoRNAs. Some of these snoRNA derived sRNAs (sdrRNAs) enter the miRNA pathway and produce miRNA-like sdrRNAs that repress translation of seed matching target mRNAs. This mechanism does not require

drosha, but does require Dicer activity. Indeed, miRNA-like sdRNAs are found in metazoans lacking the microprocessor, suggesting that they may represent an ancient and conserved RNAi mechanism. (111, 112) Deep sequencing shows that miRNA-like sdRNAs bind Ago1 in drosophila.(113, 114) As miRNA-like sdRNAs are functional in other metazoans, and so deeply conserved, they are presumed to be functional in *Drosophila* as well.(111) Protein coding gene transcripts can also produce miRNAs. In 2007, deep sequencing of *Drosophila* sRNAs revealed miRNA like sRNAs mapping to the splice acceptor and donor sites of certain introns. These sRNAs were thus dubbed mirtrons. Careful analysis revealed that following splicing, debranched introns can form secondary structures that are exported from the nucleus via exportin-5 and cleaved by Dcr1. Unlike canonical miRNA biogenesis, export and Dcr1 cleavage of mirtrons do not require drosha, as the hairpin structure formed by the debranched intron is innately compatible with with these processes. (115, 116) Biogenesis of some mirtrons requires 3' trimming by the exosome. In these cases, the debranched intron base pairs with itself such that a long 3' tail extends beyond the hairpin. The exosome then trims this tail back to the hairpin, yielding the pre-miRNA.(117) However, regardless of exosome involvement, mirtrons are loaded into Ago1, and regulate target mRNAs like miRNAs in flies.(115-117). Mirtrons were subsequently found in worms, birds, and mammals, suggesting that they are evolutionarily ancient.(118-120) As imperfectly base paired hairpin RNA structures abound in animal cells, but only a fraction of these hairpins yield miRNAs, a mechanism must exist for funneling certain hairpins and not others into the miRNA pathway. A recent report indicates that several sequence features within

and downstream of the pri-miRNA hairpin contribute to entry of a hairpin into the microprocessor in humans. However, no single sequence feature or combination of sequence features identified in this study ensured that an arbitrary hairpin would be processed into a miRNA. It therefore appears that the transcriptional origin, sequence features, and structure all contribute to routing hairpin RNAs into the miRNA pathway.(121) The rapid increase in acquisition and availability of sRNA sequencing data continually reveals examples of novel mechanisms for miRNA biogenesis. It is therefore unlikely that the mechanisms described here represent the totality of ways that miRNAs are generated, though they are likely to be the most important in *Drosophila*.

miRNA mediated repression of mRNAs is achieved through several mechanisms in *Drosophila*. Unlike Ago2, which binds siRNAs and has a strong slicer activity, Ago1 drives miRNA mediated silencing by recruiting other proteins that inhibit translation, destabilize mRNAs, or both. The repertoire of proteins comprising and associating with miRISCs is non-uniform and dynamic. Recent work demonstrates that the protein complement of *Drosophila* miRISCs changes in response to extracellular signaling, and that these changes can modulate miRNA mediated silencing of target mRNAs.(66) Following loading of the mature miRNA into Ago1 by the miRISC loading complex, which contains the miRNA, Ago1, Dcr1 and loqs, at least two types of active miRISCs can form. These miRISC types are defined by the proteins that associate with Ago1 within them. In the first type, following loading, Ago1 releases Dcr1 and loqs, and associates with GW182. As it contains GW182, a mature miRNA, and Ago1, this complex is known as G-miRISC. In

the second type, following loading of the mature miRNA into Ago1, Dcr1 dissociates from Ago1, but loqs is retained, yielding a miRISC composed of the mature miRNA, Ago1, and loqs. As loqs containing miRISCs sediment with polysomes, such miRISCs are termed P-miRISCs. GW182 and loqs binding to Ago1 are mutually exclusive. Further, G-miRISCs and P-miRISCs interact with distinct sets of proteins, and thereby silence target mRNAs in mechanistically different ways. In G-miRISCs, GW182 localizes the miRISC and its bound target mRNA to cytoplasmic foci containing mRNAs and proteins called P-bodies. P bodies contain enzymes that degrade mRNAs, and are regions in which mRNA silencing occurs. GW182 interacts with poly-A binding protein (PABP), and recruits the CCR4:NOT deadenylase and DCP1:DCP2 decapping enzymes to the mRNA-miRISC complex. Shortening of the poly-A tail by CCR4:NOT leads to decapping, and subsequent 5' to 3' degradation of the mRNA.(122, 123) GW182 can be recruited to mRNAs by proteins other than Ago1, and GW182 recruitment to mRNAs leads to silencing and degradation, even in the absence of Ago1. GW182 thus links the mRNA degradation machinery to miRNA mediated Ago1 binding of target mRNAs.(66, 122) However, GW182 can also induce translational silencing independent from its role in mRNA degradation. Teathering of GW182 to mRNAs prevents 48s and 80s ribosome formation, and therefore inhibits initiation of translation.(124, 125) However, miRNA silencing can occur without GW182. P-miRISCs contain a mature miRNA, Ago1 and loqs-PB, but not GW182, and silence targeted mRNAs by inhibiting translation elongation.(124) P-miRISCs associate with polysomes and other dense, non-translating mRNA-protein complexes. While G-miRISC silencing leads to target mRNA destruction, P-miRISC

induced silencing leaves ribosomes poised on the target mRNA, is reversible, and is thought to be involved in keeping certain mRNAs transcriptionally silent during intracellular transport. (66, 126) In S2 cells, the relative abundance of the two active miRISC types is altered by lipid and PKC signaling. Thus, the regulatory consequence of miRNA mediated silencing is responsive to extracellular cues. Additional study is required to fully understand the mechanisms involved in miRNA mediated silencing, and how these mechanisms are themselves regulated. miRNAs are unquestionably vital players in animal gene regulation, and resolving such questions is therefore a major priority in many disciplines within the life sciences. (66, 124, 127)

Biogenesis And Function Of piRNAs

piRNAs are 24-32nt RNAs that act as guides for piwi clade argonaute proteins exclusively, and primarily function in transcriptionally silencing repetitive elements and transposons through heterochromatin formation and maintenance. However, piRNAs can also target protein coding genes for silencing, and trigger PTGS. As is the case with other sRNA pathways, piRNA mediated silencing is a deeply conserved mechanism. Mobilization of transposons in the germ line poses a major threat to genomic integrity, and correspondingly, piRNAs are highly expressed in *Drosophila* ovaries and testis. The ovaries have therefore been the primary *Drosophila* tissue in which piRNAs are studied.(128, 129) Once transcribed, the gypsy family of transposons can reinsert themselves into the genome.

Moreover, gypsy family transposons can form particles capable of infecting other cells. Gypsy transposons are thus targeted for piRNA mediated silencing in both oocytes and the somatic ovarian follicle cells, which surround oocytes and support their development. Loss of the piRNA pathway in *Drosophila* ovaries or testis leads to elevated transposon expression and subsequent sterility. Two distinct mechanisms for piRNA biogenesis exist. Primary piRNA biogenesis involves only piwi, and not Aub or Ago3, and is the only mechanism used in follicle cells. In germ cells, an additional mechanism involving Aub and Ago3 amplifies piRNAs. Mounting evidence indicates that both mechanisms are active in somatic cells outside of reproductive tissues. In *Drosophila*, most piRNAs arise from specific loci termed piRNA clusters. piRNA clusters are repositories of transposons and transposon fragments. Deletion of these clusters results in derepression of transposons throughout the genome. Introduction of novel transposons can lead to incorporation of their sequences into piRNA clusters. However, piRNA clusters also exist in widely dispersed euchromatic transposons, and in sequences within the 3'UTRs of some protein coding genes. In oocytes and sperm cells, most clusters are transcribed, frequently bidirectionally. In follicle cells, the *flamenco* piRNA cluster, and the 3'UTR of the traffic jam (tj) gene are the major source of piRNAs. The *flamenco* cluster contains sequences from long terminal repeat (LTR) transposons, including the gypsy family. The cluster is transcribed via PolII from a single promoter, and generates a long single stranded piRNA precursor RNA that is antisense to the transposon sequences within the cluster. (61, 130) The other major piRNA cluster in follicle cells resides within the 3' UTR of tj.(128, 129) Interestingly,

tj is a transcription factor that drives piwi expression. tj derived piRNAs have a high degree of complementarity to FasIII transcripts, and FasIII is ectopically expressed in tj or piwi mutants. Thus, the tj gene and its piRNA cluster are intimately involved in piRNA mediated silencing.(131) A variety of other genes also produce piRNAs from their 3'UTRs, but the regulatory significance of these has not been well studied.(132) piRNA precursors are exported to the cytoplasm, where they undergo primary processing by a mechanism that is not yet fully understood. However, the available evidence supports a model for primary piRNA biogenesis in which the 5' end of piRNAs is defined via cleavage by a mitochondrial membrane associated endonuclease named Zucchini (Zuc). Structural and genetic studies of Zuc also support this view. In vitro cleavage of RNAs by Zuc also leaves a 5' monophosphate, which is a feature of piRNAs. (128, 129, 133, 134) In somatic follicle cells, 5' cleaved primary piRNA intermediates then enter perinuclear cytoplasmic loci of piRNA processing termed Yb bodies. Yb bodies contain several proteins which are required for piRNA biogenesis and transposon silencing. These proteins include the TUDOR domain containing proteins Yb and Vreteno (vret), the helicase Armitage (Armi), the coshaperone shut down (shu), and piwi. In the Yb body, piwi preferentially binds 5' cleaved piRNA intermediates bearing a 5' Uridine.(135) At this stage, the 3' end of the primary piRNA intermediate extends beyond the 32nt' upper limit of typical piRNA length. The 3' end of the primary piRNA intermediate is then trimmed to the 24-32nt size range typical of piRNAs by an unknown exonuclease. piwi interaction with the precursor protects bases within the piwi footprint from removal by this nuclease. 3' trimming is also coupled to 3' O-

methylation of the piRNA by Hen1. The mature piRNA loaded piwi is then imported to the nucleus, where it triggers transcriptional silencing guided by the piRNA. In the germ line and in somatic cells of non-reproductive tissues, piRNA biogenesis differs in a number of ways. First, the *flamingo* locus does not dominate piRNA production. In germ cells and somatic cells of non-reproductive tissues, all piRNA clusters are active, and guide silencing of an expanded set of targets beyond those present in *flamingo*. Unlike *flamingo*, these clusters are often transcribed bidirectionally. Importantly, in these cell types, Aub and Ago3 act together to amplify piRNAs targeting active transposons through a mechanism termed the ping-pong cycle. The ping-pong amplification loop is initiated by loading of Aub with primary piRNAs that are antisense to active transposons, either produced within the cell, or already bound to Aub and maternally deposited into oocytes. (136-138) Such primary piRNAs base pair with transcripts from active transposons and guide cleavage by Aub via its slicer activity. This cleavage yields a new piRNA whose 5' end is defined by the cleavage site, and that is loaded onto Ago3. As primary piRNAs have a uridine bias at the 5' nucleotide, and transposon cleavage by Aub occurs 10nt downstream from the uridine, Ago3 bound piRNAs have an adenosine bias at position 10, and are in the sense orientation. As is the case in primary piRNA biogenesis, the 3' end of the new Ago3 loaded piRNA precursor extends beyond 32nt at this stage. In the current model for ping-pong piRNA amplification, an unknown exonuclease then trims the 3' end of the Ago3 bound precursor to the binding footprint of Ago3. As in primary biogenesis, trimming and 3' 2'O-methylation by Hen1 are coupled. Ago3 can then cleave antisense piRNA cluster transcripts,

thereby defining the 5' end of a new piRNA precursor destined for Aub. Once loaded onto Aub, this new antisense piRNA precursor is trimmed and 3' 2'O-methylated as in the case of Ago3, thereby yielding a mature piRNA and completing the ping-pong loop. (129, 137, 139, 140) Unlike follicle cells, germ cells and somatic cells outside of reproductive tissues do not express Yb, and therefore do not have Yb bodies.

Instead, piRNA processing in these cells takes place in the nuage, a perinuclear locus related to Yb bodies. However, Handler et al show that the Yb related proteins Brother of Yb (BoYb) and Sister of Yb (SoYb) are required for primary piRNA biogenesis in the germ line, indicating that this family of TUDOR domain containing proteins plays a critical role in piRNA biogenesis.(141) The nuage contains many of the same proteins as Yb bodies, including vret, Armi, and shu. The nuage also contains Ago3 and Aub, and loading of these proteins with piRNAs is thought to occur within it. Transposon silencing via ping-pong derived piRNAs obviously occurs posttranscriptionally, as cleavage of transposon transcripts is required for their biogenesis. However, piwi mediated silencing occurs transcriptionally. Piwi's slicer activity is dispensable for silencing by piRNAs, and nuclear localization is required for silencing by piwi. (131, 142-144) In the nucleus, piRNAs act as guides, directing piwi mediated silencing by base pairing with targets. Genomic context determines the mechanism by which piwi acts. PolII transcription occurs in euchromatin, and piwi bound piRNAs base pair with nascent transposon transcripts in this context. In heterochromatin, which is transcriptionally silent, piwi bound piRNAs directly base pair with single stranded DNA. In either case, piwi recruits factors that lead to heterochromatin formation and maintenance. The histone

methyltransferase Su(var)3-9 is recruited by piwi, either directly, or through recruitment of Hp1a, and methylates H3K9.(145-147) H3K9 methylation is a heterochromatin mark, and blocks PolII transcription. Maelstrom (mael) influences H3K9 methylation spreading from marks established by piRNA targeting. H3K9 methylation spreading in mael mutants is not however associated with silencing. Transposons in such regions are transcribed, leading to the conclusion that mael acts downstream of H3K9 methylation to silence transcription. Further, genes nearby piRNA targets can be silenced, though they themselves are not subjected to H3K9 methylation, demonstrating the existence of factors other than H3K9 methylation in piRNA mediated silencing.(145) In addition to its role in transcriptional silencing, piwi may also act via PTGS. However, it is difficult to distinguish a direct role for piwi in PTGS from its role in piRNA biogenesis, and subsequent Aub and Ago3 mediated PTGS.(128) At a cellular level, the piRNA pathway has been implicated in canalization, a process by which phenotypic traits are maintained despite genetic or environmental differences. In this role, it appears that piwi mediated silencing of transposons helps suppress emergence of new genetic variation, and piwi mediated heterochromatin maintenance helps suppress expression of cryptic genotypes. (148, 149) Additionally, growing evidence indicates that the piRNA pathway plays a critical role during development and in maintaining proper cellular differentiation. As was previously mentioned, maternally deposited piRNAs program silencing mechanisms in oocytes. In this case, maternal piRNAs targeting a protein coding gene convert the gene to a piRNA producing locus. The gene continues to be transcribed, but its transcripts are used

as substrates for the ping-pong cycle, and the transcript derived piRNAs can thereby spread silencing to other loci with sequences similar to those of the transcript.(138) Thus, in addition to their role in genome defense, piRNAs are a mechanism by which the genome can be epigenetically programmed for silencing.(150) Further, knockdown of piwi leads to failure of germ line stem cell maintenance. (142, 151) Ectopic expression of piwi in somatic cells exacerbates tumor growth and leads to acquisition of germ line stem cell traits. (62, 152) Taken together, these studies indicate that the piRNA pathway drives cellular programs that induce or maintain a less differentiated developmental state, and encourage proliferation.

miRNAs In Neurophysiology And Behavior

Insect and human brains differ by orders of magnitude in complexity and cognitive capacity. Yet the difference in gene number between the two is much less substantial. This demonstrates the essential role gene regulation plays in achieving structural and functional complexity of the brain. The nervous system of an animal is composed of cell types that in some cases differ from each other subtly, and in others dramatically. Moreover, within a given cell type, considerable diversity of behavior and responsiveness to external signals exists. Glia and neurons must develop and function together. Neurons must wire properly, and must maintain largely stable wiring diagrams, yet they must also retain the capacity to strengthen or establish new connections, and to weaken or eliminate others. Further, neurons must send and respond to signaling appropriately. Gene regulation lies at the heart

of all of these processes. Though much is understood about gene regulation generally, neurons have unique requirements. Some genes are expressed only within neurons. Interestingly, RNA binding proteins, such as the *Drosophila* embryonic lethal abnormal vision (ELAV) gene product, comprise the majority of known neuron specific genes. Many genes undergo brain specific splicing, and certain transcripts are localized to particular regions within neurons.(153) Recent work in many organisms has shown that sRNAs play central roles in many such neuron specific regulatory mechanisms. The protein products of several genes that are causative in diseases of the nervous system, such as fragile X mental retardation protein (Fmr1) and ataxin-2 (Atx-2), interact with RISC components and are involved in miRNA mediated silencing in neurons.(68) miRNAs were originally identified in the context of their temporal regulation of developmental processes. It therefore comes as no surprise that miRNAs regulate many aspects of neurodevelopment in *Drosophila*. miR-8 negatively regulates neuroepithelial expansion and neuroblast transition via control of the epithelial growth factor receptor (EGFR) signaling pathway in glia of the optic lobe. miR-8 silences spitz (spi) via target sites in its 3'UTR. Spitz is a transforming growth factor α (TGF- α) like ligand for the *drosophila* EGFR gurken (grk), and expression of spitz in miR-8 positive glia is required for neuroepithelial cell expansion, neuroblast generation, and thereby normal optic lobe development.(154) Data from our lab, as well as from others, demonstrate that spatio-temporally regulated expression of the miRNA let-7 controls neuronal differentiation, and is required for normal MB formation. The insect hormone ecdysone regulates many aspects of development, and pulses of

ecdysone that occur during larval and pupal stages initiate major developmental programs. In the developing brain, temporally controlled ecdysone signaling drives *let-7* expression, which in turn negatively regulates the transcription factor *abrupt* (*ab*). *Abrupt* negatively regulates the cell adhesion molecule fasciclin II (*FasII*). *FasII* is expressed in the α/β and γ lobes of the MB, but not in the α'/β' lobes. Tight regulation of *FasII* expression is required for normal MB development, and *ab* mutants exhibit ectopic *FasII* expression in the α'/β' lobes. Expression of *FasII* in α'/β' lobes results in α'/β' neurons invading the α/β lobes, and conversely expression of *ab* in α/β lobes leads to innervation of α'/β' lobes by α/β neurons. α/β neurons are the last MB neurons generated during the pre-pupal to pupal transition, a period corresponding to elevated ecdysone and *let-7* levels. Decreasing levels of the transcription factor *chronically inappropriate morphogenesis* (*chinmo*), in post-mitotic MB neurons specifies the sequential differentiation of each of the MB cell types. *Chinmo* is a *let-7* target, and the decline in its expression corresponds to increasing *let-7* expression. Thus, *let-7* controls neuronal differentiation and wiring of the *Drosophila* brain.(155, 156) MicroRNAs also play a vital role in modulating synaptic efficacy. There are now numerous examples in many organisms of microRNA mediated mechanisms that regulate neuronal function. The microRNAs of the *Drosophila* miR-310, miR-311, miR-312, and miR-313 cluster (mir-310-313) negatively regulate kinesin heavy chain 73 (*Khc-73*) through target sites in its 3'UTR.(70) Kinesins are involved in transport of various cargoes to synapses, and *Khc-73* is involved in transport along microtubules and interacts with *Rab-5* containing vesicles.(157, 158) Loss of mir-310-313 in *Drosophila* motor neurons

results in increased Khc-73 expression, accumulation of the presynaptic active zone marker Bruchpilot (brp), and increased calcium influx. In this way, mir-310-313 negatively regulates the efficacy of neuro-muscular junctions (NMJ) in *Drosophila* motor neurons by reducing Khc-73 mediated transport.(70, 157, 159) In an example of the direct role microRNAs play in learning and memory, expression of the *Drosophila* miR-276a in the MB is required for LTM formation. Dopaminergic signaling in the MB via the dopamine receptor DopR is also required for LTM formation. miR-276a regulates DopR, and removing a single copy of the DopR gene rescues LTM defects in miR-276a mutants. miR-276a acts by tuning DopR levels, and this regulatory pairing is required for LTM.(160) MicroRNA regulation of synaptic efficacy is dynamic, and responds to neuronal signaling. In *Aplysia*, the CNS enriched miR-124 is rapidly down-regulated in response to serotonin induced MAPK signalling, and does not return to baseline levels for 12 hours. miR-124 negatively regulates CREB, and reduced silencing of CREB by miR-124 results in lasting enhancement of synaptic efficacy.(161) As CREB is a transcriptional regulator, reduced translational silencing by miR-124 initiates lasting changes in expression of many genes beyond the set it directly silences, even after it returns to baseline levels. More recently, Nesler et al reported that chronic activation of motor neurons in *Drosophila* larvae induces changes in expression levels of several miRNAs. They also report that these changes result in alterations in synaptic size and number at the NMJ.(162) However, chronic activation does not resemble normal patterns of activity in the CNS, and NMJs differ from synapses in the brain in many important ways. Therefore, this result may not reflect changes in miRNA

expression induced by activity relevant to memory formation. Nonetheless, this result indicates that neural activity can induce complex changes in microRNA expression patterns, and that examination of single microRNAs may not be sufficient to understand the regulatory contribution of miRNAs in response to neural activity. Neurons have perhaps the most elaborate morphology of any cell type, and can extend processes to great lengths, even a meter or more. Rapid and lasting changes in synaptic plasticity would be unachievable in such cases if it were to require transport to synapses of proteins newly synthesized in the soma. As such, localized synthesis of proteins is a particularly important feature of neuronal physiology. Neurons localize certain mRNAs and microRNAs to distinct cellular regions, including at synapses. Dendrites and axons contain different sets of translationally silenced mRNAs, poised for local protein production in response to appropriate patterns of neural activity. However, while a large complement of mRNAs is present at dendrites, they are largely excluded from axons. Preferential localization of mRNAs within dendrites reflects the importance of postsynaptic local translation. Indeed, translation within dendrites isolated from the soma can effect lasting changes in synaptic efficacy. Such local translation enables modulation of synaptic efficacy within selected dendritic branches or even at individual synapses, while leaving more distant synapses within the same cell unchanged. Synapse specific, or dendritic branch specific plasticity is thought to play a vital role in memory formation, as it allows a neuron to selectively tune the strength of those synapses receiving learning relevant input, while maintaining relatively stable connectivity with its other uninvolved synaptic partners. (163-167) In neurons, mRNAs are

selectively transported to specific cellular locations by forming ribonucleoprotein (RNP) complexes with RNA binding proteins (RBPs). These RNPs are incorporated into transport granules, and trafficked to their destinations along microtubules. RBPs involved in localizing mRNAs within neurons largely depend on interactions with sequence elements present in the 3'UTR. Transport granules also contain RISC components, and some RBPs involved in mRNA localization physically interact with RISC component proteins. Interactions between mRNAs and their RBP partners, as well as interactions between RBPs are modulated by neuronal activity.(153) Some mRNAs are delivered to dendrites in a translationally silenced state. When protein synthesis is dictated by synaptic activity, local signaling leads to covalent modification of RBPs, and subsequent changes in translation of proximal mRNAs. However, our understanding of how synaptic activity is transduced into local changes protein synthesis remains incomplete. Results from several studies indicate that microRNA mediated silencing controls local translation of specific mRNAs at or near synapses, and that this silencing can be modulated in response to neural activity. A recent study in the rat brain demonstrated that activity induced changes in miRNA expression do not directly translate into changes in levels of miRISC bound miRNAs.(168) This suggests that association of microRNAs with argonaute proteins is itself regulated in response to neuronal activity in a way that is independent from changes in miRNA biosynthesis. However, this study did not examine whether microRNA-argonaute association is controlled by changes in miRISC loading or microRNA degradation. Interestingly, certain pre-miRNAs and the microRNA biogenesis factor Dicer, are present in rat hippocampal

synaptoneuroosomes. In rats, pre-miR-134 is localized to dendrites through the interaction of sequences present in its terminal loop with the DEAH-box helicase DHX36. Further, silencing of target mRNAs by miR-134 in dendrites is DHX36 dependent, and knockdown of DHX36 leads to enlarged dendritic spines.(169) miR-134 silences the LIM domain containing kinase LimK1, and loss of LimK1 mRNA silencing by miR-134 leads to increased dendritic spine size.(170) It is therefore possible that at least some pre-microRNAs are processed into mature microRNAs in dendrites, and that regulation of microRNA processing provides a control point for modulating microRNA mediated translational silencing near synapses. Additionally, microRNA and miRISC interactions with targeted mRNAs are modulated by signaling pathways known to control synaptic plasticity. Experiments in our lab, and by Banerjee et al provide evidence that local degradation of miRISC components at synapses in response to neuronal activity leads to synthesis of proteins involved in synaptic plasticity. (126, 171) In mice, the RISC component MOV10 is required for translational silencing of CaMKII α , Limk1, and Lypla1. MOV10 is degraded at synapses in a proteasome dependent manner in response to NMDA receptor signaling. Using a fluorescent reporter of protein synthesis harboring the Lypl1a 3'UTR, the authors showed that degradation of MOV10 corresponds to new synthesis of the reporter at synaptic sites following NMDA receptor stimulation.(126) Subsequent work showed that degradation of proteins in the rat amygdala via the ubiquitin-proteasome (UPS) pathway is required for long term fear memory, and that NMDA receptor signaling during memory formation and retrieval triggers UPS mediated degradation of MOV10.(172) A recent study of

MOV10's interaction with mRNAs and other RISC component proteins indicates that MOV10 functions as an RNA helicase that facilitates UPF1 mediated 5' to 3' exonucleolytic decay via XRN1 and XRN2 by unwinding secondary structures that inhibit such decay.(173) 5' to 3' exonucleolytic decay via XRN1 is a known feature of miRNA mediated silencing. MicroRNA mediated silencing may also be controlled in ways other than regulated destruction of miRISC components. MicroRNA-target binding is regulated by RBPs aside from miRISC components. In mice, NMDA receptor signaling leads to activation of the kinase mTORC1, which in turn inhibits binding of the RBP HuD to mRNA encoding the voltage gated potassium channel Kv1.1. In the absence of HuD, miR-129 silences Kv1.1 mRNA. Without mTORC1 activity, HuD binds Kv1.1 mRNA, and thereby relieves it from miR-129 induced silencing.(174) Similarly, the HuD related protein HuR relieves target mRNAs from silencing by let-7. HuR binds sequences in the 3'UTR, and this binding results in displacement of miRISC from the target mRNA. Sequences bound by HuR can be tens of bases distant from microRNA target sites, and relief from microRNA mediated silencing by HuR is dependent on its ability to oligomerize. Further, HuR is unable to displace miRISC proteins that are directly tethered to mRNAs, and the ability of HuR to displace miRISC is reduced if microRNA-target complementarity is perfect. These results indicate that HuR, and perhaps other similar proteins such as HuD and the *Drosophila* protein ELAV, relieve microRNA mediated silencing by interfering with basepairing of microRNAs with their targets.(175) The activity of ELAV family proteins including HuD and HuR has been shown to be modulated by covalent modification and proteolytic cleavage. (174, 176, 177) Thus, ELAV family

proteins may be a major factor in transducing synaptic signaling into changes in microRNA mediated translational silencing. Several RBPs have been implicated in control of microRNA mediated translational control at synaptic sites. For instance, fragile X syndrome, the most common monogenetic form of intellectual disability is caused by loss of the RBP FMRP. FMRP is found at synapses, and is involved in localizing mRNAs to dendrites and in control of dendritic protein synthesis. In its phosphorylated state, FMRP associates with the mammalian microRNA effector argonaute protein, and leads to formation of a FMRP-miR-125a-miRISC that silences mRNA encoding the postsynaptic density protein 95 (PSD-95). Signaling from metabotropic glutamate receptors (mGluR) triggers dephosphorylation of FMRP, subsequent dissociation of FMRP-miR-125a-miRISC from PSD-95 mRNA, and leads to rapid translation of PSD-95.(178) Given the many RNA-protein and protein-protein interactions that occur along the 3'UTR of mRNAs, it is likely that additional mechanisms controlling miRNA mediated silencing in response to neuronal activity will continue to be discovered for some time to come. However, existing work has demonstrated that reversible silencing of mRNAs by microRNAs is an important feature of translational control at or near synapses, and is involved in lasting forms of memory. Intriguingly, recent work demonstrates that microRNAs also act via multiple mechanisms as intercellular signaling molecules. MicroRNAs are packaged into extracellular vesicles, and are secreted by cells. Several microRNAs, including let-7b, are ligands for the toll like receptor TLR7. TLR7 can directly activate the transient receptor potential cation channel TRPA1. TLR7 stimulation with let-7b induces inward currents and action potentials in cells coexpressing TLR7 and

TRPA1. TRPA1 is expressed in nociceptive neurons in mice, and its activation causes pain. Injected and endogenous intercellular let-7b triggers a pain response that is sequence dependent, and can be blocked by inhibitors of TRPA1 or pretreatment with let-7b inhibitors. Interestingly, let-7b is highly expressed in nociceptive neurons in mice, and is secreted in response to neuronal activity in these cells.(179) Thus, let-7b and other microRNAs harboring a specific shared nucleotide sequence act as signaling molecules in neurons. Secreted microRNAs can also be taken up by recipient cells where they are capable of silencing mRNAs.(180) MicroRNAs released in exosomes at synaptic sites in response to neuronal activity are taken up by glia and can alter expression of genes involved in regulating neurotransmitter levels in the synaptic cleft, thereby modulating synaptic efficacy.(181) While microRNAs are known to be essential for many biological processes, and in virtually all cell types in eukaryotes, they have uniquely diverse and vital functions in neurons. Underscoring the importance of microRNAs in proper function of the nervous system are numerous examples of intellectual disability and neuromuscular diseases caused by mutations in genes that encode miRISC interacting proteins. Moreover, the pathologies and symptoms of diseases caused by mutations in these genes are strongly conserved from insects to humans, demonstrating the ancient and central function of the microRNA pathway in the nervous system. However, in order to fully understand the contributions to neuronal function of microRNAs, additional studies examining the activity of all microRNAs simultaneously are needed. Previous work has studied the plasticity relevant dynamics of individual microRNAs in several model animal systems, but

the full spectrum of microRNA regulation during learning and memory formation remains largely unstudied and is poorly understood. Thus, the importance of microRNAs in synaptic plasticity and LTM is well established, but the complex interplay of posttranscriptional regulation by hundreds of microRNAs requires additional study to fully understand their function in these processes.

esiRNAs, piRNAs, And Novel sRNAs In Neurophysiology

Studies of sRNA activity in the nervous system have thus far focused almost exclusively on microRNAs, and have left the potential roles of other classes of sRNAs in neurons largely unexplored. The initial discovery of piRNAs in reproductive tissues at first biased the exploration of their function away from somatic cells, let alone the nervous system. More recently, piRNAs have been shown to be well expressed in a variety of somatic tissues from several species.(58, 60, 61, 137) Neurons of the *Drosophila* brain express piRNAs and the piRISC components Aub and Ago3. Interestingly, expression of Aub and Ago3 is lower in $\alpha\beta$ neurons of the MB than in neighboring MB neurons. The most well studied function of the piRNA pathway is in maintaining genome integrity by posttranscriptionally silencing transposon expression. Accordingly, transposon expression is elevated in $\alpha\beta$ neurons. Expression of retrotransposons can lead to their mobilization, and formation of genomic lesions at the sites of excision and disruption of genes at insertion sites. In $\alpha\beta$ neurons, de novo transposon insertion into exons preferentially occurs within genes annotated with neural gene ontology (GO) terms.

Insertion into promoter regions occurs largely within promoters that are active in $\alpha\beta$ neurons. These findings indicate that preferential reduction of piRNA pathway activity within neurons required for LTM drives genetic diversity amongst these cells, and likely results in differences in their physiology.(59) piRNAs may also play an important role in activity dependent changes in gene regulation. piRNAs are found in the *Aplysia* CNS, including piRNAs matching a CpG island in the promoter of the CREB2 gene. Following exposure of CNS neurons to serotonin, the CREB2 promoter matching piRNAs are induced. Consistent with their known ability to transcriptionally silence genomic regions with which they are complementary, induction of these piRNAs leads to methylation of the CREB2 promoter, and a resulting piwi dependent reduction in CREB2 expression. CREB2 inhibits CREB1 driven transcription, thereby negatively regulating memory formation.(60) This finding therefore implicates the piRNA pathway in transcriptional regulation involved in lasting memory formation. No other example of such a piRNA mediated mechanism controlling gene expression in response to memory relevant signaling has been published to date. It is possible that the mechanism may be unique to transcription dependent memory in *Aplysia*, but the dearth of published studies examining piRNA expression in the brain leaves open the possibility of continuing discoveries in this vein. While reports of piRNA involvement in neurophysiology are few and far between, studies of esiRNA involvement are rarer still. However, esiRNAs are expressed in the CNS of nematodes, insects, and mammals. Though research into the functions of esiRNAs in neurons is in its infancy, evidence supporting their involvement in processes relevant to memory formation already

exists.(72, 113, 114, 182) In *C. elegans*, the guanylyl cyclase ODR-1 is required for odor sensation. Adaptation, a process in which prolonged exposure to a given odorant while undergoing nutritional starvation results in decreased attraction to that odorant, requires downregulation of ODR-1.(183) Odor adaptation also requires expression of the siRNA effector argonaute protein NRDE-3 in olfactory sensory neurons. esiRNAs complementary to *odr-1* coimmunoprecipitate with NRDE-3 in these cells. esiRNAs matching *odr-1* are upregulated following odor adaptation, and this upregulation corresponds with a decrease in *odr-1* mRNA. esiRNAs are thought to act cotranscriptionally, and exert silencing through heterochromatin formation. Following odor adaptation, the heterochromatin binding protein HPL-2 is found at the *odr-1* gene locus. Thus, it appears that prolonged stimulation of olfactory sensory neurons leads to production of esiRNAs targeting *odr-1*, a gene required for olfactory sensation. This in turn results in heterochromatin formation at the *odr-1* locus, reduced ODR-1 protein expression, and lasting attenuated sensitivity to odor.(72) The mouse hippocampus produces a variety of sRNAs, including esiRNAs that map to predicted hairpin RNA forming regions within genes. Interestingly, genes annotated with the GO term synapse are overrepresented in this set. Following olfactory discrimination training of mice, hippocampal expression esiRNAs as a class, and sRNAs derived from snoRNAs and other non-coding RNAs increased.(71) However, this study did not use statistical analyses sophisticated enough to determine whether the expression esiRNAs from any given locus changed following training. Nor did it examine expression of any of the putative mRNA targets of the esiRNAs identified. As few studies examining

esiRNA function in synaptic plasticity or memory have been published, the existing evidence does not conclusively support or reject the existence of such a mechanism. The demonstration of esiRNA involvement in nematode odor adaptation is encouraging, and argues for further study of the possibility that esiRNAs are involved in synaptic plasticity or memory in higher animals, though significant differences between nematode, mammalian, and insect esiRNA pathways exist. A similar situation exists in the case of piRNAs, with the tantalizing possibility that CREB2 regulation by piRNAs in mollusks is only one example of a broader mechanism for formation of stable memory. Furthermore, the recent advent and continuing improvement of high throughput sequencing methods has revealed a huge array of other previously unknown sRNAs. Little is known about the biological significance of most of these novel sRNAs, and it is possible that some may be involved in memory formation. Unbiased surveys of sRNA expression during memory formation remain lacking, and could provide significant insight into the ways in which gene expression is controlled during this process.

Memory formation is the result of alterations in patterns of activity in networks of neurons. Such alterations are achieved through making, breaking, and tuning the efficacy of synaptic connections between neurons. While fleeting memories can result from covalent modification of existing proteins at synapses, lasting memories are formed through regulated production of new proteins, and changes in gene expression programs in neurons. MicroRNAs have emerged as central players in neuronal function, governing expression of genes in key pathways

involved in synaptic plasticity and memory formation. In addition to their essential activities in the soma, mounting evidence indicates that reversible silencing of translation by microRNAs in dendrites is an important feature of stable memory formation. To date, most research into the function of microRNAs in neurons has focused on individual microRNA-target regulatory pairings. However, as an individual microRNA may have many mRNA targets, and an individual mRNA may be targeted by multiple microRNAs, it is clear that a more systems based approach is needed. The recent advent and rapid evolution of high throughput sequencing technologies has greatly expanded our understanding of microRNA genetics, biogenesis, function, and regulation. Using this technology, one can measure the expression of all microRNAs simultaneously, even in very small tissue samples. Further, unlike other technologies such as RT-PCR or hybridization, high throughput sequencing does not require a priori selection of the sequences to be studied. This feature of high throughput sequencing has led to the rapid expansion of the catalog of known microRNAs, and uncovered unexpected aspects of their biology. It has also unveiled a great diversity of previously unknown functional sRNAs including piRNAs and esiRNAs. The regulatory potential of these new classes of sRNAs is only beginning to be understood, and substantial opportunities for discoveries in this area exist, particularly in the context of synaptic plasticity and memory formation. In chapter II, I will describe experiments directed at understanding how sRNAs are regulated during formation of long-term memory in *Drosophila*, and identify several microRNAs, piRNAs, and esiRNAs exhibiting a response to aversive olfactory conditioning.

Part III: The Beta Secretase Beta-Site APP-Cleaving Enzyme (BACE) In Memory Formation and Cognitive Impairment

Proteolytic Processing Of Amyloid Precursor Protein (APP) Family Proteins Is Involved In Alzheimer's Disease Pathology

Our experience of life occurs largely in retrospect, with virtually all that happens to us immediately contextualized with what has come before. Whether this takes the form of a whiff of fragrance that quickly brings to mind a narrative recollection of an important time with a loved one, or in the form of our ability to unthinkingly find our way home from the office, the past is constantly brought into the present and often dictates our actions. Remembering skills acquired in the past and faithfully repeating the actions they involve is perhaps the greatest evolutionary advantage humans possess. Our ability to form supportive social groups is our other major evolutionary advantage, and our navigation of these groups largely defines modern life. Social interactions are entirely dependent on being able to learn new names and associate them with faces in the future, to recall past conversations, details of shared experience, and to remember facts about the people one meets. Accordingly, injuries or diseases that disrupt memory acquisition and retrieval are amongst the most crippling afflictions for those affected and are a great burden for their caretakers. Alzheimer's disease (AD) and related neurodegenerative dementias are among the most common late onset cognitive

impairments, affecting tens of millions of people world wide, and are predicted to increase in prevalence as life expectancies rise in developing countries. Substantial effort has been directed at understanding the disease mechanisms of these conditions. Much of what we know about AD comes from studies of brain tissue of patients from families exhibiting heritable susceptibility to early onset of the disease. This work has described features associated with disease progression. In AD, the microtubule associated protein tau (Tau), which is involved in regulating axonal transport, becomes hyperphosphorylated in neurons, and this results in formation of Tau aggregates termed tangles. Accumulation of these tangles disrupts transport along microtubules, and results in cell death. This condition is termed tauopathy, and is a shared feature of neurodegenerative diseases other than AD. As AD has unique aspects, and dementia is not a feature of all diseases featuring tauopathy, it is logical to assume that tauopathy is a result, and not the root cause of AD. Progression of AD is also associated with formation of extracellular senile plaques near synaptic sites. Senile plaques are largely composed of aggregates of a single 42-43 amino acid peptide termed amyloid- β ($A\beta$), which is derived from cleavage of the amyloid precursor protein (APP) by one of two beta-site APP cleaving enzymes (BACE1 and BACE2). As AD is strongly linked to trisomy of chromosome 21 in humans, APP was first identified by screening cDNA libraries generated from brain tissue collected from individuals with this genetic abnormality using oligonucleotide probes generated based upon the sequence of $A\beta$.⁽¹⁸⁴⁾ This approach lead to the identification of an mRNA encoding a 695 amino acid transmembrane protein. Subsequent work showed that $A\beta$ is derived from a region

near the C-terminus that includes extracellular and intramembrane portions of the APP sequence. APP is sequentially cleaved by multiple proteases, yielding several functional peptides. APP processing occurs in one of two pathways that are distinguished by the initial cleavage event, and yield metabolites with distinct activities. The A β peptide is the product of processing initiated by cleavage of APP by BACE1 or BACE2, membrane-bound aspartic proteinases termed β -secretases. This cleavage defines the N-terminus of A β , and liberates the soluble extracellular N-terminal domain of APP termed sAPP β . The 99aa membrane bound C-terminal fragment produced by β -secretase cleavage is termed C99 and includes the A β sequence. Subsequently, C99 is cleaved within the membrane by a membrane-bound aspartic proteinase termed γ -secretase. This second cleavage event yields A β , and a C-terminal intracellular domain fragment (AICD). A β can move into extracellular space, where it can lead to senile plaque formation. AICD is transported to the soma and enters the nucleus, where it can act as a transcriptional regulator. However, β -secretase initiated APP processing is the exception, and most APP processing is instead initiated by ADAM10, an α -secretase type zinc metalloproteinase. Cleavage by the α -secretase occurs within the A β region, and liberates the extracellular N-terminal domain and part of the A β sequence from the cell surface. This metabolite is termed soluble APP α (sAPP α). As cleavage by α -secretase occurs within the A β sequence, A β generation is precluded. The 83aa α -secretase generated C-terminal fragment is termed C83, and like C99, is cleaved within the membrane by γ -secretase. This second cleavage event yields a short membrane bound peptide termed P3, and AICD. However, unlike AICD production

initiated by β -secretase cleavage of APP, AICD produced as a result of cleavage by α -secretase is largely excluded from the nucleus and degraded, and does not induce the same transcriptional changes as β -secretase derived AICD.(185) AICD is rapidly degraded in the cytosol, but BACE1 is largely found in recycling endosomes, and trafficking to the nucleus via the endosomal pathway protects AICD, thereby allowing it to regulate transcription. (186-188) The coupling of $A\beta$ production and AICD mediated changes in transcription suggests the possibility that accumulation of senile plaques may not in itself drive AD, and is instead a symptom of misregulated β -secretase activity and downstream signalling. This view is supported by the observation that cognitive decline in human AD is not halted even when senile plaques are cleared following administration of monoclonal antibodies directed against $A\beta$. Further, $A\beta$ plaques can be found in brains from non-demented patients, and dementia can occur in the absence of significant $A\beta$ plaque accumulation.(189) Work in mammalian model systems further complicates the picture. For instance, endogenous APP and $A\beta$ is required for LTP in the mouse hippocampus, indicating that $A\beta$ production is a function of normal neurophysiology.(190) Indeed, though there is substantial evidence implicating the amyloidogenic pathway as causal in AD, and despite significant efforts in academic and industry labs, no therapy targeting this pathway has yet proven successful enough for use in treating AD in humans. This may be in part due to the extensive use of cell culture and in vitro methods to study the function of APP and its metabolites. Numerous differences between in vitro and in vivo results in studies of APP processing and function have been documented. This fact is indicative of the

need for studies to be conducted in animals in order to better understand the role of APP and its processing in normal neurophysiology, and in AD. As APP cleavage by BACE generates A β , and has been shown to be required for LTP, mechanisms regulating APP cleavage by BACE, and events downstream of this cleavage that regulate synaptic plasticity are of particular interest.

APPL Is The Drosophila Homologue Of APP, And Its Processing And Functions Are Conserved

Recently, *Drosophila* has been developed as a model organism in which to study AD. The relative simplicity and accessibility of the *Drosophila* brain, as well as the ease of measuring memory formation and retention in large numbers flies using behavioral studies, makes experimental approaches possible that would be prohibitively time consuming or difficult in mammals. Further, the availability of existing reagents with which to genetically dissect AD in flies makes rapid advances in our understanding of the underlying disease mechanisms possible. APPL, The *Drosophila* homologue of APP, is processed similarly to APP, and when human APP is expressed in flies, it too is processed much as it would be in humans. As is the case in mammals, processing of APP and APPL in *Drosophila* can occur along either an α -secretase or β -secretase initiated pathway. The α secretase pathway involves Kuzbanian (Kuz) and Presenillin (Psn), homologues of α and γ -secretases respectively. (191-195) Kuz and Psn have been extensively studied in the context of proteolytic processing of the *Drosophila* signaling protein Notch, which also involves

sequential cleavage by α and γ -secretases. Similar to AICD production from APP, Notch cleavage also produces a transcription regulating C-terminal fragment, dubbed NICD. The Notch pathway is one of the most well studied systems regulating growth and development, and thus provides an excellent intellectual scaffold upon which to build, and a large set of tools with which to work.(195) More recently, a homologue of the human BACE genes (dBACE) was identified in *Drosophila*. While mammals possess two BACE genes (BACE1 and BACE2), dBACE is the only *Drosophila* homologue identified thus far. Cleavage of APPL by Kuz or dBACE occurs within the extracellular domain, and liberates a large soluble fragment (sAPPL). Both Kuz and dBACE cleave at positions toward the C-terminal end of APPL, near the cell surface. While the human α - cleavage site of APP is closer to the C-terminus than the β -cleavage site, the situation is reversed in *Drosophila*, with dBACE cleaving closer to the C-terminus than Kuz.(194) Human APP expressed in *Drosophila* cells is cleaved by dBACE, and this cleavage initiates amyloidogenic processing similar to that occurring in humans. Mirroring AD pathology, transgenic expression of human APP or A β in the fly brain leads to plaque formation, neurodegeneration, and behavioral defects. (193, 194, 196) Though the A β sequence is not conserved in APPL, aged flies overexpressing APPL and young flies co-overexpressing APPL and dBACE in the brain exhibit formation of plaques that are Thioflavin-S positive, a classic marker of senile plaques in mammals. Plaque formation in flies induced by pan-neuronal co-overexpression of APPL and dBACE is associated with behavioral defects that worsen with age.(194) The relatively late discovery of dBACE long discouraged use of *Drosophila* as a model organism in

which to study the role of APP processing in synaptic plasticity, neurodegeneration, memory, and behavioral defects. However, with the addition of dBACE to the list of conserved elements of the APPL processing machinery, it is now clear that the complete pathway is evolutionarily ancient, and that work in flies can provide unique contributions to our understanding memory formation and AD.(reviewed in (197) and (198))

Proteolytic APPL Processing

APPL and its homologues are expressed in many tissues, and are involved in regulating diverse processes including growth, calcium, insulin and glucose homeostasis, apoptosis, mitochondrial function, and synaptic plasticity. (199-201) This conserved family of transmembrane proteins appear to act as both receptors and ligands in these processes, interacting with other cells through their extracellular domains, and transducing signals to the nucleus through their intracellular domain. Proteolytic processing of APPL is a major aspect of its activity, and generates several functional metabolites.(reviewed in (197, 200)) The extracellular domain of APPL includes 2 regions of extensive conservation dubbed E1 and E2. The E1 region is closest to the N-terminus, and includes a heparin-binding/growth-factor-like domain, and copper and zinc binding domains, and may be involved in functional interactions such as dimerization, ligand binding, and transfer of metal ions to other proteins. The E2 conserved region dimerizes in solution, such that the N-terminal end of one E2 region packs with the C-terminal

end of the other. Thus, the conserved structures within the extracellular domain of APPL suggest that it is functional, and is involved in protein-protein interactions.(202, 203) As previously mentioned, the α and β cleavage sites lie just outside of the cell surface, and cleavage by dBACE or Kuz liberate nearly the entire extracellular domain (sAPPL $^{\beta}$ and sAPPL $^{\alpha}$, respectively). The resulting C-terminal fragments are ~100aa long, and include the transmembrane domain, and a highly conserved intracellular domain. The transmembrane domain contains the γ cleavage site, which is cleaved by Psn. γ cleavage by Psn yields a highly conserved AICD and an unconserved short peptide whose N-terminus is defined by either the α or β cleavage site. The β -cleavage derived peptide (dA β), though dissimilar to A β in sequence, forms toxic aggregates, suggesting that it is functionally similar to A β . The AICD also includes a G $_{\alpha}$ -binding domain, an internalization signal, and a conserved YENPTY motif, which mediates binding with the adapter proteins X11/Mint and Disabled (Dab).

Though the presence of AICD has been demonstrated in *Drosophila*, its functions remain unknown.(195) However, the extensive sequence conservation within the intracellular region of APPL and the similarity of APPL processing and function to that of APP suggest strongly that *Drosophila* AICD functions analogously to human AICD. (194, 200, 204, 205) In humans, APP and BACE-1 are trafficked to synaptic sites in separate vesicle types, and remain segregated until appropriate signaling triggers their convergence. BACE-1 is localized largely to recycling endosomes, and thus prevented from interacting with APP. AICD contains an internalization signal that is required for endocytosis of APP, and for colocalization of APP and BACE-1.

Mutation of the APP internalization signal, or blockade of endocytosis also reduce A β production.(206) BACE-1 activity is optimal in an acidic environment such as that found within endosomes. Furthermore, the γ -secretase cleavage site differs when cleavage occurs at the plasma membrane vs. in endosomes. Thus, BACE-1 initiated processing of APP can be regulated independently of ADAM10 initiated processing through endocytosis of APP.(206, 207) Such a mechanism could explain why nuclear localization of AICD is largely driven by β -secretase initiated processing. Though segregation into recycling endosomes of dBACE initiated processing of APPL has not yet been demonstrated, the functional conservation of dBACE suggests strongly that this mechanism is also conserved.

APPL And Its Metabolites Are Involved In Neurodevelopment, And Regulate Synaptic Structure

Proper APPL expression is required for normal synapses in *Drosophila*. Synapses can form in flies carrying a mutation that eliminates APPL expression (APPL^d), but these flies produce fewer synaptic boutons at larval NMJs, while flies overexpressing APPL exhibit increased synaptic bouton number and defects in synaptic bouton size at larval NMJs. Expression in *Drosophila* neurons of APPL processing products or of APPL transgenes carrying mutations that affect the production and function of these processing products, shows that APPL processing regulates synaptic number, structure, and function through several pathways.(204) The initial step in APPL processing is cleavage by either Kuz or dBACE, and liberates

the corresponding N-terminal fragments sAPPL^α or sAPPL^β from the membrane. Conserved domains within the extracellular region of APPL suggest that, once liberated from the cell surface, it may function as a diffusible ligand. Alternatively, cleavage of APPL by Kuz or dBACE could be the functional event, initiating intracellular signaling events, and extracellular APPL fragment production only an intermediate step on the way to its degradation following cleavage. Torroja et al. found that expression of a transgene encoding the APPL extracellular domain, and with a C-terminus that does not include the α or β cleavage sites (sAPPL), in larval motor neurons is hampered by rapid turnover, and produces no phenotype at the NMJ unless expression is strongly driven by multiple copies of the transgene. Even when expression is driven at high levels, no change in the number of synaptic boutons is evident, though the number of satellite boutons is reduced. Conversely, expression of a transgene encoding APPL with both α and β cleavage sites deleted (APPL^{sd}) mimics the phenotype produced by overexpression of APPL, and yields increased numbers of both satellite and parent boutons. Further deletion of either the E1 or E2 regions of APPL^{sd} (APPL^{sdΔE1} and APPL^{sdΔE2} respectively) eliminates the increase in parent boutons produced by APPL^{sd} expression, but still yields increased satellite boutons. However, expression of APPL^{sd} mutated such that the C-terminal intracellular domain is deleted (APPL^{sdΔC}) produces no change from wild type in synaptic structure at the NMJ.(192, 204, 208) These results indicate that APPL influences NMJ structure largely through its highly conserved AICD, though the extracellular domain contains activities that can influence AICD mediated control of NMJ structure. APPL also regulates neurite outgrowth in cultured *Drosophila*

neurons. Curiously, cultured neurons from flies overexpressing APPL, and from APPL^d flies, both exhibit reduced neurite outgrowth.(209) Functional dissection of the APPL protein in cultured neurons from flies expressing APPL variant transgenes elucidates this result. Cultured neurons from APPL^{sd} or APPL^{sdΔC} flies exhibit excess neurite branching and protuberances resulting from actin filament and microtubule abnormalities. Neurites of sAPPL neurons branch less than do those of wild type neurons. Coculture of neurons expressing sAPPL and APPL^{sd} decreases neurite branching in APPL^{sd} cells and reduces the number of APPL^{sd} cells with protuberances.(209) This suggest that membrane bound APPL can act as a receptor that positively regulates neurite outgrowth and branching, and that sAPPL is a ligand that inhibits this activity. If so, overexpression of APPL leads to increased sAPPL levels, and this inhibits any signaling that increases neurite outgrown, even when extra APPL is present.(197) APPL has also been shown to regulate neuronal morphology in the adult brain. Overexpression of APPL in neurons of the adult brain results in dramatically increased axonal arborization. This increase requires the presence of the conserved YENPTY motif within AICD, the adapter protein Dab, and activation of the Dab interacting dAbl kinase. dAbl is known to regulate the cytoskeleton, and is involved in axon guidance.(210) APPL and the products of its proteolytic processing influence numerous aspects of neuronal structure and function. The regulatory functions of APPL processing products also appear to interact in complex ways, and sometimes counteract each other. APPL and its metabolites are able to modulate several signaling pathways, and can alter transcriptional programs. It is therefore possible for changes in the regulation of

the proteolytic events involved in APPL processing to have profound consequences for neuronal connectivity, function, and ultimately behavior.

APPL Processing Regulates Neuronal Activity.

APPL regulates the morphology of neurons, and the number of boutons present at a synapse.(204) As synaptic boutons are the sites of neurotransmitter release, their number is related to the efficacy of presynaptic neurons. However, soluble APPL metabolites can also modulate the excitability of neurons by altering ion channels. The presence of sAPPL in cell culture media reduces excitability of cultured *Drosophila* neurons by enhancing K⁺ currents. Conversely, spontaneous excitatory postsynaptic potentials (EPSP) are enhanced in neurons expressing APPL^{sd} or APPL^{sdΔC}. Coculture of neurons expressing sAPPL and APPL^{sd} restores excitability to near wild type levels in APPL^{sd} cells.(209) In mammals, sAPP^α activates K⁺ channels and suppresses NMDAR currents via PKG, thereby reducing excitability. (211, 212) These findings indicate that sAPPL can decrease neuronal activity by enhancing K⁺ channel activity, and that sAPPL production can provide negative feedback against increased excitability that is driven by membrane bound APPL. Though the conservation of many aspects of APPL function suggest that sAPPL^α is responsible for enhancing K⁺ currents in flies, reliance on the sAPPL transgene in work thus far does not allow the relative importance of sAPPL^α or sAPPL^β in modulating neuronal activity to be evaluated. However, in mammals, the

C-terminal end of sAPP α , which differs from that of sAPP β , is required for these activities.(211)

A β also regulates excitability of neurons in mammals, though the underlying mechanisms differ from those involving sAPP α . Both acute and chronic application of physiological concentrations of A β reduce the excitability of neurons of the rat prefrontal cortex. Interestingly, while application of high concentration A β initially reduces excitability, prolonged exposure enhances excitability, and can lead to tonic firing.(213) As is the case with sAPP α , A β 's effects may result from altered K $^+$ channel activity and expression. However, A β also directly binds and alters the behavior of nicotinic acetylcholine receptors (nAChR). (214, 215) Cholinergic signaling is also controlled presynaptically by A β via alterations in K $^+$ conductance, leading to reduced acetylcholine release.(216) Further, A β alters calcium influx via MAPK phosphorylation of L-type voltage dependent Ca $^{++}$ channels.(217) Evidence also indicates that A β inhibits NMDAR signaling, though this likely occurs downstream of the receptor, and may be related to the presence of APP in the multiprotein NMDAR complex.(218, 219) As dBACE and dA β were discovered only recently, the role of dA β in regulation of neuronal activity remains poorly understood. However, given the similarity of phenotypes generated by A β and dA β manipulations, one might expect that dA β has many of the same effects on neuronal activity as A β .

In addition to the rapid, and perhaps direct effects on neuronal activity just discussed, APPL and its homologues regulate synaptic plasticity via slower and more persistent mechanisms. Internalization of APPL family proteins can induce

NMDAR subunit substitution. The C-terminal intracellular domain of APPL family proteins contains sites for interaction with G_o, and numerous adapter proteins. Ligand binding to the extracellular domain of APP alters G_o signaling and other pathways including MAPK, and the Jun N-terminal kinase (JNK) pathway. APP cleavage is not required to activate G_o signaling, but cleaved AICD can do so in the absence of the extracellular domain. Furthermore, transcriptional changes driven by nuclear AICD can alter expression of genes involved in synaptic plasticity, including APP itself, BACE1, and the A β degrading enzyme Neprilysin. Transcriptional regulation by AICD can involve the activity of the histone acetyl transferase Tip60. However, transcriptional changes induced by APPL family proteins and their metabolites remain poorly understood.(Reviewed in (197, 201, 207)) As these findings demonstrate, APPL family proteins and their metabolites are potent regulators of neuronal activity and synaptic plasticity, and act through modulation of a complex set of mechanisms.

Processing Of APPL And Its Homologues Is Regulated By Neuronal Activity

Expression and proteolytic processing of APPL and its homologues can be stimulated in response to a number of conditions including hypoxia, stress, traumatic brain injury, and importantly, neuronal activity.(reviewed in (197, 200, 201)) As previously discussed, APPL and its metabolites can alter neuronal activity. Thus, changes in APPL processing induced by neuronal activity can in turn alter activity, thereby setting up complex feedforward and feedback loops that can

stabilize or profoundly alter the behavior of neural circuits. Torroja et al showed that intense neuronal activity driven in the hyperexcitable *eag sh* double mutant results in altered APP localization and perhaps processing.(204) Many studies indicate that A β is produced in response to various forms of neuronal activity, including electrical stimulation and pharmacological manipulation of cultured neurons, brain slices, and intact brains. APP is processed in response to muscarinic M1 acetylcholine receptor signaling, releasing sAPP α and downregulating A β production.(220-223) A similar result is observed following exposure of cultured neurons to AMPAR antagonists.(187) However, in virtually all of these studies either pathogenically high levels of activity were induced, APP was overexpressed, animals carrying APP mutations driving aberrant APP processing were used, or some combination of these conditions were present.(224-226) Such manipulations may not reflect APP processing in any condition relevant to normal brain function or AD. However, studies of APP processing following more careful manipulation of neuronal activity show that the picture is more complex. Prolonged, and perhaps pathogenic NMDAR activity stimulates APP expression and A β production in cultured mouse neurons.(226) In a seemingly contradictory result, NMDAR antagonists or blockade of Ca²⁺ channels reduce baseline α -secretase driven APP processing. Also, in contrast to prolonged NMDAR stimulation, brief NMDAR stimulation in cultured neurons induces sAPP α release, and reduces A β levels in a calcium dependent manner.(188) Further complicating the picture, experiments in intact brains differ sharply from those in cell culture. Verges et al. used microdialysis to manipulate NMDAR activity and to measure APP metabolite levels

in interstitial fluid (ISF) of intact brains of awake and freely moving mice. These experiments show that low doses of NMDA trigger A β production, while high doses result in decreased A β levels in ISF. Both NMDA concentrations trigger synaptic activity, but activate distinct signaling cascades, and drive APP processing down opposing pathways. Preadministration of tetrodotoxin (TTX), which inhibits voltage gated sodium channels, thereby blocking action potentials, reduces basal A β levels and prevents A β production in response to low dose NMDA. However, preadministration of TTX does not prevent reduction in A β resulting from high dose NMDA. This reveals that NMDA induced A β production is driven by the events triggered by action potentials, while inhibition of A β production driven by high dose NMDA does not. High levels of Ca²⁺ influx via intense NMDAR stimulation activates several second messenger mediated signaling pathways independent of action potentials. Preadministration of compounds that inhibit one such mechanism, the extracellular regulated kinase (ERK) pathway, transforms the response to NMDA administration such that both low and high dose NMDA drive A β production. ERK inhibits γ -secretase, and activates α -secretase, thereby reducing A β production.(227) Thus, while NMDAR signaling induces APP processing, the duration or intensity of NMDAR activity, and cellular setting governs the type of APP processing induced. The view that the balance of α and β cleavage initiated processing is governed by a competition between mechanisms involved in action potentials that drive A β production, and ERK driven α -processing, is supported by recent work showing that activity induction leads to clathrin dependent endocytosis of APP and processing by BACE1 in recycling endosomes.(206) BACE1 is normally

localized to recycling endosomes, and thus segregated and prevented from acting on APP. Action potentials drive neurotransmitter release via vesicle fusion with the plasma membrane, and clathrin dependent recycling endocytosis. This results in APP internalization, and trafficking such that it is colocalized with BACE1 in pH conditions optimal for BACE1 activity. This leads to β and γ cleavage, and results in AICD production.(206) Under certain conditions, neuronal activity can drive intracellular calcium levels high enough to initiate signaling cascades not triggered by neuronal activity at lower calcium concentrations. Such calcium dependent signaling can drive α -cleavage and inhibit γ -cleavage of APP.(227) Such processing precludes APP processing that results in A β and AICD production. Thus, APP processing is regulated by the level and nature of neuronal signaling. As the various metabolites of APP family proteins exert different effects on neuronal activity, the nature of activity induced APP processing provides important regulatory feedback. Our understanding of the mechanisms governing the regulation of APP processing is incomplete. This is due in large part to the difficulty of work in intact mammalian brains. The biochemical and functional similarity of APPL processing to that of APP may permit discoveries in *Drosophila* to inform our understanding of these processes in humans. The ease with which neuronal activity and gene expression can be manipulated in selected circuits of intact fly brains could provide a means by which to address remaining questions regarding the regulation of APP processing and its consequences in mammals.

Tight Control Of APPL Expression And Processing Is Required For Drosophila LTM

In addition to the well established role it plays in AD, many studies have demonstrated that APP and its appropriate processing are required for normal memory in mammals.(reviewed in (201, 228, 229)) Similarly, genetic, biochemical, and behavioral studies in *Drosophila* demonstrate that APPL has major functions in memory as well. APPL is highly expressed in the MB of adult flies, and is required during development for normal neurite morphology in the MB. Manipulations of APPL processing, or expression of human APP fragments in the drosophila brain results in structural defects in the MB.(209) Thus, both proper APPL expression and processing are required for normal MB structure. Defects in MB structure or function disrupt olfactory memory formation in *Drosophila*. Therefore, high levels of APPL expression confined to this structure strongly implicate this gene in olfactory memory. Experiments in which siRNAs directed against APPL are inducibly expressed only within the α/β and γ neurons of the adult MB at various time points during several aversive olfactory classical conditioning paradigms show that APPL is not required for learning, STM, or ARM, but is required for LTM. Conditional overexpression of human APP in the adult MB disrupts LTM as well(230) Thus, LTM requires that APPL expression in the MB falls within a suitable range. As transcription is also specifically required for LTM, this suggests the intriguing possibility that APPL cleavage leading to AICD mediated transcriptional changes may be a key event in LTM formation. If this is the case, one would expect that Psn and dBACE mutants also affect memory formation. Proper Psn activity is required for courtship memory in male flies.(231) Psn acts on targets other than

APPL, including Notch, so it is possible that the memory defects observed in Psn mutant flies are not the result of disrupted APPL processing alone. However, pan-neuronal overexpression of human APP or human BACE1 also resulted in disruption of courtship memory, but not of learning. These defects could be suppressed by administration of a γ -secretase inhibitor.(232) BACE1 homozygous mutant mice exhibit memory defects using several behavioral paradigms. These memory deficits can be ameliorated by expressing transgenes encoding APP and Presenillin mutants that result in elevated AICD production.(233) As AICD nuclear signaling is largely driven by β -secretase initiated processing of APP, it stands to reason that the LTM specific memory defects resulting from APPL knockdown observed in *Drosophila* could result from the loss of AICD nuclear signaling.(185, 186, 234) However, The complexities of APPL processing and resultant signaling preclude drawing such a conclusion based upon existing evidence.

The cognitive defects resulting from AD burden its sufferers and their caretakers in uniquely difficult ways. Though the exploration of AD pathology has yet to lead to effective treatments, it has added to our understanding of the basic mechanisms involved in regulating neural circuits, synaptic plasticity, and memory formation. Substantial evidence implicates aberrant APP processing as causal in AD. However, the role of proteolytic processing of APP and its homologues in normal neuronal function is poorly understood. Recent work indicates that β -secretase initiated processing of APP family proteins is vital for normal brain function, and not solely a disease causing event. In fact, mounting evidence suggest that it is a

consequence of normal neuronal activity. Research in *Drosophila* has contributed to our knowledge of these systems, and with the recent discovery of dBACE and dA β , research in flies is poised to make growing contributions. Behavioral genetics studies in *Drosophila* unveiled detailed aspects of memory formation that were previously unknown. Recently, APPL was shown to be required specifically for long term memory formation. In chapter III, I will discuss results of our work, indicating that dBACE is also required specifically for LTM, is upregulated as a result of an aversive olfactory conditioning paradigm that produces long term memory, and that this upregulation results in increased AICD production.

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Chapter II

Drosophila Olfactory Long Term Memory Formation Alters Short Non-Protein Coding RNA Profiles

Summary

Long term memory (LTM) formation involves changes in the activity of neural circuits brought about by alterations in neuronal structure and synaptic efficacy. Changes in transcription and protein synthesis are required for LTM (reviewed in (1, 2)). Short non-protein coding RNAs (sRNAs) transcriptionally and post-transcriptionally regulate expression of genes, including those involved in synaptic plasticity and LTM formation.(3-5) Previous studies have identified individual sRNAs that are regulated in response to neural activity.(6, 7) To date, few studies have examined genome wide regulation of any class of sRNA, let alone all sRNAs, during memory formation. In this study, we sought to thoroughly catalog microRNAs and other sRNAs expressed in *Drosophila* heads, and to describe any changes in their expression during LTM formation. We conducted an extensive analysis of sRNA regulation in response to aversive olfactory classical conditioning of *Drosophila*. Following conditioning, we find that miR-312-3p is downregulated, and that miR-314-3p, miR-956-3p, miR-958-3p, and miR-958-5p are upregulated. Our use of high-throughput sequencing (HTS) technology also allowed us to profile isoMiRs, untemplated nucleotide additions to microRNAs, and instances of RNA editing of microRNAs. Similarly, we were able to catalog endogenous short interfering RNAs (esiRNAs) and piwi interacting RNAs (piRNAs) expressed in the *Drosophila* head, and to identify several loci producing these sRNAs that respond to conditioning. Lastly, by examining changes in sRNA expression resulting from presentation of only the unconditioned stimulus (US) or only the conditioned

stimulus (CS), we show that observed changes in sRNA expression following conditioning are primarily driven by the US.

Introduction

While memories that last only a short time can be formed quickly, and after only a brief presentation of the thing to be remembered, formation of lasting memories is slower, and usually requires practice. Even after practice, formation of lasting memories can, for a time, be disrupted by distracting information. However, if the memory persists beyond this labile period, it becomes largely immune to such distracting information, and can last a lifetime.(8) In the initial minutes following learning, the ability to correctly recall fleeting or lasting memories decays rapidly. In the case of a fleeting memory this rapid decay continues until, after a few minutes, the memory is lost. However, though a lasting memory also decays rapidly in the minutes following learning, the memory is not lost, and the decay becomes much more gradual in subsequent hours.(9) These behavioral observations reflect fundamental and evolutionarily conserved aspects of the biology underlying memory formation. Initially, formation of fleeting and lasting types of memories involve the same mechanisms. However, formation of lasting memories activates additional mechanisms that stabilize the memory.(2)

Memories are encoded in a process that involves alterations in the activity of neural circuits. Such alterations in activity occur through changes in the number, structure, or efficacy of synaptic connections. Initially, these changes occur through the modification of proteins present at existing synapses. Such modifications are not stable, and resembling short-term memory (STM), they decay on the order of

minutes. LTM formation initially also involves modification of proteins already in place at existing synapses. However, LTM formation requires the slower processes of transcription and protein synthesis. Newly synthesized proteins and altered transcriptional programs produce lasting changes in neural excitability, connectivity, and synaptic efficacy or structure.(2) Some aspects of gene regulation involved in the formation of LTM formation have been characterized, but it is clear that significant work remains in this area.

Much of what is known of the genetic requirements of memory formation comes from studies of *Drosophila*. Though the *Drosophila* brain is orders of magnitude less complex than mammalian brain, it is still capable of generating a diverse set of behaviors. In addition, *Drosophila* can form lasting memories in a variety of learning paradigms. *Drosophila* memory is readily evaluated through observation of the behavior of individuals or of large groups of flies. Though many methods for training flies and evaluating learning have been developed, aversive olfactory classical conditioning is the most widely used and best understood training paradigm.(10) In this paradigm, groups of flies are placed in a chamber containing a grid of electrodes and in which odors can be presented and removed at will. During conditioning, flies receive the unconditioned stimulus (US) in the form of a mild shock to their feet via an electrode grid while being presented with the conditioned stimulus (CS) in the form of an odorant of choice. (11-13) Memory in flies trained using this method is typically evaluated using a T maze, in which one arm of the T contains the conditioned odorant, and the other arm of the T contains a

neutral odorant. Memory is scored by observing the fraction of flies present in each arm at the end of the trial. A single pairing of shock and odor produces an associative memory that lasts on the order of minutes. Repeated sessions pairing the odor and shock, with periods of rest between sessions, produces robust associative memory that can last for days.(9) In classic work using these techniques, Tully and Quinn identified several genes and neural circuitry required during each phase of LTM formation.(14-17) Of note, they showed that the ~5000 neurons of the mushroom body (MB) of the *Drosophila* brain comprise the major site of associative olfactory memory storage (Reviewed in (18)).

Previous studies have shown that microRNAs and the multiprotein complexes they associate with (termed RNA induced silencing complexes (RISCs)) regulate the physiology of neurons, synaptic plasticity, and behavior, by controlling protein synthesis.(19-21) MicroRNAs are involved in the control of protein expression from mRNAs localized at or near synapses, and therefore may have uniquely important functions in regulating synaptic plasticity and memory.(4) Additionally, several microRNAs and RISC associated proteins have been shown to be regulated by neural activity.(20, 22, 23) Because microRNA sequences specify which mRNAs are targeted for RISC induced silencing, changes in microRNA expression profiles during memory formation result in altered patterns of protein synthesis.(6) Complicating matters, many if not most mRNAs are predicted targets of multiple microRNAs.(24) Though changes in the expression of individual

microRNAs following learning have been documented, genome wide changes in microRNA expression during memory formation remains poorly studied.

The development and application of high throughput sequencing (HTS) of sRNAs has revealed that a diversity of sRNAs are produced from microRNA precursors. Though in most cases, a single 'canonical' sequence from each of the 5' and 3' arms of the pre-microRNA hairpin represent the overwhelming majority of sequencing reads derived from a given microRNA precursor, examination of 'non-canonical' microRNA reads can provide insight into the biogenesis and regulation of microRNAs. Changes in the proportions of canonical and non-canonical microRNA reads derived from a given pre-microRNA may also have important consequences for the regulatory output of the precursor.(25) The biological significance of non-canonical microRNA reads is poorly understood, and largely unknown in the context of synaptic plasticity or memory.

Recently, other classes of sRNAs including endogenous short interfering RNAs (esiRNAs) and piwi interacting RNAs (piRNAs) have been implicated in synaptic plasticity and memory formation in nematodes, mollusks, and mammals.(3, 5, 26) Similar to microRNAs, esiRNA and piRNA sequences specify the targets for silencing via proteins incorporated in RISCs. In *Drosophila*, esiRNAs and piRNAs both suppress mobilization of retrotransposons.(27, 28) Silencing of retrotransposons via the piRNA pathway is non-uniform in the *Drosophila* brain, and in the MB in particular. Retrotransposon mobilization leads to genetic diversity

in the *Drosophila* brain, and preferentially affects genes involved in neural physiology.(29) esiRNAs are also known to target mRNAs for post-transcriptional silencing in *Drosophila*.(30, 31) To date, no published study has examined regulation of esiRNAs or piRNAs during memory formation in *Drosophila*.

Here, I present the results of profiling genome wide sRNA expression in *Drosophila* heads, and identify changes in sRNA expression during LTM formation resulting from aversive olfactory classical conditioning. We profile sRNA expression in heads of flies similarly treated, but exposed only to the US (shock), or to the CS (odor). Our results indicate that changes in sRNA expression during LTM formation are primarily driven by the US, which involves dopaminergic signaling in the MB.

In section I of this chapter, I present a detailed analysis of microRNA expression in the fly head, and the changes in microRNA profiles during LTM formation. I show that miR-312-3p is significantly downregulated, and that miR-314-3p, miR-956-3p, miR-958-3p, and miR-958-5p are upregulated during LTM formation. Using microRNA target prediction and gene ontology (GO) analyses, we have explored the potential regulatory consequences resulting from the observed changes in the expression of these microRNAs. We document an extensive set of non-canonical microRNA reads, but find that LTM formation does not significantly alter the proportion of microRNA reads that they represent.

In section II of this chapter, I discuss our analysis of esiRNA and piRNA profiles in the *Drosophila* head, and changes in their expression during LTM formation. I catalog esiRNA producing loci, and identify 6 that are regulated during LTM formation or US exposure, and none that change significantly following CS exposure. Of the 6 significantly regulated loci, 4 map to lysozyme family genes in a region within an intron of the multiple wing hairs (*mwh*) gene. In some cases these esiRNA producing lysozyme family genes overlap each other on opposite strands. By examining predicted secondary structures of lysozyme mRNAs, and of the *mwh* intron, I show that esiRNAs produced from these loci are likely derived from long hairpin structures within the *mwh* intron, and not from overlapping transcription of the lysozyme genes themselves. Reads derived from this region do not map elsewhere in the genome, and are therefore likely to be involved only in regulation of the lysozyme family genes, or of *mwh*. The *mwh* intron from which these esiRNAs are produced is not included in all *mwh* transcripts, suggesting that *mwh* promoter choice may influence the expression of lysozyme genes.⁽³²⁾ Lastly, I identify piRNA producing loci, and examine piRNA expression during LTM formation. I find that no piRNA loci are upregulated, and 10 are downregulated during LTM formation. *Invader3* and *Dm88* transposons map within downregulated piRNA loci, both of which are long terminal repeat (LTR) type retrotransposons. This work is, to my knowledge, is the first genome wide analysis of sRNA expression in the context of LTM formation in *Drosophila*.

Results

Section I: Analysis of microRNA expression in the *Drosophila* head during long-term memory formation

microRNA expression in the Drosophila head during LTM formation

Tissue-specific profiles of microRNA expression have been reported, and the expression of certain microRNAs is restricted to a single tissue or sets of cells.(33-35) microRNA expression also responds to environmental stimuli and signaling cascades.(36) Some microRNAs have been shown to be regulated by neural activity, and the expression of a limited set of microRNA precursors or individual mature microRNAs has been shown to change during LTM formation.(37-39) However, genome wide regulation of mature microRNAs has not been studied in the context of *Drosophila* behavior or memory paradigms. To obtain a genome wide profile of microRNA expression in the *Drosophila* brain and to understand changes in microRNA expression during LTM formation, we conducted a high throughput sequencing study of 15-35nt RNAs extracted from heads of flies that had been subjected to aversive olfactory classical conditioning. Training followed the olfactory LTM conditioning paradigm described by Tully and Quinn(13) In order to understand the contributions of the US (spaced shocks) and CS (odor only) to changes in microRNA expression following conditioning, we also sequenced 15-35nt RNAs extracted from heads of flies that had been subjected to training identical to

that of LTM conditioned flies, but received either the US alone, the CS alone, or neither (control). We chose to use whole head lysates in this study as opposed to exclusively brain tissue because our primary goal was to identify changes in sRNA expression during LTM formation, rather than simply identifying all microRNAs expressed in the brain alone. The brain and eyes comprise most of the volume of the *Drosophila* head, though muscle, hemolymph, tracheal, and other tissues are also present. We reasoned that the variability introduced by the delicate and time consuming dissection of the brain from the fly head would be more problematic than inclusion of non-neuronal tissues. This also made it possible to greatly increase the number of flies represented in each sequencing library, and as a result, improved the uniformity of RNA extracts used to prepare sequencing libraries. We took great care to ensure that flies in each treatment group differed only in the stimuli to which they were exposed during training. Fly husbandry and handling were carried out meticulously to ensure that all flies used in this work had as closely matching life experiences prior to training as is possible. For each matched set of samples, a single age matched set of flies was split into the four treatment groups. All four treatments occurred simultaneously, and in close proximity, through the use of an automated olfactory conditioning apparatus developed in our lab.(40) This apparatus is under computer control, and produces highly replicable training sessions. Heads from 10 male and 10 female flies in each condition were dissected and flash frozen 2 hours after conditioning. Conditioned flies were tested for memory of the pairing of shock and odor 24 hours after conditioning. Sequencing libraries were prepared only from matched treatment sets in which flies in the LTM

condition displayed memory formation 24 hours after conditioning. Because of the great care we took in fly handling, training, and sequencing library preparation, we feel confident in asserting that, despite including tissues of the head other than the brain in our samples, any observed differences in sRNA expression between treatment groups at least primarily reflect differences in neural activity induced by the stimuli used in each treatment group.

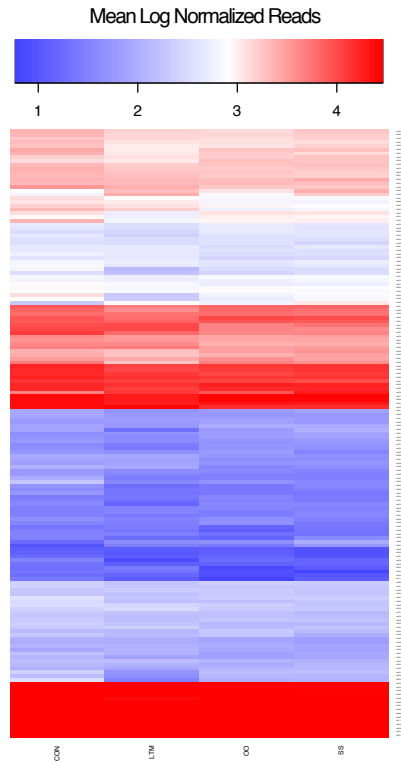
Sequencing was conducted on the Illumina Genome Analyzer Iix platform. Sequencing reads were filtered for quality, and adapter sequences were removed using the FASTX toolkit. Sequencing reads were aligned to the pre-microRNA sequences annotated in miRBase. Counts of reads that aligned to pre-microRNAs were normalized using the upper quartile method of edgeR. Reads that precisely matched the mature microRNA sequences annotated in miRBase were designated as canonical mature microRNAs. While many sequencing reads other than canonical mature microRNA sequences also aligned to pre-microRNAs, we left such reads out of our initial analysis for several reasons. Non-canonical microRNA reads are diverse in origin, span the entire 15-35nt size range we sampled, and are therefore sometimes incompatible with Ago1 loading. Thus, the functional significance of non-canonical microRNA reads may vary greatly, and their inclusion in this part of our analysis would have hindered interpretation. Existing microRNA target predictions are available only for canonical mature microRNAs, thereby restricting downstream analysis of the consequences of any changes in microRNA expression detected. Lastly, previous studies of microRNA function and regulation in the

context of synaptic plasticity and memory have examined only canonical mature microRNAs. Therefore interpreting our results in the context of previous work would be impossible if non-canonical microRNA reads were included. We will discuss our detailed analysis of non-canonical microRNA reads later in this section.

We detected 317 of the 426 mature microRNAs found in the miRBase microRNA database.(Figure S2.1) MiRBase has been curated with data from the modencode consortium, which has used a very large set of publicly available sequencing data to identify and validate candidate microRNAs. Thus, the miRBase catalog of *Drosophila* microRNAs is considered to represent the complete set of microRNAs expressed in flies. We found that a handful of mature microRNAs represent the vast majority of all sequencing reads mapping to microRNA precursors.(Figure 2.1)

Figure 2.1. The 10 most highly expressed mature microRNAs comprise ~90% of all mature microRNA reads

A.



B.

The 10 most abundant mature microRNAs as fractions of all normalized mature microRNA reads

| miRNA | Con | LTM | OO | SS | Mean |
|-------------|--------|--------|--------|--------|--------|
| miR-184-3p | 0.3645 | 0.2932 | 0.5728 | 0.5560 | 0.4466 |
| miR-1-3p | 0.3107 | 0.3651 | 0.2514 | 0.2397 | 0.2917 |
| miR-8-3p | 0.0507 | 0.0523 | 0.0296 | 0.0353 | 0.0420 |
| miR-276a-3p | 0.0471 | 0.0454 | 0.0172 | 0.0214 | 0.0328 |
| bantam-3p | 0.0312 | 0.0346 | 0.0182 | 0.0193 | 0.0258 |
| let-7-5p | 0.0263 | 0.0264 | 0.0236 | 0.0249 | 0.0253 |
| miR-957-3p | 0.0261 | 0.0300 | 0.0154 | 0.0176 | 0.0223 |
| miR-263b-5p | 0.0160 | 0.0199 | 0.0111 | 0.0115 | 0.0146 |
| miR-8-5p | 0.0118 | 0.0146 | 0.0067 | 0.0084 | 0.0104 |
| miR-193-5p | 0.0111 | 0.0129 | 0.0048 | 0.0058 | 0.0087 |

Sequencing was conducted on 15-35nt RNAs isolated from heads of drosophila in each of the treatment conditions (Con: Control, LTM: Odor + Shock, OO: Odor only, SS: Shock Only). Sequencing reads were aligned to the pre-microRNA sequences annotated in miRBase. Counts of reads that aligned to pre-microRNAs were normalized using the upper quartile method of edgeR. Reads that precisely matched the mature microRNA sequences annotated in miRBase were designated as canonical mature microRNAs. A: heatmap of mature microRNA expression for mature microRNAs with at least 10 reads amongst all samples. B: The top 10 canonical mature microRNAs contributed ~90% of all mature microRNA reads in all conditions.

We used the edgeR differential expression analysis software package to identify mature microRNAs that exhibit changes in expression following treatment. edgeR incorporates functionality that includes batch effects and differing library sizes, in addition to treatment groups in its analysis. This functionality, when combined with our paired sample experimental design, yields a powerful method for detection of truly differentially expressed microRNAs, while minimizing false positives. Recently, our selection of a paired sample experimental design and choice of edgeR was shown to be the best available methodology for differential expression analysis from sequencing data.⁽⁴¹⁾ Using this approach, we compared expression of mature microRNAs following LTM conditioning, odor only training (OO), or shock only training (SS), to expression in the control group. Broadly speaking, the profile of LTM condition most strongly differed from controls, while the OO and SS conditions showed fewer changes. Many changes in mature microRNA expression were observed in the various treatment conditions, but only 5 mature microRNAs displayed statistically significant changes in any treatment group.(Figure S2.2) We identified one significantly downregulated mature microRNA, and 4 significantly upregulated mature microRNAs following treatment.(Figure 2.2)

Figure 2.2: Mature microRNAs with statistically significant changes in expression following treatment

| microRNA | LTM | | OO | | SS | |
|------------|-----------------|---------------|-----------------|---------------|-----------------|---------------|
| | Log Fold Change | FDR adj. pVal | Log Fold Change | FDR adj. pVal | Log Fold Change | FDR adj. pVal |
| miR-958-3p | 2.314706243 | 8.01E-05 | 0.186303652 | 1 | 1.265770139 | 0.357331616 |
| miR-958-5p | 2.158785348 | 0.000363861 | 1.090557475 | 1 | 1.897431458 | 0.043614936 |
| miR-956-3p | 1.812438154 | 0.008384943 | 0.394817835 | 1 | 1.490435064 | 0.143442265 |
| miR-314-3p | 1.443989577 | 0.040044656 | 0.548143742 | 1 | 1.477616407 | 0.185118642 |
| miR-312-3p | -1.659199692 | 0.042035678 | -0.705716964 | 1 | -1.318339372 | 0.832161977 |

We identified 5 mature microRNAs with significantly changed expression following treatment. Changes following shock only training resembled those produced by LTM conditioning, while changes produced by odor only training were smaller and not statistically significant. All 5 regulated mature microRNAs responded significantly to LTM conditioning, while only the change in miR-958-5p expression was statistically significant in the shock only condition.

The microRNAs with statistically significant changes in expression following LTM showed similar changes in the SS condition, but only miR-958-5p changed significantly in SS. No mature microRNAs showed statistically significant changes in expression in the OO condition.

Target analysis for microRNAs regulated during LTM formation

Having identified mature microRNAs that are significantly regulated during LTM formation, we examined the downstream consequences of these changes. We used microRNA target predictions from the DIANA microT algorithm, as it uses modern prediction methods, and scans entire mRNAs for target sites as opposed to restricting the search to the 3'UTR as is the case with all other publicly available target prediction databases.(24) The full catalog of predicted targets for microRNAs regulated following LTM conditioning is available in Figure S2.3. Together, the

significantly regulated microRNAs are predicted to target transcripts of 1090 genes. Using the microT target predictions, we sought to delineate genes targeted by more than one of the microRNAs regulated following LTM conditioning from those targeted by only one. miR-312-3p has far more predicted targets than any of the other significantly regulated microRNAs. miR-312-3p is predicted to target transcripts of 562 genes, of which 484 are not targeted by any other significantly regulated microRNA. Significantly upregulated microRNAs together target 606 genes, of which 528 are not also targeted by miR-312-3p. 49 genes are targeted by more than one up-regulated microRNA. Of those, 10 genes were also targeted by the lone significantly down-regulated microRNA, miR-312-3p (Figure 2.3).

Figure 2.3. Genes targeted by 2 or more upregulated microRNAs

| FBgnBase ID | Symbol | Name | dme-miR-312-3p | dme-miR-314-3p | dme-miR-956-3p | dme-miR-958-3p | dme-miR-958-5p |
|-------------|------------|---|----------------|----------------|----------------|----------------|----------------|
| FBgn0003507 | srp | serpent | + | + | - | + | - |
| FBgn0261986 | RASSF8 | NA | + | + | + | - | - |
| FBgn0029761 | SK | small conductance calcium-activated potassium channel | + | + | + | - | + |
| FBgn0039078 | CG4374 | NA | + | + | + | - | - |
| FBgn0035229 | CG7852 | NA | + | + | - | + | - |
| FBgn0030680 | CG8944 | NA | + | + | + | - | - |
| FBgn0032341 | Reps | NA | + | - | + | + | - |
| FBgn0262738 | norPA | no receptor potential A | + | + | + | - | - |
| FBgn0038341 | CG14869 | NA | + | + | + | - | - |
| FBgn0011582 | Dop1R1 | Dopamine 1-like receptor 1 | + | + | + | + | - |
| FBgn0024234 | gbb | glass bottom boat | - | + | + | - | - |
| FBgn0262579 | Ect4 | Ectoderm-expressed 4 | - | + | + | - | - |
| FBgn0040388 | boi | brother of ihog | - | + | + | - | - |
| FBgn0023081 | gek | genghis khan | - | + | + | - | - |
| FBgn0037336 | CG2519 | NA | - | + | + | + | - |
| FBgn0263218 | NA | NA | - | + | + | - | - |
| FBgn0260499 | qvr | quiver | - | + | + | - | - |
| FBgn0085400 | CG34371 | NA | - | + | + | - | - |
| FBgn0004622 | Takr99D | Tachykinin-like receptor at 99D | - | + | + | - | - |
| FBgn0003071 | Pfk | Phosphofruktokinase | - | + | - | - | + |
| FBgn0027512 | CG10254 | NA | - | + | + | - | - |
| FBgn0004892 | sob | sister of odd and bowl | - | + | - | + | - |
| FBgn0028550 | Atf3 | Activating transcription factor 3 | - | + | + | - | - |
| FBgn0016059 | Sema-1b | Sema-1b | - | + | + | - | - |
| FBgn0031632 | CG15628 | NA | - | + | + | - | - |
| FBgn0036317 | CG10948 | NA | - | + | - | + | - |
| FBgn0028506 | CG4455 | NA | - | + | + | - | - |
| FBgn0038829 | CG17271 | NA | - | + | - | + | - |
| FBgn0038595 | CG7142 | NA | - | + | + | - | - |
| FBgn0053558 | mim | missing-in-metastasis | - | + | + | - | - |
| FBgn0030432 | CG4404 | NA | - | + | - | - | + |
| FBgn0024963 | GluClalpha | GluClalpha | - | + | - | - | + |
| FBgn0036522 | CG7372 | NA | - | + | + | - | - |
| FBgn0036446 | CG9384 | NA | - | + | - | + | - |
| FBgn0035085 | CG3770 | NA | - | + | - | - | + |
| FBgn0032378 | CycY | Cyclin Y | - | + | + | + | - |
| FBgn0031637 | CG2950 | NA | - | + | - | + | - |
| FBgn0016641 | PTP-ER | Protein tyrosine phosphatase-ERK/Enhancer of Ras1 | - | - | + | - | + |
| FBgn0040089 | meso18E | meso18E | - | - | + | + | - |
| FBgn0259938 | cwo | clockwork orange | - | - | - | + | - |
| FBgn0036202 | CG6024 | NA | - | - | + | + | - |
| FBgn0038890 | CG7956 | NA | - | - | + | + | - |
| FBgn0085446 | CG34417 | NA | - | - | + | + | - |
| FBgn0262734 | Rbp2 | RNA-binding protein 2 | - | - | + | - | + |
| FBgn0000395 | cv-2 | crossveinless 2 | - | - | + | - | + |
| FBgn0037659 | Kdm2 | Lysine (K)-specific demethylase 2 | - | - | - | + | + |
| FBgn0030758 | CanA-14F | Calcineurin A at 14F | - | - | - | + | + |
| FBgn0032312 | CG14071 | NA | - | - | - | + | + |
| FBgn0036732 | Oatp74D | Organic anion transporting polypeptide 74D | - | - | - | + | + |

Target predictions were obtained from the DIANA microT algorithm for mature microRNAs displaying statistically significant changes in expression following LTM conditioning. 49 genes are targeted by 2 or more significantly upregulated microRNAs. Of these, 10 are also targeted by the lone significantly downregulated mature microRNA miR-312-3p.

Interestingly, the Dop1R1 dopamine receptor is targeted by all significantly regulated mature microRNAs except miR-958-5p. Expression of this gene in the mushroom body is required for olfactory memory formation.(42) The small

conductance calcium-activated potassium channel (SK) gene is targeted by all significantly regulated mature microRNAs except miR-958-3p. This gene is involved in courtship STM and LTM.(43) Cyclin Y and the poorly conserved gene CG2519 are targeted by miR-314-3p, miR-956-3p, and miR-958-3p.

Gene ontology analysis of targeted genes

While some target genes such as Cyc-Y, SK, Dop1R1 and CG2519 are obvious candidates for regulated silencing during LTM formation, we hoped to identify other, less immediately obvious candidate genes for further study. We also sought a better understanding of the cellular processes potentially regulated by microRNA mediated silencing during LTM formation. To accomplish both of these goals, we obtained gene ontology annotations for genes targeted by significantly regulated microRNAs from the PANTHER database.(44) When the list of all genes targeted by significantly regulated microRNAs is examined for pathways whose component genes are over or underrepresented in the list, only the “Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway” annotation is over or underrepresented. In a list of this size, 3.38 genes in this pathway would be expected to be found, while the actual list of targeted genes includes 12 (FDR adjusted P-val = 0.0367) (Figure S2.4) Interestingly, miR-958-5p is the only significantly regulated microRNA that does not target any of the 12 genes in this pathway(Figure 2.4).

Figure 2.4. Targeting of genes in the Gi alpha and Gs alpha mediated pathway by significantly regulated microRNAs

| FlyBase ID | Symbol | miR-312-3p | miR-314-3p | miR-956-3p | miR-958-3p | miR-958-5p |
|-------------|-----------|------------|------------|------------|------------|------------|
| FBgn0003371 | sgg | - | + | - | - | - |
| FBgn0250910 | Octbeta3R | + | - | - | - | - |
| FBgn0024814 | Clc | + | - | - | - | - |
| FBgn0011582 | Dop1R1 | + | + | + | + | - |
| FBgn0028433 | Ggamma30A | - | - | - | + | - |
| FBgn0000253 | Cam | + | - | + | - | - |
| FBgn0024941 | RSG7 | - | - | + | - | - |
| FBgn0015129 | | + | - | - | - | - |
| FBgn0051960 | CG31960 | - | + | - | - | - |
| FBgn0031995 | CG8475 | - | + | - | - | - |
| FBgn0000037 | mAcR | + | - | - | - | - |

Pathway annotations for all genes targeted by significantly regulated microRNAs were obtained from the PANTHER database. With 12 genes, the “Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway” was overrepresented in the set of targeted genes (FDR Adj P-val 0.0367). Genes targeted by a given microRNA are marked with a “+”.

We repeated the ontology analysis on the set of all genes targeted by significantly regulated microRNAs, but this time examined the classes of proteins encoded by targeted genes, their molecular functions, and the biological processes they participate in. (Figure S.2.5). 17 protein class annotation terms are significantly over or underrepresented (FDR Adj P-val < 0.05). These correspond to 14 categories of molecular functions. 29 biological processes are significantly overrepresented (FDR Adj P-val < 0.05) in the set of all genes targeted by significantly regulated microRNAs (Figure 2.5).

Figure 2.5. Overrepresented biological process annotations for all genes targeted by significantly regulated microRNAs

| Biological Process | All Drosophila Genes With Annotation | Targeted Genes | Expected Genes | over/under | FDR Adj. P-value |
|---|--------------------------------------|----------------|----------------|------------|------------------|
| cell communication | 1873 | 262 | 150.88 | + | 3.68E-17 |
| cellular process | 2904 | 357 | 233.94 | + | 9.96E-16 |
| signal transduction | 1739 | 242 | 140.09 | + | 2.63E-15 |
| developmental process | 1193 | 179 | 96.1 | + | 1.53E-13 |
| system development | 735 | 121 | 59.21 | + | 3.61E-11 |
| neurological system process | 1109 | 162 | 89.34 | + | 4.06E-11 |
| transcription | 1049 | 155 | 84.5 | + | 6.08E-11 |
| transcription from RNA polymerase II promoter | 1048 | 154 | 84.42 | + | 1.12E-10 |
| system process | 1210 | 171 | 97.47 | + | 1.21E-10 |
| intracellular signaling cascade | 631 | 107 | 50.83 | + | 1.76E-10 |
| ectoderm development | 555 | 93 | 44.71 | + | 1.09E-08 |
| regulation of transcription from RNA polymerase II promoter | 781 | 118 | 62.91 | + | 1.51E-08 |
| Unclassified | 6079 | 388 | 489.7 | - | 5.99E-08 |
| nervous system development | 522 | 87 | 42.05 | + | 6.04E-08 |
| cell surface receptor linked signal transduction | 835 | 121 | 67.26 | + | 1.02E-07 |
| muscle contraction | 188 | 44 | 15.14 | + | 1.50E-07 |
| mesoderm development | 454 | 76 | 36.57 | + | 6.84E-07 |
| muscle organ development | 215 | 46 | 17.32 | + | 9.53E-07 |
| cell adhesion | 476 | 78 | 38.35 | + | 1.05E-06 |
| cell motion | 347 | 60 | 27.95 | + | 1.04E-05 |
| cell-cell signaling | 517 | 78 | 41.65 | + | 2.94E-05 |
| apoptosis | 442 | 66 | 35.61 | + | 3.70E-04 |
| vesicle-mediated transport | 526 | 73 | 42.37 | + | 1.33E-03 |
| transmembrane receptor protein tyrosine kinase signaling pathway | 93 | 22 | 7.49 | + | 1.98E-03 |
| protein modification process | 765 | 96 | 61.63 | + | 2.93E-03 |
| induction of apoptosis | 120 | 25 | 9.67 | + | 4.25E-03 |
| nucleobase, nucleoside, nucleotide and nucleic acid metabolic process | 2117 | 218 | 170.54 | + | 1.32E-02 |
| MAPKK cascade | 129 | 25 | 10.39 | + | 1.32E-02 |
| synaptic transmission | 393 | 55 | 31.66 | + | 1.40E-02 |
| sensory perception of sound | 44 | 13 | 3.54 | + | 1.42E-02 |
| embryonic development | 168 | 29 | 13.53 | + | 2.67E-02 |

Biological process annotations for all genes targeted by significantly regulated microRNAs were obtained from the PANTHER database. 29 biological processes are significantly overrepresented (FDR Adj P-val < 0.05) in this set, including the terms “neurological systems process” and “synaptic transmission”.

Of note, the terms “neurological systems process” and “synaptic transmission” are significantly overrepresented. All genes annotated with “synaptic transmission” are also included in the “neurological systems process” annotation, as the latter is one of the parent GO terms for genes expressed in neurons or glia. The 154 target genes with this annotation are attractive candidates for subsequent investigation. We therefore further parsed the 154 neurological systems process genes by the microRNAs that target each gene (Figure S2.6). 68 of 154 genes with the “neurological systems process” annotation were uniquely targeted by miR-312-3p, and 68 genes with this annotation were targeted by one or more significantly upregulated microRNA, but not by miR-312-3p. 26 “neurological systems process” genes were targeted by 2 or more significantly regulated microRNAs (Figure 2.6).

Figure 2.6. Genes annotated with the term “neurological systems process” and targeted by multiple significantly regulated microRNAs

| FlyBase ID | Symbol | miR-312-3p | miR-314-3p | miR-956-3p | miR-958-3p | miR-958-5p |
|-------------|------------|------------|------------|------------|------------|------------|
| FBgn0011582 | Dop1R1 | + | + | + | + | - |
| FBgn0032341 | Reps | + | - | + | + | - |
| FBgn0000448 | Hr46 | + | + | - | - | - |
| FBgn0003870 | ttk | + | - | + | - | - |
| FBgn0004242 | Syt1 | + | + | - | - | - |
| FBgn0004622 | Takr99D | - | + | + | - | - |
| FBgn0013733 | shot | + | - | - | + | - |
| FBgn0014870 | Psi | + | - | - | - | + |
| FBgn0015774 | NetB | + | - | - | + | - |
| FBgn0016059 | Sema-1b | - | + | + | - | - |
| FBgn0017549 | Ric | + | + | - | - | - |
| FBgn0024963 | GluClalpha | - | + | - | - | + |
| FBgn0026086 | Adar | + | - | + | - | - |
| FBgn0028550 | Atf3 | - | + | + | - | - |
| FBgn0028704 | Nckx30C | + | + | - | - | - |
| FBgn0029508 | Tsp42Ea | + | - | - | + | - |
| FBgn0031760 | Tsp26A | + | - | - | + | - |
| FBgn0034433 | endoB | + | - | - | + | - |
| FBgn0038890 | CG7956 | - | - | + | + | - |
| FBgn0039431 | CG6490 | + | - | + | - | - |
| FBgn0040388 | boi | - | + | + | - | - |
| FBgn0085446 | CG34417 | - | - | + | + | - |
| FBgn0259231 | CCKLR-17D1 | + | + | - | - | - |
| FBgn0262350 | bru-3 | + | + | - | - | - |
| FBgn0262737 | mub | + | - | + | - | - |
| FBgn0263218 | Dscam2 | - | + | + | - | - |

154 genes targeted by significantly regulated microRNAs are annotated with the term “neurological systems process”. Of these, 26 are targeted by more than one significantly regulated microRNA. Genes targeted by a given microRNA are marked with a “+”.

We conducted similar gene ontology enrichment analyses on the sets of predicted targets of each microRNA individually, and for the set of targets unique to miR-312-3p, the set of targets of only upregulated microRNAs, and for the set of targets shared by miR-312-3p and at least one upregulated microRNA. However, these analyses did not yield additional insights as the smaller sets of genes reduced the statistical power of the enrichment analysis, and those annotations that were found to be significantly over or underrepresented were also found in the analysis of the set of all genes targeted by significantly regulated microRNAs.

Expression of non-canonical microRNA sequences in the Drosophila head during LTM formation

In addition to canonical mature microRNAs, a variety of other sequencing reads map to pre-microRNAs. Previous studies have suggested that such non-canonical reads may be indicative of altered microRNA processing, stability, or RNA editing activity. Many non-canonical reads, which are known as isomiRs, will have substantially different regulatory function than their corresponding canonical mature microRNAs. We therefore sought to profile non-canonical microRNA reads in *Drosophila* heads, and to determine if this profile changes during LTM formation. In order to detect non-canonical microRNA reads, we aligned our sequencing reads to pre-microRNA hairpin sequences from miRBase, such that up to two nucleotide mismatches were permitted. Only alignments with scores equal to the highest alignment score for a given read were retained. This approach permits alignment of reads featuring untemplated or edited nucleotides, while minimizing the chances of spurious alignments being retained. Instances of more than two untemplated nucleotide additions to microRNA reads have been documented by other groups, but such reads would be rejected by our methodology. The computational requirements of such an analysis are beyond the scope of this study, and previous work has demonstrated that reads with one or two untemplated nucleotides dominate tailed species, and that addition of up to two untemplated nucleotides indicates the activation of tailing mechanisms.⁽⁴⁵⁾ We therefore felt that our approach would capture a representative fraction of tailing events, while not imposing excessive computational burdens. In addition to canonical mature microRNAs, we observed several classes of non-canonical microRNA reads using

our approach. 3' offset reads have a canonical 5' end, and no untemplated nucleotides, but are either shorter or longer than the canonical mature microRNA. Similarly, 5' offset reads have canonical 3' ends and no untemplated nucleotides, but differ from canonical mature microRNAs at their 5' end. Overlap reads have no untemplated nucleotides, and overlap the position of their corresponding canonical mature microRNA, but have noncanonical 3' and 5' ends. 3' tailed reads have canonical 5' ends, may have a noncanonical 3' end, and up to 2 untemplated 3' nucleotides. 5' tailed reads have canonical 3' ends, and up to 2 untemplated nucleotides at the 5' end. Substitution reads contain up to 2 untemplated nucleotides at positions more than 2 nucleotides away from both the 5' and 3' ends. Hairpin loop reads map to the region between the 5' and 3' canonical species derived from a given pre-microRNA. Mix reads exhibit more than one of the above feature types(Figure 2.7).

Figure 2.7. Examples of isomiR classes.

```

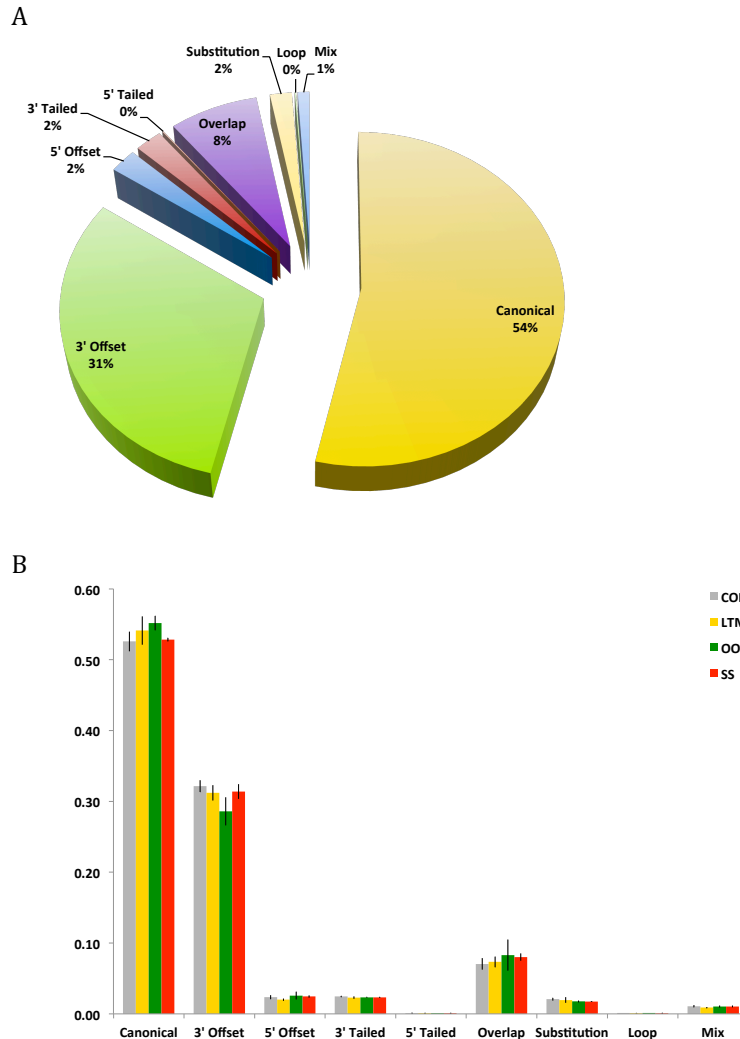
>bantam
AUUUGACUACGAAACCGGUUUUCGAUUUGUUUGACUGUUUUUCAUCAAGUGAGAUCAUUUUGAAAGCUGAUUUUGUCA
-----UGAGAUCAUUUUGAAAGCUGAUU----- 3' Species Canonical:      191233
-----UGAGAUCAUUUUGAAAGCUGA----- 3' Species 3' -2nt Offset:    18124
-----UGAGAUCAUUUUGAAAGCUGAUUU----- 3' Species 3' +1nt Offset:    1143
-----CCGGUUUUCGAUUUGUUUGACU----- 5' Species Canonical:      1054
-----GAGAUCAUUUUGAAAGCUGAUU----- 3' Species 5' +1nt Offset:    522
-----UGAGAUCAUUUUGAAAGCUGAUUa----- 3' species 3' Tail:         454
-----UGAGAUuAUUUUGAAAGCUGAUU----- 3' species substitution:    435
-----GAGAUCAUUUUGAAAGCUGAU----- 3' species 5' & 3' Offset:    202
-----cGAGAUCAUUUUGAAAGCUGAUU----- 3' Species 5' Tail         88
-----UGAGAUuAUUUUGAAAGCUGA----- 3' Species Mix             74
-----UGUUUUUCgUuCAAG----- Hairpin Mix             1

```

Sequencing reads were aligned to pre-microRNA hairpins such that up to 2 mismatches were permitted. Only alignments with alignment scores equal to the best score for a given read were retained. Read counts were upper quartile normalized. 3' offset reads have a canonical 5' end, and no untemplated nucleotides, but are either shorter or longer than the canonical mature microRNA. 5' offset reads have canonical 3' ends and no untemplated nucleotides, but differ from canonical mature microRNAs at their 5' end. Overlap reads have no untemplated nucleotides, and overlap the position of their corresponding canonical mature microRNA, but have noncanonical 3' and 5' ends. 3' tailed reads have canonical 5' ends, may have a noncanonical 3' end, and up to 2 untemplated 3' nucleotides. 5' tailed reads have canonical 3' ends, and up to 2 untemplated nucleotides at the 5' end. Substitution reads contain up to 2 untemplated nucleotides at positions more than 2 nucleotides away from both the 5' and 3' ends. Hairpin loop reads map to the region between the 5' and 3' canonical species derived from a given pre-microRNA. Mix reads exhibit more than one of the above feature types.

Canonical mature microRNAs dominate microRNA reads. However, 3' offset reads represent a significant fraction of reads mapping to pre-microRNAs. None of the treatment conditions we subjected flies to produced a significant change in the proportions of all pre-microRNA mapping reads that fell into each category (Figure 2.8).

Figure 2.8. Noncanonical microRNA reads as a proportion of all reads mapping to pre-microRNAs in each condition

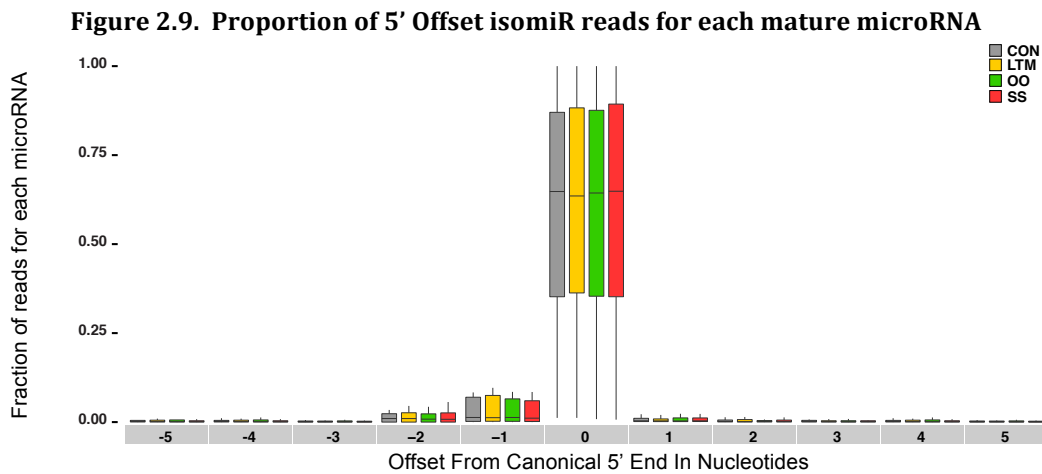


The fraction of each category of noncanonical microRNA reads was calculated for each library. A. Mean fractions of normalized reads from all conditions falling into each isomiR category. B. proportions of each isomiR class for each treatment condition. No treatment condition produced a statistically significant change in the proportion of all pre-microRNA mapping reads falling into any of these categories. Error bars represent SEM.

Offset isomiR analysis for individual pre-microRNAs

Having found no significant changes in the overall proportions of the various classes of isomiR reads amongst all pre-microRNA mapping reads, we next sought to

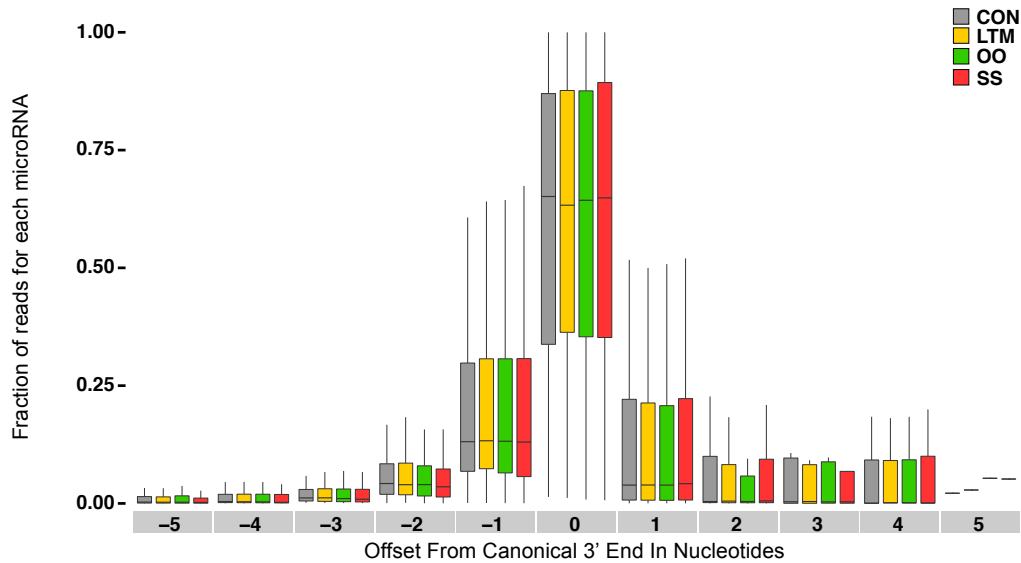
investigate isomiR read features in detail. As microRNA target specificity is largely determined by the 5' seed sequence, any changes in the position of the 5' end of a microRNA will greatly affect the set of mRNAs it targets. In general, microRNA processing is highly precise at the 5' end. Of those microRNAs with a substantial fraction of 5' isomiR reads, most 5' offset isomiRs are offset by 1-2nt (Figure 2.9).



For each mature microRNA, the proportion of reads with a 5' end at each nucleotide position was calculated. The overwhelming majority of microRNA reads have a canonical 5' end. Of those with a 5' offset, reads extending 1 or 2 nucleotides 5' of the canonical 5' position are most common. Rectangles mark the 25th and 75th percentile. Whiskers are 5th and 95th percentile, dots denote outliers.

A comparison of 5' isomiR fractions for each microRNA in all of our treatment groups shows that no treatment produces a statistically significant change in the fraction of 5' isomiR reads for any microRNA. While the 5' ends of microRNA reads overwhelmingly correspond to the canonical 5' position, substantial diversity in the 3' ends of microRNA reads exists. 25% or more of microRNA reads have noncanonical 3' ends for 105 mature microRNAs. For most microRNAs, 3' isomiRs are primarily truncated by 1nt. However, extension of the 3' end by as much as 4nt are not uncommon for many microRNAs (Figure 2.10).

Figure 2.10. Proportion of 3' Offset isomiR reads for each mature microRNA



For each mature microRNA, the proportion of reads with a 3' end at each nucleotide position relative to the canonical position was calculated. While most microRNA reads have a canonical 3' end, a substantial fraction of reads do not. Of those with a 3' offset, reads extending 1 nucleotide 5' or 1 nucleotide 3' of the canonical 3' position are most common. Rectangles mark the 25th and 75th percentile. Whiskers are 5th and 95th percentile, dots denote outliers.

Analysis of untemplated nucleotide tailing

Previous studies have demonstrated that untemplated nucleotide addition affects the stability and function of microRNAs. While the 5' seed sequence largely determines target specificity for microRNAs, 3' nucleotide addition is thought to stabilize or mark microRNAs for destruction. Specifically, 3' adenylation is thought to stabilize animal microRNAs and affect their targeting, while 3' uridination is thought to destabilize microRNAs (35, 46-49). We therefore sought to profile such modifications to microRNA reads in our libraries. Using the same alignment method as was used for the offset analysis, we calculated the fraction of all reads mapping to each mature microRNA locus that feature 3' untemplated adenines or uridines. For

our analysis of 3' untemplated nucleotide addition, we considered only microRNAs that had more than 100 normalized reads per sample. We found that 16 microRNAs had 1% or more of their reads 3'adenylated in any condition, and only miR-927-3p has more than 20% of its reads 3' adenylated in any condition (Figure 2.11 A).

Figure 2.11. 3' Tailing of microRNA reads

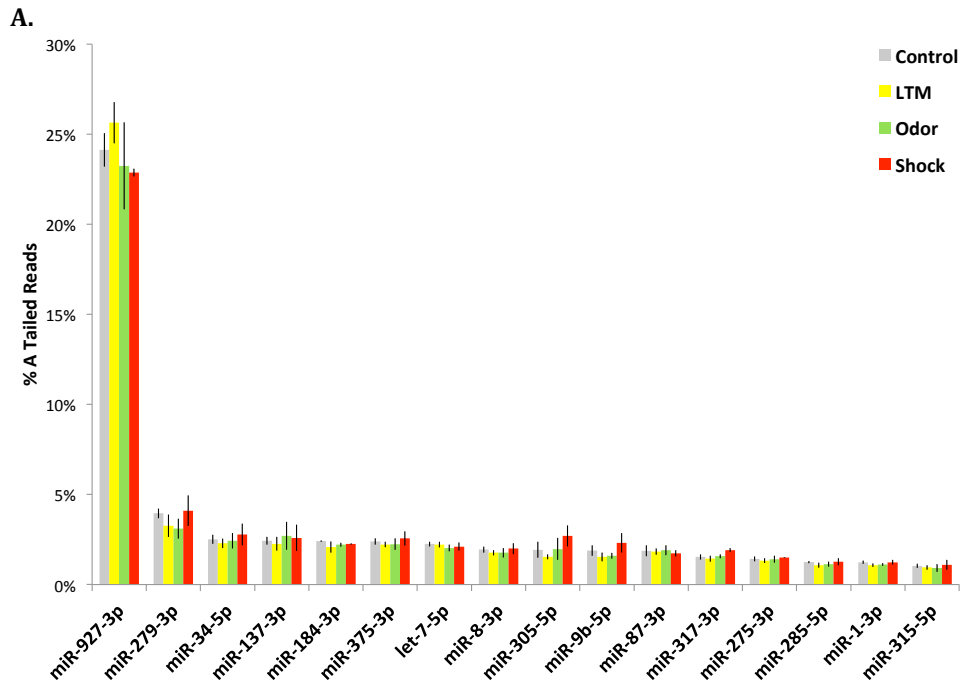
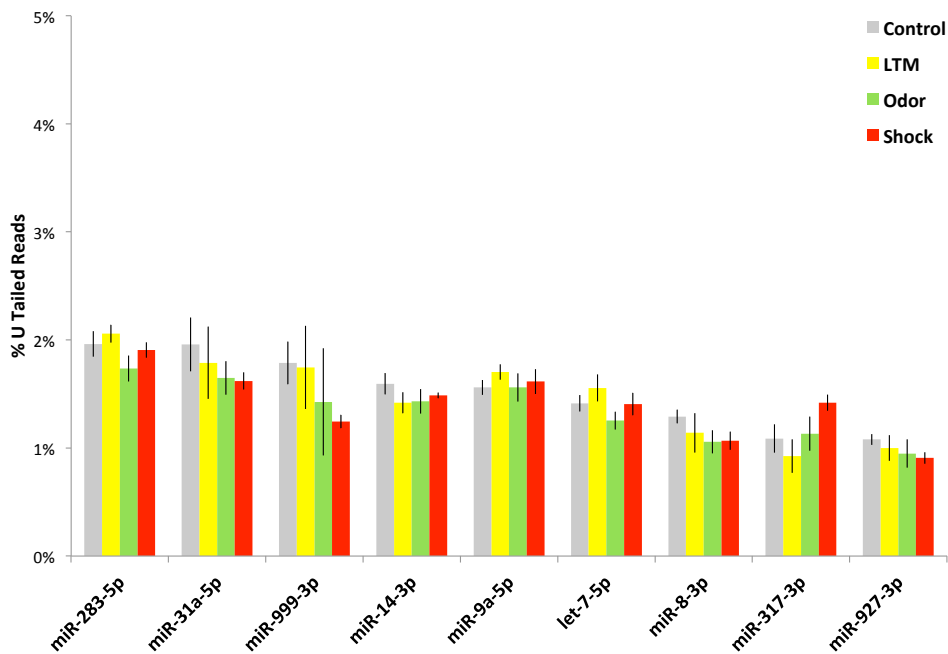


Figure 2.11 (Continued)

B.



Reads mapping at mature microRNA loci were examined for 3' untemplated nucleotide addition. Read counts were upper quartile normalized, and only microRNAs with an average of 100 or more normalized reads per sample were considered in our analysis of 3' tailing. A. 16 microRNAs had greater than 1% of their reads featuring mono or polyadenylation in any treatment condition. B. 9 microRNAs had greater than 1% of their reads featuring mono or polyuridination in any treatment condition.

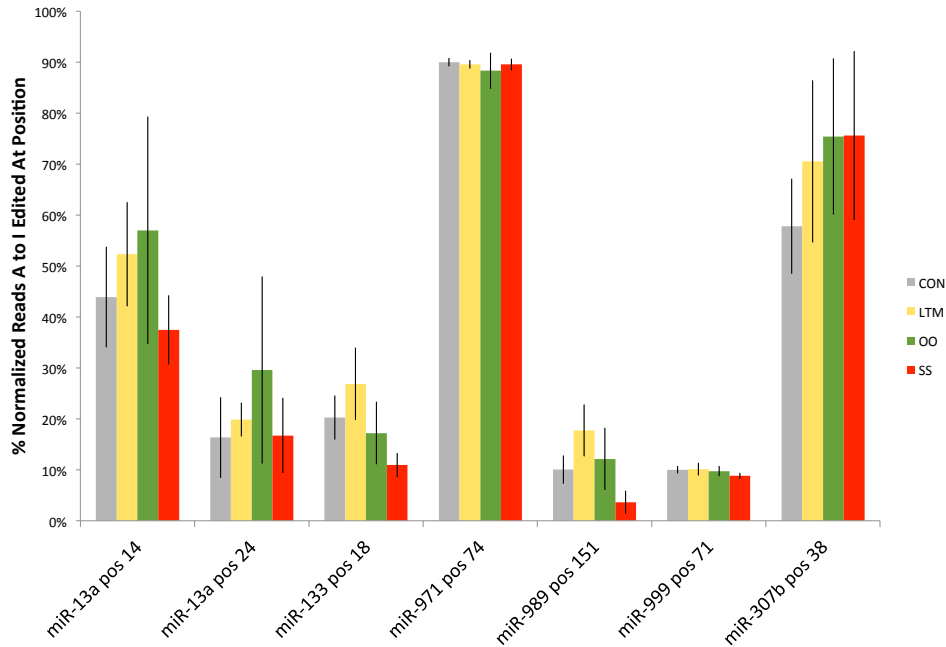
We found that 4 microRNAs had 2% or more of their reads 3' uridinated in any condition, and only miR-970-3p has more than 10% of its reads 3' uridinated in any condition (Figure 2.12 B). Interestingly, miR-927-3p also had greater than 1% of its reads 3' uridinated. We also looked for statistically significant changes in the fractions of 3' adenylated or 3' uridinated reads for all microRNAs. No microRNA displayed a statistically significant change in 3' A or U tailing in any of our treatment conditions. A similar examination of 5' untemplated additions to microRNA reads found such modifications to be essentially absent in our libraries.

Analysis of microRNA editing

Previous studies have found that the RNA editing enzyme adenosine deaminase acting on RNA (ADAR) is highly expressed in the *Drosophila* CNS, and particularly in the MB. Further, ADAR interacts with RISC components, and regulates synaptic structure in *Drosophila*. We therefore examined our sequencing libraries for evidence of pre-microRNA or microRNA editing. Again, we aligned our sequencing reads to pre-microRNA hairpin sequences from miRBase, such that up to two mismatches were allowed, and only alignments with scores equal to the best alignment score for a given read were retained. We then examined instances in which untemplated nucleotides occurred greater than 2nt away from both the 3' and 5' ends of a read. This step was to ensure that we did not include tailed microRNA reads in our analysis, as terminal untemplated nucleotide addition occurs via a mechanism distinct from ADAR activity. Illumina sequencing technology reads inosine nucleotides as guanine. If nearly all of our reads feature a nucleotide that differs from the reference sequence at a given position, we would consider this to be a single nucleotide polymorphism, and not the result of ADAR activity. Conversely, if few reads feature a nucleotide that differs from the reference sequence at a given position within a pre-microRNA, we would consider this to be the result of sequencing noise. Thus, we considered instances in which between 10% and 90% of reads mapping to a given pre-microRNA nucleotide featured an A to G substitution to be a genuine editing event. Further, we required at least 15 normalized reads to have mapped to the position in all libraries in order to include

the position in our editing analysis. Using these criteria, we identified 7 genuine editing events within pre-microRNA sequences (Figure 2.12).

Figure 2.12. A to I editing instances within pre-microRNAs



Sequencing reads were mapped to pre-microRNA sequences from miRBase such that up to 2 mismatches were permitted. Only mismatches at least 2nt from both the 5' and 3' ends of the read were considered. The fraction of reads mapping at each position along pre-microRNAs that featured an A to G substitution at that position was computed. Positions with at least 15 normalized reads mapped in all libraries and having between 10% and 90% of reads featuring an A to G substitution at that position were considered genuine editing events. Error bars represent SEM.

Having identified several apparently genuine editing events, we next examined our data for evidence that any of our treatments caused a change in A to I editing rates at these positions. Using a student's t-test, we found that none of the observed differences between conditions in the fraction of A to I edited reads at a given position were statistically significant.

Section II: Analysis of esiRNA and piRNA expression in the *Drosophila* head during long-term memory formation

The importance of microRNA mediated translational control in synaptic plasticity and memory formation is well established. Recently, several publications have indicated that esiRNAs and piRNAs are involved in these processes as well in nematodes, mollusks, and mice. (5, 26, 50-52) piRNAs and esiRNAs are expressed in the *Drosophila* head, leaving open the possibility that these classes of sRNAs could be involved in synaptic plasticity and memory formation in flies as well.(53, 54) Though several studies have demonstrated their presence in *Drosophila* heads, none have directly addressed whether esiRNAs or piRNAs are involved in memory formation. More broadly, the potential roles of esiRNAs and piRNAs in memory formation in any animal species remain poorly understood. We therefore sought to profile expression of these sRNA classes in *Drosophila* heads, and to identify any changes in these profiles during memory formation. With these goals in mind, we constructed the sRNA sequencing libraries described in section I of this chapter such that we could capture esiRNAs and piRNAs in addition to microRNAs. The catalog of microRNAs expressed by *Drosophila* is well understood and thoroughly curated.(35, 54) However this is not the case for other sRNA classes. Furthermore, while microRNAs are highly conserved, and transcribed from known genomic locations, esiRNAs and piRNAs are generated from less well defined regions within repetitive elements, mobile elements, and in concert with heterochromatin

formation. Thus, studying these classes of sRNAs demands computational approaches that differ from those for microRNAs.

Identification of esiRNA producing loci

To study esiRNA and piRNA expression, we first had to identify loci that produce these sRNAs. To eliminate loci that could potentially confound our analysis, we first filtered our sequencing data for reads mapping perfectly to pre-microRNA hairpins, tRNAs, rRNAs, snRNAs, and snoRNAs. We then mapped the filtered reads to the *Drosophila* reference genome available from FlyBase such that no mismatches were allowed, and all perfect alignments to the genome were retained.(32) To capture all possible loci, we merged the combined alignments from all of our sequencing libraries into 'read-contigs'. All alignments that overlap or are immediately adjacent to each other form a read-contig. This process yielded 874802 read-contigs. However, this number is inflated, as many identical loci are produced by repetitive elements. To reduce this number, and to generate more useful read-contigs, we further merged all read-contigs within 100nt of each other, yielding 219482 read-contigs. This number still includes read-contigs comprising a single read in one library. To further refine our search, we filtered our read-contigs such that only read-contigs with at least 250 reads in our combined sequencing libraries were retained. This criteria translates to roughly 15 reads per locus per library. As reads within 100nt of each other are bridged, this threshold is still quite low. We used this set of filtered read-contigs as the basis for subsequent analysis. In addition to other

characteristics, esiRNAs and piRNAs are known to have distinctive size ranges. esiRNAs are almost entirely 21-22nt in length. Therefore, we identified read-contigs in which at least 75% of reads from all libraries are 21-22nt as esiRNA loci. This approach yielded 368 esiRNA loci (Figure 2.13).

Figure 2.13. Properties of read-contigs

A

| Contig Type | Count | Avg. Contig Length | SEM Contig Length |
|--------------------------|--------|--------------------|-------------------|
| All Contigs | 219482 | 190.74 | 1.36 |
| All Contigs >= 250 Reads | 7581 | 2176.43 | 28.83 |
| esiRNA (21-22nt) | 368 | 4536.40 | 231.73 |

B

| | Total | Intron | Exon | 5' UTR | 3' UTR | Intergenic | Transposon | Gene | Repeat | Gene w/o Transposon | Gene w/o Repeat |
|-----------------------------|---------|--------|--------|--------|--------|------------|------------|--------|--------|---------------------|-----------------|
| # of esiRNA Loci | 368 | 192 | 51 | 71 | 41 | 177 | 214 | 212 | 354 | 67 | 14 |
| Fraction of all esiRNA Loci | 100.00% | 52.17% | 13.86% | 19.29% | 11.14% | 48.10% | 58.15% | 57.61% | 96.20% | 18.21% | 3.80% |

Read-contigs were generated by merging all sequencing reads from all of our libraries whose ends fall within 100nt of each other into a single contig. We refined this list of read-contigs by retaining only those with at least 250 reads from our combined libraries. esiRNA loci were defined as those read-contigs with >= 75% of mapped reads from our combined libraries being 21-22nt in length. A: esiRNA loci are longer than read-contigs. B: The types of genomic features overlapping each esiRNA locus were obtained using bedtools to intersect feature position lists with esiRNA loci positions. We then calculated the fraction of esiRNA loci overlapping each type of feature.

esiRNA expression profile in the Drosophila head

Closer inspection of the esiRNA loci we identified reveals a variety of sources for esiRNAs, each with unique features (Figure 2.14).

Figure 2.14 examples of esiRNA loci

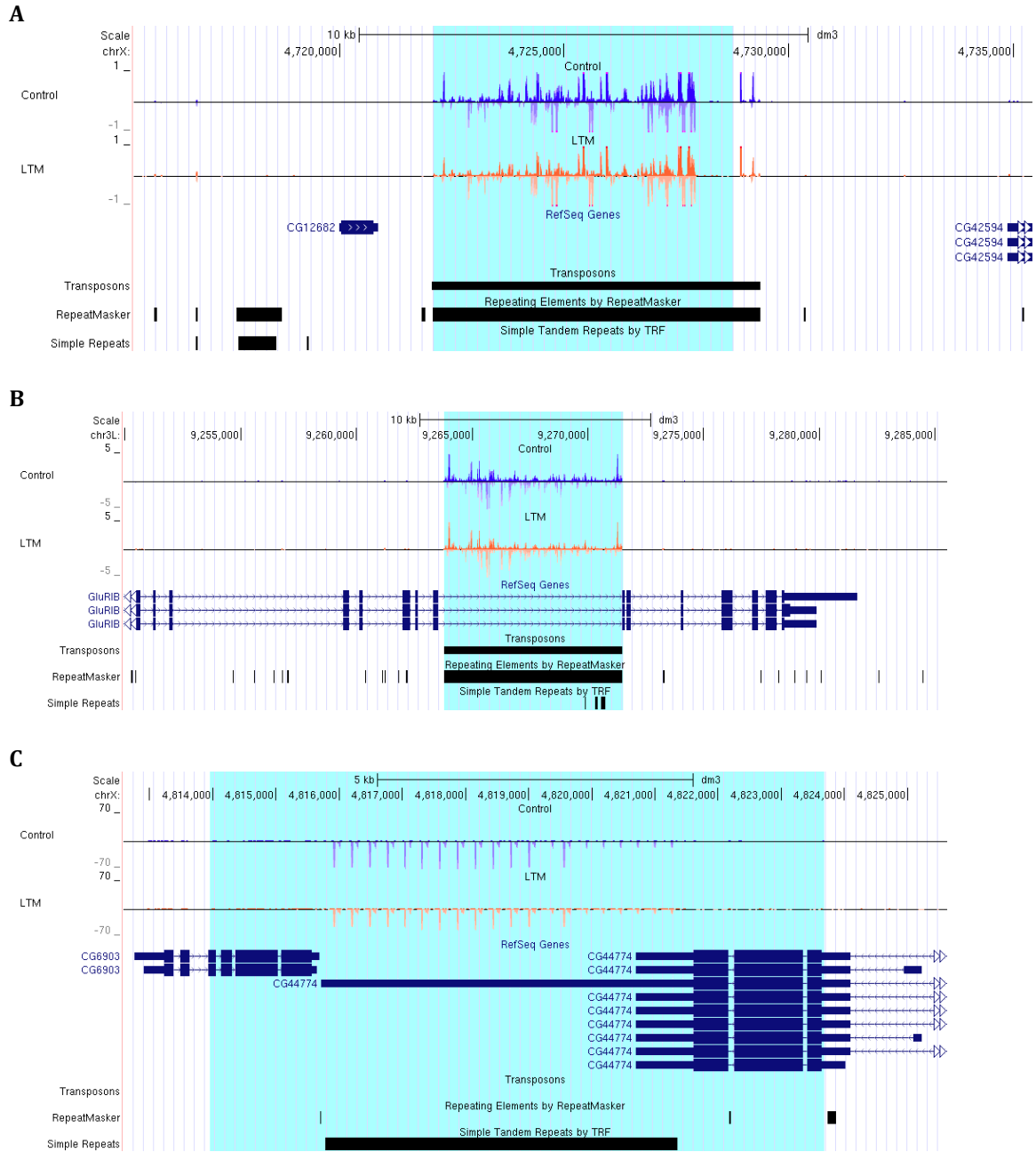
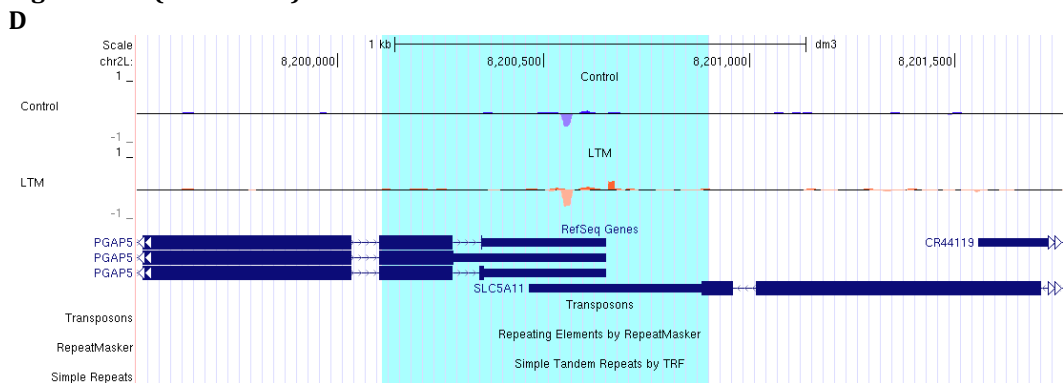


Figure 2.14 (Continued)



Reads were mapped to the *Drosophila* genome such that no mismatches were permitted, and all perfect alignments were retained. Read counts were upper-quartile normalized across all libraries, and average normalized read counts for each condition were computed at every nucleotide position of the genome. These average read depth values were then plotted across the genome using the UCSC genome browser. Read depth values above the black line in each condition represent alignments on the + strand, while values below the line represent alignments on the - strand. Numeric read depth values on the Y-axis represent per-condition mean normalized reads. The light blue shaded region indicates the location of the esiRNA locus. A: Example of an esiRNA locus that maps to an isolated transposon. B: Example of an esiRNA locus that maps to a transposon within an intron of the *GluR1B* gene. C: Example of an esiRNA locus that maps to a long tandem repeat in the 3' UTR of the *CG44774* gene and the adjacent *CG6903* gene. These genes are transcribed from opposing strands, but do not overlap. D: Example of an esiRNA locus that maps to the Post-GPI attachment to proteins 5 ortholog (*PGAP5*) and Sodium/solute co-transporter-like 5A11 (*SLC5A11*) genes. A stretch of overlapping convergent transcription within the 3' UTRs of both genes produces substantially more reads than the surrounding region, and unlike the surrounding region, a substantial number of reads map to both strands.

The bulk of esiRNA loci include repetitive elements and transposons, both of which can reside in intergenic regions, or within genes (Figure 2.14 A, B, & C). Such loci produce large numbers of reads, and can span several Kb. In the case of inverted repeats. The esiRNA loci we identified also include regions of overlapping transcription on opposite strands, so called cis-natural antisense transcripts (cis-NATs) (Figure 2.14 D). As reported in other studies, the only examples of cis-NATs that exhibited a strong 21-22nt bias in our libraries are cases in which transcription on the two strand is convergent.⁽⁵⁵⁾ Our esiRNA loci that map to cis-NATs often extend beyond the region of overlap between the two genes. However, in such cases most of the reads within the esiRNA locus still map within the region of overlap.

The extension of cis-NAT esiRNA loci is due to the presence of reads nearby, but outside of the overlap, and bridging of even single reads within 100nt of each other during the generation of our read-contigs.

Previously reported profiles of esiRNA expression in various *Drosophila* tissues and cell lines have noted several interesting regions that produce esiRNAs (33, 54, 56-59). The esiRNA loci we identified also include many of these same regions. For instance, the production of esiRNAs from the cis-NAT region encompassing the Ago2 and CG7739 genes produces abundant 21-22nt reads. Our methods identify 4 esiRNA loci in this region. Our analysis also identified 2 esiRNA loci mapping to the pseudogene CG18854, which overlaps the Inositol 1,4,5-triphosphate kinase 1 (IP3K1) gene on the opposite strand. CG18854 contains an inverted repeat that produces esiRNAs that were shown to be functional by Okamura et al (Fig 2.15).(59)

Figure 2.15. Comparison of previously published esiRNA loci and those identified in this study

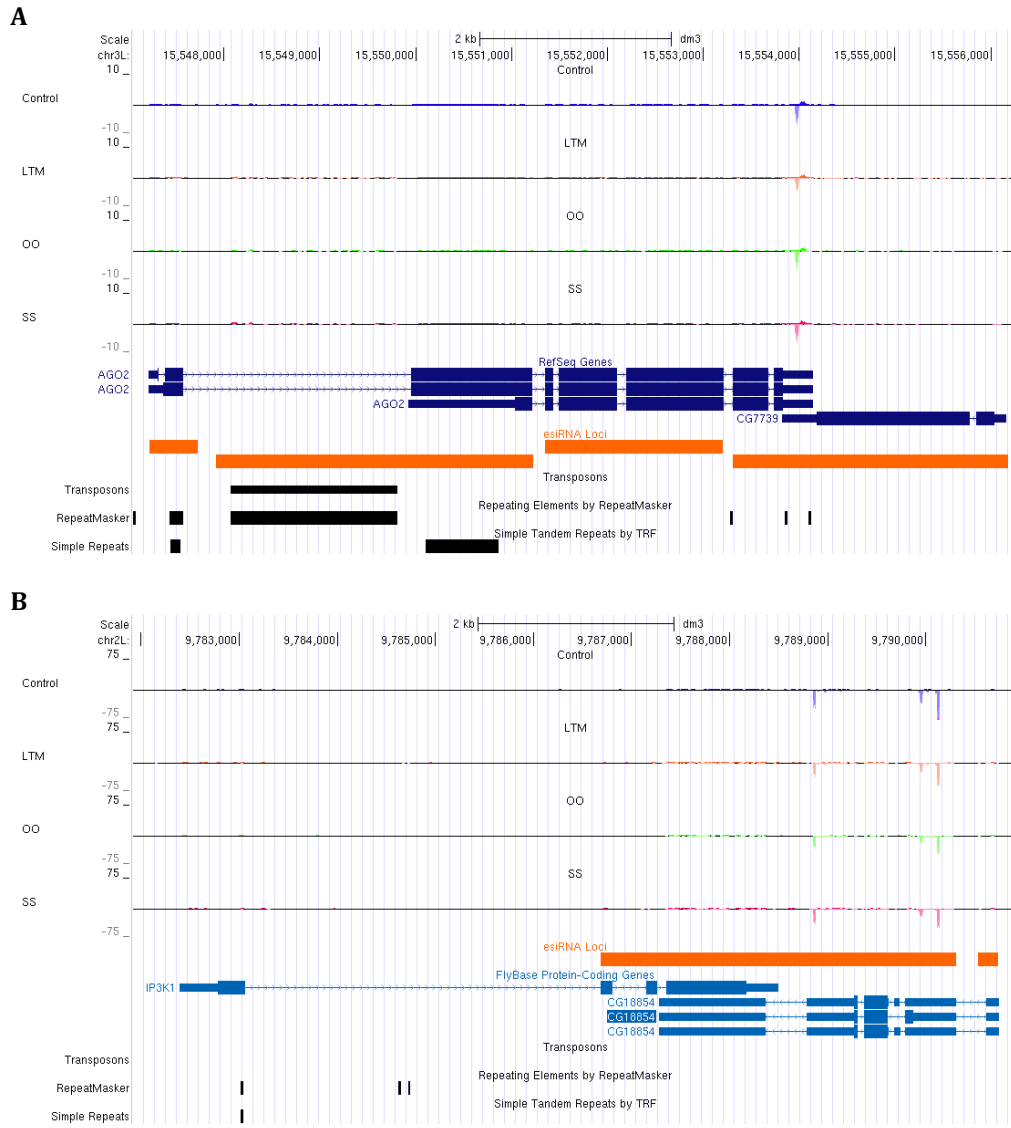
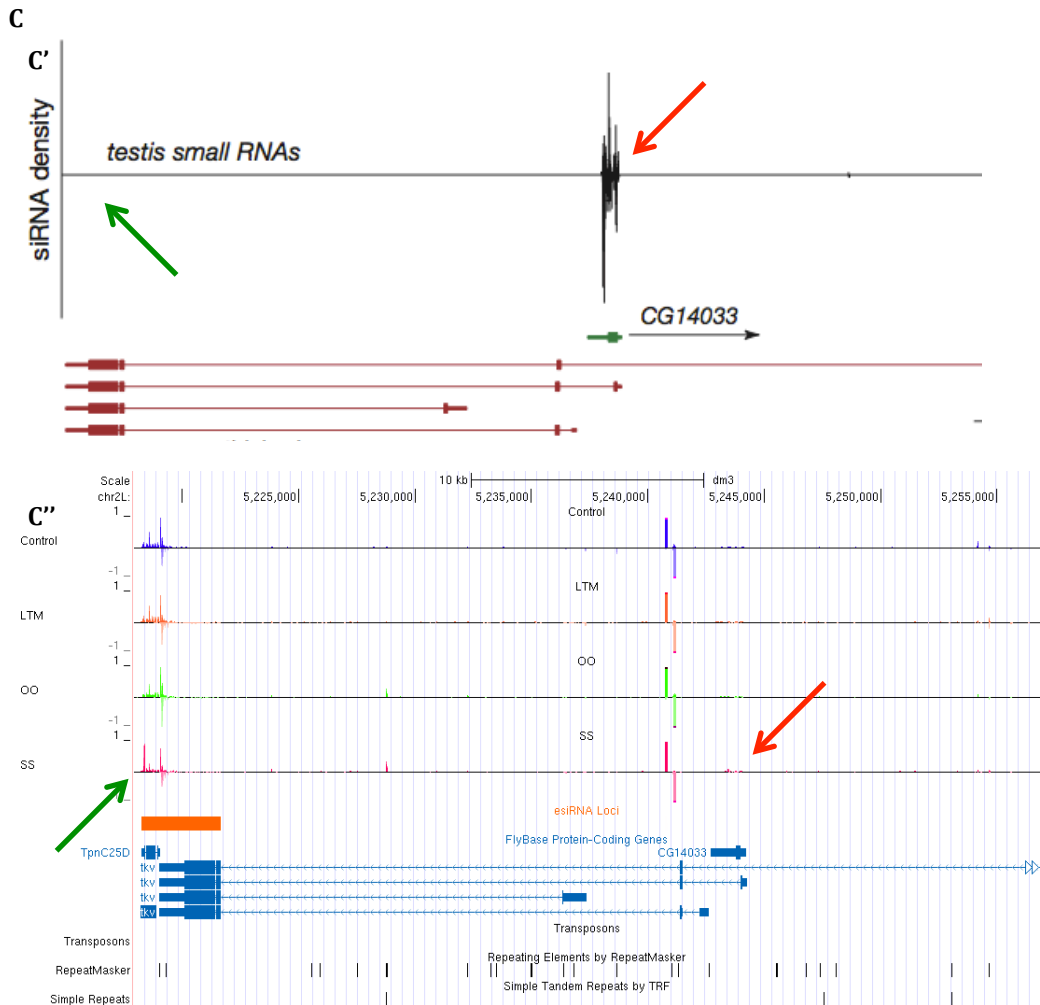


Figure 2.15. (Continued)



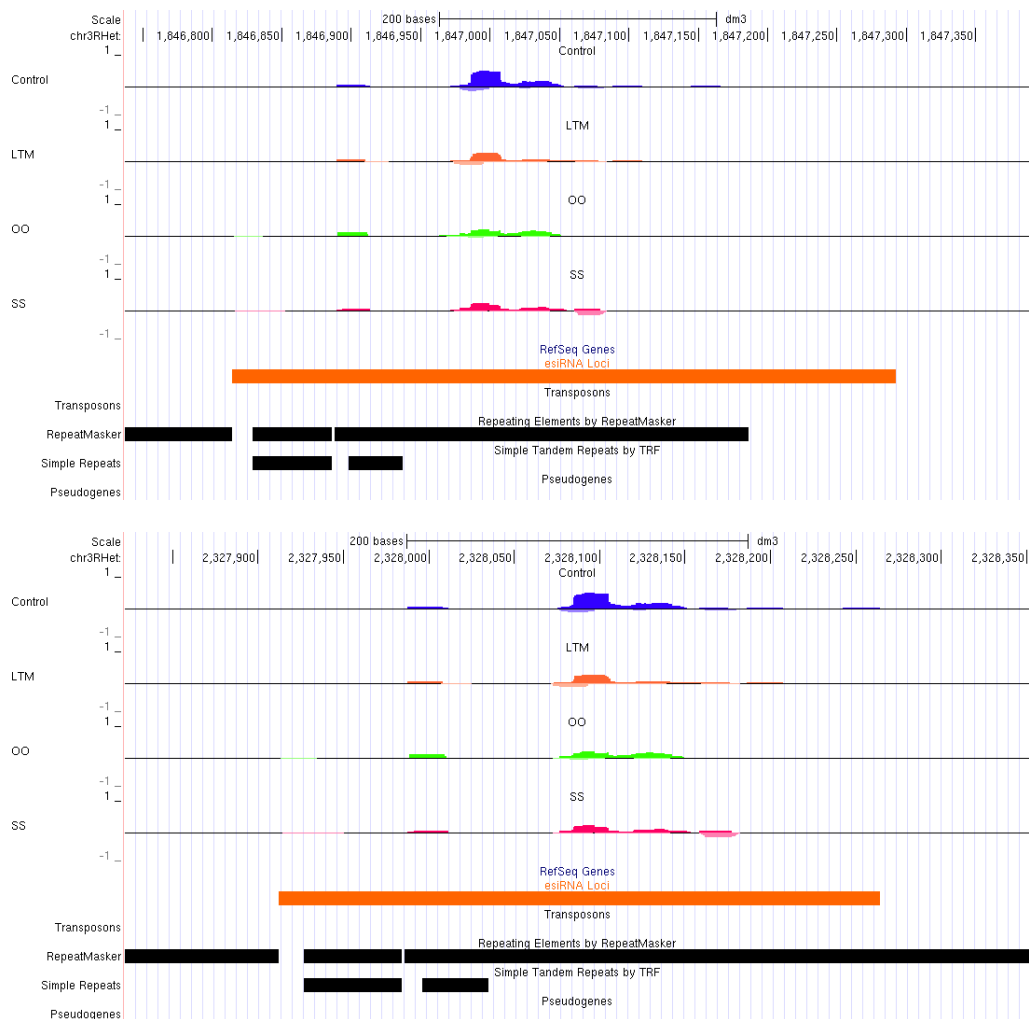
Sequencing reads were aligned to the *Drosophila* genome such that no mismatches were permitted, and all perfect alignments were retained. Read counts were upper-quartile normalized across all libraries, and average normalized read counts for each condition were computed at every nucleotide position of the genome. These average read depth values were then plotted across the genome using the UCSC genome browser. Read depth values above the black line in each condition represent alignments on the + strand, while values below the line represent alignments on the - strand. Numeric read depth values on the Y-axis represent per-condition mean normalized reads. The positions of the esiRNA loci we identified are represented in orange. A: As reported elsewhere, our data shows that region encompassing the esiRNA effector Argonaute protein Ago2 produces abundant esiRNAs from the coding strand. The region of overlap between the Ago2 3'UTR and the CG7739 produces abundant esiRNAs from both strands, a hallmark of esiRNA production from cis-NATs. B: The pseudogene CG18854 overlaps the 3' end of the IP3K1 gene and produces abundant esiRNAs from 2 esiRNA loci. esiRNAs produced from this region are known to be functional. C: The pattern of esiRNA expression at the 3' end of the tkv gene in our libraries differs from that reported elsewhere. C': Czech et al report abundant esiRNA production corresponding to the CG14033 locus (red arrow) and little or no esiRNA production from the 3' tkv exon, which is proximate to TpnC25D (green arrow). C'': In our libraries, few esiRNAs are produced from the CG14033 locus (red arrow), but abundant esiRNAs are produced from the 3' exon of the tkv gene (green arrow).

In other cases, our data shows features not reported elsewhere. For instance, Czech et al. reported abundant esiRNA production from the pseudogene CG14033, which is proximate to the 5' exon of the thickveins-C (tkv-C) transcript in libraries prepared from *Drosophila testis*.(57) However, these authors did not report substantial esiRNA production from the 3' end of the tkv gene, which is proximate to the Troponin C at 25D (TpnC25D) gene on the opposite strand. This esiRNA expression pattern is also reported in many of the modencode sRNA tracks. While we see reads at both positions, our data shows that the 3' tkv/TpnC25D locus produces far more reads than does the tkv/CG14033 locus. In fact, the tkv/CG14033 locus produces so few reads, that our methods do not identify it as an esiRNA locus.

Changes in esiRNA expression during LTM formation

We next sought to identify changes in esiRNA expression during LTM formation at the loci we identified. Read counts at esiRNA loci were obtained using BEDTools. We used edgeR in its GLM mode to test esiRNA loci for differential expression. 6 esiRNA loci display statistically significant changes in esiRNA expression during LTM formation. 2 Loci mapping to identical repetitive elements in the heterochromatin of chromosomal arm 3R display significantly reduced read counts in the LTM condition, but not in the odor or shock conditions (Fig 2.16).

Figure 2.16. esiRNA loci downregulated during LTM formation



Sequencing reads were aligned to the *Drosophila* genome such that no mismatches were permitted, and all perfect alignments were retained. Read counts were upper-quartile normalized across all libraries, and average normalized read counts for each condition were computed at every nucleotide position of the genome. These average read depth values were then plotted across the genome using the UCSC genome browser. Read depth values above the black line in each condition represent alignments on the + strand, while values below the line represent alignments on the – strand. Numeric read depth values on the Y-axis represent per-condition mean normalized reads. The positions of the esiRNA loci we identified are represented in orange. Each esiRNA locus was tested for differential expression using edgeR. 2 esiRNA loci mapping to identical repetitive elements in the heterochromatin of chromosomal arm 3R showed significantly reduced read counts in the LTM condition. Although read counts within these loci were also reduced in the odor only (OO) and shock only (SS) conditions, edgeR did not find these changes to be statistically significant.

As these loci are identical in sequence, reads mapping within them map to both loci.

However, these reads do not map elsewhere in the genome. Both down-regulated

loci are separated from known coding and regulatory sequences by tens of Kb. Therefore, these loci and the reads mapping within them do not have obvious regulatory functions. The 4 esiRNA loci displaying statistically significant increased read counts all map to lysozyme family genes residing within a single intron of the multiple wing hairs (mwh) gene (Fig 2.17A).

Figure 2.17. esiRNA Loci mapping to lysozyme family genes within an intron of the mwh gene are significantly up-regulated during LTM formation.

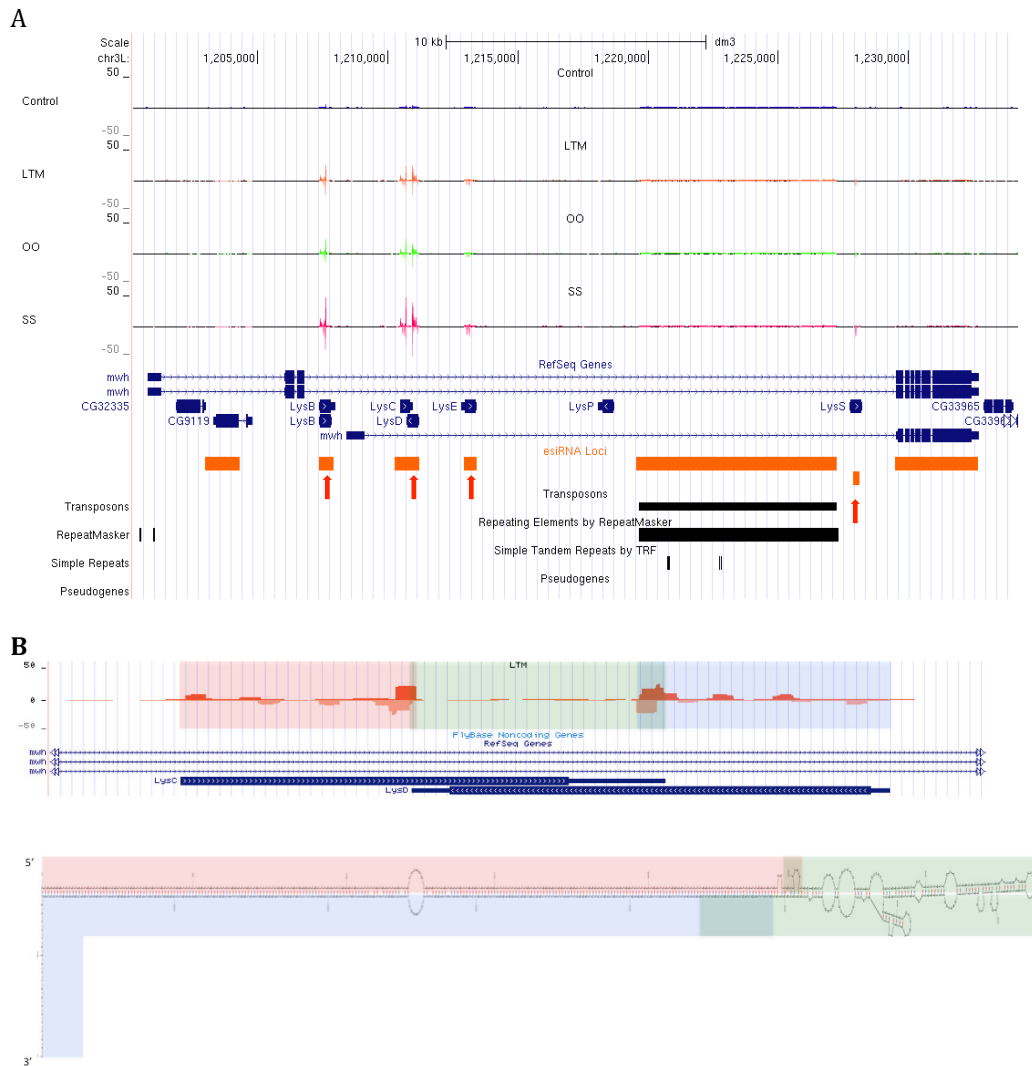


Figure 2.17. (Continued)

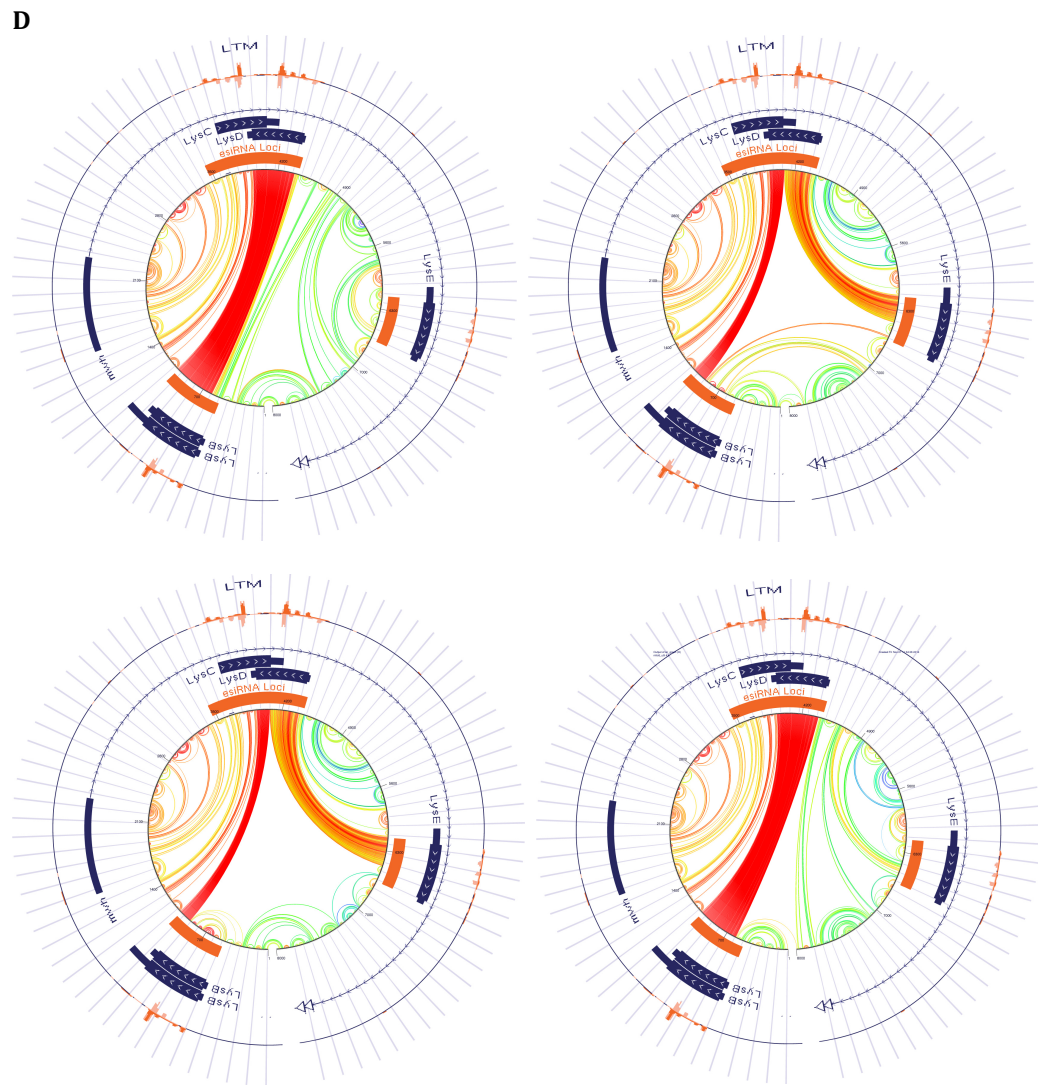
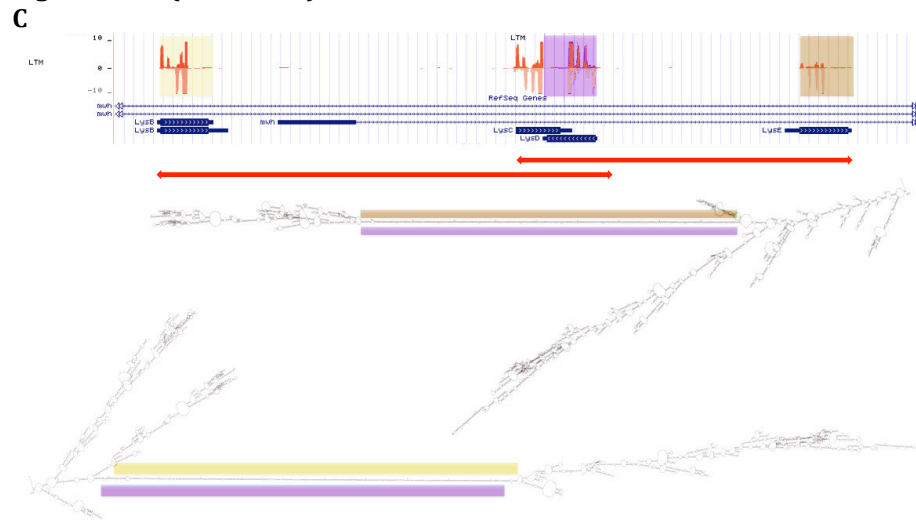


Figure 2.17. (Continued)

4 esiRNA loci display significantly increased read counts in the LTM and Shock only conditions ($p = <.00005$). These loci also have increased read counts in the odor only condition, but the increase is smaller and not statistically significant. A: All up-regulated esiRNA loci map to lysozyme family genes residing within a single intron of the multiple wing hairs (mwh) gene (indicated by red arrows). B: Most reads mapping to the esiRNA locus within the lysozyme C (LysC) and lysozyme D (LysD) genes fall outside of the region of overlap between the two genes. The regions producing the most reads (highlighted in red and blue) correspond to a long stretch of perfect duplex formed by a hairpin structure in the mwh intron (shown in the lower panel), while the region of overlap between the two genes (highlighted in green), and largely corresponding to the hairpin loop, produces far fewer reads. C: The mwh intron produces several hairpin structures with long stretches of perfectly complementary duplex forming between LysB and LysD (highlighted in yellow and purple, respectively), or LysE and LysD (highlighted in orange and purple, respectively). The lower panel depicts the two predicted secondary structures for the mwh intron with the highest scores, but several other valid predicted secondary structures also form perfect duplexes in the same regions. The red markers denote the extents of RNA sequences depicted in the structures in the lower panel. D: Circle plots showing 4 examples of 50 high confidence structure predictions for an RNA encoded by the 5' 8Kb of the mwh intron. The intron's 5' end is at the 6 o'clock position, and is transcribed clockwise around the circle. Arcs connect base paired nucleotides across the center of the circle plot. Red, orange, and yellow arcs indicate high confidence predictions, while greens and blues indicate lower confidence predictions. Read coverage for the LTM condition is depicted in orange outside of the circle plot, as are the positions of transcripts and esiRNA loci.

Initially, the overlapping antisense arrangement of the LysC/D locus suggested that the reads mapping to the highly similar lysozyme family genes within the mwh intron might all arise from this locus via the cis-NATesiRNA mechanism. However, closer inspection of the LysC/LysD locus unexpectedly reveals that the region of overlap between the two genes produces far fewer reads than do the regions of LysC and LysD outside of the overlap (Fig 2.17 B). We therefore sought to determine if transcription at this locus might yield a hairpin secondary structure that could be a substrate for the esiRNA biogenesis machinery. We used mFold to predict secondary structures for a conceptual RNA whose 5' end corresponds to the transcription start site (TSS) of LysC, and whose 3' end is the LysD TSS.(60) The mFold predicted structure for this RNA

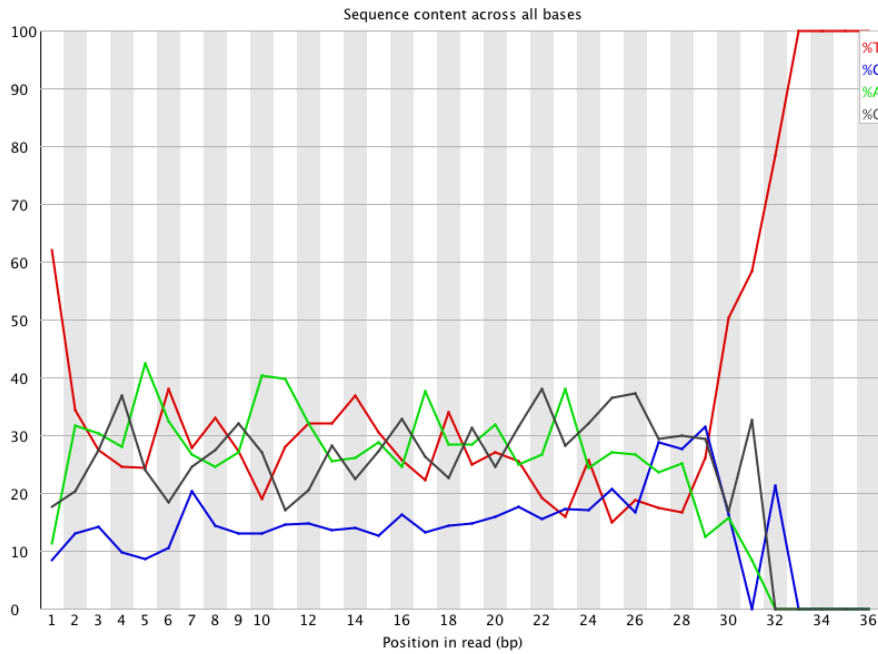
Exhibits a long hairpin structure with a long region of perfect duplex (Fig 2.17 B, lower panel). This duplex region corresponds nearly perfectly to the regions of LysC and LysD that do not overlap, and produce the most reads. The area of reduced read production in the region of overlap between LysC and LysD corresponds to a segment of the secondary structure that contains the hairpin loop, and a number of bulges where no duplex is formed. Thus, it seems likely that reads mapping to the LysC/LysD esiRNA locus are the product of the hairpin esiRNA pathway, and not of cis-NAT driven esiRNA biogenesis. The LysB, LysE and LysS esiRNA loci, also reside within the same mwh intron, and therefore might also correspond to regions of secondary structure duplex formed by the mwh intron. To test this idea, we submitted segments of the mwh intron to mFold. The maximum sequence length capacity of mFold is 9Kb. While LysB, LysC/LysD, and LysE fall within the 5' 7Kb of the mwh intron, LysS is ~15Kb 3' of LysE, which prevented us from testing for secondary structures in which LysS forms duplexes with the other up-regulated esiRNA loci. As the lysozyme family genes closely resemble each other in sequence, we expected that duplexes might form between different combinations of intron sequences corresponding to these genes. Therefore, we first predicted structures for mwh intron sequences spanning individual pairings of esiRNA loci. Interestingly, while the hairpin formed within the LysC/LysD esiRNA locus did not form extensive duplexes that include the region of overlap between the genes, mwh intron sequences that span the region of LysB through LysD, or LysC through LysE both form long hairpins with extensive duplexes that span the entire length of LysD, including the region of overlap between LysC and LysD (Fig 2.17 C). We next

obtained secondary structure predictions for the 5' 8Kb of the mwh intron using mFold. The structures predicted for this 8Kb sequence all exhibit extensive duplexes between the LysB, and LysC/LysD, and between LysC/LysD and LysE loci, though the particular regions of each locus involved in duplex formation varied between predictions. Lastly, we could not conduct such a secondary structure analysis for the LysS esiRNA locus, as it is too distant from the other esiRNA loci within the mwh intron. However, we note that unlike the other up-regulated lysozyme gene family esiRNA loci, LysS reads are dominated by just two reads. Both dominant LysS reads also map to all other up-regulated lysozyme gene family esiRNA loci. Thus, LysS mapping reads, and their up-regulation may actually reflect esiRNA production from the hairpin structures formed by the 5' end of the mwh intron.

A profile of piRNA expression in the Drosophila head

In a manner similar to that which we used to identify esiRNA loci, we collected piRNA loci from our read-contigs by selecting those read-contigs having at least 250 mapped reads from our combined libraries, with at least 75% of these reads being 24-29nt. This size range corresponds to the known lengths of *Drosophila* esiRNAs. Using these criteria, we identified 82 likely piRNA loci. Reads mapping within piRNA loci display a strong bias for uridine at the 5' nucleotide, and adenosine at the 10th nucleotide, a hallmark of the ping-pong piRNA mechanism (Fig 2.18).

Figure 2.18. Per-base sequence composition of reads mapping to piRNA loci



Reads mapping to piRNA loci were selected using bedtools, and the fraction of reads with each base at each nucleotide position were then plotted using FastQC. piRNA mapping reads have a strong bias for a 5' uridine, and adenosine at position 10, a hallmark of the ping-pong mechanism.

These biases argue in favor of the validity of our designation of these loci as piRNA producing regions. piRNA loci are shorter and have fewer mapped reads than esiRNA loci. 95.06% of the piRNA loci we identified map to repetitive elements (Fig 2.19).

Figure 2.19. Characteristics of piRNA loci

A

| Contig Type | Count | Avg. Contig Length | SEM Contig Length |
|--------------------------|--------|--------------------|-------------------|
| All Contigs | 219482 | 190.74 | 1.36 |
| All Contigs >= 250 Reads | 7581 | 2176.43 | 28.83 |
| esiRNA (21-22nt) | 368 | 4536.40 | 231.73 |
| piRNA (24-29nt) | 82 | 1453.13 | 275.05 |

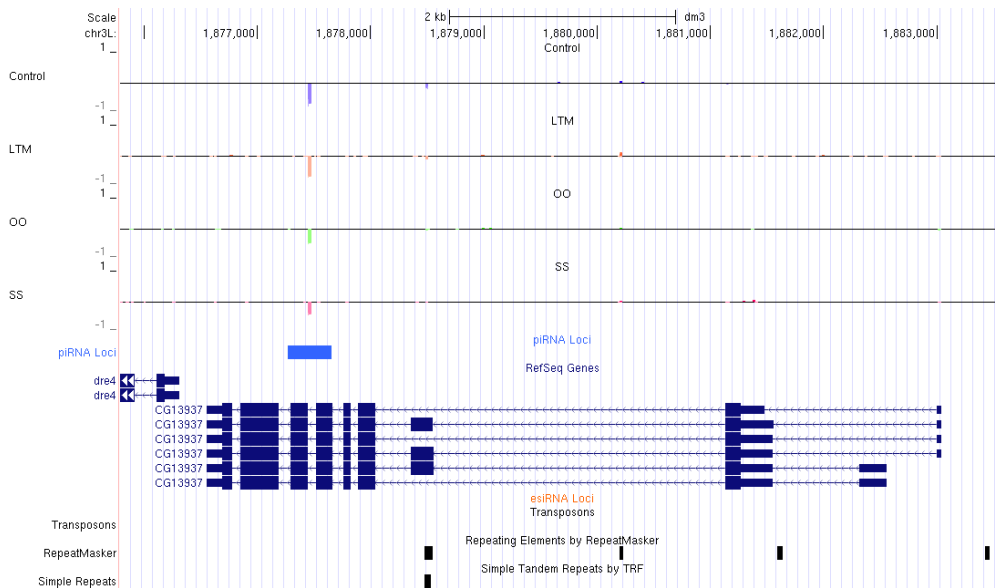
B

| | Total | Intron | Exon | 5' UTR | 3' UTR | Intergenic | Transposon | Gene | Repeat | Gene w/o Transposon | Gene w/o Repeat |
|----------------------------|---------|--------|-------|--------|--------|------------|------------|--------|--------|---------------------|-----------------|
| # of piRNA loci | 82 | 34 | 7 | 10 | 3 | 45 | 16 | 36 | 77 | 33 | 1 |
| Fraction of all piRNA Loci | 100.00% | 41.98% | 8.64% | 12.35% | 3.70% | 55.56% | 19.75% | 44.44% | 95.06% | 40.74% | 1.24% |

piRNA loci were collected from our read-contigs by selecting those contigs having 75% mapped reads 24-29nt in length. A: mean piRNA locus length is shorter than that for all read-contigs, or esiRNA loci. B: The types of genomic features overlapping each piRNA locus were obtained using bedtools to intersect feature position lists with piRNA loci positions. We then calculated the fraction of piRNA loci overlapping each type of feature.

Surprisingly, more piRNA loci overlap genes than overlap transposons. However, many piRNA loci overlapping genes correspond to repetitive elements that fall entirely within long introns. The one piRNA locus mapping within a gene but not to a repetitive element is dominated by a single 26nt sequence, rather than being comprised of a variety of sequences spread out across the locus as is typically the case. This sequence maps to the 3' end of an intron of the CG13937 gene, and nowhere else in the genome (Fig 2.20).

Figure 2.20. piRNA locus mapping to CG13937 and outside of repetitive elements



The lone piRNA locus mapping within a gene but outside of repetitive elements is dominated by a single sequence. This sequence maps to the 3' end of an intron of the CG13937 gene.

CG13937 is annotated in FlyBase as a HNK-1 sulfotransferase based upon its amino acid sequence, and is expressed in the larval and adult CNS. However, this gene has no known function. Only two piRNA loci map entirely within exons, both of which correspond to separate instances of the same short repetitive element. Instances of this element occur at several locations across the genome, including in the 3' UTR of

the Phosphoglycerate mutase 5-2 (Pgam5-2) gene, which resides on chromosome arm 2R. The only gene other than Pgam5-2 that this element maps to is the PHD finger protein 7 ortholog (phf7), where it falls within a protein coding exon (Fig 2.21).

Figure 2.21. A repetitive element produces piRNAs antisense to exons



Only one piRNA locus maps completely within exons. This piRNA locus corresponds to a short repetitive element found in several locations throughout the genome. A: The repetitive element generates piRNAs that are antisense to the 3' UTR of the Pgam5-2 gene. B: The same reads mapping to Pgam5-2 also map antisense to a protein coding region within 3' exons of the Phf7 gene.

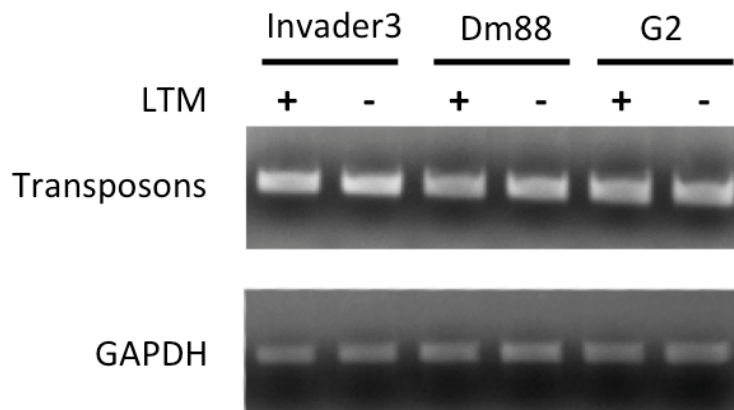
In both instances, piRNAs produced by this short repeat map in the opposite direction of the gene in which the repeat is present.

Changes in piRNA expression during LTM formation

Having identified sites of piRNA production across the genome, we next sought to determine if expression at any of these loci changes during LTM formation. Further, we hoped to resolve any distinct contributions of the US or CS to such changes. We used edgeR in its GLM mode to test for statistically significant differential expression between our experimental conditions. EdgeR analysis identifies 10 piRNA loci exhibiting significant changes in expression during in the LTM condition, but no changes in the shock only or odor only conditions are significant. When compared to control, all 10 of these loci have decreased read counts in the LTM condition. Further, all 10 significantly down-regulated piRNA loci correspond to intergenic repetitive elements. 2 transposons, Dm88 and Invader3, are present in the repetitive elements exhibiting significantly decreased read counts. Both belong to the long terminal repeat (LTR) class of retrotransposons. As such, decreased piRNA expression at these loci might result in increased expression of their corresponding transposons. As our libraries were prepared from size selected sRNAs, we used semi-quantitative RT-PCR to examine expression of these transposons in the same total RNA samples from which our sequencing libraries were prepared. We used random hexamers to prime the reverse transcription reaction, and primers directed at sequences in the Invader3 and Dm88 LTR

retrotransposons, as well as the non-LTR transposon G2 to amplify the cDNA. Primers directed at a GAPDH sequence were used as an internal control. In 3 replicates, expression of all 3 transposons examined remained essentially unchanged (Fig. 2.22).

Figure 2.22. Examination of transposon expression using semi-quantitative RT-PCR



Semi-quantitative RT-PCR was used to examine expression of the LTR retrotransposons Invader3 and Dm88, as well as the non-LTR transposon G2 during LTM Formation. GAPDH was used as an internal control for all reactions. In all cases, transposon expression remained essentially unchanged.

Discussion

Formation of lasting memories is known to involve synaptic plasticity mediated by changes in transcriptional programs and protein synthesis. sRNAs influence both of these mechanisms in a number of ways. A substantial and growing body of evidence demonstrates the critical importance of translational control via the microRNA pathway in regulating synaptic plasticity, and in memory formation. Much of what is known about the involvement of microRNAs in memory formation comes from studies of individual microRNAs. However, the many microRNAs

expressed in a single cell engage in complex and overlapping regulatory cascades. In many cases, target sites for multiple microRNAs are present within a single mRNA. Thus, a comprehensive understanding of the changes in gene expression driven by microRNAs during memory formation requires simultaneous observation of all microRNAs present in the brain. To our knowledge, this work is the first genome wide examination of microRNA expression during memory formation in *Drosophila*.

The profile of microRNA expression in the fly head that we obtained differs somewhat from that of Berezikov et al, which is perhaps the most comprehensive examination of microRNA expression in *Drosophila* to date.(35) In both cases, the most highly expressed mature microRNAs represent a large fraction of all microRNA reads. However, we find that miR-184-3p is the most highly expressed mature microRNA, accounting for ~36% of all raw microRNA reads, while Berezikov et al find that miR-184-3p is only the 30th most highly expressed mature microRNA, representing just ~0.3% of all raw mature microRNA reads. Similarly, Berezikov et al find that let-7 is the most highly expressed mature microRNA in head lysates, comprising ~25% of raw mature microRNA reads, while it is the 7th most abundant mature microRNA read in our libraries, representing just ~3% of such reads. In both studies, the 10 most highly expressed microRNAs constitute the overwhelming majority of mature microRNA sequences, representing ~90% and ~75% of mature microRNA reads in this study and in Berezikov et al., respectively. It is likely that both data sets suffer from cloning artifacts that could have been introduced at the

adapter ligation or PCR amplification steps. Such artifacts are known to be produced by the Illumina sRNA prep kit v1.5, which both studies utilized. Further complicating comparison of the two studies, the Berezikov et al data includes several libraries produced by the authors themselves, as well as several previously published head specific data sets. The libraries produced by the authors were prepared and sequenced similarly to our libraries, but those obtained from previous work were more varied, including libraries sequenced using 454 technology and others that had undergone beta-elimination. Thus, the overrepresentation of the most highly expressed microRNAs in the Berezikov et al data may be somewhat reduced by this diversity of source material, while the more uniform libraries in our study likely exacerbated such artifacts. However, when comparing mature microRNA expression between the libraries used in this study, we find that expression ranks are largely consistent. This suggests that if overrepresentation of certain microRNAs in our libraries is an artifact, it is systematic. Berezikov et al do not provide per-library counts, and it is therefore not clear how consistent microRNA expression is across the libraries they used.

In addition to profiling miRNA expression in the *Drosophila* head, we sought to identify changes in this profile resulting from LTM formation. Further, our use of the well characterized aversive olfactory classical conditioning paradigm allowed us to examine changes in microRNA expression uniquely induced by the CS or US, as well as those that result from the combination of CS and US during conditioning.

Again, this work is, to our knowledge, the first genome-wide examination of changes in microRNA expression to make such a distinction.

Using this approach, we show that miR-312-3p is the lone mature microRNA significantly down-regulated during LTM formation. While miR-312-3p expression is also reduced in the odor only and shock only conditions, our analysis did not find these changes to be statistically significant. We had 6 samples in the control condition, 5 samples in the LTM condition, but just 3 samples each in the shock only and odor only conditions. Thus, the lack of statistical significance in the odor only and shock only conditions may simply reflect the reduced power of our analysis in these groups. The reduction in miR-312-3p expression is more pronounced in the LTM and shock only conditions than in the odor only condition (-1.66, -1.32, and -0.71 log fold change in concentration respectively). These findings suggest that reduced miR-312-3p expression in the *Drosophila* brain during LTM formation results largely from signaling via the US circuitry, which involves dopaminergic signaling in the MB. We examined the predicted targets of miR-312-3p in the hope of identifying candidate genes for future study. Interestingly, miR-312-3p targets the dopamine receptor Dop1R1. Previous work demonstrates that both microRNA and dopamine receptor expression in the corresponding areas of the brain are required for LTM in *Drosophila*.⁽⁴²⁾ miR-312-3p also targets the octopamine receptor Octbeta3R. Octopamine signaling is known to be activated in the *Drosophila* brain during conditioning.⁽⁶¹⁾ Additionally, a recent publication by Wu et al demonstrates that octopaminergic circuitry in the *Drosophila* MB is involved in

ARM formation.(62) Lastly, miR-312-3p negatively regulates synaptic efficacy at the NMJ via silencing of kinesin heavy chain expression.(21) Thus, our finding that miR-312-3p is significantly down-regulated during LTM formation fits with a model in which LTM relevant neuronal activity increases synaptic efficacy in appropriate circuits in part by relieving key pathways from miR-312-3p mediated silencing. Additional experiments will be required to determine the validity of such a model, and to identify the neurons involved. We also identified 4 mature microRNAs up-regulated during LTM formation. miR-314-3p, miR-956-3p, miR-958-3p, and miR-958-5p all display statistically significant up-regulation in the LTM condition. Only miR-958-5p is significantly up-regulated in the shock only condition. No change in the expression of any microRNA is statistically significant in the odor only condition. However, of the microRNAs significantly up-regulated in the LTM condition, miR-958-5p increases the most in all three experimental conditions. Observed changes in expression of microRNAs in the odor only condition are generally smaller than in the shock only or LTM conditions. This may simply reflect the activation of a smaller set of neurons by odor than by shock. However, it may also reflect the nature of signaling activated, as the US is known to trigger activity in the dopaminergic circuitry of the MB. We generated our libraries from whole head lysates, and we are therefore unable to determine which cell types contribute to observed changes in microRNA expression. It is possible that a small number of cells are exhibiting large changes, or that a larger set of cells undergoes more modest changes. Further, we are unable to determine if all changes in microRNA expression occur in the same cells, or whether different cell types are responsible

for each significantly altered microRNA. However, previous studies suggest that alterations in neural activity in a single cell type can induce changes in the expression of many microRNAs simultaneously.(38) Nonetheless, results from this study point the way for future investigations using other methods better able to resolve such distinctions. Further, our data strongly suggest a number of genes for further investigation in the context of microRNA activity during memory formation. A number of mRNAs are targeted by more than one of the mature microRNAs that are significantly regulated in the LTM condition. mRNAs of 10 genes are targeted 3 or more of the 5 significantly regulated microRNAs that we identified. This set includes Dop1R1, the small conductance calcium activated potassium channel (SK), and No receptor potential A (NorpA), all of which directly modulate neural activity. Intriguingly, the dopamine receptor Dop1R1 is targeted by 3 out of 4 upregulated microRNAs as well as by miR-312-3p. This may reflect the importance of post-transcriptional regulation of Dop1R1 in memory formation. While it may seem contradictory that several microRNAs whose expression changes with the opposite sign following LTM training would target the same gene, the combined regulatory output of these microRNAs may work synergistically to facilitate olfactory memory. It may be that the up- and down-regulated microRNAs are expressed in different sets of cells. One could conceive that neurons involved in the memory trace might need enhanced sensitivity to dopamine, while those that are not require reduced dopamine sensitivity. Competitive regulation of Dop1R1 by these 4 microRNAs in the same cells might also have important effects. It is also important to note that, while Dop1R1 is targeted by 4 regulated microRNAs, only dme-mir-312-3p targets

Dop1R2 and Oct β 3R. Thus, the combined effect of dme-mir-312-3p and the 3 upregulated microRNAs that also target Dop1R1 may be to shift dopamine reception to Dop1R2 and to enhance sensitivity to octopamine. Determining which, if any, of these models is correct will require further experimentation. A key step will be determining the expression patterns of regulated microRNAs within the brain. Whether or not these microRNAs are expressed in the same cells will obviously affect the regulatory consequences of changes in their expression levels. Another key set of experiments will be to examine how response to dopamine is modulated by expression of these microRNAs, and to determine if such an effect is important for memory formation.

To better understand which pathways are subject to regulation by microRNAs whose expression change during memory formation, we conducted a gene ontology analysis on the sets of genes targeted by these microRNAs. We find that genes involved in G protein coupled signaling are overrepresented in this set. G protein coupled signaling regulates synaptic activity and efficacy in important ways, most directly through a variety of G protein coupled receptors present at synapses. MicroRNA mediated control of local translation could provide a way by which G protein coupled signaling is selectively tuned for subsets of the many synaptic connections in which a given neuron engages. Such selective alterations in neural circuits are integral to models of memory formation, but the molecular mechanisms involved are not fully understood. We also identified a number of genes annotated with the term “neurological systems process” that are targeted by more than one of

the microRNAs significantly regulated during LTM formation. Genes in these sets are obvious avenues for an in depth examination of the function of microRNAs in regulating synaptic plasticity and memory.

Though microRNA sequences are dominated by a few canonical mature microRNAs, a diversity of non-canonical microRNAs are present in cells.(33) Previous studies of microRNA involvement in *Drosophila* synaptic plasticity and memory have exclusively focused on canonical mature microRNAs. Our use of massively parallel sequencing allowed us to examine our reads for signs of changes in microRNA processing across our experimental conditions. Such changes might provide a mechanism through which microRNA activity could be regulated. We observed numerous instances of non-canonical 3' end formation for several microRNAs, but very few instances of non-canonical 5' ends for all but a few miRNA. These results largely resemble those reported for a similar analysis by Berezikov et al.(35) For instance, we observe that ~46% of miR-79-3p reads are 5' offset, while Berezikov et al report the rate to be ~30%. Some differences do exist between our results and those reported by Berezikov et al, but their analysis of non-canonical microRNA ends pooled reads from many tissue types, cell lines, and mutant strains.(35) The substantial similarity between the results of our non-canonical microRNA end analysis and those of Berezikov et al therefore supports the view that for most microRNAs, 5' end precision is strictly maintained. As the 5' seed sequence is the major determinant of microRNA target specificity, 5' precision is vital for proper microRNA function. The overwhelming precision of 5' ends we observed

shows that imprecise 3' cleavage, and not offset microRNA processing, is the major cause of non-canonical 3' ends present in our data. Though we document extensive 3' imprecision, we observe no statistically significant changes in microRNA processing between our experimental conditions. Massively parallel sequencing also permitted investigation of untemplated nucleotide addition to microRNA reads. Such modification is thought to reflect the activity of mechanisms that regulate the stability of microRNAs.(49, 63) We find that 5' tailing is extremely rare, and that 3' tailed reads represent a small fraction of microRNA reads, with only miR-927-3p having greater than 5% of its reads tailed. miR-927-3p tailing events are almost exclusively instances of mono- and poly-adenylation. miR-927-3p is the minor species produced from the mir-927 hairpin. For microRNAs expressed at high enough levels in both our work and head libraries from Berezikov et al, we find similar rates of 5' and 3' tailing.(35) Though we were able to observe 3' tailing of microRNAs, we did not observe any statistically significant changes in 3' tailing between our treatment conditions. Our analytical techniques exclude instances of 3' modification extending beyond 2nt. However, previous studies have demonstrated that microRNAs with untemplated extensions beyond 2nt also have a substantial number of 2nt tailing events.(45, 64) Thus, we believe that our methods allow us to obtain a representative view of 3' tailing events.

As massively parallel sequencing allows observation of untemplated nucleotides, we were able to search for instances of microRNA editing via ADAR activity. ADAR is known to influence expression of mRNAs in the brain, and

influences neuronal activity and behavior in *Drosophila*.(65, 66) Furthermore, modification of microRNA precursors by ADAR regulates expression of polycistronic microRNAs.(67) We observed what appears to be genuine A to I editing events in 7 microRNAs. Both our analysis and berezikov et al. identify a candidate editing event in miR-971.(35) However, the other 6 candidate editing events we identify and 3 candidate editing events identified by Berezikov et al in wild type head libraries do not overlap. This difference is likely do at least in part to our exclusive use of Canton-S flies, and the inclusion of libraries from Oregon-R fly heads in their analysis. Several of the candidate editing events we identify occur at positions within the mature microRNA sequence, and are therefore likely to influence the silencing activity of these microRNAs. However, published validated microRNA target predictions are based solely upon the canonical microRNA sequence. As de-novo target prediction or validation is beyond the scope of this work, we are unable to comment on the potential downstream regulatory consequences of these editing events. Instances in which editing falls outside of the mature microRNA sequence may lead to altered expression of mature microRNAs, but again we are unable to predict the outcome of such editing. None of the microRNA editing events we observed exhibited statistically significant differences between treatment groups. Thus, our data does not indicate that RNA editing is a major mechanism regulating microRNA activity during memory formation.

Taken together, our profile of microRNA sequences present in the *Drosophila* head, and characterization of the changes in these sequences resulting from

aversive olfactory classical conditioning, represent the most comprehensive view of microRNA response to long term memory formation in the insect brain to date. Our data provide clear direction for future work towards an understanding of microRNA involvement in memory. We recognize that our use of whole head lysates likely obscures subtle changes in many neurons, and perhaps significant changes in a few neurons. However, our inclusion of all cell types present in the brain at the least provides the opportunity to detect such changes, if they do occur. While we cannot rule out such possibilities, the statistically significant changes in microRNA expression that we were able to detect likely represent key regulatory events, and should be pursued further. To our knowledge, this study is the first detailed examination of non-canonical microRNA sequences in the context of insect memory formation. Though we did not identify any statistically significant changes in the classes of non-canonical sequences we examined in any of our treatment groups, the regulatory implications of observed microRNA editing events and other modifications are unknown, and may yet yield new insight.

Though others have previously profiled expression of esiRNAs or piRNAs in the *Drosophila* head, this study is, to our knowledge, the first examination of the expression of these classes of sRNAs during memory formation in insects. We identified a set of putative esiRNA loci and profiled sRNA expression at these loci in each of our treatment groups. Preliminary examination of our data revealed a substantial number of what appeared to be esiRNA reads outside of loci identified in previous studies. One such example occurs within the tkv esiRNA producing gene.

Czech et al report a large esiRNA peak corresponding to a region of overlap between tkv and the pseudogene CG14033, which are located on opposite strands.(57)

Though we too observe 21nt reads in this region of overlap, we do not see a large peak as reported by Czech et al. However, we observe a large number of 21nt reads mapping to both strands at the 3' end of the tkv gene, which is in a cis-NAT arrangement with the TpnC25D gene. Such a signal is the characteristic signature of a cis-NAT esiRNA locus. Czech et al do not report a peak in reads at this cis-NAT region. This discrepancy is likely due to our use of head lysates in this study and the use of testis by Czech et al. The paucity of reads we observe at the CG14033 overlap may result from preferential use of the tkv-B and tkv-D isoforms in heads, whose transcription start sites are 3' of the tkv sequence overlapping CG14033, and therefore do not yield transcripts complementary to CG14033. Alternatively, the CG14033 pseudogene may not be strongly coexpressed with tkv in the head. Similarly, the absence of a peak at the region of overlap between tkv and TpnC25D in the Czech et al data may result from a lack of tkv/TpnC25D cotranscription in the testis.

Many studies reporting the genomic locations of esiRNA production use RNAs extracted from gonads, as *Drosophila* esiRNAs were first recognized in studies of sRNAs expressed in these tissues. We reasoned that reliance on previously reported esiRNA loci might therefore exclude potentially interesting head specific loci, or loci only revealed by neural activity induced during classical conditioning. Accordingly, we performed de novo esiRNA locus identification. Our identification

of several previously unreported esiRNA loci within lysozyme family genes prove the value of this step. Our differential expression analysis shows that the lysozyme family loci are the only esiRNA producing regions mapping at or near genes that whose expression changes significantly during LTM formation. While reads are present at these loci in the control condition, read counts increase dramatically in the LTM and shock only conditions. These changes are highly significant ($p \leq 0.0005$). Read counts at these loci also increase in the odor only condition, but this increase is smaller, and not statistically significant. Differential expression analysis shows that, though read counts are higher in the shock only condition than in LTM, the difference between LTM and shock only is not statistically significant. This indicates that neural activity driven by the US (shock) is responsible for the observed increase in lysozyme family esiRNA expression. This circuitry has been characterized in the *Drosophila* brain, and is largely dopaminergic. Accordingly, this population of cells is an obvious target for investigation into the significance of lysozyme family esiRNAs.

LysC and LysD are in a cis-NAT arrangement, and all lysozyme family genes exhibiting increased read counts in the LTM and shock only conditions share stretches of sequence identity. One might therefore expect that reads mapping to these lysozyme family genes are the product of LysC and LysD cotranscription. However, careful examination of read coverage across the LysC/LysD locus reveals an unusual signature in which read coverage is dramatically higher in the non-overlapping segments of these genes than in the region of overlap. Long inverted

repeats that form RNA hairpins are known to produce functional esiRNAs in the region of perfect duplex, but not from the hairpin loop.(59) Such an arrangement might better explain the pattern of reads at the *LysC/LysD* locus than a cis-NAT mechanism. To explore such a possibility, we predicted secondary structures for several conceptual RNAs spanning this locus. In all cases, the RNAs formed extensive stretches of perfect duplex at sequences corresponding to the areas of the *LysC/LysD* locus producing the most reads. Further, the overlapping portion of the *LysC/LysD* locus, which produces few reads, corresponded to the hairpin loop and adjacent bulged duplexes. These observations argue strongly that esiRNA like reads at the *LysC/LysD* locus are produced via the hairpin pathway, and not by a cis-NAT mechanism. Most of the reads mapping to *LysB* and *LysE* also map within the *LysC/LysD* locus. *LysB* and *LysE* reads could primarily be of *LysC/LysD* origin. We noted that the pattern of coverage peaks across *LysB* and *LysE* also resemble the pattern of peaks across the *LysC/LysD* locus. However, the patterns of peaks do not match closely enough to be explained simply by multi-mapping reads. This observation raised the intriguing possibility that a much longer progenitor RNA spanning the *LysB – LysE* region might form duplexes that yield esiRNAs via the hairpin mechanism. Indeed, Mfold predicts hairpin structures spanning this region, with duplexes between *LysC/LysD* and *LysB* or *LysE*. *LysC/LysD*, *LysB-LysC/LysD*, and *LysE-LysC/LysD* duplexes are mutually exclusive, as these structures involve overlapping segments of *LysD*. No single structure out of those just discussed is capable of producing the observed pattern of read coverage across the *LysB-LysE* region. Thus, It is likely that esiRNA production from this region involves a

combination of several hairpin structures. LysS is located too far away from the other lysozyme family genes in this region to permit structure prediction using Mfold. However, we note that the coverage peak mapping within LysS corresponds to a handful of unique read sequences that are also present in the LysB-LysE region. It is therefore likely that reads mapping to LysS are actually the result of esiRNA production from LysB-LysE hairpins, and not from LysS itself. The regulatory function of esiRNA production from the LysB-LysE region is unclear. esiRNAs produced from this region should downregulate LysB, LysC, LysD, LysE and LysS. However, lysozymes primarily function in defense against bacterial infection by catalyzing the hydrolysis of peptidoglycans present in bacterial cell walls. As such, the reason that reduced Lysozyme expression would be needed during LTM formation is not obvious. An alternate explanation for increased LysB-LysE esiRNA production during LTM formation could be that the increase reflects increased expression of the mwh-B or mwh-C isoforms. These isoforms both include the intron that spans the full LysB-LysS region, while the TSS for the mwh-A isoform is downstream of LysB, and therefore does not include this intron. Mwh is a G-protein binding domain-formin homology 3 (GBD-FH3) protein in the frizzled pathway that negatively regulates actin polymerization and filament formation, and is a planar cell polarity (PCP) effector.(68, 69) Thus, one possible interpretation of this result is that increased production of LysB-LysE esiRNAs reflects regulation of mwh in the course of reorganizing the cytoskeleton to accommodate altered neural connectivity. Mwh has been studied largely in the context of PCP during wing hair formation, and no role for mwh in synaptic plasticity has yet been demonstrated.

However, as a regulator of the cytoskeleton, *mwh* is a more obvious target for regulation in the course of memory formation than are lysozyme family genes. This possibility should be further investigated, as it would be the first instance of an esiRNA mediated change in gene regulation in response to memory formation documented in insects. Further, such investigation would require exploration of the possibility that lysozyme family genes are regulated during memory formation. While a negative result would support *mwh* involvement in synaptic plasticity, a positive result would necessitate a reexamination of the functions of lysozyme genes in the *Drosophila* head. Both possibilities provide the potential for novel insights into the basic mechanisms of memory.

Our methods also allowed us to identify genomic regions that produce sequencing reads with characteristics typical of piRNAs, including length distribution and a 5' uridine bias. However, our study did not include methods such as β -elimination that would more definitively categorize these reads as genuine piRNAs. Nonetheless, the fact that many of our piRNA loci correspond to those identified in studies that did include these steps gives us confidence that our methods yield meaningful piRNA profiles.(33, 54) We observe only subtle changes in piRNA profiles in the LTM treatment group, and insignificant changes in the odor only or shock only conditions. Those piRNA loci exhibiting statistically significant changes in the LTM condition exclusively mapped to repeats and LTR retrotransposons. In all such instances, reads decreased in the LTM condition vs. control. None of these loci produce reads that map perfectly to mRNAs or to known

regulatory elements. Furthermore, semi-quantitative RT-PCR did not detect significant changes in the levels of transcripts corresponding to these LTR retrotransposons. Thus, there are no obvious consequences for gene or transposon regulation stemming from changes in piRNA expression at these loci. We did however identify piRNA loci with interesting features, perhaps warranting further investigation. In one instance, a 26nt read uniquely maps to the 3' end of an intron of the CG13937 gene. This read does not correspond to a repeat or transposon, and is therefore atypical for a piRNA, if it is in fact loaded into piwi proteins. The 3' end of the read maps precisely to the 5' end of a CG13937 exon, suggesting that this might be a miRtron. However, at 26nt, this read is longer than would be typical for a microRNA, and this sequence is not found in the miRbase catalog of *Drosophila* microRNAs, which includes miRtrons. Again, it is difficult to speculate on what regulatory significance this sequence has. Another interesting piRNA locus corresponds to a short repeat found within exons of the Pgam5-2 and Phf7 genes. In both cases, the read is antisense to the overlapping gene. While no statistically significant change in the expression of this read is observed in any of our treatment conditions, the unique characteristics of this locus may warrant further study. Rajasethupathy et al. show that piRNAs are expressed in *Aplysia* neurons, and are regulated by memory relevant neurotransmitters.(5) Serotonin exposures that induce LTP result in downregulation of the transcriptional repressor CREB2. This occurs via piwi and DNA methyltransferase (DNMT) dependent methylation of the CREB2 promoter. Antisense inhibitors of piRNAs mapping at the translational start site of CREB2 relieve this repression. Thus, their results support the idea that

piRNAs are involved in memory relevant gene regulation, and lead us to search for evidence of such a mechanism in our data. However, differences exist in the piRNA pathways of *Aplysia* and *Drosophila*. Furthermore, DNA methylation in *Drosophila* appears to occur at different locations, and via a mechanism that differs substantially from those of other model organisms. *Drosophila* do not possess a DNMT-1 homologue, and DNMT-2 is dispensable for methylation of their DNA.(70) Thus, it is possible that no mechanism analogous to piwi/piRNA mediated CREB2 regulation in *Aplysia* exists in *Drosophila*. piRNA function in the fly brain remains poorly studied, and may yet prove relevant to memory. Though our methods failed to detect changes in expression of piRNAs that would have obvious implications for gene regulation, they may have limited sensitivity. Therefore, our results do not preclude the possibility that piRNAs mediate aspects of memory formation in *Drosophila*.

Expanding our examination of sRNA expression in the fly head beyond microRNAs to include esiRNAs and piRNAs allowed us the first view of how these classes of sRNAs respond during memory formation in insects. Though we observe few statistically significant changes, our data hint at previously unidentified regulatory functions for these sRNAs, and at novel ways in which gene regulation is altered by neural activity and memory formation. The extensive conservation of the esiRNA and piRNA pathways indicates that both have vital functions in animal biology. Though much has been learned about their roles in defense of the genome, a great deal remains to be understood about how they influence gene expression.

Further, much of what is known about esiRNAs and piRNAs comes from studies in *Drosophila* gonads, tissues with unique properties that may prohibit the generalization of findings from these studies to cell types from other organs. As such, a great deal of work remains to be done before these sRNA classes are fully understood. Due to its central role in the discovery of esiRNAs and piRNAs, and the available repositories of sequencing data produced in those efforts, *Drosophila* remains one of the best model organisms in which to study these classes of sRNAs. This study is one of the first attempts to examine how esiRNA and piRNA expression changes in response to the demands of altered physiology in wild type animals. The genetics of learning, memory, and behavior are perhaps better understood in *Drosophila* than in any other model organism. Accordingly, the fly brain will likely prove to be a productive system in attempts to identify the possible functions of esiRNAs or piRNAs in regulating gene expression in neurons. Results from this study support such a view.

Defects in posttranscriptional control of gene expression are emerging as the underlying causes of many diseases of the brain and nervous system. sRNAs are central players in mechanisms that govern the stability, localization, and translation of mRNAs. Numerous cases of individual microRNAs required for normal neural function, memory, and behavior have been identified in many model organisms, and in humans. Yet we know that microRNAs do not operate in isolation. As such, a better understanding of the functions of microRNAs in these processes will require genome wide approaches. Further, the roles that esiRNAs and piRNAs play in

neurons remain poorly understood. As few cases of individual genes strongly regulated by these sRNA classes have been identified, let alone well characterized, esiRNAs and piRNAs may still be best studied through genome wide surveys of their expression in various experimental settings. This study is an early attempt to address these needs. Future work with a tighter focus on specific cell types, sRNA/target pairings, or other refinements may better inform our understanding of sRNA biology in neurons, and in memory formation more broadly. Indeed, as this study does not include experiments examining sRNA/target interactions, or behavioral studies in which the regulated sRNAs we identify are misexpressed, we can not draw direct conclusions about the relevance to LTM of the changes in sRNA expression we observe following conditioning. However, this study does provide foundational information, based upon which such experiments can be designed.

Materials and Methods

Drosophila rearing

CS-Quinn flies were reared on standard cornmeal medium at 25 °C under a 12hr light/dark cycle. 1 Day old adults were used in all experiments.

Training and memory assay

Training was conducted using a semi automated conditioning apparatus as described in (40). Briefly, a single batch of flies was split into one of four groups: Control, odor only, shock only, or shock + odor (LTM), and housed in bottles containing no food for one hour prior to training. Flies were then loaded into vials that permit the flow of air from the apparatus and contain electrode grids. Odor and shock delivery are controlled by a computer. Following training, flies were returned to bottles containing no food, and allowed to rest for 2 hours before dissection.

Memory formation in LTM flies was evaluated using a T-maze apparatus as described in (13). The performance index (PI) was calculated as the number of flies avoiding the conditioned odor minus the number of flies avoiding a control odor divided by the total number of flies in the experiment.

Tissue processing and total RNA extraction

For each sample, heads of 10 male and 10 female flies were dissected and immediately flash frozen and stored at -80 °C while awaiting results from memory testing. Only samples from matched batches of flies in which the LTM trained flies

had a PI ≥ 20 were used in subsequent steps. Heads were homogenized in Qiazol (Qiagen) using a motorized tissue homogenizer. Total RNA was extracted using the Qiagen miRNEasy micro kit, according to the manufacturer's protocol. RNA concentration was obtained using a Nanodrop and Bioanalyzer. RNA quality was determined by Bioanalyzer analysis.

sRNA sequencing

Prior to sequencing library preparation, total RNA used for sRNA sequencing was size selected by PAGE, as recommended in the Illumina sRNA sample prep kit (v1.5) protocol. Gel slices containing only 15-35nt RNAs were excised and used in downstream library preparation steps. sRNA sequencing libraries were prepared using the Illumina sRNA sample prep kit (v1.5) according to the manufacturer's protocol. Library quality and concentration were determined by Bioanalyzer analysis. sRNA sequencing was conducted by Harvard systems biology core facility staff, using an Illumina Genome Analyzer IIX.

Sequencing data analysis

Adapter sequences were removed using the FASTX toolkit. For microRNA analysis, sequencing reads were aligned to miRBase release 19 Drosophila hairpin sequences using Bowtie v0.12.7.(71, 72) Custom software written in the R and AWK languages were used for canonical and isomiR analysis. For esiRNA and piRNA analysis, reads were aligned to the FlyBase Drosophila melanogaster genome (v5.48) using Bowtie v0.12.7.(71). SAMtools was used to convert alignments to BAM format.(73)

BedTools v2.18.2 was used to count reads aligning within a given feature, and to merge reads into read-contigs.(74) The UCSC genome browser was used to visualize sequencing data.(75)

Differential expression analysis

The Bioconductor package edgeR v3.1.9 was used in its GLM mode for read count differential expression analysis. Read counts were normalized using the upper quartile method.(76) For isomiR analysis, the percentage of normalized reads with a given non-canonical feature was computed for each miRNA. Two tailed students t-tests were used to test for statistical significance of differences in these percentages. P-values were then adjusted for multiple testing using the Holm-Bonferroni method.

Target prediction

DIANA microT v4 predictions were used for microRNA target analysis.(24)

Gene ontology analysis

The PANTHER gene ontology (GO) database was used to assign GO terms and search for over/underrepresented terms.(44)

RNA secondary structure prediction

Mfold was used to predict RNA secondary structures.(60)

Semiquantitative RT-PCR

First strand cDNA was reverse transcribed from 500ng input total RNA, using random hexamer primers (Invitrogen) and the Superscript II kit (Invitrogen). Reverse transcription occurred at 42 °C for 1 hour. Oligonucleotide pairs spanning transposon sequences were used to prime 20 cycles of PCR amplification. Perfect Taq (5 prime) polymerase was used for PCR amplification.

Primer sequences:

Gapdh-FWD: AGCTGATCTCTTGGTACGACAAC
Gapdh-REV: ATGCTTATGAGTCGGCATTTTTA

Inv3-FWD: CCTTTAGCCAACTTCACGACGG
Inv3-REV: GGAATTCGAATTGCCCTAACGG

G2-FWD: GGCAATCAAACTCTCACGGATG
G2-REV: GGGGATTTGCTAGCCTTTAGG

DM88-FWD: GGATACTCTGATGCTTCTAAGGG
DM88-REV: CACTGCAAAGACCCATTTTGAC

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Chapter III

Beta-Site APP-Cleaving Enzyme Is Required For Long Term Memory In *Drosophila*

Summary

Alzheimer's disease (AD) and related dementias are among the most widespread age related neurodegenerative diseases. Brains of AD patients exhibit tauopathy, accumulation of senile plaques, and cell death, resulting in progressive memory impairment and cognitive defects. Senile plaques are largely composed of a peptide termed amyloid-beta ($A\beta$), which is derived from cleavage of the Beta-Amyloid Precursor Protein (APP) by membrane-bound aspartic proteinases termed β -secretases. Such cleavage and resultant $A\beta$ production were long thought to be essentially toxic, and to be the central disease mechanism underlying AD. However, recent work demonstrates that AD symptoms such as dementia are not always coupled to the presence of senile plaques in humans, and that $A\beta$ is required for LTP in the mouse hippocampus. *Drosophila* express homologues of all of the genes thought to be central in AD progression. Using aversive olfactory classical conditioning, I show that LTM formation upregulates the *Drosophila* β -secretase dBACE, resulting in increased proteolytic processing of the APP homologue APP-Like (APPL). Further, using inducible RNAi knockdown of dBACE in the brain, we demonstrate that dBACE is required for LTM, but not for learning, or for STM.

Introduction

Memories are encoded in the synaptic connections of the brain's neural circuitry. An organism's ability to retain learned information over long periods of time, while remaining capable of acquiring new memories, requires a finely tuned balance of mechanisms that build, destroy, strengthen and weaken synapses. Misregulation of these processes can have profound effects on health, and can alter memory, cognition, and behavior. AD and related dementias are examples of how defects in the molecular mechanisms governing synaptic plasticity can cause profound changes in mental health. Common features of AD brains are tauopathy and the accumulation of the APP cleavage product A β into senile plaques. Tauopathy is a feature of many neurodegenerative diseases, while senile plaques are closely associated with Down syndrome and AD, both of which affect memory and cognition. As such, understanding the function of APP and its processing have been major areas of research into AD. Previous studies reveal that APP influences synaptic plasticity in complex ways, and that APP expression and processing must be exquisitely controlled for proper synaptic connectivity and memory.

The APP pathway is evolutionarily conserved from insects to humans. However, rodent A β does not form amyloid deposits. As such, researchers are forced to use exogenous human APP or A β in studies of AD in mice and rats. Recently, interest in *Drosophila* as a model organism in which to study the APP pathway and AD has grown, following the discovery that proteolytic processing of

the fly homologue of APP (APPL) yields a peptide functionally similar to A β (dA β).⁽¹⁾ The *Drosophila* APPL pathway appears to function similarly to the human APP pathway, and many of the components from both species are mechanistically compatible. Expression of human APP rescues memory defects exhibited by *Drosophila* mutants of the APP homologue APPL.⁽²⁾ The APPL processing machinery present in *Drosophila* cleaves human APP much as would occur in its endogenous setting. Overexpression of human A β in *Drosophila* leads to formation of aggregates resembling senile plaques, neurodegeneration, and memory defects. Co-overexpression of APPL and dBACE also leads to production of aggregates resembling senile plaques, despite the fact that APPL does not contain a sequence similar to A β . This demonstrates that the APP pathway is functionally conserved, even though some amino acid sequences of its components are not.⁽¹⁾ These findings and others show that *Drosophila* can serve as a model organism for understanding APP family protein metabolism and its function in synaptic plasticity and memory (reviewed in (3, 4)).

APPL undergoes proteolytic processing along two pathways, each initiated by a characteristic cleavage event. Processing of APPL initiated by dBACE is considered amyloidogenic, as this pathway results in dA β production and formation of deposits resembling senile plaques. APPL cleavage by dBACE liberates a soluble extracellular N-terminal fragment, and a membrane bound C-terminal fragment (β CTF). Subsequent cleavage of β CTF within its transmembrane domain by the γ -secretase presenillin (Psn) produces dA β . Due to the high degree of sequence

conservation within the CTF and functional conservation of the pathway, it is presumed that cleavage of β CTF by Psn also releases the APPL intracellular C-terminal domain (AICD). *Drosophila* AICD contains a highly conserved G_o-interacting domain, and the perfectly conserved YENPTY internalization sequence.(5) The high degree of sequence similarity between *Drosophila* and human AICD supports the assumption that they are functionally similar as well. In humans, AICD can translocate to the nucleus and induce transcriptional changes.(3, 4) Though AICD production has been demonstrated in *Drosophila*, the resultant downstream effects remain uncharacterized.(6) Cleavage of APPL by the α -secretase Kuzbanian (Kuz) also liberates the N-terminal extracellular domain, and yields a C-terminal fragment (α CTF) that appears unable to generate dA β or aggregates.(1) The APPL processing pathway initiated by Kuz is therefore termed nonamyloidogenic. In humans, APP processing is overwhelmingly initiated by α -secretase. However, AICD production is largely unaffected by α -secretase inhibitors. Furthermore, β -secretase initiated processing leads to greater nuclear accumulation of AICD.(7-9) These differences in AICD derived from α - γ -processing or β - γ -processing likely result from spatial segregation of α -secretase and β -secretase activity primarily to the cell surface and recycling endosomes, respectively. AICD is rapidly degraded in the cytosol. However, endosomes are transported along microtubules to the perinuclear area. AICD liberated by γ -secretase cleavage in this region is therefore less likely to be degraded before entering the nucleus than is AICD produced at the cell surface. This model is supported by the finding that manipulations driving endosome localization to the cellular periphery reduce AICD

nuclear accumulation.(7) Localization of β -secretase to endosomes also provides a mechanistic link between neural activity and AICD production. Action potentials trigger fusion of neurotransmitter containing vesicles with the plasma membrane, and subsequent clathrin dependent recycling endocytosis. This process leads to colocalization of APP previously present at the plasma membrane and β -secretase already residing within endosomes.(10) Thus, neural activity drives β - γ -secretase processing of APP. The functional conservation of the APP pathway in *Drosophila* suggests that β - γ -processing of APPL may also be stimulated by neural activity. Activity induced colocalization of dBACE and APPL could shift processing of APPL toward the amyloidogenic pathway, but this shift would be limited to the duration of increased synaptic vesicle release and recycling, were no other modulation of the pathway to occur. APPL levels and the relative abundance of its various metabolites can profoundly alter synaptic number and structure.(5) How APPL levels and the balance of the amyloidogenic and nonamyloidogenic pathways are altered or maintained remain poorly understood, and largely unstudied in *Drosophila*.

Here, I present results from our study of APPL processing in the course of LTM formation. This work was initiated after sRNA sequencing from head lysates of flies that had been subjected to olfactory aversive classical conditioning revealed a class of genes with an unusual sRNA signature. Genes in this class have a high degree of read coverage on the coding strand of exons. We therefore conclude that this signal is derived from mature transcripts, and designate this set as high exon coverage transcripts (HECTs). sRNA reads mapping to HECTs do not have features

of known sRNA classes such as esiRNAs, piRNAs, or microRNAs. A subset of HECTs display statistically significant changes in the HECT signal following conditioning, hinting at memory relevant regulation. Amongst regulated HECTs, dBACE has the largest change. I show that the increase in the dBACE signal is linked to increased dBACE expression and APPL processing. dBACE expression increases rapidly following LTM training, and remains elevated 24 hours later. This study is, to my knowledge, the first demonstration of activity dependent regulation of dBACE expression. I examine regulated HECTs for common sequence features, and show that intronless genes are overrepresented amongst upregulated HECTs, and underrepresented amongst downregulated HECTs. I show that most regulated HECTs carry a sequence element known to facilitate nuclear export of intronless transcripts via a pathway that involves components of the splicing machinery. I also present work from our lab demonstrating that dBACE expression is required for LTM, but not for short term memory (STM) or for learning. As far as we are aware, this is the first report of a requirement for dBACE in memory formation.

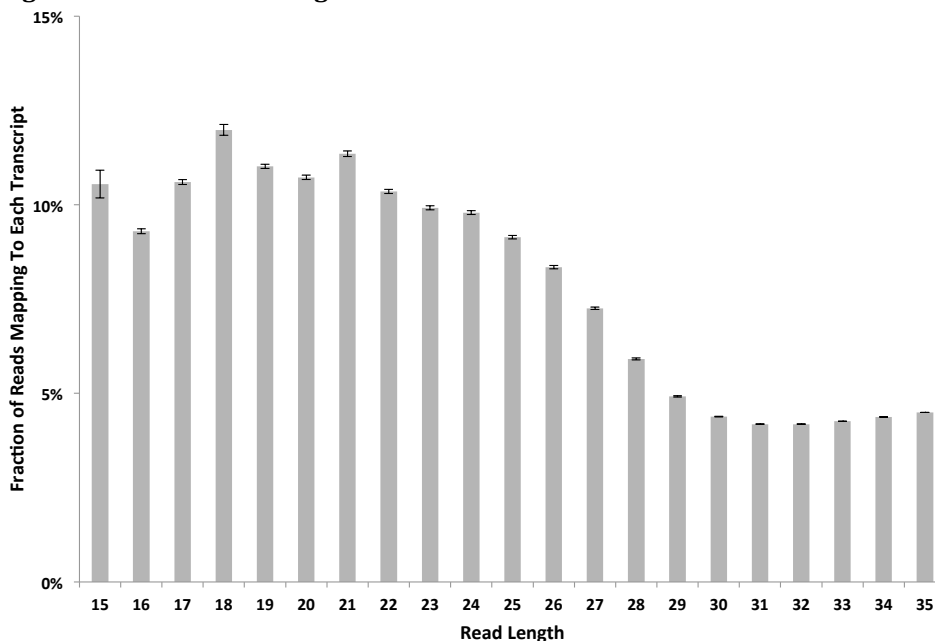
Results

sRNAs are produced from highly expressed transcripts

In chapter II, I describe experiments directed at characterizing changes in sRNA expression during LTM formation. In this effort, we conducted massively parallel sequencing of 15-35nt sRNAs extracted from heads of *Drosophila* subjected to aversive olfactory classical conditioning, or to the component stimuli (odor or electric shock) used in such training. While the primary goal was to profile microRNAs, esiRNAs and piRNAs, we also hoped to identify novel sRNA signals in our data. We aligned our sequencing reads to the *Drosophila* genome such that no mismatches were allowed, and all perfect alignments were retained. We then visualized these alignments using the UCSC genome browser.⁽¹¹⁾ Superficial visual inspection of our data using the UCSC browser revealed a set of genes exhibiting near complete read coverage across exons on the coding strand, and a near complete absence of reads mapping to introns. Further, a subset of these high exon coverage genes exhibits a large increase in reads vs. control in the LTM and/or shock only conditions. The near exclusive mapping of reads to exons of these genes suggests that these reads are derived from spliced mRNAs. We therefore remapped our reads to all spliced transcripts annotated in FlyBase. This step ensured that reads spanning exon junctions would be captured, and allowed us to more easily compare transcripts that include overlapping sets of exons. The hallmark features of high exon coverage transcripts (HECTs) are near complete coverage on the

coding strand, and a high sense:antisense read count ratio. Accordingly, we established $\geq 95\%$ read coverage, and a 10:1 sense:antisense read count ratio as parameters for designating transcripts as HECTs. We noted that some HECTs exhibit few reads at all in some experimental conditions. We therefore combined the reads from all of our sequencing libraries for the purposes of classifying transcripts as HECTs, so as to ensure that those HECTs displaying a dramatic difference in read counts between conditions would also be included in our subsequent analysis. These steps yielded 780 HECTs representing 465 genes (Figure S3.1). We next sought to determine if HECT reads had characteristics of known sRNA classes. Defined size ranges are a feature of known sRNAs. Accordingly, we computed read length distributions for each HECT. HECT reads have a much broader length distribution than miRNAs, which are 21~24nt, esiRNAs, which are almost exclusively 21-22nt, or piRNAs, which are largely 24-29nt (Fig 3.1).

Figure 3.1. Mean read length distribution for HECTs



Sequencing reads from all libraries were aligned to all FlyBase transcripts such that no mismatches were allowed, and all perfect alignments were retained. Those transcripts with $\geq 95\%$ coverage on the coding strand and $\geq 10:1$ sense:antisense read count ratio were designated as HECTs. Read length distributions were then computed for each HECT. HECT reads have a broad length distribution, unlike known sRNA classes. Bars represent mean read length distribution for HECTs. Error bars represent SEM.

This analysis indicates that HECT reads do not correspond to known sRNA classes.

We note that many HECT genes are ubiquitously expressed, including Gapdh1, Gapdh2, Act5C, several ribosomal proteins, ATP synthases, and mitochondrial cytochrome c oxidase subunits. To test if ubiquitously expressed genes are in fact overrepresented in the set of HECT genes, we conducted gene ontology (GO) analysis using PANTHER.(12) This analysis shows that ribosomal proteins, Krebs cycle components, and cytoskeleton genes are overrepresented in the HECT gene set, while transcription factors are underrepresented (Figure 3.2).

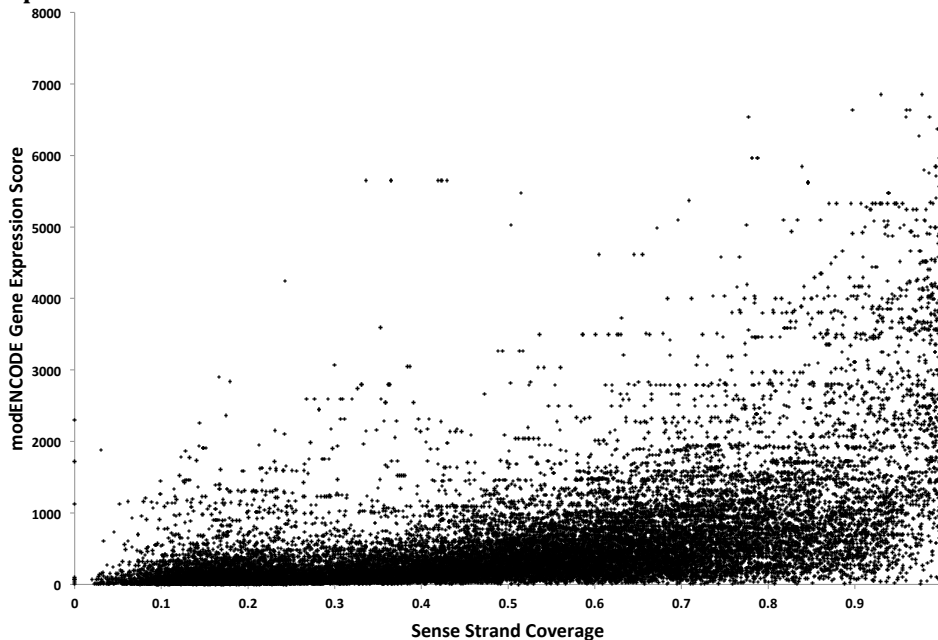
Figure 3.2. PANTHER gene ontology analysis of HECT genes

| PANTHER Protein Class | Drosophila melanogaster | HECT Genes | Expected | over/under | P-value |
|-----------------------------------|-------------------------|------------|----------|------------|----------|
| ribosomal protein | 164 | 61 | 5 | + | 2.08E-43 |
| oxidoreductase | 619 | 63 | 18.86 | + | 1.51E-14 |
| dehydrogenase | 252 | 36 | 7.68 | + | 7.77E-12 |
| ATP synthase | 44 | 13 | 1.34 | + | 3.23E-07 |
| nucleic acid binding | 1520 | 87 | 46.3 | + | 1.09E-06 |
| transcription factor | 740 | 3 | 22.54 | - | 3.95E-05 |
| actin family cytoskeletal protein | 169 | 20 | 5.15 | + | 7.48E-05 |

HECT gene GO annotations were obtained and tested for over/underrpresentaion using the PANTHER database. Ribosomal proteins, Krebs cycle components, and cytoskeleton genes are significantly overrepresented in HECT gene protein class GO annotations, while transcription factors are significantly underrepresented. P-values include Bonferroni correction for multiple testing.

This lead to the speculation that HECT reads might represent turnover products of highly expressed transcripts. We obtained gene expression level scores for all transcripts from modENCODE, and compared these scores to transcript coverage on the sense strand (Fig 3.3).

Figure 3.3 Comparison of sense strand read coverage and modENCODE gene expression score



Comparison of sense strand coverage and modENCODE gene expression scores. Sense strand read coverage from our combined libraries was computed for each FlyBase transcript. Gene expression scores for each transcript were obtained from modENCODE. Spearman rank correlation analysis shows that sense strand coverage is correlated with modENCODE gene expression scores ($\rho = 0.59$, p -value $< 2.2e-16$).

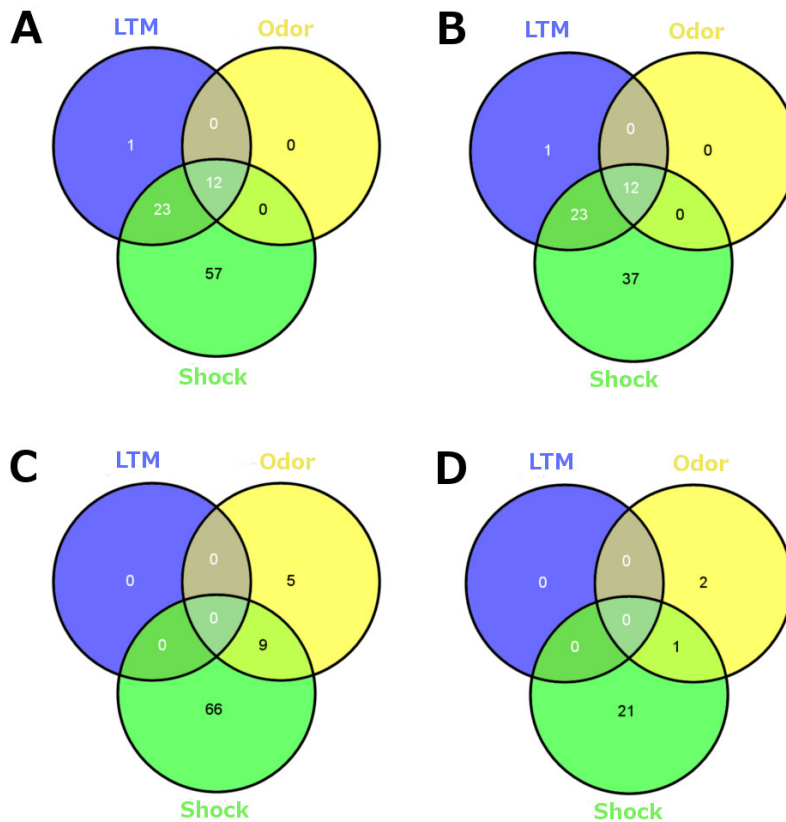
Spearman rank correlation analysis shows that sense strand coverage is correlated with modENCODE gene expression score ($\rho = 0.59$, $p\text{-value} < 2.2e-16$). Though this analysis alone does not allow us to conclude that HECT reads represent turnover products of highly expressed transcripts, it does not contradict such a view.

All three experimental treatments induce significant changes in HECT read counts

If the HECT signature does represent turnover of highly expressed transcripts, then the subset of HECTs displaying large differences in read counts between our experimental conditions might reflect gene regulation in response to neural activity. To explore this possibility, we first tested HECTs for statistically significant differences in read counts from control using the edgeR software package.⁽¹³⁾ 93 HECTs, representing 73 genes, display significantly increased read counts in the LTM, shock only or odor only conditions. 80 HECTs, representing 25 genes, display significantly decreased read counts in these experimental groups (Figure S3.2). While the shock only condition has the largest set, substantial overlap exists between experimental conditions in the sets of HECT genes with statistically significant differences in read counts from control (Fig 3.4). CG13324 is the lone HECT gene that has significantly increased reads only in the LTM condition. CG13324 reads also increase in the shock only condition, but this increase is not statistically significant. CG13324 is predicted to produce a 12.6kD polypeptide, but has no known function or conserved domains. No HECT genes have an increase in reads uniquely in the odor only condition. 37 HECT genes display a significant read

increase uniquely in the shock condition. All of these 37 HECT genes also have increased read counts in the LTM condition, though the increases in LTM are not statistically significant. 24 HECT genes have significantly decreased read counts in any condition, 21 of which are significant only in the shock only condition. No HECTs have significantly decreased read counts in the LTM condition.

Figure 3.4. Overlap between sets of HECT genes significantly regulated in each experimental condition.



HECT read counts were tested for significant differences from control using the edgeR software package. Venn diagrams representing overlap of HECTs or HECT genes found to differ significantly from control in each condition. A: Significantly upregulated HECTs. B: Significantly upregulated HECT genes. C: Significantly Downregulated HECTs. D: Significantly Downregulated HECT genes.

Proteases are enriched in the set of HECT genes with increased read counts in the LTM condition

We repeated our GO analysis on the set of HECT genes upregulated in any experimental condition. Genes involved in proteolysis are significantly overrepresented in this set (Fig 3.5).

Figure 3.5. Overrepresented molecular function annotations in the set of HECT genes significantly upregulated in any condition.

| Molecular Function | Drosophila melanogaster | Up In Any Treatment Group | Expected | Over/Under | P-value |
|--|-------------------------|---------------------------|----------|------------|----------|
| serine-type peptidase activity | 301 | 15 | 1.59 | + | 8.10E-09 |
| hydrolase activity | 1629 | 29 | 8.61 | + | 1.67E-07 |
| peptidase activity | 591 | 17 | 3.12 | + | 1.48E-06 |
| structural constituent of ribosome | 157 | 8 | 0.83 | + | 3.05E-04 |
| hydrolase activity, hydrolyzing O-glycosyl compounds | 31 | 4 | 0.16 | + | 3.85E-03 |
| amylase activity | 15 | 3 | 0.08 | + | 1.19E-02 |
| catalytic activity | 3727 | 35 | 19.7 | + | 1.58E-02 |

Significantly overrepresented molecular function annotations for the set of HECT genes significantly upregulated in any condition were obtained using PANTHER. Genes annotated with molecular terms related to proteolysis are significantly overrepresented.

Nearly half (11 out of 23) HECT genes with significantly increased reads in both the LTM and shock only conditions, but not in the odor only condition, are annotated as serine type endopeptidases. Reads significantly increase for 12 HECT genes in all three experimental conditions vs control. 10 of these HECT genes have known or predicted functions, 8 of which are proteases. Ribosomal proteins are significantly overrepresented in the set of HECT genes with significantly increased reads only in the shock only condition. Many HECT genes with significantly decreased read counts in any condition are cytoskeletal or actin binding genes. Most of these genes have fewer reads in all conditions than those with significant increases.

Intronless genes are overrepresented in the set of HECT genes with significantly increased reads in the LTM condition.

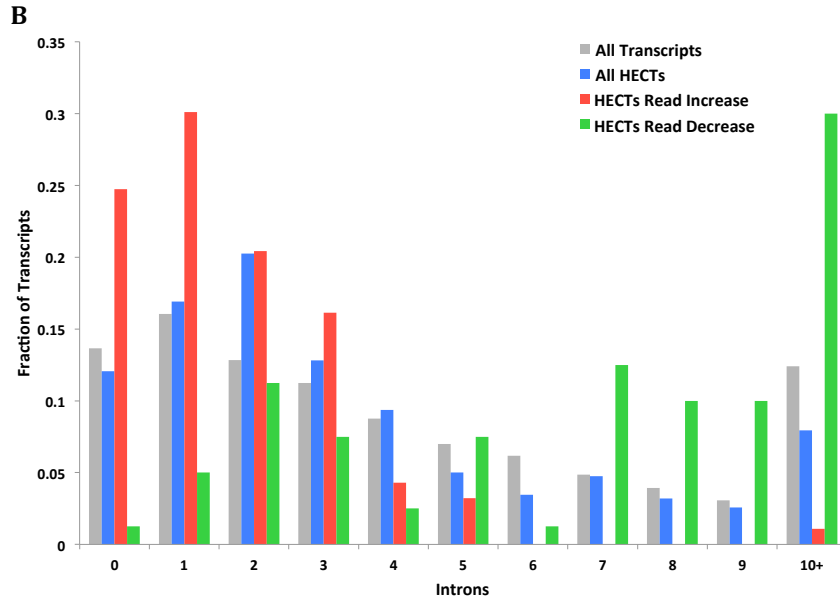
The overrepresentation of proteases amongst HECT genes displaying increased reads in the LTM condition lead us to search for other common features of these genes. Superficial inspection using UCSC genome browser visualizations lead to the observation that many of these genes also lacked introns. To check whether this phenomenon is statistically significant, we compared the intron numbers of HECTs with significantly increased reads in each condition to the intron numbers of all HECTs, and of all Drosophila transcripts using hypergeometric distributions (Fig 3.6).

Figure 3.6. Comparison of intron numbers for HECTs and all transcripts.

A

| Introns | All Transcripts | | All HECTs | | | Increased Reads | | | Decreased Reads | | |
|---------|-----------------|----------|-----------|----------|----------|-----------------|----------|----------|-----------------|----------|----------|
| | Count | Fraction | Count | Fraction | pVal | Count | Fraction | pVal | Count | Fraction | pVal |
| 0 | 4318 | 0.1366 | 94 | 0.1205 | 8.90E-02 | 23 | 0.2473 | 1.33E-02 | 1 | 0.0125 | 1.01E-03 |
| 1 | 5074 | 0.1606 | 132 | 0.1692 | 1.24E-01 | 28 | 0.3011 | 2.67E-03 | 4 | 0.0500 | 1.58E-02 |
| 2 | 4054 | 0.1283 | 158 | 0.2026 | 1.15E-08 | 19 | 0.2043 | 6.29E-02 | 9 | 0.1125 | 1.30E-01 |
| 3 | 3554 | 0.1125 | 100 | 0.1282 | 9.99E-02 | 15 | 0.1613 | 1.24E-01 | 6 | 0.0750 | 1.86E-01 |
| 4 | 2769 | 0.0876 | 73 | 0.0936 | 1.25E-01 | 4 | 0.0430 | 9.80E-02 | 2 | 0.0250 | 8.08E-02 |
| 5 | 2209 | 0.0699 | 39 | 0.0500 | 3.16E-02 | 3 | 0.0323 | 1.00E+00 | 6 | 0.0750 | 1.00E+00 |
| 6 | 1949 | 0.0617 | 27 | 0.0346 | 1.65E-03 | 0 | 0.0000 | 1.86E-02 | 1 | 0.0125 | 1.02E-01 |
| 7 | 1538 | 0.0487 | 37 | 0.0474 | 1.00E+00 | 0 | 0.0000 | 5.75E-02 | 10 | 0.1250 | 2.04E-02 |
| 8 | 1245 | 0.0394 | 25 | 0.0321 | 8.90E-02 | 0 | 0.0000 | 9.47E-02 | 8 | 0.1000 | 4.33E-02 |
| 9 | 970 | 0.0307 | 20 | 0.0256 | 6.33E-02 | 0 | 0.0000 | 5.48E-02 | 8 | 0.1000 | 1.55E-02 |
| 10+ | 3919 | 0.1240 | 62 | 0.0795 | 1.31E-04 | 1 | 0.0108 | 5.82E-04 | 24 | 0.3000 | 1.33E-04 |

Figure 3.6. (Continued)



C

| introns | All Transcripts | | All HECTs | | | LTM | | | Odor | | | Shock | | |
|---------|-----------------|----------|-----------|----------|----------|-------|----------|----------|-------|----------|----------|-------|----------|----------|
| | Count | Fraction | Count | Fraction | pVal | Count | Fraction | pVal | Count | Fraction | pVal | Count | Fraction | pVal |
| 0 | 4318 | 0.1366 | 94 | 0.1205 | 8.90E-02 | 19 | 0.5429 | 5.12E-09 | 7 | 0.5833 | 1.44E-03 | 22 | 0.2391 | 3.38E-03 |
| 1 | 5074 | 0.1606 | 132 | 0.1692 | 1.24E-01 | 11 | 0.3143 | 1.31E-01 | 4 | 0.3333 | 8.26E-01 | 28 | 0.3043 | 3.04E-03 |
| 2 | 4054 | 0.1283 | 158 | 0.2026 | 1.15E-08 | 3 | 0.0857 | 2.57E-01 | 0 | 0 | 6.47E-01 | 19 | 0.2065 | 2.17E-01 |
| 3 | 3554 | 0.1125 | 100 | 0.1282 | 9.99E-02 | 0 | 0 | 7.31E-02 | 0 | 0 | 1.00E+00 | 15 | 0.163 | 2.84E-01 |
| 4 | 2769 | 0.0876 | 73 | 0.0936 | 1.25E-01 | 1 | 0.0286 | 6.74E-01 | 1 | 0.0833 | 1.00E+00 | 4 | 0.0435 | 1.95E-01 |
| 5 | 2209 | 0.0699 | 39 | 0.0500 | 3.16E-02 | 1 | 0.0286 | 9.23E-01 | 0 | 0 | 1.00E+00 | 3 | 0.0326 | 1.64E-01 |
| 6 | 1949 | 0.0617 | 27 | 0.0346 | 1.65E-03 | 0 | 0 | 1.00E+00 | 0 | 0 | 1.00E+00 | 0 | 0 | 2.22E-01 |
| 7 | 1538 | 0.0487 | 37 | 0.0474 | 1.00E+00 | 0 | 0 | 8.78E-01 | 0 | 0 | 1.00E+00 | 0 | 0 | 6.84E-02 |
| 8 | 1245 | 0.0394 | 25 | 0.0321 | 8.90E-02 | 0 | 0 | 6.23E-01 | 0 | 0 | 1.00E+00 | 0 | 0 | 2.06E-01 |
| 9 | 970 | 0.0307 | 20 | 0.0256 | 6.33E-02 | 0 | 0 | 3.95E-01 | 0 | 0 | 7.31E-01 | 0 | 0 | 2.36E-01 |
| 10+ | 3920 | 0.1241 | 62 | 0.0795 | 1.31E-04 | 0 | 0 | 2.14E-01 | 0 | 0 | 1.00E+00 | 1 | 0.0109 | 4.96E-03 |

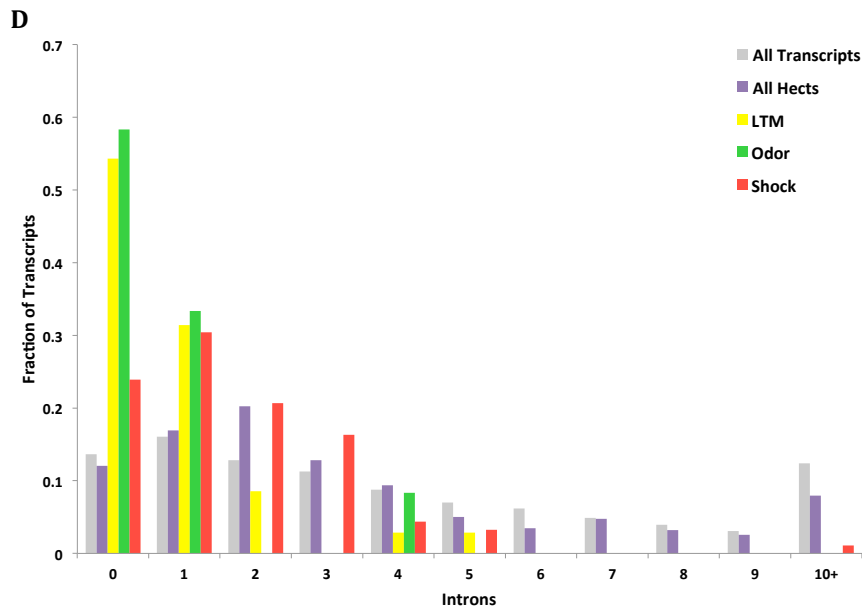
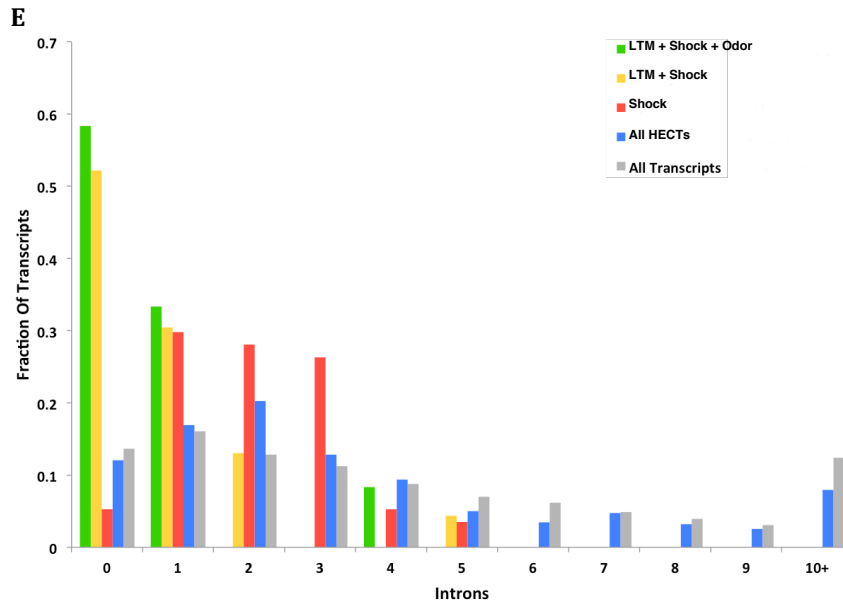


Figure 3.6. (Continued)



Intron counts for all *Drosophila* transcripts were obtained from FlyBase. Intron numbers of HECTs were compared using the hypergeometric distribution. P-values were corrected for multiple testing using the Benjamini-Hochberg method. A & B: While the intron count distribution for all HECTs largely resembles that of all transcripts, HECTs with 0 or 1 intron are over-represented amongst transcripts with increased reads following any treatment, while they are under-represented amongst transcripts with decreased reads following any treatment. C & D: Monoexonic transcripts are overrepresented amongst HECTs with increased read counts in the LTM, odor only, and shock only conditions. E: Grouping HECTs with significantly increased reads in more than one experimental condition reveals that the monoexonic overrepresentation in all conditions is due to the contribution of HECTs with a significant increase in the LTM condition.

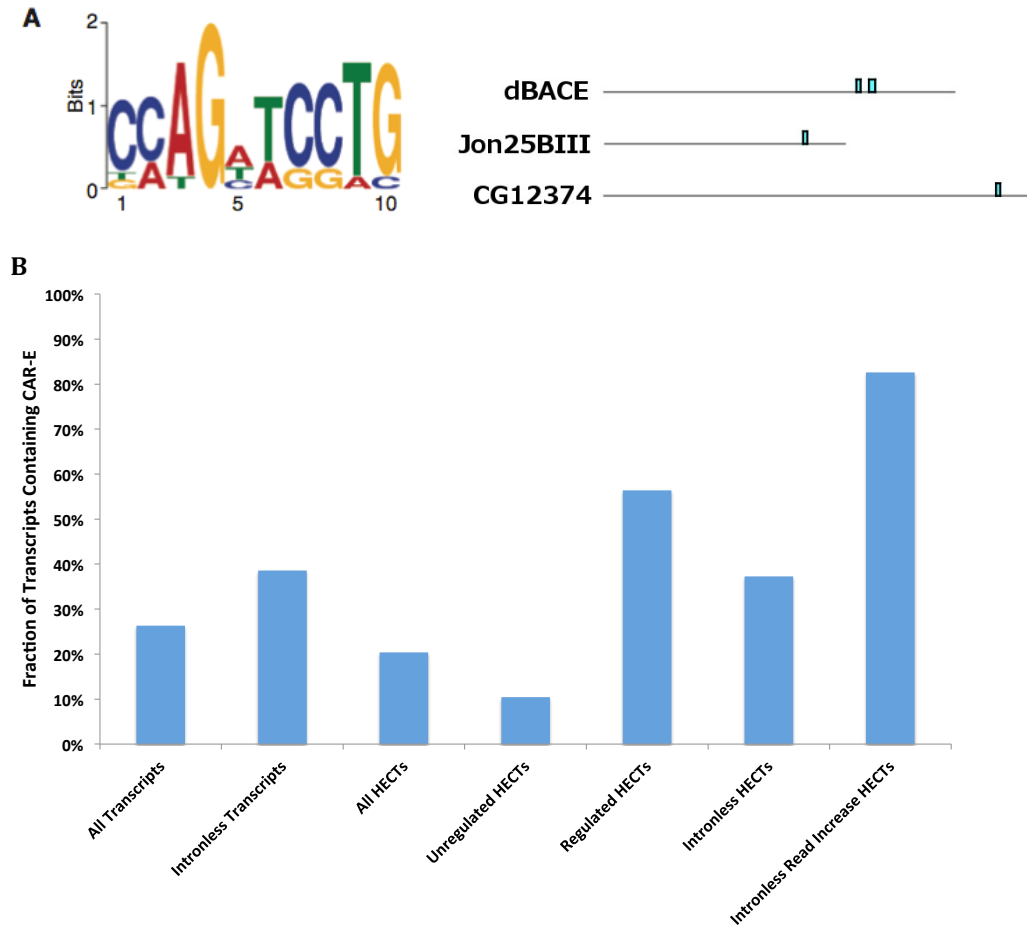
This analysis reveals that intronless transcripts are overrepresented amongst HECTs with significantly increased reads in any condition. However, examination of the overlap between conditions reveals that this overrepresentation is due to intronless HECTs with increased reads in the LTM condition (Fig.3.6 E).

Transcripts harboring a consensus sequence that facilitates nuclear export and expression of intronless mRNAs are overrepresented amongst regulated HECT genes.

We note that NMD, 5' cap formation, polyadenylation, and nuclear export are linked to splicing. Further, transcripts of naturally intronless genes, and cDNAs are less stable and are expressed at lower levels than transcripts that contain introns. This effect is tied to nuclear retention of transcripts that have not undergone splicing. In conditions of stress, expression of some intronless genes increases, while expression of genes with introns is reduced.(14) Surprisingly, the *Drosophila* splicing factor U2 small nuclear riboprotein auxiliary factor 50 (dU2AF⁵⁰) is required for nuclear export of many intronless transcripts.(15) In human cells, intronless genes and cDNAs harboring a consensus sequence element termed the cytoplasmic accumulation region element (CAR-E) are exported from the nucleus at substantially higher rates than those that do not contain the element. mRNAs bearing CAR-E tandem repeats assemble into RNPs containing the dU2AF⁵⁰ homolog U2AF2.(14, 16) The link between the CAR-E, U2AF2, and nuclear export suggests the possibility that such an element might also foster nuclear export of intronless mRNAs in *Drosophila*. If so, one might expect to find CAR-Es present in intronless HECTs. We used the FIMO and MAST algorithms to identify CAR-E occurrences in HECTs, and in all *Drosophila* transcripts.(17, 18) This analysis revealed that CAR-E containing transcripts are slightly, though significantly underrepresented amongst all HECTs. However, further parsing of HECTs reveals CAR-E containing transcripts are significantly overrepresented amongst HECTs with significant changes in read counts in any of our treatment groups. Further, CAR-E containing transcripts are significantly underrepresented amongst HECTs that do not exhibit changes in read counts. Most dramatically, 19 out of 23 (82.61%, p =

7.53 E-8) intronless HECTs with increased reads in any treatment group contain at least one CAR-E (Fig3.7).

Figure 3.7. CAR-E containing transcripts are overrepresented amongst regulated HECTs



| | All Transcripts | Intronless Transcripts | All HECTs | Intronless HECTs | Unregulated HECTs | Regulated HECTs | Intronless Read Increase HECTs |
|-----------|-----------------|------------------------|-----------|------------------|-------------------|-----------------|--------------------------------|
| Total | 70127 | 2818 | 780 | 94 | 593 | 172 | 23 |
| CAR-E | 18458 | 1087 | 159 | 35 | 62 | 97 | 19 |
| % | 26.32% | 38.57% | 20.38% | 37.23% | 10.46% | 56.40% | 82.61% |
| Adj p-Val | | 1.93E-47 | 3.70E-05 | 5.79E-03 | 1.49E-21 | 2.15E-16 | 7.53E-08 |

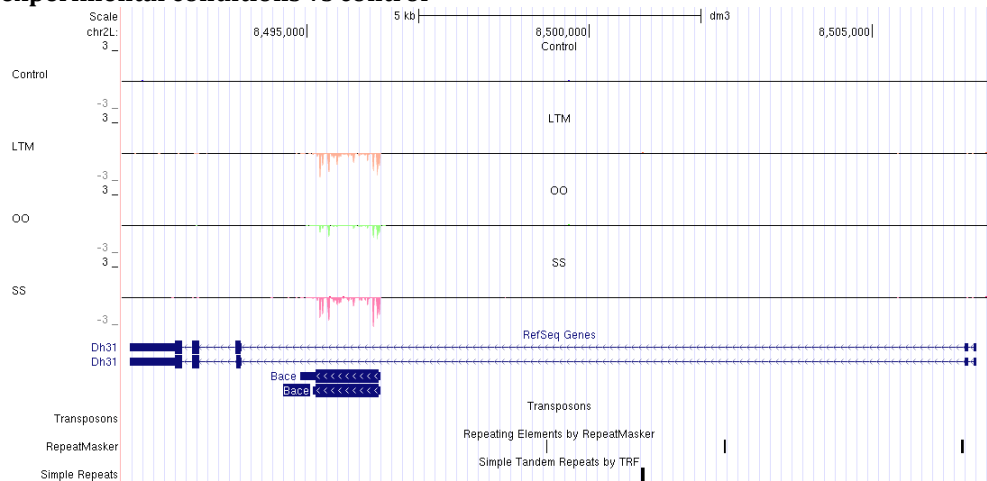
CAR-E containing transcripts are overrepresented amongst intronless HECTs with increased read counts following treatment. FIMO was used to identify CAR-E occurrences in transcripts. A: The left panel shows a sequence logo for CAR-E as reported in (16). The positions of CAR-E occurrences in the three HECTs with the largest read increases are shown in the right panel. B: Bar chart and table displaying the number of transcripts, and the number of transcripts containing at least one CAR-E in each category. P-values were computed using hypergeometric distributions, and were adjusted for multiple testing using the Holm-Bonferonni method.

Further investigation into whether CAR-Es, or U2AF⁵⁰ are involved in the HECT signature, or in the increase in reads observed amongst intronless HECTs, was beyond the scope of this study. However, the strong overrepresentation of CAR-E containing transcripts amongst intronless HECTs that have increased reads following LTM training is intriguing.

dBACE mRNA is upregulated by LTM training, and by spaced sessions of the US alone

Our investigation of HECTs began with the observation that a large number of reads mapped to the dBACE gene in all experimental conditions, but not in control libraries. This signal was immediately apparent when visually examining our data using the UCSC genome browser, and inspired our search for other genes with this signature (Fig 3.8).

Figure 3.8. sRNA sequencing reads increase across the dBACE locus in all experimental conditions vs control



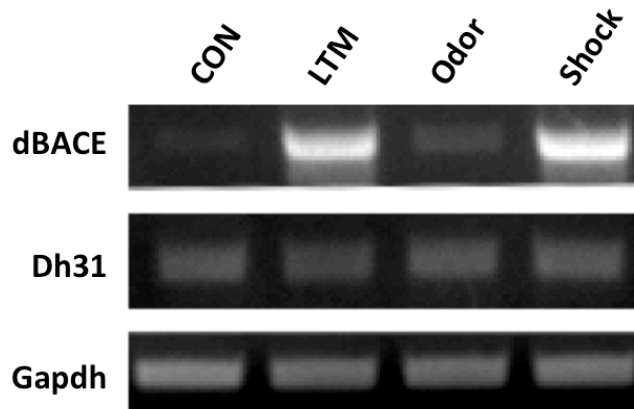
sRNA sequencing data visualized using the UCSC genome browser. Plots represent mean normalized reads mapping to each nucleotide position across the genome for each treatment group. Orange: LTM trained. Green: Odor only. Red: Shock Only. Few reads map within the Dh31 region in the control condition. The LTM, odor only, and shock only groups all exhibit significant read depth and coverage across dBACE gene on the coding strand.

Our subsequent differential expression analysis shows that dBACE is the HECT gene with the largest change in read counts vs control in all conditions (Figure S3.2).

dBACE cleaves APPL, leading to production of $dA\beta$. This pathway regulates neural morphology, synaptic plasticity, neurotoxicity, and behavior in insects, rodents, and humans.^(1, 3, 19) We therefore sought to understand how the observed increases in dBACE reads relate to changes in dBACE expression. Semiquantitative RT-PCR conducted on the same total RNA samples used in preparing our sRNA sequencing libraries shows that dBACE mRNA is strongly upregulated in the LTM and shock only conditions, but not in the odor only condition. dBACE is an intronless gene residing within an intron of the Diuretic hormone 31 (Dh31) gene. Though few reads map to Dh31 exons in any condition, we also examined the possibility that

dBACE reads reflect Dh31 regulation or mRNA levels. Dh31 mRNA remained unchanged in all conditions (Fig. 3.9).

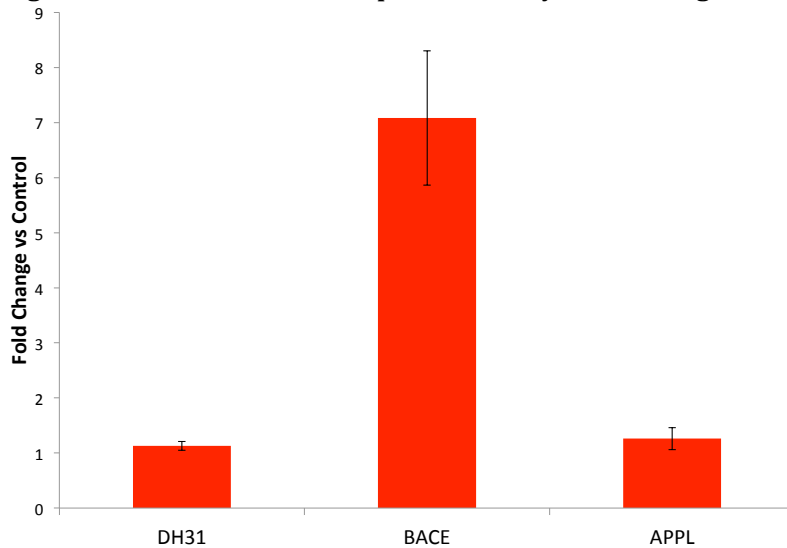
Figure 3.9. Semiquantitative RT-PCR reveals that dBACE mRNA is strongly upregulated in the LTM and shock only conditions



dBACE and Dh31 mRNA levels in the same total RNA samples used as input for sRNA sequencing library preparation were evaluated by semiquantitative RT-PCR. These samples were collected 2hrs after treatment. Poly-T oligonucleotides primed reverse transcription, and primers pairs spanning ~500bp exon sequences of dBACE, Dh31, or Gapdh were used for PCR amplification. dBACE mRNA is strongly upregulated in the LTM and shock only conditions, while it is only mildly induced in the odor only condition. Dh31 expression is not affected by any of our treatments.

Semiquantitative RT-PCR can give misleading results if the reaction is ended outside of the log phase of amplification. We therefore used quantitative real-time PCR analysis to better quantify the change in dBACE mRNA levels induced by LTM, and to ensure that Dh31 expression indeed remained unchanged. Further, dBACE expression might increase following LTM training to keep pace with increased APPL expression. Alternatively, elevated dBACE expression might reflect a shift to APPL processing along the amyloidogenic pathway. To investigate these possibilities, we examined APPL expression in these experiments as well. Again, we used the same total RNA samples from which our sRNA sequencing libraries were prepared (Fig. 3.10).

Figure 3.10. Real-Time PCR expression analysis following LTM training.



qRT-PCR shows that dBACE expression dramatically increases following LTM training, while APPL and Dh31 remain essentially unchanged. We used the same total RNA samples from which our sRNA sequencing libraries were prepared as input for these reactions. These samples were collected 2hrs after treatment. Poly-T oligonucleotides were used to prime reverse transcription. Primer pairs spanning ~500bp exon sequences were used to amplify Dh31, dBACE, APPL, or β -Tubulin. Triplicate technical repeats of each reaction were run on the same plate. This procedure was repeated for 3 independent matched sets of LTM trained and control RNA samples. Changes in expression were calculated using the $\Delta\Delta C_t$ method, with β -Tubulin serving as the internal control. Error bars represent the standard error of the mean.

qRT-PCR analysis reveals that dBACE expression significantly increases (7.08 fold vs. control, $p=2.48E-3$) 2 hours after LTM training. Meanwhile, Dh31 and APPL mRNA levels remain essentially unchanged. These results suggest that at least at 2 hours after training, increased dBACE expression could reflect a change in APPL processing towards the amyloidogenic pathway, rather than a compensatory increase in dBACE expression to keep pace with elevated APPL expression. However, if Kuz expression were also increased, the relative preference for amyloidogenic vs non-amyloidogenic APPL processing could be maintained, even if total APPL cleavage rates increased. Furthermore, APPL expression, and the

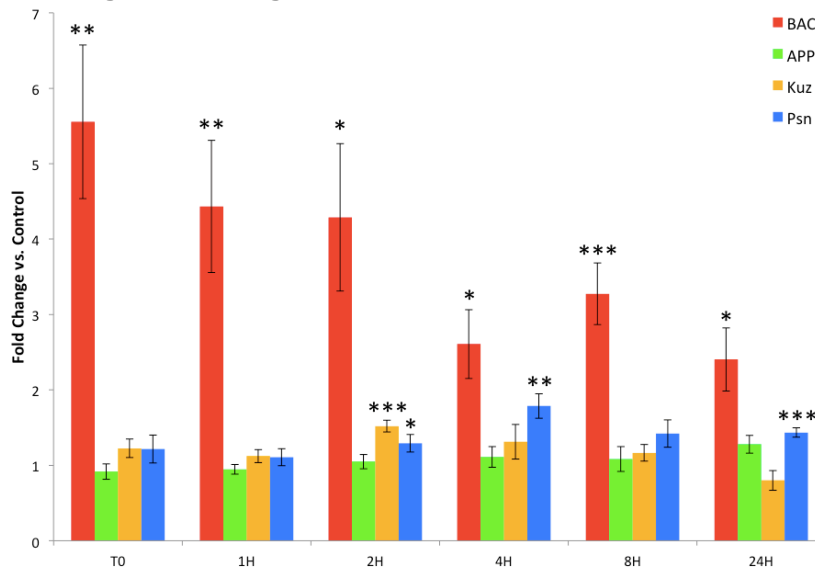
balance between APPL processing pathways may have important dynamics not apparent at the 2 hour post-training time point.

dBACE expression is rapidly upregulated following LTM training and remains elevated 24 hours post-training

The finding that dBACE is dramatically upregulated 2 hours after LTM training, or spaced sessions of US presentation, opened several lines of inquiry. First, how quickly after training is dBACE expression elevated, and how long does it stay elevated? Second, how do the other key components of the APPL pathway respond following training? The answers to these questions might provide insights into the roles of APPL and its metabolism in memory formation. We therefore conducted time course experiments, using qRT-PCR to simultaneously measure the expression of Kuz, dBACE, Psn, and APPL during the 24 hours following training. The fly handling and training of flies used in these experiments was nearly identical to that of our sRNA sequencing experiments. The only change in our procedures was that only LTM trained and control flies were prepared for the purposes of the time course experiments. We collected heads from 10 male and 10 female flies immediately after training, and again at 1, 2, 4, 8, and 24 hours after training. Total RNA was collected from LTM trained and control heads from each time point. We repeated these experiments 3 times, each time checking that a stable memory of the shock and odor pairing was present at 24 hours post-training. We performed each qRT-PCR measurement in triplicate on the same plate. Gapdh was used as an

internal control for all reactions. These experiments show that dBACE expression alone is strongly upregulated immediately after training, and decreases slowly over the course of 24 hours. Even with the decrease in expression that occurs after T0, dBACE remains elevated at the 24 hour time point (Fig. 3.11).

Figure 3.11. Time course measurement of APPL pathway component expression following LTM training.



qRT-PCR measurement of APPL pathway component expression shows that dBACE expression increases rapidly following LTM training, and remains elevated 24 hours later. Kuz expression increases mildly at the 2hr time point, but is otherwise relatively stable. Psn expression increases beginning 2hrs post training, and remains slightly, though significantly, elevated 24hrs post-training. APPL expression does not change significantly during the 24hrs following LTM training. Poly-T oligonucleotides were used to prime reverse transcription. Primer pairs spanning ~500bp exon sequences were used to amplify dBACE, APPL, Kuz, Psn, or gapdh. Triplicate technical repeats of each reaction were run on the same plate. This procedure was repeated for 3 independent matched sets of LTM trained and control RNA samples. Changes in expression were calculated using the $\Delta\Delta C_t$ method, with gapdh serving as the internal control. Error bars represent the standard error of the mean. P-values were calculated using two-tailed student's t-test. (*: $P \leq 0.05$, **: $P \leq 0.005$, ***: $P \leq 0.0005$).

While dBACE is rapidly upregulated following training, expression of the other APPL pathway members we examined remains stable at the T0 and 1hr time points. At 2hrs post training, Kuz is mildly, though significantly upregulated (1.52 fold change,

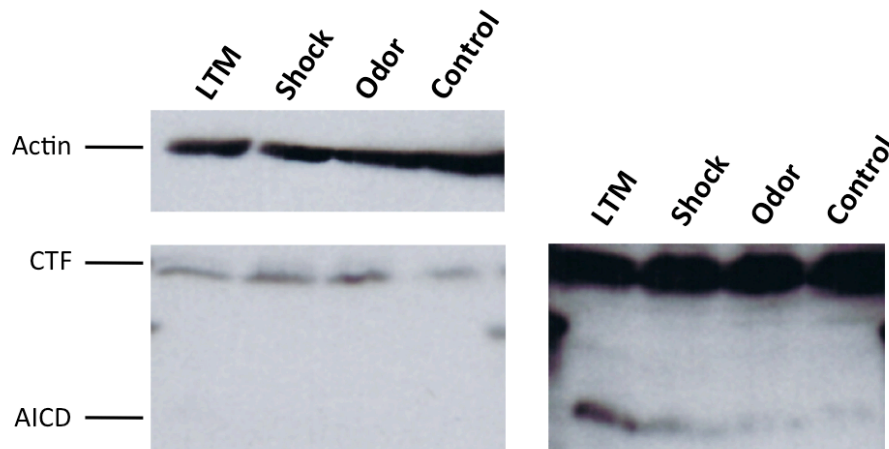
$p \leq 0.0005$). At the 4hrs post-training time point, Kuz expression has declined somewhat, and is no longer significantly above control. From 4hrs to 24hrs post-training, Kuz expression continues to decline, but differences from control are not statistically significant. Psn expression also significantly elevated at the 2hrs post-training time point (1.29 fold change, $p \leq 0.05$). Psn expression continues to rise up to ~1.8 fold ($p \leq 0.005$) above control at 4hrs post-training, and remains significantly elevated 24hrs after training (1.44 fold change, $p \leq 0.0005$). Though we observed a Psn expression level 1.42 fold higher than control at the 8hrs post-training time point, experimental variability meant that the difference was not statistically significant ($p = 0.60$). Taken together, the time course qRT-PCR data indicate that expression of APPL pathway members is rapidly altered following LTM training, such that processing via the amyloidogenic pathway becomes more likely.

APPL processing is stimulated by LTM training and spaced sessions of US exposure.

We were not able to directly measure dBACE protein levels, as no antibody was available for such experiments. We speculated that increased dBACE expression might result in altered APPL processing. To test this idea, we examined APPL expression and processing in head lysates from flies subjected to LTM training, odor only, or shock only treatments and from control flies in the previously described manner using western blots. We used an antibody raised against a conserved C-terminal peptide within the AICD of *Manduca Sexta* APPL that is able to detect the C-terminal end of *Drosophila* APPL to probe our western blots.(1, 20)

These experiments show that all three experimental conditions mildly enhance a ~15kD band, a size corresponding to the CTFs resulting from the initial cleavage of APPL by either Kuz or dBACE. Long exposures reveal that LTM training, and to a lesser degree spaced sessions of US exposure, stimulate the appearance of an approximately 6kD band, a size corresponding to the predicted molecular weight of AICD (Fig 3.12).

Figure 3.12. LTM training and spaced sessions of shocks or odor enhance APPL processing



Western blots reveal that all three experimental treatments enhance APPL processing. Heads from flies in each condition were collected 2Hrs after treatment. Membranes were probed using α -msAPPL-AICD. Actin-5C was used as a loading control. In the left panel, a 4Hr exposure shows that samples from all three experimental conditions have slightly elevated levels of an ~15kD band. This size range corresponds to the C-terminal fragments of APPL resulting from cleavage by Kuz or dBACE. In the right panel, a 36Hr exposure of the same membrane reveals the presence of an additional ~6kD C-terminal APPL band. This size range matches the predicted molecular weight of the AICD. Both LTM training and spaced sessions of shocks increase the signal from this band, but the signal is much stronger in the LTM lane.

The 6 kD size range also corresponds to that predicted for dA β . However, the antibody used in our western blots was raised against a peptide corresponding to the highly conserved YENPTY sequence present at the C-terminus of APPL. This

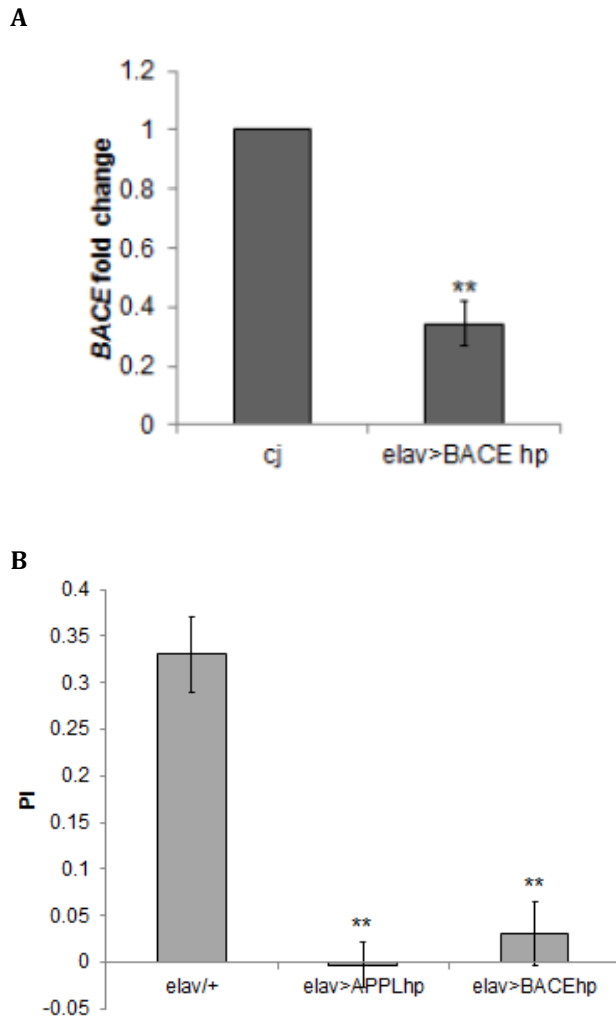
sequence resides within the AICD. Therefore, it is more likely that the 6 kD band corresponds to AICD than to dA β .

APPL and dBACE are required for aversive and appetitive LTM.

Having shown that training stimulates dBACE mRNA expression and APPL processing, we next sought to determine if memory formation is dependent on these activities. APPL knockout flies and flies overexpressing APPL or dBACE have been shown to have defects in perception of electric shock and locomotor defects that interfere with evaluation of memory using the T-maze test.(1) These defects are thought to be due to disturbances of neurodevelopment driven by such misexpression. We therefore sought to avoid such developmental defects by inducibly knocking down expression of APPL or dBACE just prior to training. To do so, we obtained *Drosophila* lines expressing RNA hairpins under UAS control directed against either dBACE (dBACE-hp) or APPL (APPL-hp). We then crossed the dBACE-hp or APPL-hp lines with enhancer trap flies expressing Gal4 driven by the panneuronal Elav enhancer, and a separate transgene in which a temperature sensitive variant of the yeast repressor Gal80 (Gal80ts) is expressed ubiquitously under the control of the tubulin promoter. In this set up, at normal temperatures Gal80ts blocks expression of the hairpins under UAS control. When flies are shifted to the restrictive temperature, Gal80ts is no longer able repress Gal4-UAS driven expression of the hairpins. The progeny of these crosses were subjected to aversive olfactory LTM training as previously described. Knockdown of either APPL or

dBACE significantly reduces PIs when these flies were tested for olfactory LTM as previously described (Fig 3.13).

Figure 3.13. Knockdown of APPL or dBACE disrupt olfactory LTM



Flies harboring the panneuronal Elav-Gal4 driver, and the ubiquitously expressed tubulin-Gal80ts temperature sensitive repressor were used to conditionally express RNA hairpins directed against either APPL or dBACE. A) RT-PCR from heads of Elav-Gal4/Tub-Gal80ts/UAS-dBACE-hp flies housed at the permissive temperature, thus expressing dBACE-hp in all neurons, have significantly reduced dBACE mRNA levels. B) Flies in which panneuronal expression APPL-hp or dBACE-hp was induced for 30min immediately prior to training were subjected to aversive classical conditioning as previously described. Memory of the pairing of shock and odor was tested 24hrs later using a T-maze. APPL and dBACE hairpins both significantly reduced olfactory LTM PIs ($p < 0.005$).

To determine if the LTM defect caused by APPL-hp or dBACE-hp expression is generalizable to other training paradigms, we subjected the *Elav-Gal4/tub-Gal80ts/UAS-APPL-hp* and *Elav-Gal4/tub-Gal80ts/UAS-dBACE-hp* flies to appetitive olfactory conditioning. In this paradigm, flies learn to associate an odor with the availability of sucrose. Flies that have been deprived of food for 20hrs prior to training are exposed to a CS (odor) in the presence of a rewarding US (sucrose). These flies are also exposed to a control odor without US presentation. Memory is then evaluated in a T-maze as previously described. Using the appetitive conditioning method, a single 2 minute session produces protein synthesis dependent LTM.(21) Our experiments show that expression of APPL and dBACE are also required for appetitive olfactory LTM (Fig 3.14).

Figure 3.14. APPL or dBACE knockdown disrupts appetitive olfactory LTM
A

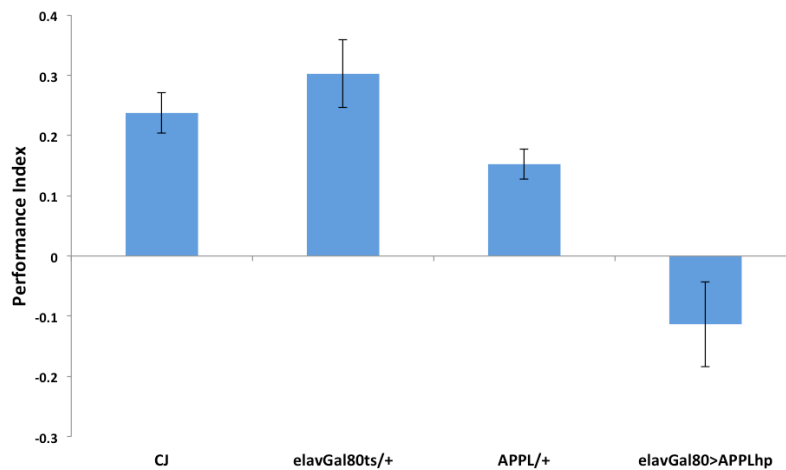
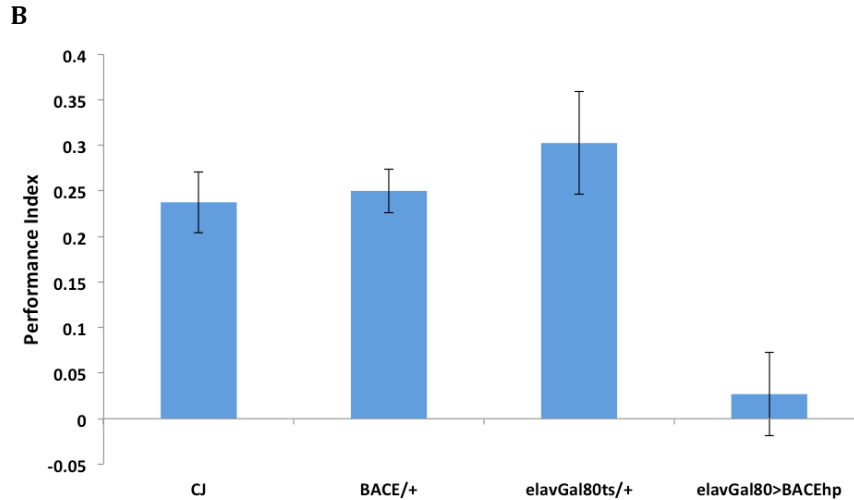


Figure 3.14. (Continued)



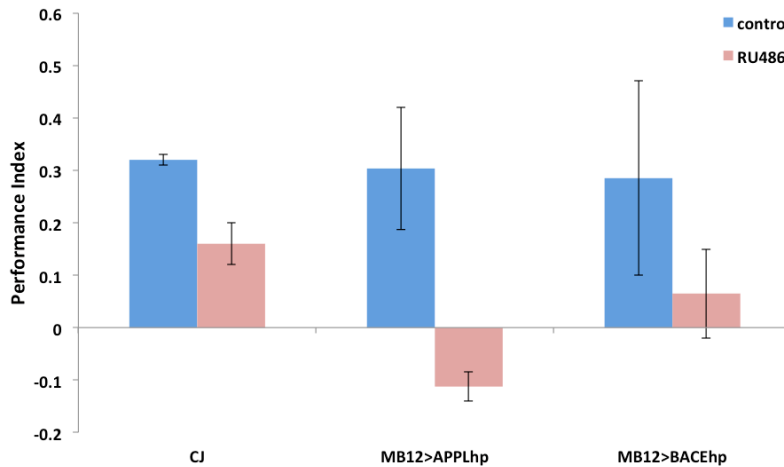
Flies in which panneuronal expression of RNA hairpins directed against either APPL or dBACE was induced just prior to training were subjected to appetitive olfactory classical conditioning. Memory of the pairing of sucrose and odor was tested 24hrs later using a T-maze. APPL (A) and dBACE (B) hairpins both significantly reduced appetitive olfactory LTM PIs.

Knockdown of APPL or dBACE in the adult MB disrupts LTM

The neural circuitry involved in *Drosophila* olfactory memory is well characterized. Lasting forms of both aversive and appetitive olfactory memory are encoded in the MB. Both involve dopaminergic signaling in the MB, but appetitive olfactory conditioning also involves octopaminergic input from APL neurons.(22, 23) MB specific misexpression of some genes is sufficient to disrupt olfactory memory. Knockdown of APPL in the adult MB reduces LTM PIs in aversive olfactory classical conditioning.(24) To determine if appetitive olfactory LTM also requires APPL and dBACE expression in the MB, we tested flies expressing APPL-hp or dBACE-hp in the MB for LTM using our sucrose reward paradigm. For these experiments, we used the same gene switch system as was used in Goguel et al. to

induce hairpin expression within the MB beginning 48hrs prior to training. The gene switch system is a binary conditional expression method that drives transcription under the control of a UAS in response to administration of the drug RU486. A construct that encodes a chimeric protein harboring the DNA binding domain of the yeast Gal4 activator, the ligand binding domain of the mammalian progesterone receptor, and an activation domain from p65, is inserted into the genome. RU486 activates this chimeric protein, which is then able to drive transcription of UAS constructs already in widespread use. In the MB12 line, the gene switch construct is placed downstream of a 247bp Dmef2 enhancer element that is known to produce expression patterns that are restricted to the MB. The combination of this spatial restriction with temporal control through RU486 administration allows for precisely defined expression of UAS constructs.(25) MB12 driven expression of APPL-hp beginning 48hrs prior to appetitive conditioning produces impairment of olfactory LTM. dBACE-hp expression in the MB also reduces appetitive olfactory LTM, though not as strongly as APPL-hp (Fig. 3.15).

Figure 3.15. Expression of APPL-hp or dBACE-hp in the MB impairs LTM



Flies harboring UAS-APPL-hp or UAS-dBACE-hp constructs driven by the MB specific MB12 gene switch have lower performance indices when fed RU486 for 48hrs prior to appetitive olfactory conditioning than flies of the same genotype that were not fed RU486.

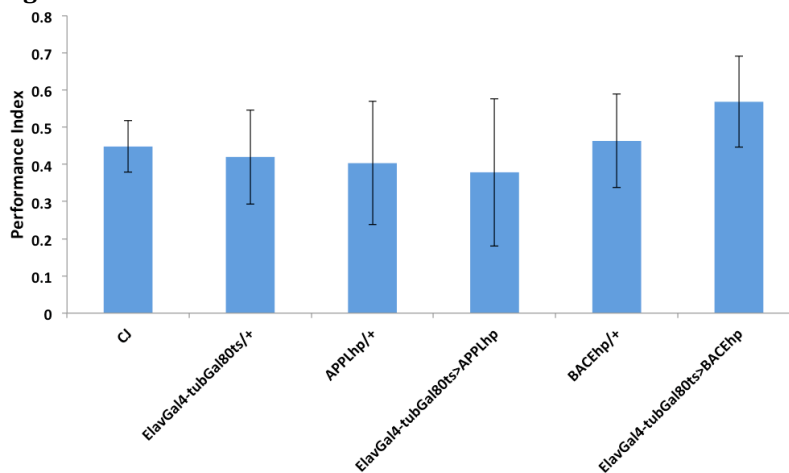
RU486 administration reduces PIs in control flies as well, but this effect alone does not account for the greater PI reductions in MB12>dBACE-hp or MB12>APPL-hp flies. Thus, our methods demonstrate that APPL expression is required in the MB for appetitive olfactory LTM, as well as for aversive olfactory LTM as reported by Goguel et al. Our data also indicate that dBACE-hp expression in the MB reduces appetitive olfactory LTM. However, the effect is smaller, and the negative impact of RU486 administration on PIs do not allow us to confidently conclude that the requirement for dBACE expression in olfactory LTM formation is confined to the MB.

APPL and dBACE are not required for STM

Our results showing that APPL is required for aversive and appetitive LTM are in agreement with a previous study in which APPL-hp was inducibly expressed

in the MB using the gene switch system.(26) Using this approach, Goguel et al. showed that knockdown of APPL in adulthood reduces LTM, but leaves STM and perception of the US intact. Similarly, we find that adult expression of dBACE-hp in neurons also disrupts aversive and appetitive olfactory LTM. This result begs the question of whether neuronal dBACE-hp expression leaves STM intact as is the case with APPL-hp. To test this, we subjected our *Elav-Gal4/tub-Gal80ts/UAS-APPL-hp* flies and *Elav-Gal4/tub-Gal80ts/UAS-dBACE-hp* flies to a single conditioning session pairing odor with shock after inducing hairpin expression. Such conditioning produces a memory of the pairing lasting on the order of hours in wild type flies. Two hours after conditioning, we tested for memory of the pairing using a T-maze as previously described. We find that neither APPL-hp or dBACE-hp significantly affects STM when expressed in neurons (Fig 3.16).

Figure 3.16. Panneuronal knock down of dBACE or APPL does not reduce STM



Flies were subjected to a single pairing of odor and shock and tested for memory of the pairing 2hrs after conditioning using a T-maze. STM scores for flies expressing APPL-hp or dBACE-hp in neurons were not significantly different from those of any of the control genotypes.

Discussion

During memory formation, synaptic efficacy and/or structure are altered. Formation of lasting memories requires that these alterations are durable. Transcription and translation are required for LTM, reflecting the need for altered gene expression programs to produce lasting changes in synaptic structure and function. After decades of study, novel aspects of gene regulation continue to be discovered. The widespread adoption of high-throughput nucleotide sequencing technology has led to several such discoveries. Soon after their discovery, microRNAs, esiRNAs, and piRNAs were all shown to regulate important aspects of neuronal function and behavior.(27-29) With this in mind, we designed our sRNA study such that we would be able to profile these well studied sRNA classes, and to simultaneously extend our analysis beyond them. This approach yielded a wealth of unexpected sRNA sequences, many of which remain to be examined in depth. Of note, we identified a set of transcripts that produce copious reads mapping along the entirety of the sense strand of their exons. Though some of these sequences are present in previously published sRNA sequencing data sets, this study is to our knowledge the first to describe these HECT sRNAs as a class.(30) Further, we identify a subset of HECTs with read counts that change significantly during LTM formation, or following spaced sessions of exposure to odor alone or electric shock alone. Monointronic and intronless genes are overrepresented in the set of HECTs with increased reads following treatment and underrepresented amongst those with decreased reads. Furthermore, genes with GO annotations related to

proteolysis are also overrepresented amongst HECTs with increased read counts following training. These findings suggest the presence of a previously unrecognized regulatory mechanism driving increased expression of proteases following training.

While our primary goal was to profile microRNA expression during memory formation, the sensitivity and capacity of Illumina sequencing is several times greater than would be required to fully profile microRNA expression in a given sample. Rather than restricting our study to microRNAs and multiplexing our samples through barcoding, we chose to use the excess “sequencing space” to expand the size range of sRNAs studied. A size range of 21-29nt would have been sufficient to encompass esiRNAs, microRNAs, and piRNAs. However, other studies indicate that sRNAs outside of this size range exist in animal cells, and may be functional.(31-33) The 15-35nt size range we selected encompasses ~18nt transcription initiation RNAs (tiRNAs) at the low end, while remaining below the 36nt maximum size recommended in the Illumina sRNA library preparation kit v1.5 protocol. Reads with lengths outside the size ranges of microRNAs, esiRNAs, or piRNAs map to diverse loci across the genome. ncRNAs including rRNAs, snoRNAs, snRNAs, and tRNAs are major sources for such sRNA reads. sRNAs derived from ncRNAs have been reported previously, and aspects of their biogenesis and functions have been explored in a number of studies. (34-39) However, aside from snoRNA derived microRNAs, the functions of ncRNA derived sRNAs remain largely unknown in *Drosophila*. We also detected abundant tiRNAs at many transcription

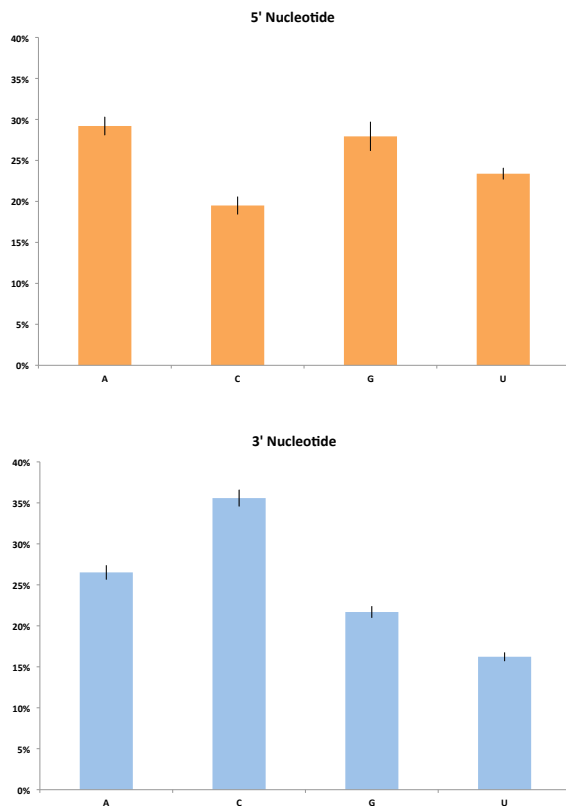
start sites (TSS). The number of unique tiRNA reads present at a TSS is correlated with expression levels of associated transcripts.(32, 40) Though we note the presence of tiRNAs in our samples, analysis of these reads remains for future work. We also detected very short sRNA reads mapping near predicted insulator sites, and enhancers, but again, analysis of these reads is beyond the scope of this study. While we recognize the potential for novel insights in studying the classes of sRNAs just mentioned in our samples, we chose to focus on the abundant sRNA reads mapping to HECTs.

HECT sRNAs have a size distribution unlike those of other classes of sRNAs. MicroRNAs, esiRNAs, and piRNAs all have characteristic size ranges spanning 5nt or less. HECT sRNAs have a far broader size distribution, with 15nt-25nt reads constituting similar fractions of all sRNA reads. Our size selection precluded examination of RNAs shorter than 15nt, but shorter HECT sRNAs may also be present in animals. At the upper end of the HECT sRNA size range, reads longer than 26nt are less abundant, but 35nt reads are readily detectible. Longer HECT sRNAs may also occur, but again were outside of the range of our size selection, and thus do not appear in our data. The broad size range and transcript origin of HECT sRNAs suggests that they may be products of mRNA degradation. Previous studies in which parallel analysis of read ends (PARE) was used to explore the degradome of human cells suggest that this may be the case. The PARE method is used to determine the 5' end of cleaved or degraded mRNAs. PARE libraries are generated from polyadenylated mRNA fragments. The 5' PARE adapter is ligated using

chemistry that requires a 5' monophosphate on the poly-A tailed mRNA fragment. Such studies find monophosphorylated 5' ends mapping across transcripts. Bracken et al. show that higher PARE read counts are correlated with higher mRNA levels, but not with mRNA stability.(41) However some well expressed genes produce few if any PARE reads. As PARE relies on the presence of a 5' monophosphate, the absence of these well expressed genes in PARE libraries likely reflects degradation via mechanisms that do not leave a 5' monophosphate, and not necessarily greater mRNA stability.(41) Thus, existing evidence suggests that our analysis showing HECT read counts are correlated with modencode expression scores is indeed valid. Using PARE, read counts increase towards the 3' end of transcripts with multiple PARE reads, reflecting stalled processive 5'-3' degradation via the exonuclease Xrn1.(41, 42) While we do not observe accumulation of reads at the 3' end of HECTs, this distinction could reflect either or both the underlying biology, and the differences between PARE and our sRNA sequencing methods. Reverse transcription during PARE library prep is primed by poly-T oligonucleotides. Thus, our methods capture products of endonuclease cleavage, and 3'-5' exonuclease activity that are not present in PARE libraries. Nonetheless, the patterns of sRNA read coverage across a given HECT is highly stereotyped amongst our samples. This may reflect aspects of the biogenesis of HECT sRNAs, but exploration of this idea will have to wait for future work. The preference for certain 5' or 3' sites for HECT sRNA reads of varying lengths does however support such a view. If HECT sRNAs are in fact produced via degradation of mRNAs, preferential mapping of 5' or 3' ends of reads to specific sites may be due to sequence or structural motifs involved in the

degradative process. We examined the possibility that mRNA secondary structure is related to HECT read coverage peaks using Mfold predictions for HECTs. This search was not comprehensive, but we were unable to identify any obvious relationship between structure predictions and read coverage peaks. We also looked for 5' and 3' nucleotide biases in HECT reads. We found that most HECT sRNAs have a 5' purine, and that cytidine is the least common 5' nucleotide. We also found that HECT sRNAs have a mild 3' bias for cytidine and against uridine. However, the significance of these biases is not immediately apparent. (Fig 3.17).

Figure 3.17: 3' and 5' nucleotide occurrence for HECT sRNAs



5' and 3' nucleotides were tallied for HECT sRNA reads in each library. These totals were divided by the total number of reads in the corresponding library. HECT sRNAs display a 5' preference for purines. Fewer sRNA reads have a 5' cytidine than any other nucleotide, while cytidine is the most common 3' nucleotide. Uridine is the least common 3' nucleotide.

Lastly, A+U rich elements (ARE) can affect the stability of their mRNA hosts. We conducted a search for AREs amongst 50 HECTs and did not find that transcripts containing AREs are overrepresented. Taken together, these results indicate that HECT sRNAs are not related to known classes of regulatory sRNAs, and are in fact degradation products of highly expressed mRNAs. However the nature of the processes that lead to HECT sRNA production remain to be elucidated in future work.

A subset of HECTs display statistically significant changes in read counts following training. Intriguingly, genes with GO annotations related to proteolysis are overrepresented in the set of HECTs with increased read counts. Further, HECT genes with increased read counts following treatment are largely intronless or have a single intron. Parsing this result further, we find that intronless genes are strongly overrepresented amongst HECTs with increased read counts in the LTM condition. Splicing and nuclear export are linked, and expression of intronless genes or transcripts of cDNAs is generally reduced in the absence of sequence motifs that allow for nuclear export via atypical mechanisms.(14) Previous work in human and *Drosophila* cells indicates that expression of intronless genes is regulated differently than genes whose transcripts undergo splicing. Conditions of cellular stress generally lead to translational arrest for most genes. However, in *Drosophila*, mRNAs for a set of intronless genes that are retained in the nucleus and thus poorly expressed under normal conditions, become cytoplasmically localized and escape translational silencing under conditions of stress.(15) In human cells, intronless

genes harboring the CAR-E consensus sequence in their coding regions are exported from the nucleus and expressed at far higher levels than those that do not contain a CAR-E.(16) We conducted a transcriptome wide search for the consensus CAR-E, and found that CAR-E containing transcripts are significantly overrepresented amongst regulated HECTs, and underrepresented amongst unregulated HECTs. Furthermore, we found that greater than 80% of intronless HECTs with increased read counts following training contain the consensus CAR-E. While these results strongly suggest that the CAR-E regulates expression of intronless HECTs following training, proof of such a model will depend on direct measurement of HECT gene expression and mutational analysis of the CAR-Es present in regulated HECTs.

The HECT gene with the largest read increase in all treatment groups was dBACE, a homologue of the human BACE genes. dBACE initiates amyloidogenic processing of APPL. In humans, misregulation of this pathway is thought to be the causal mechanism in AD progression. We find that increased dBACE reads reflect increased dBACE transcript levels. This increase corresponds to accumulation of APPL metabolites. To our knowledge, this study is the first to show that classical conditioning induces dBACE expression and APPL processing. Expression and proteolytic processing of APP family proteins influences neural morphology, synaptic structure, plasticity, and memory in complex ways. AD progression is typically associated with accumulation of senile plaques composed largely of A β . However, the view that β -secretase initiated processing of APP and resultant A β production is inherently toxic has been countered by recent studies in rodents and

humans (reviewed in (43)). Accumulating evidence indicates that APP expression and processing along the amyloidogenic and nonamyloidogenic pathways must all occur, and be exquisitely balanced for proper neural function and behavior.

However, the difficulties of studies in humans and the absence of endogenous senile plaque forming A β in rodents has hampered efforts to understand the fundamental mechanisms by which APP controls synaptic plasticity, neural function, memory, and how these mechanisms are misregulated in AD. Recently, *Drosophila* has been shown to possess homologues of all of the key components of the APP pathway, and that APPL metabolism is functionally conserved. Importantly, β - γ -cleavage of APPL yields a peptide termed dA β that forms aggregates similar to senile plaques and, though its sequence is not conserved, is functionally similar to A β .(1, 6, 26) These findings demonstrate that studies of APPL and its proteolytic processing can inform our understanding of APP processing and its role in AD. *Drosophila* behavioral genetics has proven instrumental in unraveling the biochemical underpinnings of many aspects of neural function, and indeed of memory itself. With these factors in mind, it seems likely that *Drosophila* will become an important model organism with which to study how processing of APP family proteins participates in memory formation. Yet, studies of APPL metabolism to date have largely relied on nonphysiologic conditions, and have focused on resulting pathologies. In this study, we examine the APPL pathway in wild type flies that have been subjected to aversive olfactory classical conditioning, or to exposure to the constituent odor (CS) or shock (US) alone. dBACE HECT sRNA reads increase significantly in the odor only, shock only, and LTM conditions. However, the increase is larger in the LTM

and shock only conditions than in the odor only condition, and semiquantitative RT-PCR shows that dBACE transcript levels are also induced much more strongly in the shock only and LTM conditions than in the odor only condition. Timecourse qRT-PCR shows that LTM training rapidly and strongly induces dBACE expression, but does not immediately alter expression of the other major APPL pathway components. At 24hrs post training, dBACE expression remains significantly elevated. By 2hrs post-training, the α -secretase Kuz is mildly induced, but this induction is fleeting, and Kuz expression is no longer significantly different from control at 4hrs post-training. Expression of the γ -secretase Psn is significantly elevated by 2hrs post-training, and remains elevated at 24hrs post-training. During the 24hrs following training, APPL expression remains largely unchanged. These qRT-PCR experiments suggest that APPL expression remains largely unchanged, but that its processing is increased and shifted towards the amyloidogenic β - γ -secretase pathway following training. If so, one would expect to observe elevated levels the β -CTF, A β , and AICD. However, previous studies have only reported the detection of β -CTF and AICD by Western blot through the use of transgenic or pharmacological interventions. Further, publications reporting the detection of dA β and AICD by Western blot have utilized overexpression, epitope tags, or both in these experiments.(1, 6) This reflects both the dearth of available reagents with which to study endogenous forms of these metabolites, and the low concentrations at which they are normally found. Accordingly, we were unable to clearly resolve distinct α -CTF and β -CTF bands in our Western blot experiments. We assume that this is due to the overwhelming preference for α -secretase cleavage of APPL and subsequent

obscurance of any β -CTF signal by the α -CTF in Western blot exposures long enough to detect the β -CTF at all. As described earlier in this chapter, AICD is highly unstable in *Drosophila* cells, and probably only accumulates when localized to the nucleus. Thus, the low molecular weight band we observe with very long western blot exposures following training may correspond to such nuclear accumulation. The ~ 6 kD size could also correspond to the $dA\beta$ fragment, as both $dA\beta$ and AICD fall in this size range. However, the antibody we used was raised against a peptide corresponding to the conserved YENPTY motif present within AICD, and not in $dA\beta$. While this fact gives us some confidence in asserting that this band is in fact AICD, additional experiments, perhaps using epitope tags, will be required to unambiguously identify it. In either case, the appearance of this band corresponds with the induction of $dBACE$, and likely reflects a shift to β - γ -secretase processing of APPL. Goguel et al. reported that APPL expression in the MB is required specifically for LTM, but not for learning, STM, or ARM.(26) Our data confirms this result, and expands upon it by showing that appetitive olfactory LTM also requires APPL expression in the MB. Further, we report reduced appetitive olfactory LTM in flies expressing $dBACE$ -hp in the adult MB. However, the effect is not as strong as with APPL-hp, and the negative impact of RU486 on LTM prevents us from unambiguously concluding that $dBACE$ -hp expression in the MB alone is sufficient to abrogate appetitive olfactory LTM. This result could be clarified through the use of other inducible gene expression systems that do not rely on RU486, such as Gal80ts. We also show that STM is retained in adult flies inducibly expressing APPL-hp or $dBACE$ -hp in neurons. Importantly, ARM and LTM are distinguished by the

requirement for protein synthesis in LTM, but not in ARM. This leaves open the possibility that the ARM/LTM distinction reported by Goguel et al. may reflect a requirement for synthesis of dBACE protein and resultant elevated β - γ -cleavage of APPL during LTM formation. Alternatively, signaling downstream of APPL processing may drive production of other proteins required for LTM. Additional experiments will be required to make such an ARM/LTM distinction for flies expressing dBACE-hp, and to explore these possible explanations. Also, the relationship between dBACE expression, the appearance of the ~6kD APPL band following training, and LTM formation will need to be explored further. If this band is in fact AICD, and elevated dBACE activity leads to its accumulation, one would expect that this band is not produced following training in dBACE-hp flies. This experiment remains for future work. Deeper exploration of APPL cleavage following conditioning could benefit from a number of other systems with which to observe APPL cleavage and trafficking that are now available. In one instance, a construct in which Gal4 is fused to C99 (a C-terminal fragment of human APP), and a separate construct expressing a reporter under UAS control are introduced into the same fly. The UAS-reporter is only expressed when C99 is cleaved by Psn, and the AICD-Gal4 fusion enters the nucleus.(44) A similar construct fusing Gal4 to full length APPL could allow for visualization of APPL cleavage and resultant nuclear signaling during LTM formation. Others have used bimolecular fluorescence complementation to explore the protein-protein interactions involved in localizing AICD.(45) Future experiments using such tools could provide key insight into the function of dBACE driven APPL processing in response to classical conditioning.

The original goals of this study were centered around developing a better understanding of how known categories of short regulatory noncoding RNAs participate in memory formation, but our methods were intentionally designed with hopes of identifying novel mechanisms as well. These hopes motivated our decision to select for a wider RNA size range than was necessary to capture microRNAs, esiRNAs, and piRNAs, and to sequence at sufficient depth to capture meaningful information across that broad size range. Similarly, our analytical methods were not constrained to those already in use for a preconceived set of sRNAs. This approach led to our identification of the unusual HECT sRNA signal, and ultimately to our exploration of dBACE's involvement in LTM formation. As such, this study is a clear demonstration of the value of genome wide survey experiments. Our data concerning the responses of microRNAs, piRNAs, and esiRNAs to classical conditioning are a valuable contribution to the knowledge base regarding sRNA function. However, the unforeseen identification of HECTs as a class, and the resulting series of experiments surrounding the activity of dBACE during LTM formation have direct bearing on a conserved mechanism known to be involved in AD. This study thereby supports the utility of *Drosophila* as a model organism with which to study AD. In the past, the relative ease and speed of fly work and the power of *Drosophila* genetics have been used to great effect in understanding the basic mechanisms of disease. Hopefully this will also be the case with AD. Further, the logical progression that led to our exploration of dBACE also yielded observations concerning the regulation of intronless genes that surely warrant

further experimentation. It may be that in casting a wide net, this study has opened a line of experimentation that will also contribute to our understanding of regulated nuclear export, and adds to the catalog of regulatory mechanisms involved in memory formation.

Materials and Methods

Drosophila rearing

Iso-CJ or CS-Quinn flies were reared on standard cornmeal medium at 25 °C under a 12hr light/dark cycle. 1-3 Day old adults were used in all experiments.

Aversive training and memory assay

Training was conducted using a semi automated conditioning apparatus as described in (9). Briefly, a single batch of flies was split into one of four groups: Control, odor only, shock only, or shock + odor (LTM), and housed in bottles containing no food for one hour prior to training. Flies were then loaded into vials that permit the flow of air from the apparatus and contain electrode grids. Odor and shock delivery are controlled by a computer. Following training, flies were returned to bottles containing no food, and allowed to rest for 2 hours before dissection.

Memory formation in LTM flies was evaluated using a T-maze apparatus as described in (8). The performance index (PI) was calculated as the number of flies

avoiding the conditioned odor minus the number of flies avoiding a control odor divided by the total number of flies in the experiment.

Appetitive training and memory assay

48 hrs before training, flies were pre-starved by transferring them into bottles without food, but containing a kimwipe on which 1.5ml of 2% sucrose had been soaked. Flies were then starved for 24hrs prior to training by transferring them to a bottle containing only a dampened kimwipe. Pre-starvation and starvation occurred at 25° C, unless the temperature sensitive Gal80ts allele was being induced, in which case starvation occurred at 30° C. Training occurred in tubes containing either dampened filter paper (control) or filter paper containing 2M sucrose solution (LTM trained). First, all flies are placed into control tubes, and exposed to the CS- odor for 2min. Flies are then transferred into new tubes. Control flies are transferred to a new control tube, and LTM trained flies are transferred to a tube containing filter paper soaked in sucrose. Flies are then exposed to the CS+ odor for 2min. At this point, the handling of flies for LTM testing and STM testing will differ. For LTM, flies are transferred to regular food for 2 hrs and then move to a starvation bottle again for 18-20 hrs before testing. For STM, flies are transferred to an empty tube and connect to training apparatus and exposed to fresh air for 2 min. Memory formation was evaluated using a T-maze apparatus as described in (8). The performance index (PI) was calculated as the number of flies avoiding the conditioned odor minus the number of flies avoiding a control odor divided by the total number of flies in the experiment.

Tissue processing and total RNA extraction

For each sample, heads of 10 male and 10 female flies were dissected and immediately flash frozen and stored at -80 °C while awaiting results from memory testing. Only samples from matched batches of flies in which the LTM trained flies had a PI ≥ 20 were used in subsequent steps. Heads were homogenized in Qiazol (Qiagen) using a motorized tissue homogenizer. Total RNA was extracted using the Qiagen miRNEasy micro kit, according to the manufacturer's protocol. RNA concentration was obtained using a Nanodrop and Bioanalyzer. RNA quality was determined by Bioanalyzer analysis.

sRNA sequencing

Prior to sequencing library preparation, total RNA used for sRNA sequencing was size selected by PAGE, as recommended in the Illumina sRNA sample prep kit (v1.5) protocol. Gel slices containing only 15-35nt RNAs were excised and used in downstream library preparation steps. sRNA sequencing libraries were prepared using the Illumina sRNA sample prep kit (v1.5) according to the manufacturer's protocol. Library quality and concentration were determined by Bioanalyzer analysis. sRNA sequencing was conducted by Harvard systems biology core facility staff, using an Illumina Genome Analyzer IIx.

Sequencing data analysis

Adapter sequences were removed using the FASTX toolkit. For microRNA analysis, sequencing reads were aligned to miRBase release 19 *Drosophila* hairpin sequences using Bowtie v0.12.7.(45, 46) Custom software written in the R and AWK languages were used for canonical and isomiR analysis. For esiRNA and piRNA analysis, reads were aligned to the FlyBase *Drosophila melanogaster* genome (v5.48) using Bowtie v0.12.7.(45). SAMtools was used to convert alignments to BAM format.(47) BedTools v2.18.2 was used to count reads aligning within a given feature, and to merge reads into read-contigs.(48) The UCSC genome browser was used to visualize sequencing data.(49)

Differential expression analysis

The Bioconductor package edgeR v3.1.9 was used in its GLM mode for read count differential expression analysis. Read counts were normalized using the upper quartile method.(50) For isomiR analysis, the percentage of normalized reads with a given non-canonical feature was computed for each miRNA. Two tailed students t-tests were used to test for statistical significance of differences in these percentages. P-values were then adjusted for multiple testing using the Holm-Bonferroni method.

Gene ontology analysis

The PANTHER gene ontology (GO) database was used to assign GO terms and search for over/underrepresented terms.(14)

Genomic information

Genome wide annotations, including intron counts, were obtained from FlyBase (r5.48).

RNA secondary structure prediction

Mfold was used to predict RNA secondary structures.(33)

Western blot

For each sample, heads of 10 male and 10 female flies were dissected and immediately flash frozen and stored at -80 °C for 12hrs. Heads were homogenized in Laemmli buffer containing protease inhibitors (Bio-Rad) using a motorized tissue homogenizer. Samples were electrophoresed on 4-20% gradient Tris-HCl-acrylamide gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad).

Semiquantitative RT-PCR

First strand cDNA was reverse transcribed from 500ng input total RNA, using poly-T primers (Invitrogen) and the Superscript II kit (Invitrogen). Reverse transcription occurred at 42 °C for 1 hour. Oligonucleotide pairs spanning transposon sequences were used to prime 20 cycles of PCR amplification. Perfect Taq (5 prime) polymerase was used for PCR amplification.

qRT-PCR

First strand cDNA was reverse transcribed from 500ng input total RNA, using poly-T primers (Invitrogen) and the Superscript II kit (Invitrogen). Reverse transcription

occurred at 42 °C for 1 hour. Oligonucleotide pairs spanning ~500nt transcript sequences were used to prime amplification. iTaq™ universal SYBR® Green supermix (Bio-Rad) was used for real-time amplification. The reactions were incubated in a 96-well optical plate at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. The threshold cycle (Ct) values were determined using default threshold settings. The Ct is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. For each RNA preparation, qRT-PCR reactions (including no template control), were performed in triplicate. The Ct values determined for each sample were normalized to the average Ct obtained for Gapdh, calculated from triplicate reactions. qRT-PCR was performed using an MJ Opticon (PTC-200 DNA Engine Cycler and CFD-3200 Opticon Detector) from Bio-Rad (formerly MJ research).

PCR Primers

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Summary and Conclusion

sRNA profiles are altered by classical conditioning

The advent and widespread adoption of massively parallel nucleotide sequencing has pushed RNA to the forefront of modern biology. An ever growing catalog of previously unknown or underappreciated RNAs highlights how much remains to be understood concerning the fundamental mechanisms of life. sRNAs are the subject of intense study due to their important functions in gene regulation and the evolutionary conservation of the pathways they participate in. MicroRNAs have become a major area of research in biology generally, and within neuroscience in particular. Despite the increasing availability of massively parallel sequencing technology, most studies of microRNA function in neurons to date have focused on individual microRNAs. To our knowledge, no studies that examine genome wide microRNA expression in the context of *Drosophila* memory formation or behavior have been published. Significant efforts are also directed at understanding the functions of esiRNAs and piRNAs in somatic cells. Though a few published reports indicate that these classes of sRNAs also regulate important aspects of neural function and even behavior, such functions remain poorly studied.(1, 2) Analyses of the now substantial set of publicly available sRNA sequencing data has also revealed a variety of other sRNA types aside from microRNAs, esiRNAs, and piRNAs that either have regulatory functions themselves, or that reveal the activity of other regulatory mechanisms.(3-6) Though much is now known of the basic mechanisms of sRNA function, how neurons utilize these mechanisms during memory formation

remains poorly understood. My efforts have been directed at identifying changes in gene regulation during the course of memory formation that involve sRNAs.

The literature now contains numerous instances in which individual microRNAs regulate aspects of synaptic plasticity and even memory in animals from nematodes to humans. However, microRNAs do not operate in isolation, and far fewer studies have examined the totality of microRNA involvement in these processes at once. Nonetheless, microRNAs are perhaps the best understood and most thoroughly cataloged class of sRNAs. Thus, a primary goal of my work was to profile genome wide microRNA expression in the *Drosophila* head, and to identify changes in this profile during memory formation. Here, I report in detail the expression of microRNA mapping sRNA reads in fly heads, and explore differences in expression between naïve flies, flies that have been subjected to olfactory aversive classical conditioning, and flies subjected to the conditioning protocol but exposed to only the US (Shock) or CS (Odor). I report expression levels for 316 of 427 known mature microRNAs, and show that 5 canonical mature microRNAs have significant changes in expression levels following LTM training. No microRNA was significantly regulated in the odor only condition, and only miR-958-5p changed significantly in the shock only condition. However, the odor only and shock only conditions each had only 3 samples, and additional replicates may yet show that some changes observed in these conditions are indeed significant. If not, the changes in microRNA expression that are significant only with the combination of US and CS may reflect important regulatory events involved in the integration

olfactory and noxious stimuli into a stable memory. In general, changes observed in the shock only condition resemble those of LTM trained flies. This likely reflects the greater influence on microRNA expression induced by the activity of dopaminergic US circuitry than that by activity in the olfactory input to the MB. This makes sense conceptually, as the fly continually receives olfactory stimuli, but only stimuli associated with a US warrant remembering. Thus, it might be expected that US signaling would trigger changes in microRNA expression, though the absence of a salient CS with which to associate the US might trigger such changes in a variety of circuits that respond to many different stimuli. The overwhelming salience provided by the CS odor during LTM training might then refine US induced changes in microRNA expression through strong activation of circuits responding to the CS odor. Following this logic, the coordinated activation of specific circuits by the CS odor ensures that US induced changes in microRNA expression occur within a sufficiently large number of cells expressing a defined set of microRNAs to allow us to detect changes in that set. My observation that significant changes occur only in LTM trained flies might therefore persist even if more replicates were to be added to the shock only and odor only groups. While my results concerning distinctions between LTM conditioning and shock only or odor only treatments are less certain, the differences observed between LTM and control benefit from more replicates and are robust. Of note, I show that miR-312-3p is significantly downregulated following LTM training, and is the only microRNA significantly downregulated in any condition. miR-312-3p has been shown to regulate synaptic plasticity at the NMJ by controlling expression of the motor protein kinesin heavy chain.(7)

Therefore, my result fits with what is known regarding the function of miR-312-3p in neurons. However, the set of microRNAs I identify as being regulated following classical conditioning are together predicted to target transcripts of over 1000 genes. Beyond simply enumerating these targets, I hoped to gain insight into the regulatory output of microRNA modulation during memory formation. I present the results of a detailed target gene set analysis for regulated microRNAs, indicating that the G protein coupled signaling pathway is modulated through their activity. Of particular interest is the targeting of the dopamine receptor Dop1R1 by 4 out of 5 regulated microRNAs. That the US input into the MB is dopaminergic clearly raises the question of whether the significant changes in microRNA expression I report reflect feedback and feedforward mechanisms that tune dopamine response in the MB. As such, the relationship between LTM, changes in the expression of these microRNAs, and expression of Dop1R1 warrants investigation.

My analysis extends beyond canonical mature microRNAs, and documents the extensive presence of offset reads, occurrences of untemplated nucleotides, and tailing of microRNA reads. Such reads may reflect altered microRNA biogenesis, or the activity of mechanisms regulating the turnover of microRNAs.(8-10) Changes in the relative abundance of noncanonical microRNA reads might therefore reveal mechanisms coupling neural activity and microRNA production or turnover. Though noncanonical reads are common in my datasets, I do not observe statically significant changes in the relative abundance of any class for any microRNA. Thus, it

is not likely that microRNA processing or turnover are major avenues through which neural activity alters microRNA mediated silencing in *Drosophila*.

Recent work has demonstrated that the RNA editing enzyme ADAR influences differential expression of polycistronic microRNAs, and can alter microRNA processing and expression.(11, 12) The miRISC associating protein FMRP interacts with and modulates the activity of ADAR in *Drosophila*.(13) ADAR is active in the nervous system of adult *Drosophila*, where it is required for normal behavior, and affects neural excitability.(14, 15) These observations prompted me to examine my data for evidence of regulated microRNA editing during LTM formation. While I was able to detect what appear to be RNA editing events in microRNAs, the fraction of reads exhibiting editing at these sites did not change significantly in any of our experimental groups. Nonetheless, my analyses add to existing knowledge concerning noncanonical microRNAs, and the tissue specific nature of my data will be of use in future investigations of microRNA expression and editing in the *Drosophila* brain.

While my work provides a first genome wide glimpse of microRNA regulation during LTM formation in *Drosophila*, a number of questions will need to be addressed by further experimentation. As hoped, I was able to detect simultaneous changes in the expression levels of several microRNAs following LTM training. However, identifying which cells express each regulated microRNA will be a necessary step toward understanding how these changes together affect gene

expression. If regulated microRNAs are expressed in non-overlapping sets of cells, then expression of each microRNA's target genes can be considered independently. If some or all of the upregulated microRNAs are coexpressed, this may indicate that the set of target genes they share are subject to activity dependent silencing. If cells coexpress miR-312-3p and any of the upregulated microRNAs, interpretation may be more difficult. However, In this case, it may be fruitful to compare expression of genes targeted by miR-312-3p and upregulated microRNAs to those exclusively targeted by one or the other. As mentioned in chapter II, it may be worthwhile following training to compare expression of Dop1R1, which is targeted by miR-312-3p and 3 upregulated microRNAs, and Dop1R2 or Oct β 3R, which are targeted only by miR-312-3p. Differential regulation of these genes could support a model in which changes in microRNA expression tune the MB response to US signaling following training.

My data and existing evidence from previously published studies clearly show that microRNAs participate in activity dependent gene regulation in neurons (Reviewed in (16)). Far less evidence is available in support of such a role for esiRNAs or piRNAs. This is in part due to their more recent discovery. It also stems from the greater number and diversity of esiRNA and piRNA sequences, as these sRNAs are produced from larger and less well defined regions of the genome. Indeed, while it is thought that most if not all expressed *Drosophila* microRNAs have now been identified and validated, esiRNA and piRNA expression remains poorly understood.(17, 18) Moreover, how esiRNAs and piRNAs respond to neural activity

in *Drosophila* remains largely unknown. Mechanistically, it is also less clear how these sRNA classes might function in memory relevant gene regulation. It may be that they act by modulating heterochromatin. Alternatively, they might silence genes post-transcriptionally. To address such questions, I hoped to profile expression of esiRNAs and piRNAs in the *Drosophila* head, and to identify any changes in their expression following conditioning. I designed my sequencing library construction such that a size range of sRNAs encompassing esiRNAs and piRNAs would be captured in addition to microRNAs. Rather than solely relying on previously reported esiRNA and piRNA producing loci, I conducted computational analyses directed at identifying loci with features that are characteristic of these sRNA classes. In this way, I generated expression profiles for esiRNAs and piRNAs, and then conducted differential expression analysis for the loci I identified.

I found 368 esiRNA producing loci, some of which have been reported elsewhere, and some of which are novel.(19, 20) 6 esiRNA loci display significant changes in read counts following LTM training. 4 of 6 regulated esiRNA loci correspond to a novel region of esiRNA production mapping to lysozyme family genes transcribed on both strands, and residing within an intron of the planar cell polarity (PCP) effector gene multiple wing hairs (mwh). Through analysis of reads mapping to multiple locations within this region, as well as through RNA secondary structure predictions, I show that these esiRNAs are likely derived from long hairpin structures formed by the mwh intron, and not from bidirectional transcription of lysozyme family genes. Lysozyme family genes participate in defense against

bacterial infection by catalyzing the hydrolysis of peptidoglycans present in bacterial cell walls, and it is therefore unclear how their regulation would be relevant to memory formation. However, *mwh* is a G-protein binding domain-formin homology 3 (GBD-FH3) protein in the frizzled pathway. It negatively regulates actin polymerization and filament formation, which are processes involved in cytoskeletal reorganization needed to produce structural plasticity at synaptic sites.(21, 22) Thus, a model in which lysozyme family esiRNA production reflects regulation of *mwh* has far clearer logic than one in which lysozyme protein expression participates in memory formation. However, these ideas remain to be tested. *Mwh* has largely been studied in the context of PCP in the wing, and has not been directly implicated in synaptic plasticity or memory formation in *Drosophila*. It will therefore be important to evaluate the impact of *mwh* knockdown on neural function or memory. Such experiments could and should be designed such that the effects of perturbing the different splicing variants of *mwh* are evaluated. The TSS of *mwh*-RA is located between *LysB* and *LysC*, and may therefore be selectively silenced by production of lysozyme family esiRNAs. *mwh*-RB and *mwh*-RC have TSSs that are several kb upstream of *LysB*, and include the full lysozyme gene containing intron. Thus *mwh*-RA may be regulated differently from the other *mwh* variants. RNA hairpins directed against each *mwh* isoform could be conditionally expressed in specific brain regions at various time points around training. *Mwh* cDNA, which would presumably escape regulation by lysozyme family esiRNAs, could be similarly expressed in the brain following training. Such experiments could shed light on the function of *mwh* during memory formation.

piRNAs were first discovered and perhaps best characterized in *Drosophila*. The functions of piRNAs have been most extensively studied in the gonads, as the first phenotypes identified for piRNA pathway mutants related to gametogenesis and fertility. Early studies showed that piRNA pathways primarily function in defending the genome from selfish genetic elements in gametes (Reviewed in (23)). However, piRNAs are also produced in somatic cells, and can function as triggers for epigenetic silencing. Further, piRNAs that target the 3' UTRs of mRNAs have also been identified.(24, 25) Recent publications now implicate piRNAs in control of genetic diversity within the *Drosophila* brain, and even in memory relevant transcriptional silencing.(1, 26) I therefore sought to profile piRNA expression in *Drosophila* heads, and to identify piRNA loci that might respond to LTM training. I identified 82 likely piRNA loci, and conducted differential expression analysis on them. Many of the piRNA loci I identified have been previously reported, supporting the validity of the novel loci I identified. Regulated piRNA loci mapped to LTR retrotransposons, and in all instances exhibited decreased read counts in LTM trained flies vs. control, suggesting that learning might relieve these transposons from silencing via piRNAs. However, my RT-PCR experiments did not show any change in LTR retrotransposon levels. As piRNAs are largely derived from repetitive elements and transposons that are inserted at multiple locations throughout the genome, and tend to be expressed in abundance at these sites, my whole head lysate approach may have hampered my ability to detect changes only

occurring in circuits involved in olfactory memory. Methods permitting collection of piRNAs from specific cell types might overcome such obstacles in future work.

Identification of HECT sRNAs

The primary focus of my work was to identify changes in the expression of microRNAs, piRNAs, and esiRNAs during memory formation. However, novel classes of and functions for sRNAs continue to be discovered. These novel sRNAs can be functional, or merely reflect the activity of other regulatory mechanisms.(4, 6, 27, 28) Such discoveries have often been the result of careful reanalysis of publicly available data sets, or from the examination of reads that are typically discarded during filtering of sRNA data. With this in mind, I have developed sequencing and analytical approaches that capture this type of information. In doing so, I sought to identify novel sRNAs and report their response to LTM training. My sequencing data includes many reads mapping to tRNAs, rRNAs, and other classes of known ncRNAs. The sheer volume of data returned from my sequencing experiments required me to focus my efforts elsewhere, but my preliminary examination suggests that tRNAs may produce sRNAs that are regulated by training. However, further exploration of this possibility remains for future work. My efforts toward identification of novel sRNAs lead me to describe HECT sRNAs as a class, and to report changes in read abundance for a subset of HECTs following LTM training. My work suggests that HECT sRNAs are degradative products from a subset of highly expressed mRNAs. What degradative mechanisms might drive HECT sRNA

production remain unclear, as do the reasons that HECTs are subject to such degradation while other transcripts are not. Degradome sequencing studies are shedding light on the intricacies of mRNA turnover, and could help answer these questions in the near future.(29, 30) However, I found evidence that regulation of HECTs may be tied to the presence of a sequence element termed the cytoplasmic accumulation region element (CAR-E) that is known to increase accumulation and nuclear export of intronless transcripts. However, the significance of this finding remains unclear, and further experimentation will be required to determine if LTM induced changes in HECT expression are related to the presence of the CAR-E in transcripts. Mutation of the CAR-E within regulated HECTs would be relatively straightforward, and could reveal whether training induced changes in HECT expression are dependent on its presence.

dBACE is upregulated during LTM formation

My analysis of HECT sRNAs spurred a deeper analysis of dBACE expression following LTM training. dBACE has the largest increase in HECT sRNAs of any gene following LTM training. dBACE is also the homologue of human BACE genes, which are strongly implicated in Alzheimer's disease pathology. dBACE cleaves the amyloid precursor protein (APP) and its *Drosophila* homologue APPL such that toxic senile plaque forming peptides are liberated. However, senile plaque formation appears to occur only with aberrant APPL and/or dBACE expression. Recent work in mammals suggests that BACE activity is required for normal neurophysiology,

behavior, and memory.(31-33) APP family proteins and the products of their proteolytic processing affect neural morphology, synaptic plasticity, and behavior (Reviewed in (34, 35)). Importantly, the highly conserved C-terminal fragment of APP family proteins is preferentially produced via β - γ -cleavage, and is a modulator of transcriptional programs.(36-38) My work produced evidence that increased dBACE expression following LTM training reflects a shift toward amyloidogenic processing of APPL, and leads to accumulation of AICD. These results are supported by behavioral experiments showing that dBACE and APPL expression are required for LTM, but not for STM or learning. Thus, the work presented here suggests strongly that LTM formation in flies involves a shift toward β - γ -processing of APPL, primarily driven by increased dBACE expression.

My efforts toward understanding the significance of HECT sRNA production, and related changes in dBACE expression have produced intriguing results, and prompt questions that will require additional experiments to address. Importantly, the identity of the APPL fragment that I suggest may be AICD must be confirmed. If the induced fragment is indeed AICD, this would suggest a line of questioning directed at understanding how AICD accumulation is coupled to synaptic plasticity and memory. It will also be critical to understand where in the brain dBACE is expressed, and in which cells its expression increases following training. Our data showing that dBACE and APPL expression in the MB is required for LTM suggests that such a search should begin in this brain region, but greater specificity could prove informative. Reporters of APPL cleavage and AICD nuclear localization

similar to those described in (39) could be used explore the transcriptional regulating activities of AICD during memory formation. In such experiments, nuclear localization of AICD would be revealed by the expression of a reporter, driven by the fusion of Gal4 to the C-terminus of APPL. Presumably, nuclear accumulation of AICD would largely be driven by β -secretase initiated APPL processing. This presumption could be checked through the use of dBACE mutants or knockdown.

Concluding Remarks

The original goal of my work was to understand how microRNA expression changes during memory formation. In designing experiments to tackle this issue, it became clear that a far greater variety of sRNAs could be sequenced, while still obtaining microRNA expression profiles. The unanticipated identification of training induced changes in lysozyme family esiRNAs and dBACE HECT sRNAs demonstrates the prudence of this approach. My work thus underscores the continuing value of survey type experiments in an era of abundant publicly available sequencing data. I had hoped to obtain a clear view of the contributions to sRNA regulation following conditioning from the CS and US separately, and in combination. However, the limited number of samples in the shock only and odor only conditions hampered this effort. Though the similarities between the shock only and LTM trained conditions suggests that most changes in sRNA expression

arise as a result of activity in US circuits, stronger claims in this regard will require additional samples in the shock only and odor only conditions.

The importance of microRNAs in controlling synaptic plasticity is now well established, though not fully understood. My work adds to what is known about microRNA participation in memory formation in that is, to my knowledge, the first simultaneous genome wide examination of microRNA expression during memory formation in *Drosophila*. However, the goal of such studies should be to examine expression of all microRNAs within particular neural circuits, or cells, rather than in whole head lysates. Further, experiments connecting genome wide changes in microRNA expression to changes in target gene expression, and ultimately to changes in synaptic plasticity will be necessary.

My examination of esiRNA expression during memory formation was productive in that it yielded the strongly regulated mwh/LysB-LysS esiRNA locus. However, that so few esiRNA loci display statistically significant changes in expression may support the view that esiRNAs are not a major mechanism for regulating synaptic plasticity. However, existing evidence is not sufficient to conclude one way or the other on this matter. As such, continued study of esiRNA involvement in memory formation may be warranted. Similarly, my work does not indicate that piRNAs are key players in memory formation. However, previous work in mollusks does indicate that piRNAs do regulate memory relevant gene expression.(1) Further, piRNAs do appear to have important functions in the *Drosophila* brain.(26, 40) Thus, it is possible that piRNAs participate in *Drosophila* memory in ways that my work was unable to reveal.

My findings concerning regulation of dBACE during memory formation add to what is known about the mechanisms underlying AD. My work also bolsters *Drosophila* as a model organism with which to study AD. Indeed, *Drosophila* seems poised to make important contributions in this area. Recently, it was shown that cleavage of APPL by dBACE is required non-cell autonomously for glial survival in *Drosophila*.(41) This finding agrees with current research highlighting the contributions of glia to AD progression in mammals. (42-44) The power of *Drosophila* behavioral genetics could yield important mechanistic insight regarding the causes of AD. Moreover, the ease of work in flies, and the abundance of high-throughput *Drosophila* behavioral paradigms could make them a valuable system for use in drug development. β -secretase inhibitors have been a major area of effort for AD treatment. My work underscores the relevance of *Drosophila* for such studies, and supports the view that these interventions may not be the best approach for treating AD. In the future, *Drosophila* may prove valuable in efforts to understand and treat cognitive diseases such as AD. It is my hope that my work will be of use both in understanding AD, and in developing *Drosophila* as a model organism with which to study cognitive disease.

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Appendix
Supplementary Figures

Figure S2.1. Mean normalized canonical miRNA counts

| miRNA | CONTROL | LTM | ODOR | SHOCK |
|-------------|-------------|-------------|-------------|-------------|
| bantam-3p | 269888.5882 | 247239.1294 | 325369.4314 | 293019.7451 |
| bantam-5p | 1806.843137 | 1957.905882 | 1368.215686 | 1153.254902 |
| let-7-3p | 28.3627451 | 17.95294118 | 14 | 26.15686275 |
| let-7-5p | 309420.3627 | 262324.6118 | 376999.0392 | 363086.4118 |
| miR-1-3p | 1995685.892 | 2003480.788 | 1851741.353 | 1851741.353 |
| miR-1-5p | 2.946078431 | 1.558823529 | 2.294117647 | 4.450980392 |
| miR-10-3p | 7796.303922 | 9919.682353 | 8627.72549 | 6699.352941 |
| miR-10-5p | 5675.323529 | 7560.682353 | 6427.411765 | 5229.215686 |
| miR-100-3p | 17.06372549 | 12.48823529 | 17.09803922 | 10.3627451 |
| miR-100-5p | 1952.372549 | 1501.235294 | 2022.411765 | 1739.941176 |
| miR-1000-3p | 99.48529412 | 119.8 | 90.6372549 | 83.42156863 |
| miR-1000-5p | 11138.79412 | 11271.92941 | 8991.901961 | 8849.509804 |
| miR-1001-3p | 12.14705882 | 13.79411765 | 21.30392157 | 15.68627451 |
| miR-1001-5p | 3277.166667 | 2795.647059 | 3148.666667 | 3769.098039 |
| miR-1002-5p | 1.710784314 | 1.911764706 | 1.450980392 | 1.862745098 |
| miR-1003-3p | 597.6960784 | 655.9058824 | 449.627451 | 596.0588235 |
| miR-1003-5p | 129.2205882 | 181.8588235 | 165.9411765 | 167.6568627 |
| miR-1004-3p | 213.0098039 | 312.9058824 | 159.6764706 | 201.5098039 |
| miR-1005-3p | 832.4607843 | 799.1411765 | 569.254902 | 609.0784314 |
| miR-1006-3p | 732.9411765 | 636.8470588 | 730.1372549 | 778.9607843 |
| miR-1006-5p | 49.53431373 | 78.04117647 | 36.95098039 | 32.01960784 |
| miR-1007-3p | 517.127451 | 293.5176471 | 647.1176471 | 582.9803922 |
| miR-1007-5p | 1.598039216 | 9.852941176 | 3.019607843 | 5.431372549 |
| miR-1008-3p | 74.65686275 | 70.72941176 | 50.32352941 | 66.85294118 |
| miR-1009-3p | 950.6568627 | 650.8705882 | 1136.588235 | 840.9803922 |
| miR-1010-3p | 9151.147059 | 10476.97647 | 10832.84314 | 9574.509804 |
| miR-1010-5p | 207.9215686 | 242.0705882 | 203.6862745 | 162.4215686 |
| miR-1011-3p | 35.53921569 | 44.34117647 | 37.03921569 | 38.17647059 |
| miR-1012-3p | 2926.696078 | 3159.329412 | 3637.921569 | 2605.588235 |
| miR-1012-5p | 1949.862745 | 2090.458824 | 1604.607843 | 1319.529412 |
| miR-1013-3p | 284.3333333 | 266.8 | 283.3333333 | 323.1568627 |
| miR-1014-5p | 1.12745098 | 1 | 1 | 1 |
| miR-1015-3p | 12.4754902 | 10.87058824 | 11.66666667 | 10.12745098 |
| miR-1016-3p | 5.352941176 | 4.841176471 | 4.676470588 | 6.539215686 |
| miR-1016-5p | 2.284313725 | 3.611764706 | 3.323529412 | 3.470588235 |
| miR-1017-3p | 328.1176471 | 350.4941176 | 282.9803922 | 240.6470588 |
| miR-11-3p | 30490.45098 | 31048.29412 | 36667.84314 | 35492.09804 |
| miR-11-5p | 424.3529412 | 266.0294118 | 312.9509804 | 369.3137255 |
| miR-12-3p | 50.89705882 | 59.56470588 | 59.31372549 | 74.84313725 |
| miR-12-5p | 17877.08824 | 18915.84706 | 13321.03922 | 14502.88235 |

Figure S2.1 (Continued)

| | | | | |
|-------------|-------------|-------------|-------------|-------------|
| miR-124-3p | 3082.411765 | 2138.317647 | 3240.392157 | 3192.509804 |
| miR-124-5p | 299.5784314 | 247.7764706 | 260.4607843 | 306.8627451 |
| miR-125-3p | 221.2941176 | 206.3529412 | 142.4803922 | 155.3529412 |
| miR-125-5p | 14943.22549 | 16668.29412 | 12903.84314 | 10783.19608 |
| miR-133-3p | 1488.196078 | 1321.188235 | 1143.980392 | 1122.54902 |
| miR-133-5p | 158.9754902 | 173.9470588 | 113.6372549 | 102.9019608 |
| miR-137-3p | 7772.245098 | 5412.670588 | 7624.098039 | 7564.019608 |
| miR-137-5p | 19.89215686 | 18.91764706 | 19.02941176 | 14.40196078 |
| miR-13a-3p | 290.5294118 | 206.3470588 | 298.8921569 | 328.372549 |
| miR-13a-5p | 26.86764706 | 9.5 | 23.38235294 | 46.02941176 |
| miR-13b-3p | 3849.019608 | 3897.364706 | 2922.098039 | 2645.137255 |
| miR-13b-5p | 43.78921569 | 28.58235294 | 37.64705882 | 32.40196078 |
| miR-14-3p | 30708.79412 | 28182.16471 | 40402.11765 | 32289.01961 |
| miR-14-5p | 2400.401961 | 1895.394118 | 4965.529412 | 3585.431373 |
| miR-184-3p | 2571464.049 | 2369941.694 | 2715408.588 | 2715408.588 |
| miR-184-5p | 36.38235294 | 40.98235294 | 31.11764706 | 26.44117647 |
| miR-190-3p | 6.362745098 | 4.023529412 | 5.588235294 | 4.882352941 |
| miR-190-5p | 8708.5 | 6099.529412 | 8375.54902 | 9725.72549 |
| miR-193-3p | 206.8137255 | 133.9647059 | 199.6960784 | 218.2156863 |
| miR-193-5p | 106518.5196 | 110043.5059 | 90377.17647 | 84208.58824 |
| miR-210-3p | 40945.17647 | 43440.24706 | 34552.76471 | 33130.56863 |
| miR-210-5p | 293.8137255 | 282.2588235 | 259.745098 | 241.5098039 |
| miR-219-3p | 47.10784314 | 44.55882353 | 109.2843137 | 93.81372549 |
| miR-219-5p | 210.8235294 | 83.04705882 | 406.5882353 | 454.7843137 |
| miR-2279-3p | 3.166666667 | 3.111764706 | 6.12745098 | 5.62745098 |
| miR-2279-5p | 22.13235294 | 13.54705882 | 19.15686275 | 33.30392157 |
| miR-2282-3p | 11.93137255 | 6.982352941 | 10.08823529 | 12.6372549 |
| miR-2283-3p | 3.691176471 | 1 | 4.068627451 | 1 |
| miR-2283-5p | 1.401960784 | 1 | 2.039215686 | 1.470588235 |
| miR-2489-3p | 375.3333333 | 297.1176471 | 277.5490196 | 344.2156863 |
| miR-2490-5p | 3.264705882 | 3.358823529 | 1.803921569 | 3.470588235 |
| miR-2491-3p | 1.225490196 | 1 | 1 | 1 |
| miR-2491-5p | 1.220588235 | 1.5 | 1 | 1 |
| miR-2492-3p | 1.411764706 | 1 | 3.303921569 | 1.431372549 |
| miR-2492-5p | 1.117647059 | 1 | 1.529411765 | 1 |
| miR-2494-3p | 1.220588235 | 1.258823529 | 1.450980392 | 1.431372549 |
| miR-2496-3p | 3.362745098 | 2.305882353 | 2.254901961 | 2.745098039 |
| miR-2496-5p | 1.318627451 | 2.005882353 | 2.196078431 | 2.803921569 |
| miR-2497-3p | 3.176470588 | 2.017647059 | 4.049019608 | 1 |
| miR-2497-5p | 1.651960784 | 1 | 1 | 1 |
| miR-2499-3p | 1 | 1.188235294 | 1 | 1 |
| miR-2500-3p | 58.59803922 | 60.22352941 | 39.66666667 | 46.16666667 |

Figure S2.1 (Continued)

| | | | | |
|--------------|-------------|-------------|-------------|-------------|
| miR-2500-5p | 11.20098039 | 13.07058824 | 5.578431373 | 12.17647059 |
| miR-2501-3p | 1 | 1 | 1 | 1 |
| miR-2501-5p | 2.43627451 | 2.464705882 | 3.588235294 | 3.431372549 |
| miR-252-3p | 24.17156863 | 21.27647059 | 21.08823529 | 18.7745098 |
| miR-252-5p | 61393.30392 | 54307.17647 | 67713.07843 | 52283.17647 |
| miR-2535b-3p | 222.7156863 | 237.2705882 | 270.4509804 | 252.8627451 |
| miR-263a-3p | 54.42156863 | 51.92941176 | 53.3627451 | 69.17647059 |
| miR-263a-5p | 694123.7745 | 850055.1765 | 883104.0588 | 802718.7647 |
| miR-263b-3p | 27.34803922 | 42.13529412 | 13.23529412 | 18.64705882 |
| miR-263b-5p | 182405.8725 | 230427.3176 | 227987.5686 | 227987.5686 |
| miR-274-3p | 2.029411765 | 1.3 | 2.411764706 | 1 |
| miR-274-5p | 33074.0098 | 30223.70588 | 33611.2549 | 32909.52941 |
| miR-275-3p | 18434.06863 | 19709.95294 | 14208.66667 | 11670.62745 |
| miR-275-5p | 63.40196078 | 61.28823529 | 49.33333333 | 40.54901961 |
| miR-276a-3p | 343515.1471 | 311215.6941 | 195637.8824 | 225696.098 |
| miR-276a-5p | 795.0882353 | 721.8058824 | 808.1666667 | 929.1862745 |
| miR-276b-3p | 58134.47059 | 45172.36471 | 50924.4902 | 58941.31373 |
| miR-276b-5p | 795.0882353 | 721.8058824 | 808.1666667 | 929.1862745 |
| miR-277-3p | 27744.10784 | 31049.62353 | 24599.05882 | 25123.35294 |
| miR-277-5p | 432.2254902 | 391.1647059 | 397.3627451 | 373.8431373 |
| miR-278-3p | 2266.941176 | 1340.105882 | 3220.411765 | 2886.901961 |
| miR-278-5p | 3720.607843 | 2150.276471 | 9258.901961 | 5221.72549 |
| miR-279-3p | 5503.127451 | 5218.047059 | 5433.235294 | 5882.784314 |
| miR-279-5p | 2.431372549 | 2.664705882 | 2.607843137 | 1 |
| miR-281-3p | 1195.083333 | 1200.6 | 1532.470588 | 1273.235294 |
| miR-281-5p | 20.5 | 18.71764706 | 34.99019608 | 29.31372549 |
| miR-282-3p | 1753.441176 | 1572.282353 | 1336.470588 | 1447.529412 |
| miR-282-5p | 543.9019608 | 228.3705882 | 688.0784314 | 896.8627451 |
| miR-283-3p | 70.74509804 | 85 | 68.58823529 | 56.53921569 |
| miR-283-5p | 7270.833333 | 7987.341176 | 7039.588235 | 8488.215686 |
| miR-284-3p | 1.357843137 | 1 | 1 | 1 |
| miR-284-5p | 10105.55882 | 10222.91765 | 9835.607843 | 8898.764706 |
| miR-285-3p | 2746.313725 | 2599.247059 | 2306.352941 | 2091.980392 |
| miR-285-5p | 8039.362745 | 9449.517647 | 9842.156863 | 8746.882353 |
| miR-286-3p | 86.76470588 | 78.74117647 | 104.6862745 | 106.9901961 |
| miR-286-5p | 1 | 1.194117647 | 1 | 1.901960784 |
| miR-2a-3p | 11483.29412 | 11069.70588 | 6968.176471 | 7525.098039 |
| miR-2a-5p | 49.59803922 | 33.51176471 | 59.30392157 | 57.1372549 |
| miR-2b-3p | 7414.666667 | 7309.952941 | 6643.352941 | 5662.803922 |
| miR-2b-5p | 22.89705882 | 28.57058824 | 20.3627451 | 19.83333333 |
| miR-2c-3p | 1235.235294 | 1394.364706 | 859.4509804 | 866.1176471 |
| miR-2c-5p | 1421.764706 | 1196.282353 | 2189.176471 | 1768.862745 |

Figure S2.1 (Continued)

| | | | | |
|-------------|-------------|-------------|-------------|-------------|
| miR-3-3p | 3.137254902 | 1.105882353 | 2.803921569 | 4.519607843 |
| miR-303-3p | 1.240196078 | 1 | 1 | 1 |
| miR-304-3p | 18.66176471 | 18.31764706 | 14.79411765 | 12.93137255 |
| miR-304-5p | 3219.22549 | 2723.882353 | 3986.529412 | 4209.254902 |
| miR-305-3p | 3303.421569 | 3745.4 | 2440.960784 | 2156.882353 |
| miR-305-5p | 12786.78431 | 10331.49412 | 10390.43137 | 12706.45098 |
| miR-306-3p | 316.5784314 | 310.2588235 | 244 | 200.2843137 |
| miR-306-5p | 2625.764706 | 904.0941176 | 3177.215686 | 3717.490196 |
| miR-307a-3p | 7328.441176 | 7120.258824 | 5340.352941 | 6171 |
| miR-307a-5p | 569.1078431 | 702.1411765 | 372.4901961 | 560.8039216 |
| miR-307b-3p | 25.30882353 | 22.72352941 | 17.46078431 | 20.21568627 |
| miR-307b-5p | 9.12745098 | 6.764705882 | 3.637254902 | 2.637254902 |
| miR-308-3p | 198.9411765 | 152.8294118 | 211.8431373 | 327.4901961 |
| miR-308-5p | 65.25490196 | 14.34117647 | 61.40196078 | 110.9019608 |
| miR-310-3p | 1.367647059 | 1.105882353 | 1 | 1 |
| miR-310-5p | 1.617647059 | 1.429411765 | 1 | 1 |
| miR-311-3p | 20.03921569 | 10.00588235 | 16.2254902 | 8.058823529 |
| miR-311-5p | 1.617647059 | 1.241176471 | 1 | 1 |
| miR-312-3p | 180.377451 | 41.90588235 | 77.54901961 | 63.64705882 |
| miR-312-5p | 1 | 1.241176471 | 1 | 1 |
| miR-313-3p | 1.740196078 | 1 | 1 | 1 |
| miR-313-5p | 1.31372549 | 1 | 1.450980392 | 1.431372549 |
| miR-314-3p | 997.1470588 | 2452.635294 | 1329.705882 | 2701.784314 |
| miR-314-5p | 14.8872549 | 36.41764706 | 29.45098039 | 65.67647059 |
| miR-315-3p | 21.70098039 | 12.8 | 15.51960784 | 30.12745098 |
| miR-315-5p | 39317.79412 | 31872.94118 | 40866.37255 | 42066.23529 |
| miR-316-3p | 320.627451 | 143.5647059 | 786.3529412 | 794.3137255 |
| miR-316-5p | 1669.132353 | 1603.082353 | 1989.352941 | 2183.411765 |
| miR-317-3p | 665699.8333 | 752790.0706 | 443856.6275 | 520425 |
| miR-317-5p | 294.1372549 | 286.7647059 | 239.9803922 | 240.1960784 |
| miR-318-3p | 135.4019608 | 49.14705882 | 47.97058824 | 54.41176471 |
| miR-318-5p | 1.098039216 | 1 | 1 | 1 |
| miR-31a-3p | 20.93627451 | 23.36470588 | 14.74509804 | 14.82352941 |
| miR-31a-5p | 8185.754902 | 6890.623529 | 17307.62745 | 10114.13725 |
| miR-31b-5p | 294.9019608 | 331.3294118 | 305.0392157 | 330.0784314 |
| miR-33-3p | 151.3088235 | 149.0941176 | 120.9607843 | 150.127451 |
| miR-33-5p | 15429.79412 | 15969.95294 | 13147.27451 | 15074.88235 |
| miR-34-3p | 2231.352941 | 1952.905882 | 1581.215686 | 1250.921569 |
| miR-34-5p | 33071.82353 | 45512.77647 | 25863.72549 | 27768.58824 |
| miR-3641-5p | 1.549019608 | 1.105882353 | 2.294117647 | 2.37254902 |
| miR-3642-3p | 1.093137255 | 1.241176471 | 1 | 1 |
| miR-3642-5p | 1.833333333 | 1.970588235 | 1.450980392 | 2.333333333 |

Figure S2.1 (Continued)

| | | | | |
|-------------|-------------|-------------|-------------|-------------|
| miR-3643-3p | 1 | 1.194117647 | 1 | 1 |
| miR-3643-5p | 1.220588235 | 1.264705882 | 1.450980392 | 1.431372549 |
| miR-3644-3p | 1 | 1.241176471 | 1 | 1 |
| miR-3645-3p | 2.740196078 | 2.547058824 | 4.333333333 | 2.333333333 |
| miR-3645-5p | 3.392156863 | 2.629411765 | 3.098039216 | 4.343137255 |
| miR-375-3p | 5032.22549 | 4334.2 | 4893.647059 | 5223.980392 |
| miR-375-5p | 256.9117647 | 436.5529412 | 291.1176471 | 283.8235294 |
| miR-4-3p | 2.534313725 | 4.052941176 | 1 | 2.284313725 |
| miR-4-5p | 1 | 1 | 1 | 1 |
| miR-4908-3p | 1.367647059 | 1 | 1 | 1 |
| miR-4910-5p | 4.411764706 | 3.535294118 | 3.382352941 | 3.87254902 |
| miR-4911-3p | 1 | 1 | 1 | 1 |
| miR-4912-5p | 1.117647059 | 1.447058824 | 1 | 1.901960784 |
| miR-4913-3p | 16.39705882 | 10.55882353 | 11.2254902 | 16.97058824 |
| miR-4914-5p | 1.406862745 | 1 | 1 | 1 |
| miR-4915-5p | 2.259803922 | 4.029411765 | 2.31372549 | 1.901960784 |
| miR-4916-3p | 31.47058824 | 16.45882353 | 24.8627451 | 35.01960784 |
| miR-4919-5p | 21.28431373 | 24.60588235 | 9.607843137 | 7.5 |
| miR-4939-3p | 1 | 1 | 1 | 1 |
| miR-4940-3p | 4.700980392 | 4.423529412 | 10.52941176 | 4.343137255 |
| miR-4940-5p | 19.24019608 | 20.42352941 | 29.42156863 | 30.70588235 |
| miR-4941-5p | 1 | 1.258823529 | 1 | 1 |
| miR-4942-3p | 2.401960784 | 4.170588235 | 8.039215686 | 2.637254902 |
| miR-4943-3p | 4.848039216 | 10.23529412 | 12.19607843 | 6.470588235 |
| miR-4946-5p | 2.848039216 | 1.264705882 | 1 | 1 |
| miR-4947-5p | 1 | 1.241176471 | 1 | 1.901960784 |
| miR-4949-3p | 1.62745098 | 1 | 1.450980392 | 1.431372549 |
| miR-4949-5p | 1.892156863 | 1.982352941 | 1 | 1 |
| miR-4950-3p | 1.098039216 | 1.741176471 | 1.450980392 | 1.431372549 |
| miR-4951-3p | 4.338235294 | 3.941176471 | 2.137254902 | 6.107843137 |
| miR-4951-5p | 589.4607843 | 559.7882353 | 579.6470588 | 505.2352941 |
| miR-4952-3p | 5.31372549 | 6.294117647 | 3.058823529 | 2.696078431 |
| miR-4952-5p | 39.62254902 | 35.08823529 | 38.06862745 | 32.90196078 |
| miR-4955-3p | 1.357843137 | 1.794117647 | 2.31372549 | 3.549019608 |
| miR-4956-3p | 6.568627451 | 5.623529412 | 9.921568627 | 5.549019608 |
| miR-4956-5p | 1.465686275 | 1 | 1 | 1 |
| miR-4957-3p | 2.230392157 | 2.470588235 | 2.529411765 | 1.431372549 |
| miR-4957-5p | 1.705882353 | 2.341176471 | 1.745098039 | 5.401960784 |
| miR-4958-5p | 2.254901961 | 2.388235294 | 1.294117647 | 1.81372549 |
| miR-4959-5p | 1.215686275 | 1.188235294 | 1 | 1 |
| miR-4960-3p | 210.2156863 | 232.9529412 | 241.2745098 | 203.5098039 |
| miR-4961-3p | 5.019607843 | 2.129411765 | 7.137254902 | 1 |

Figure S2.1 (Continued)

| | | | | |
|-------------|-------------|-------------|-------------|-------------|
| miR-4961-5p | 3.470588235 | 3.705882353 | 4.156862745 | 9.578431373 |
| miR-4962-3p | 12.84313725 | 14.67647059 | 34.44117647 | 39.80392157 |
| miR-4962-5p | 1 | 1 | 1.294117647 | 1 |
| miR-4963-3p | 6.754901961 | 6.2 | 7.519607843 | 9.843137255 |
| miR-4964-3p | 2.681372549 | 3.352941176 | 3.764705882 | 1 |
| miR-4965-5p | 1 | 1 | 1 | 1.901960784 |
| miR-4966-3p | 1 | 1 | 3.029411765 | 1 |
| miR-4966-5p | 1 | 1 | 1 | 1.882352941 |
| miR-4968-5p | 2.235294118 | 2.347058824 | 3.607843137 | 1 |
| miR-4969-5p | 242.3529412 | 238.4588235 | 326.1764706 | 188.9803922 |
| miR-4971-5p | 5.651960784 | 8.352941176 | 7.107843137 | 3.205882353 |
| miR-4972-3p | 1 | 1 | 1.529411765 | 1 |
| miR-4972-5p | 1 | 1 | 1 | 1 |
| miR-4973-3p | 3.593137255 | 2.788235294 | 1.803921569 | 1.431372549 |
| miR-4973-5p | 14.51470588 | 24.95294118 | 8.803921569 | 8.176470588 |
| miR-4974-3p | 2.401960784 | 1.264705882 | 1.450980392 | 1 |
| miR-4974-5p | 1 | 1 | 1.450980392 | 1 |
| miR-4975-5p | 3.799019608 | 4.011764706 | 5.117647059 | 5.196078431 |
| miR-4976-5p | 7.848039216 | 5.923529412 | 2.470588235 | 10 |
| miR-4977-3p | 5.137254902 | 7.082352941 | 5.245098039 | 7.245098039 |
| miR-4978-5p | 1.093137255 | 1 | 1 | 1 |
| miR-4979-5p | 1.240196078 | 1 | 1 | 1 |
| miR-4980-3p | 1.460784314 | 1.3 | 1.450980392 | 1.431372549 |
| miR-4981-3p | 1.632352941 | 2.411764706 | 1.529411765 | 1.901960784 |
| miR-4982-3p | 1.338235294 | 1 | 1.745098039 | 1 |
| miR-4982-5p | 2.058823529 | 1 | 1 | 4.450980392 |
| miR-4983-3p | 3.25 | 2.270588235 | 3.833333333 | 1.901960784 |
| miR-4983-5p | 1.215686275 | 1.723529412 | 1.745098039 | 2.696078431 |
| miR-4984-3p | 4.774509804 | 6.147058824 | 2.31372549 | 5.401960784 |
| miR-4985-3p | 1 | 1.194117647 | 1 | 1 |
| miR-4985-5p | 2.019607843 | 3.811764706 | 1.745098039 | 2.637254902 |
| miR-4987-3p | 1.098039216 | 1.723529412 | 1.745098039 | 1.431372549 |
| miR-5-3p | 1.240196078 | 2.570588235 | 1.509803922 | 2.725490196 |
| miR-5-5p | 66.77941176 | 57.55882353 | 66.7745098 | 78.80392157 |
| miR-6-3p | 4.475490196 | 5.347058824 | 6.205882353 | 7.254901961 |
| miR-6-5p | 1.62745098 | 1.5 | 1.529411765 | 1 |
| miR-7-3p | 21.84803922 | 13.98823529 | 26.67647059 | 26.40196078 |
| miR-7-5p | 32133.58824 | 36601.18824 | 56724.98039 | 32061.17647 |
| miR-79-3p | 567.5686275 | 425.5058824 | 625.3137255 | 782.9019608 |
| miR-79-5p | 113.7598039 | 97.27058824 | 97.02941176 | 104.7352941 |
| miR-8-3p | 475534.1078 | 516768.0471 | 472690 | 472690 |
| miR-8-5p | 75451.55882 | 94960.6 | 79513.09804 | 84893.92157 |

Figure S2.1 (Continued)

| | | | | |
|------------|-------------|-------------|-------------|-------------|
| miR-87-3p | 6998.088235 | 8694.647059 | 5280.117647 | 4679.784314 |
| miR-87-5p | 100.0833333 | 130.3176471 | 95.90196078 | 78.58823529 |
| miR-927-3p | 11275.22549 | 10322.94118 | 14784.03922 | 13672.60784 |
| miR-927-5p | 18099.2451 | 15007.85882 | 16780.5098 | 18550.86275 |
| miR-929-3p | 572.3039216 | 397.4117647 | 574.5490196 | 669.2352941 |
| miR-929-5p | 6247.980392 | 5004.258824 | 5652.039216 | 7010.647059 |
| miR-92a-3p | 57.75980392 | 57.57647059 | 54.54901961 | 43.56862745 |
| miR-92a-5p | 396.4019608 | 505.1176471 | 481.6666667 | 396.4117647 |
| miR-92b-3p | 354.4460784 | 406.1411765 | 399 | 294.0882353 |
| miR-92b-5p | 1 | 1.258823529 | 1 | 1 |
| miR-932-3p | 201.5147059 | 201.5882353 | 259.8823529 | 165.6078431 |
| miR-932-5p | 20411.69608 | 14287.11765 | 18935.17647 | 19626.11765 |
| miR-954-3p | 1.794117647 | 2.505882353 | 1.803921569 | 2.696078431 |
| miR-954-5p | 251.0196078 | 310.6117647 | 248.254902 | 207.3137255 |
| miR-955-3p | 7.421568627 | 9.064705882 | 10.3627451 | 5.607843137 |
| miR-955-5p | 13.76470588 | 13.91176471 | 5.578431373 | 5.382352941 |
| miR-956-3p | 9160.323529 | 19173.57647 | 15355.17647 | 33107.5098 |
| miR-956-5p | 49.44117647 | 143.5117647 | 91.39215686 | 249.2352941 |
| miR-957-3p | 254726.8039 | 229169.0941 | 262903.0196 | 279107.1176 |
| miR-957-5p | 55.68627451 | 49.29411765 | 60.61764706 | 39.6372549 |
| miR-958-3p | 735.6666667 | 2945.164706 | 973.0784314 | 1975.960784 |
| miR-958-5p | 360.9901961 | 1213.647059 | 765.3921569 | 1519.490196 |
| miR-959-3p | 13.23529412 | 13.95882353 | 13.65686275 | 7.794117647 |
| miR-959-5p | 8.225490196 | 7.3 | 6.735294118 | 6.343137255 |
| miR-960-3p | 8.617647059 | 12.31764706 | 3.460784314 | 5.960784314 |
| miR-960-5p | 12.18627451 | 23.99411765 | 18.23529412 | 19.23529412 |
| miR-961-3p | 1.225490196 | 2.123529412 | 1 | 1.862745098 |
| miR-961-5p | 2.965686275 | 3 | 1.803921569 | 2.803921569 |
| miR-962-5p | 2.362745098 | 3.347058824 | 1.529411765 | 1.431372549 |
| miR-963-3p | 1.12745098 | 1 | 1 | 1 |
| miR-963-5p | 1.519607843 | 1.723529412 | 1.803921569 | 1 |
| miR-964-3p | 1.607843137 | 1 | 1 | 1.431372549 |
| miR-964-5p | 4.06372549 | 4.982352941 | 5.882352941 | 6.81372549 |
| miR-965-3p | 29.42647059 | 25.44117647 | 28.2745098 | 50.73529412 |
| miR-965-5p | 2482.45098 | 2453.694118 | 2022.843137 | 1566.215686 |
| miR-966-3p | 1.117647059 | 1.435294118 | 1 | 1 |
| miR-966-5p | 139.7058824 | 158.9764706 | 173.254902 | 154.1372549 |
| miR-967-3p | 1 | 1.970588235 | 1 | 2.284313725 |
| miR-967-5p | 128.5 | 53.88235294 | 233.7941176 | 191.8431373 |
| miR-968-5p | 1.191176471 | 1.264705882 | 1.450980392 | 1.431372549 |
| miR-969-3p | 77.87254902 | 69.34117647 | 60.62745098 | 64.87254902 |
| miR-969-5p | 11.76470588 | 17.1 | 5.411764706 | 11.06862745 |

Figure S2.1 (Continued)

| | | | | |
|------------|-------------|-------------|-------------|-------------|
| miR-970-3p | 5603.833333 | 7218.529412 | 3799.901961 | 3751.392157 |
| miR-970-5p | 99.5245098 | 129.9176471 | 133.2941176 | 109.7745098 |
| miR-971-3p | 37.55392157 | 43.62941176 | 57.26470588 | 32.97058824 |
| miR-971-5p | 119.2156863 | 127.5294118 | 88.54901961 | 77.64705882 |
| miR-972-3p | 15.78921569 | 19.85882353 | 32.6372549 | 14.44117647 |
| miR-973-3p | 1.946078431 | 1.905882353 | 2.529411765 | 1.901960784 |
| miR-973-5p | 2.421568627 | 2.729411765 | 1.901960784 | 1 |
| miR-974-5p | 1.220588235 | 1.623529412 | 4.784313725 | 1.862745098 |
| miR-975-5p | 1.529411765 | 1.3 | 1.450980392 | 1.431372549 |
| miR-976-3p | 17.78921569 | 14.77647059 | 8.264705882 | 8.519607843 |
| miR-977-3p | 1.828431373 | 1.729411765 | 2.921568627 | 1.901960784 |
| miR-977-5p | 1.705882353 | 1.452941176 | 1 | 1 |
| miR-978-3p | 3.485294118 | 2.594117647 | 4.980392157 | 5.490196078 |
| miR-978-5p | 1 | 1.194117647 | 1 | 1 |
| miR-979-3p | 1 | 1.105882353 | 1.529411765 | 2.294117647 |
| miR-979-5p | 1.12745098 | 1 | 1 | 2.31372549 |
| miR-980-3p | 16.84803922 | 7.511764706 | 15.7254902 | 20.67647059 |
| miR-980-5p | 423 | 491.6823529 | 463 | 414.5882353 |
| miR-981-3p | 13124.55882 | 9919.082353 | 11153.35294 | 11725.35294 |
| miR-981-5p | 50.83333333 | 59.32941176 | 43.12745098 | 53.44117647 |
| miR-982-3p | 1 | 1 | 1 | 2.764705882 |
| miR-982-5p | 50.43137255 | 60.35882353 | 58.18627451 | 48.76470588 |
| miR-983-3p | 1.093137255 | 1.294117647 | 2.254901961 | 1.862745098 |
| miR-983-5p | 183.745098 | 152.7176471 | 195.2156863 | 213.3333333 |
| miR-984-3p | 1.519607843 | 1 | 1.529411765 | 1 |
| miR-984-5p | 2.083333333 | 3.023529412 | 3.588235294 | 2.294117647 |
| miR-985-3p | 1.950980392 | 1.5 | 1 | 1 |
| miR-986-3p | 4.289215686 | 2.782352941 | 5.392156863 | 6.029411765 |
| miR-986-5p | 4.289215686 | 2.129411765 | 3.460784314 | 6.343137255 |
| miR-987-3p | 53.04901961 | 52.44705882 | 30.74509804 | 23.56862745 |
| miR-987-5p | 52767.4902 | 53998.37647 | 51946.80392 | 51495.41176 |
| miR-988-3p | 4269.186275 | 3619.223529 | 2441.078431 | 2185.823529 |
| miR-988-5p | 1878.303922 | 1642.141176 | 1548.176471 | 1426.568627 |
| miR-989-3p | 252.1911765 | 43.72941176 | 89.40196078 | 56.35294118 |
| miR-990-3p | 3.931372549 | 2.647058824 | 2.558823529 | 3.470588235 |
| miR-990-5p | 501.4607843 | 575.4352941 | 497.2941176 | 461.3921569 |
| miR-991-3p | 2.931372549 | 2.411764706 | 2.852941176 | 3.950980392 |
| miR-992-3p | 1 | 1 | 1 | 1 |
| miR-993-3p | 587.0588235 | 517.2117647 | 630.5686275 | 345 |
| miR-993-5p | 176.1421569 | 190.1647059 | 184.0098039 | 162.372549 |
| miR-994-3p | 1.691176471 | 1 | 3.588235294 | 3.480392157 |
| miR-994-5p | 21.38235294 | 2.223529412 | 11.1372549 | 2.245098039 |

Figure S2.1 (Continued)

| | | | | |
|--------------|-------------|-------------|-------------|-------------|
| miR-995-3p | 463.5686275 | 521.0588235 | 353.1568627 | 436.5882353 |
| miR-995-5p | 45.08823529 | 40.93529412 | 28.06862745 | 25.19607843 |
| miR-996-3p | 3538.078431 | 2966.552941 | 3319.098039 | 4547.156863 |
| miR-996-5p | 497.754902 | 586.0352941 | 427.4705882 | 516 |
| miR-998-3p | 530.1764706 | 567.4705882 | 473.7647059 | 490.1764706 |
| miR-998-5p | 1435.539216 | 1119.670588 | 1164.078431 | 1050.882353 |
| miR-999-3p | 57430.87255 | 30981.56471 | 67990.92157 | 74947.27451 |
| miR-999-5p | 15.73039216 | 18.65294118 | 13.25490196 | 13.81372549 |
| miR-9a-3p | 76.34803922 | 75.74117647 | 83.82352941 | 73.24509804 |
| miR-9a-5p | 60806.7549 | 59099.83529 | 62831.60784 | 86406.7451 |
| miR-9b-3p | 639.5784314 | 637.5058824 | 488.0588235 | 481.9411765 |
| miR-9b-5p | 5425.529412 | 4859.094118 | 4516.352941 | 5527.235294 |
| miR-9c-3p | 3.745098039 | 2.617647059 | 6.068627451 | 10.2254902 |
| miR-9c-5p | 61227.17647 | 69701.94118 | 49921.11765 | 55877.29412 |
| miR-iab-4-5p | 22.57352941 | 10.71176471 | 42.15686275 | 15.82352941 |
| miR-iab-8-5p | 4.642156863 | 2.817647059 | 4.882352941 | 1.901960784 |

Canonical microRNA reads were counted for each library. These values were normalized using the quantile method of the limma Bioconductor package.⁽¹⁾ Normalized read counts were then averaged within each condition.

Figure S2.2 Differential expression analysis for canonical miRNAs

| miRNA | logCPM | LTM vs. Control | | | Odor vs. Control | | | Shock vs. Control | | |
|--------------|-------------|-----------------|-------------|-------------|------------------|-------------|-----|-------------------|-------------|-------------|
| | | logFC | PValue | FDR | logFC | PValue | FDR | logFC | PValue | FDR |
| bantam-3p | 15.23585737 | -0.058641294 | 0.880146895 | 1 | 0.273408894 | 0.570653575 | 1 | -0.032526346 | 0.946404969 | 1 |
| bantam-5p | 7.748545166 | 0.099950208 | 0.818797211 | 1 | -0.300485769 | 0.585134712 | 1 | -0.585055405 | 0.288202159 | 0.936915813 |
| let-7-3p | 1.685256517 | -0.578969888 | 0.291292912 | 1 | -1.289216401 | 0.075772142 | 1 | -0.542052855 | 0.433739651 | 1 |
| let-7-5p | 14.65036861 | 0.153503373 | 0.688802168 | 1 | -0.049012437 | 0.92491275 | 1 | -0.330208098 | 0.532671216 | 1 |
| miR-1-3p | 18.28477604 | 0.330378844 | 0.51708143 | 1 | 0.030615613 | | 1 | -0.331340128 | 0.333907745 | 1 |
| miR-10-3p | 9.635691567 | 0.244281423 | 0.562583455 | 1 | 0.087128527 | 0.869317418 | 1 | -0.039922339 | 0.939414573 | 1 |
| miR-10-5p | 8.712946695 | 0.518800502 | 0.204711108 | 1 | 0.337279151 | 0.513043657 | 1 | 0.154533213 | 0.76577772 | 1 |
| miR-100-5p | 8.107038715 | -0.204086467 | 0.630663781 | 1 | -0.459303405 | 0.393002767 | 1 | -0.543708535 | 0.313811019 | 1 |
| miR-1000-3p | 3.73856449 | 0.141021582 | 0.770623235 | 1 | -0.104405035 | 0.862542529 | 1 | -0.416024234 | 0.493700551 | 1 |
| miR-1000-5p | 9.426926455 | 0.110611297 | 0.786898747 | 1 | -0.164019215 | 0.748577324 | 1 | -0.289947021 | 0.571550241 | 1 |
| miR-1001-5p | 2.554266029 | -0.57406814 | 0.280446351 | 1 | -0.815268596 | 0.23460798 | 1 | -0.927554277 | 0.168795431 | 1 |
| miR-1003-3p | 6.607792263 | 0.224754893 | 0.611693062 | 1 | -0.163545309 | 0.770820322 | 1 | 0.07739698 | 0.888438459 | 1 |
| miR-1004-3p | 5.378139854 | 0.330525642 | 0.47500414 | 1 | -0.335100477 | 0.570030178 | 1 | -0.086727602 | 0.879477299 | 1 |
| miR-1005-3p | 7.265455106 | -0.152635307 | 0.73227475 | 1 | -0.486778394 | 0.401630996 | 1 | -0.545551959 | 0.339866723 | 1 |
| miR-1006-3p | 7.067273225 | -0.172446262 | 0.688336684 | 1 | 0.063577008 | 0.905390947 | 1 | -0.02590386 | 0.961230673 | 1 |
| miR-1007-3p | 4.216328839 | -1.032799917 | 0.033862413 | 0.501779388 | -0.714916274 | 0.280451393 | 1 | -0.846293431 | 0.213563485 | 1 |
| miR-1009-3p | 7.239357425 | -0.382926733 | 0.36600589 | 1 | 0.145004747 | 0.784570492 | 1 | -0.045450692 | 0.933527356 | 1 |
| miR-1010-3p | 10.62132796 | -0.020360865 | 0.960892584 | 1 | -0.197465523 | 0.696858631 | 1 | -0.384481711 | 0.448443559 | 1 |
| miR-1011-3p | 2.682050373 | 0.401221925 | 0.423291755 | 1 | -0.04780464 | 0.939967688 | 1 | 0.161616628 | 0.79467726 | 1 |
| miR-1012-3p | 9.273458653 | -0.069019624 | 0.868611295 | 1 | 0.099402241 | 0.846092447 | 1 | -0.38147816 | 0.45851096 | 1 |
| miR-1017-3p | 5.208223806 | -0.084661172 | 0.853331791 | 1 | -0.19990445 | 0.729791681 | 1 | -0.584863983 | 0.311466824 | 1 |
| miR-11-3p | 12.09822583 | -0.174423621 | 0.666587895 | 1 | -0.219256108 | 0.660904305 | 1 | -0.42432024 | 0.397301287 | 1 |
| miR-11-5p | 5.519117565 | -0.43976099 | 0.319838071 | 1 | -0.621314317 | 0.279155453 | 1 | -0.671593258 | 0.241421439 | 1 |
| miR-12-3p | 3.501241861 | 0.223367693 | 0.639491591 | 1 | 0.400112499 | 0.509226225 | 1 | 0.557129005 | 0.352114284 | 1 |
| miR-12-5p | 10.89944788 | 0.102212953 | 0.814337535 | 1 | -0.661650428 | 0.244770336 | 1 | -0.456950407 | 0.425242237 | 1 |
| miR-124-3p | 7.267934818 | -0.101714527 | 0.809850565 | 1 | 0.098257782 | 0.853978653 | 1 | -0.144910433 | 0.786148493 | 1 |
| miR-124-5p | 5.399853181 | -0.021541978 | 0.961434188 | 1 | -0.303392888 | 0.589604817 | 1 | -0.254123281 | 0.65105351 | 1 |
| miR-125-3p | 4.698702483 | -0.339145188 | 0.475267596 | 1 | -0.688202529 | 0.264204034 | 1 | -0.561414236 | 0.356376048 | 1 |
| miR-125-5p | 11.28574705 | -0.037567174 | 0.925230477 | 1 | 0.010880454 | 0.982720545 | 1 | -0.286649132 | 0.568966393 | 1 |
| miR-133-3p | 7.449865748 | -0.000846242 | 0.998451187 | 1 | -0.477885449 | 0.383441468 | 1 | -0.574396948 | 0.301261071 | 1 |
| miR-133-5p | 3.841850651 | 0.014216382 | 0.977784559 | 1 | -0.504979788 | 0.431559153 | 1 | -0.675069985 | 0.295330611 | 1 |
| miR-137-3p | 10.43541844 | -0.470830186 | 0.247289438 | 1 | -0.301468712 | 0.54850661 | 1 | -0.407454375 | 0.418650447 | 1 |
| miR-137-5p | 1.595784662 | -0.294348005 | 0.598366234 | 1 | 0.028197966 | 0.968776516 | 1 | -0.702717172 | 0.327851782 | 1 |
| miR-13a-3p | 3.533710139 | -0.103707445 | 0.833821927 | 1 | -0.254637777 | 0.689037917 | 1 | -0.140466199 | 0.826325974 | 1 |
| miR-13b-1-5p | 2.413520346 | -0.529332729 | 0.304892713 | 1 | -0.137752014 | 0.827448767 | 1 | -0.569765251 | 0.384402643 | 1 |
| miR-13b-2-5p | 2.612289157 | -0.24032295 | 0.639420172 | 1 | -0.323361543 | 0.620225516 | 1 | 0.001110794 | 0.999726672 | 1 |
| miR-13b-3p | 8.725166527 | 0.06207687 | 0.881736551 | 1 | -0.088653213 | 0.864723321 | 1 | -0.331061588 | 0.526400705 | 1 |
| miR-14-3p | 12.0537345 | -0.001839887 | 0.996454858 | 1 | -0.091346829 | 0.85494692 | 1 | -0.190125855 | 0.719348914 | 1 |
| miR-14-5p | 8.041557128 | -0.115341883 | 0.780109392 | 1 | 0.002502451 | 0.996945078 | 1 | -0.492755038 | 0.390688238 | 1 |
| miR-184-3p | 18.32086626 | -0.256756256 | 0.511246169 | 1 | -0.197257539 | 0.041560447 | 1 | -0.619105305 | 0.125966855 | 1 |
| miR-190-5p | 7.389700671 | -0.586467432 | 0.173556832 | 1 | -0.287675342 | 0.586193395 | 1 | -0.394142478 | 0.466183792 | 1 |
| miR-193-3p | 3.998883668 | -0.072517416 | 0.879788213 | 1 | -0.374011105 | 0.553586279 | 1 | -0.347879403 | 0.575747906 | 1 |
| miR-193-5p | 13.64050009 | -0.000274807 | 0.9993906 | 1 | -0.284372923 | 0.564620292 | 1 | -0.371495782 | 0.452486724 | 1 |
| miR-210-3p | 8.609659059 | -0.099246989 | 0.809411624 | 1 | 0.229880784 | 0.659101942 | 1 | 0.174973087 | 0.735440739 | 1 |
| miR-210-5p | 2.979872847 | -0.324538082 | 0.522200907 | 1 | -0.615728315 | 0.336402279 | 1 | -0.837964068 | 0.207128718 | 1 |
| miR-219-3p | 2.727046757 | 0.143711284 | 0.775061965 | 1 | 0.432530256 | 0.536585761 | 1 | 0.137144452 | 0.829226147 | 1 |
| miR-219-5p | 3.740475205 | -1.075333531 | 0.049378954 | 0.670730793 | -1.211367214 | 0.106051779 | 1 | -1.30303325 | 0.092974656 | 1 |
| miR-2489-3p | 5.086020548 | -0.065999343 | 0.883817158 | 1 | -0.182716768 | 0.746438829 | 1 | -0.403121472 | 0.476456102 | 1 |
| miR-2500-3p | 2.494822817 | 0.093567188 | 0.853239418 | 1 | -0.145622992 | 0.825825878 | 1 | -0.212857923 | 0.74743275 | 1 |
| miR-252-5p | 13.14643863 | -0.018054799 | 0.962746767 | 1 | -0.087402212 | 0.864693773 | 1 | -0.434726706 | 0.392183215 | 1 |
| miR-263a-3p | 2.53231484 | -0.276078887 | 0.583264591 | 1 | -0.207333025 | 0.754198372 | 1 | -0.00717753 | 0.990358756 | 1 |
| miR-263b-3p | 1.969115731 | 0.523282952 | 0.338984793 | 1 | -1.083712282 | 0.145291602 | 1 | -0.364217521 | 0.61124957 | 1 |
| miR-263b-5p | 13.9697347 | 0.127116337 | 0.745528884 | 1 | -0.194612296 | 0.70916219 | 1 | -0.495722364 | 0.34577413 | 1 |
| miR-275-3p | 10.79453277 | 0.298617439 | 0.477758016 | 1 | -0.270670149 | 0.608148086 | 1 | -0.604628504 | 0.259781089 | 1 |
| miR-276a-3p | 15.51528667 | -0.137284434 | 0.726270676 | 1 | -0.339176352 | 0.482972245 | 1 | -0.374603187 | 0.439379051 | 1 |
| miR-276b-3p | 12.93522605 | -0.246854625 | 0.537350539 | 1 | -0.514966907 | 0.309569348 | 1 | -0.421847142 | 0.407461915 | 1 |
| miR-277-3p | 11.69884567 | -0.099867638 | 0.798984309 | 1 | 0.437490022 | 0.388771568 | 1 | 0.230811686 | 0.650269337 | 1 |
| miR-277-5p | 5.378827353 | -0.184264525 | 0.676340536 | 1 | 0.135003915 | 0.812340512 | 1 | -0.226730581 | 0.68610722 | 1 |
| miR-278-3p | 7.412567753 | -0.493802602 | 0.268264952 | 1 | -0.798634538 | 0.191235299 | 1 | -0.83923715 | 0.173240519 | 1 |
| miR-278-5p | 9.073283461 | -0.487447906 | 0.238756606 | 1 | 0.070951448 | 0.902035124 | 1 | -0.375823906 | 0.49601967 | 1 |
| miR-279-3p | 6.795250803 | 0.011010601 | 0.979439898 | 1 | 0.084076329 | 0.878259073 | 1 | 0.196950035 | 0.725347188 | 1 |
| miR-281-2-5p | 6.097648577 | 0.075198734 | 0.862628118 | 1 | 0.499594196 | 0.359104336 | 1 | 0.186903333 | 0.729179235 | 1 |

Figure S2.2 (Continued)

| | | | | | | | | | | |
|-------------|-------------|--------------|-------------|-------------|--------------|-------------|---|--------------|-------------|-------------|
| miR-281-3p | 3.316493205 | 0.008964708 | 0.985315433 | 1 | 0.118701798 | 0.845442804 | 1 | 0.158645041 | 0.791005686 | 1 |
| miR-282-3p | 8.367271885 | -0.05502631 | 0.894686928 | 1 | -0.083638774 | 0.872350229 | 1 | 0.083824674 | 0.871631641 | 1 |
| miR-283-5p | 8.889675463 | 0.184239889 | 0.661805509 | 1 | -0.178207501 | 0.729173429 | 1 | 0.115359253 | 0.820774353 | 1 |
| miR-284-5p | 8.528188577 | -0.015733189 | 0.969804434 | 1 | -0.243480051 | 0.637135486 | 1 | -0.26469791 | 0.61066807 | 1 |
| miR-285-3p | 6.82271699 | -0.033106621 | 0.938341073 | 1 | -0.219027771 | 0.688476053 | 1 | -0.042247156 | 0.938092165 | 1 |
| miR-285-5p | 10.23618039 | 0.123303267 | 0.764392858 | 1 | -0.150619114 | 0.765838275 | 1 | -0.347129471 | 0.495340538 | 1 |
| miR-286-3p | 3.919218741 | -0.228904075 | 0.637080464 | 1 | 0.059603695 | 0.91999009 | 1 | -0.121531342 | 0.837530498 | 1 |
| miR-2a-1-5p | 2.329380427 | -0.581042572 | 0.28083852 | 1 | -0.316037206 | 0.635322752 | 1 | -0.40795922 | 0.537260123 | 1 |
| miR-2a-2-5p | 6.423892038 | -1.095063684 | 0.017884934 | 0.323916019 | -0.824663421 | 0.16846089 | 1 | -0.731096367 | 0.232604204 | 1 |
| miR-2a-3p | 6.452557627 | -0.100257453 | 0.815826051 | 1 | 0.208321228 | 0.698449039 | 1 | -0.112623103 | 0.834475809 | 1 |
| miR-2b-1-5p | 1.370915036 | 0.002847814 | 0.997763456 | 1 | 0.439018007 | 0.578323868 | 1 | -0.128611601 | 0.860916459 | 1 |
| miR-2b-2-5p | 6.097843173 | -0.316651553 | 0.466181054 | 1 | -0.174823161 | 0.748632041 | 1 | -0.125540783 | 0.818690101 | 1 |
| miR-2b-3p | 7.264307529 | 0.017241204 | 0.967262864 | 1 | 0.182830116 | 0.733758532 | 1 | -0.082354966 | 0.877558807 | 1 |
| miR-2c-5p | 4.818247587 | 0.131526549 | 0.776875125 | 1 | 0.356948899 | 0.527478242 | 1 | -0.212473558 | 0.709919844 | 1 |
| miR-304-5p | 8.186291826 | -0.154930554 | 0.707267538 | 1 | 0.566696537 | 0.278513944 | 1 | 0.322057596 | 0.540468035 | 1 |
| miR-305-3p | 8.218779781 | -0.033589442 | 0.937399666 | 1 | -0.349772227 | 0.525145132 | 1 | -0.259848235 | 0.633843596 | 1 |
| miR-305-5p | 8.051862412 | -0.290734661 | 0.487140561 | 1 | -0.359473678 | 0.498409205 | 1 | -0.159672038 | 0.761050581 | 1 |
| miR-306-3p | 5.881937572 | -0.102994572 | 0.818094379 | 1 | -0.167856199 | 0.763742444 | 1 | -0.705557653 | 0.216918191 | 1 |
| miR-306-5p | 6.109294119 | -0.835918592 | 0.069514035 | 0.871599056 | -0.748232547 | 0.226180934 | 1 | -0.468082292 | 0.454563584 | 1 |
| miR-307a-3p | 8.499935706 | -0.318882684 | 0.44523638 | 1 | -0.593352574 | 0.255089079 | 1 | -0.329651318 | 0.524007087 | 1 |
| miR-307a-5p | 2.263030857 | 0.469968189 | 0.372834328 | 1 | 0.230172697 | 0.740051303 | 1 | 0.687452684 | 0.319573382 | 1 |
| miR-307b-3p | 1.830528351 | -0.587992357 | 0.314938562 | 1 | -0.690178655 | 0.365127322 | 1 | -0.612664914 | 0.420215664 | 1 |
| miR-308-3p | 3.191713274 | -0.875474543 | 0.089739447 | 1 | -0.003400586 | 0.994166613 | 1 | 0.661571716 | 0.320055735 | 1 |
| miR-312-3p | 3.945523735 | -1.659199692 | 0.001289438 | 0.042035678 | -0.705716964 | 0.297386889 | 1 | -1.318339372 | 0.045947594 | 0.832161977 |
| miR-314-3p | 8.37376838 | 1.443989577 | 0.000982691 | 0.040044656 | 0.548143742 | 0.280283683 | 1 | 1.477616407 | 0.003407902 | 0.185118642 |
| miR-314-5p | 2.284241089 | 1.686189628 | 0.005674363 | 0.132131584 | 0.548037252 | 0.405630076 | 1 | 1.578457236 | 0.015208886 | 0.413174731 |
| miR-315-5p | 11.46591991 | -0.188529457 | 0.641974107 | 1 | -0.315794262 | 0.526760409 | 1 | -0.401067305 | 0.422406374 | 1 |
| miR-316-3p | 5.21356517 | -0.152611281 | 0.750916013 | 1 | -0.443841244 | 0.504859243 | 1 | -0.618420609 | 0.373689784 | 1 |
| miR-316-5p | 8.090571659 | 0.09974866 | 0.810757067 | 1 | 0.077509849 | 0.883950597 | 1 | 0.189112141 | 0.719009104 | 1 |
| miR-317-3p | 13.17322299 | -0.434995885 | 0.311484714 | 1 | -1.075173331 | 0.05791588 | 1 | -0.857250889 | 0.135853947 | 1 |
| miR-317-5p | 5.230829214 | -0.150027263 | 0.737378599 | 1 | -0.154415484 | 0.785272651 | 1 | -0.313975724 | 0.576284665 | 1 |
| miR-318-3p | 3.496263345 | -1.190305361 | 0.022320504 | 0.363824212 | -1.21221768 | 0.062351367 | 1 | -1.347656284 | 0.035754045 | 0.74328897 |
| miR-31a-3p | 1.6540048 | 0.101555761 | 0.857034134 | 1 | -0.141050444 | 0.85187446 | 1 | -0.054749733 | 0.940869503 | 1 |
| miR-31a-5p | 9.9035515 | -0.071160717 | 0.861400609 | 1 | 0.246780269 | 0.659215579 | 1 | -0.099309168 | 0.850914252 | 1 |
| miR-31b-5p | 4.793109398 | 0.178603871 | 0.705975355 | 1 | -0.260080858 | 0.657233017 | 1 | -0.520813916 | 0.381931423 | 1 |
| miR-33-3p | 4.668544478 | -0.047694361 | 0.917444372 | 1 | -0.008445212 | 0.988497279 | 1 | 0.253130576 | 0.65924018 | 1 |
| miR-33-5p | 11.48048597 | 0.05256508 | 0.895185518 | 1 | -0.08285812 | 0.868027311 | 1 | 0.047676531 | 0.923706952 | 1 |
| miR-34-3p | 8.063707758 | -0.104140247 | 0.806250248 | 1 | -0.207895554 | 0.695308962 | 1 | -0.565805583 | 0.289735058 | 1 |
| miR-34-5p | 12.20270936 | 0.376297206 | 0.359275638 | 1 | -0.234880729 | 0.646506739 | 1 | -0.313659967 | 0.545020103 | 1 |
| miR-375-3p | 8.005550386 | 0.214387882 | 0.619259215 | 1 | -0.724247684 | 0.209343945 | 1 | -0.6534049 | 0.260780784 | 1 |
| miR-375-5p | 5.265727028 | 0.380188878 | 0.410860053 | 1 | -0.280214706 | 0.617798277 | 1 | -0.580253266 | 0.304522318 | 1 |
| miR-4919-5p | 1.862810986 | 0.168397763 | 0.763050042 | 1 | -0.728671933 | 0.335225358 | 1 | -1.586494309 | 0.03648044 | 0.74328897 |
| miR-4951-5p | 6.216678219 | 0.070815681 | 0.873726611 | 1 | -0.143796137 | 0.792033071 | 1 | -0.403891691 | 0.460980347 | 1 |
| miR-4960-3p | 5.131359312 | 0.118807574 | 0.795096608 | 1 | -0.131525624 | 0.816419107 | 1 | -0.384866944 | 0.496809071 | 1 |
| miR-4969-5p | 2.364800835 | 0.340384369 | 0.506821976 | 1 | 0.330983656 | 0.608654349 | 1 | -0.146377937 | 0.82181917 | 1 |
| miR-5-5p | 3.36636367 | -0.229698736 | 0.644200273 | 1 | -0.111890738 | 0.854888576 | 1 | 0.01336056 | 0.983057878 | 1 |
| miR-7-5p | 10.36805848 | -0.044280935 | 0.917068692 | 1 | -0.403319929 | 0.492821493 | 1 | -0.583803902 | 0.318043982 | 1 |
| miR-79-3p | 5.455666796 | -0.222775942 | 0.615898992 | 1 | -0.049693811 | 0.931805589 | 1 | 0.17160729 | 0.780927756 | 1 |
| miR-79-5p | 2.823556623 | -0.183766978 | 0.70733866 | 1 | 0.232569174 | 0.735856949 | 1 | 0.075290707 | 0.90821792 | 1 |
| miR-8-3p | 15.85693044 | -0.096992441 | 0.800548007 | 1 | 0.381605323 | 0.42679733 | 1 | 0.274381481 | 0.567788025 | 1 |
| miR-8-5p | 13.81169041 | 0.013230751 | 0.97354938 | 1 | -0.002452417 | 0.996016196 | 1 | -0.061808502 | 0.900152934 | 1 |
| miR-87-3p | 4.105457922 | -0.160188718 | 0.759978441 | 1 | -1.088673872 | 0.140997619 | 1 | -0.695569573 | 0.334715081 | 1 |
| miR-87-5p | 3.683581286 | 0.440664482 | 0.361164037 | 1 | -0.480123123 | 0.434929452 | 1 | -0.578005341 | 0.348691219 | 1 |
| miR-927-3p | 10.60948793 | -0.269885417 | 0.517139014 | 1 | -0.254002513 | 0.616275612 | 1 | -0.451526766 | 0.375351969 | 1 |
| miR-927-5p | 11.45401412 | -0.237436034 | 0.553127092 | 1 | -0.303940521 | 0.539705989 | 1 | -0.381314553 | 0.444711467 | 1 |
| miR-929-3p | 2.506970451 | -0.590105603 | 0.251006266 | 1 | -0.67609194 | 0.312840293 | 1 | -0.270645622 | 0.684263945 | 1 |
| miR-929-5p | 9.005498783 | -0.17753719 | 0.661417255 | 1 | 0.289837706 | 0.580221178 | 1 | 0.379299505 | 0.473262554 | 1 |
| miR-92a-3p | 3.27549448 | -0.162052517 | 0.743178332 | 1 | 0.345295557 | 0.57585119 | 1 | -0.60495265 | 0.342049836 | 1 |
| miR-92a-5p | 5.775516775 | 0.372508293 | 0.419393215 | 1 | -0.282099037 | 0.627574084 | 1 | -0.474261792 | 0.417965972 | 1 |
| miR-92b-3p | 6.107794862 | 0.062031414 | 0.889147878 | 1 | 0.134795338 | 0.80591297 | 1 | -0.504836084 | 0.366048219 | 1 |
| miR-932-3p | 4.19443899 | 0.319402631 | 0.48490241 | 1 | 0.171131805 | 0.782803292 | 1 | -0.189261317 | 0.754317639 | 1 |
| miR-932-5p | 11.37089801 | -0.325020622 | 0.419990024 | 1 | -0.249216316 | 0.615171276 | 1 | -0.1895236 | 0.701503187 | 1 |
| miR-954-5p | 4.365763248 | 0.116402063 | 0.803130427 | 1 | -0.303481373 | 0.604343773 | 1 | -0.649494914 | 0.270657713 | 1 |
| miR-956-3p | 10.91184837 | 1.812438154 | 0.000154324 | 0.008384943 | 0.394817835 | 0.413741093 | 1 | 1.490435064 | 0.001760028 | 0.143442265 |

Figure S2.2 (Continued)

| | | | | | | | | | | |
|------------|-------------|--------------|-------------|-------------|--------------|-------------|---|--------------|-------------|-------------|
| miR-956-5p | 2.948260573 | 1.496591064 | 0.009788804 | 0.199446882 | 0.550971407 | 0.382629954 | 1 | 1.589471753 | 0.010868428 | 0.357331616 |
| miR-957-3p | 14.89873089 | -7.37E-05 | 0.999872694 | 1 | -0.00478068 | 0.992153768 | 1 | -0.215858731 | 0.656442273 | 1 |
| miR-957-5p | 2.85383034 | -0.035817123 | 0.942489985 | 1 | -0.223167393 | 0.719452201 | 1 | -0.844868224 | 0.180799386 | 1 |
| miR-958-3p | 8.310831727 | 2.314706243 | 4.92E-07 | 8.01E-05 | 0.186303652 | 0.712606082 | 1 | 1.265770139 | 0.010961093 | 0.357331616 |
| miR-958-5p | 6.900025359 | 2.158785348 | 4.46E-06 | 0.000363861 | 1.090557475 | 0.038676418 | 1 | 1.897431458 | 0.000267576 | 0.043614936 |
| miR-965-3p | 2.185727415 | 0.115132026 | 0.825147595 | 1 | -0.648687471 | 0.360035701 | 1 | 0.531833202 | 0.418877148 | 1 |
| miR-965-5p | 8.673057205 | -0.040078405 | 0.923648695 | 1 | -0.130946098 | 0.802189004 | 1 | -0.428793312 | 0.413671224 | 1 |
| miR-966-5p | 3.492204108 | 0.261042147 | 0.588434242 | 1 | 0.03542036 | 0.953668679 | 1 | -0.404828471 | 0.517074282 | 1 |
| miR-967-5p | 3.800271284 | -0.596168265 | 0.21974432 | 1 | -0.2194901 | 0.737515533 | 1 | -0.506737358 | 0.428234025 | 1 |
| miR-969-3p | 3.67426177 | -0.054542991 | 0.9093359 | 1 | -0.404694795 | 0.492689429 | 1 | -0.547509734 | 0.353481786 | 1 |
| miR-970-3p | 9.889888449 | 0.363498277 | 0.378513642 | 1 | -0.052915808 | 0.918301256 | 1 | -0.148200385 | 0.774953424 | 1 |
| miR-970-5p | 2.989430389 | 0.354780971 | 0.465227858 | 1 | 0.190790354 | 0.768211605 | 1 | -0.232657342 | 0.724766606 | 1 |
| miR-971-3p | 2.745983975 | 0.032224011 | 0.948608821 | 1 | 0.967916908 | 0.124149682 | 1 | -0.136368314 | 0.835278486 | 1 |
| miR-971-5p | 4.255314796 | 0.091513821 | 0.845332306 | 1 | 0.316918761 | 0.593395279 | 1 | -0.236249476 | 0.692707371 | 1 |
| miR-980-5p | 6.239027074 | -0.126544708 | 0.78115459 | 1 | 0.098283546 | 0.860483976 | 1 | -0.277972542 | 0.620261724 | 1 |
| miR-981-3p | 8.204984709 | -0.548648316 | 0.195904533 | 1 | -0.460475873 | 0.417696922 | 1 | -0.536414522 | 0.336176169 | 1 |
| miR-981-5p | 2.327424776 | -0.027767015 | 0.958064638 | 1 | -0.56018062 | 0.409068799 | 1 | 0.139995321 | 0.829152245 | 1 |
| miR-982-5p | 2.798511974 | 0.513856202 | 0.320590307 | 1 | 0.517863506 | 0.422471861 | 1 | 0.282794795 | 0.657643077 | 1 |
| miR-983-5p | 4.459624063 | -0.192373735 | 0.682554385 | 1 | 0.014548354 | 0.980527413 | 1 | -0.03235354 | 0.955003657 | 1 |
| miR-987-5p | 12.77368322 | 0.103532919 | 0.796941135 | 1 | -0.32950657 | 0.509275876 | 1 | -0.623660981 | 0.212891317 | 1 |
| miR-988-3p | 9.120133801 | -0.192739887 | 0.646834886 | 1 | -0.324100961 | 0.537902496 | 1 | -0.485078023 | 0.357074917 | 1 |
| miR-988-5p | 6.638418198 | -0.140393635 | 0.742488919 | 1 | -0.475359725 | 0.387008862 | 1 | -0.462366792 | 0.405683546 | 1 |
| miR-989-3p | 1.982007788 | -1.614402128 | 0.005450829 | 0.132131584 | -0.209989858 | 0.759678602 | 1 | -0.647151365 | 0.350071959 | 1 |
| miR-990-5p | 5.12536801 | -0.142470623 | 0.752513182 | 1 | -0.36325654 | 0.519076221 | 1 | -0.557394543 | 0.323625118 | 1 |
| miR-993-3p | 4.95980935 | -0.158083802 | 0.724474946 | 1 | 0.336653703 | 0.561078486 | 1 | -0.639712541 | 0.279184378 | 1 |
| miR-993-5p | 4.189138895 | 0.641609359 | 0.166784246 | 1 | 0.255821688 | 0.679395815 | 1 | -0.043012814 | 0.945277263 | 1 |
| miR-995-3p | 6.371078462 | 0.345076459 | 0.431002717 | 1 | 0.084222541 | 0.87863987 | 1 | 0.019490568 | 0.971788725 | 1 |
| miR-995-5p | 1.394820354 | -0.110990302 | 0.847077846 | 1 | 0.172152181 | 0.812713162 | 1 | -0.488236208 | 0.508668712 | 1 |
| miR-996-3p | 8.912290788 | -0.253338497 | 0.537764884 | 1 | -0.081567862 | 0.873915698 | 1 | 0.110503492 | 0.82909609 | 1 |
| miR-996-5p | 4.013570169 | -0.063634969 | 0.894674482 | 1 | -0.091170523 | 0.880868253 | 1 | -0.506443425 | 0.412489136 | 1 |
| miR-998-3p | 6.479387215 | 0.170441745 | 0.694320491 | 1 | 0.017025782 | 0.975188479 | 1 | -0.150266435 | 0.783190351 | 1 |
| miR-998-5p | 5.112213837 | -0.092990825 | 0.838908985 | 1 | -0.727846552 | 0.228106048 | 1 | -0.649981224 | 0.280682624 | 1 |
| miR-999-3p | 12.96543783 | -0.650871594 | 0.10503707 | 1 | -0.317882125 | 0.527620197 | 1 | -0.294609246 | 0.558875817 | 1 |
| miR-9a-5p | 12.1277482 | -0.017140672 | 0.965272802 | 1 | 0.03524345 | 0.943533191 | 1 | -0.100000626 | 0.843449747 | 1 |
| miR-9b-3p | 6.591093721 | -0.122764621 | 0.780341338 | 1 | -0.543443031 | 0.32666275 | 1 | -0.62827422 | 0.253683262 | 1 |
| miR-9b-5p | 9.44835821 | 0.020327894 | 0.960646576 | 1 | -0.313673167 | 0.54172067 | 1 | -0.28295317 | 0.580542927 | 1 |
| miR-9c-5p | 11.62107167 | -0.008822764 | 0.982556318 | 0.999872694 | -0.400528039 | 0.42379021 | 1 | -0.404696698 | 0.420383735 | 1 |

Differential expression analysis for canonical mature microRNAs was conducted using the edgeR Bioconductor software package.(2) 5 canonical mature microRNAs are expressed at levels that differ significantly from control in any condition.

Figure S2.3 DIANA MicroT target predictions for regulated miRNAs

| FlyBase ID | Symbol | dme-miR-312-3p | dme-miR-314-3p | dme-miR-956-3p | dme-miR-958-3p | dme-miR-958-5p |
|-------------|---------|----------------|----------------|----------------|----------------|----------------|
| FBgn0000157 | Dll | 1 | 0 | 0 | 0 | 0 |
| FBgn0003382 | sha | 1 | 0 | 0 | 0 | 0 |
| FBgn0004396 | CrebA | 1 | 0 | 0 | 0 | 0 |
| FBgn0038439 | Cad89D | 1 | 0 | 0 | 0 | 0 |
| FBgn0032001 | CG8360 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034143 | CG8303 | 1 | 0 | 0 | 0 | 0 |
| FBgn0019968 | Khc-73 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036741 | CG7510 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039810 | CG15549 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033867 | Cpr50Ca | 1 | 0 | 0 | 0 | 0 |
| FBgn0038438 | Der-2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029835 | CG5921 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051122 | CG31122 | 1 | 0 | 1 | 0 | 0 |
| FBgn0085227 | CG34198 | 1 | 0 | 0 | 0 | 0 |
| FBgn0001319 | kn | 1 | 0 | 1 | 0 | 0 |
| FBgn0038073 | CG14395 | 1 | 0 | 0 | 0 | 0 |
| FBgn0035283 | CG12024 | 1 | 0 | 0 | 1 | 0 |
| FBgn0035625 | Blimp-1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051224 | CG31224 | 1 | 0 | 0 | 0 | 0 |
| FBgn0017549 | 0 | 1 | 1 | 0 | 0 | 0 |
| FBgn0033996 | CG11807 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028642 | #N/A | 1 | 0 | 0 | 1 | 0 |
| FBgn0039530 | Tusp | 1 | 0 | 0 | 0 | 0 |
| FBgn0086475 | sec3 | 1 | 0 | 0 | 0 | 0 |
| FBgn0035050 | ST6Gal | 1 | 0 | 0 | 0 | 0 |
| FBgn0015567 | #N/A | 1 | 0 | 0 | 1 | 0 |
| FBgn0259789 | vfl | 1 | 0 | 0 | 0 | 0 |
| FBgn0035144 | CG17181 | 1 | 1 | 0 | 0 | 0 |
| FBgn0033903 | CG8323 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034997 | CG3376 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036935 | CG14186 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037672 | sage | 1 | 0 | 0 | 0 | 0 |
| FBgn0015772 | Nak | 1 | 0 | 0 | 1 | 0 |
| FBgn0085250 | CG34221 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038418 | pad | 1 | 0 | 0 | 0 | 0 |
| FBgn0030921 | CG6290 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040359 | CG11380 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038815 | CG5466 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037744 | CG8417 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034509 | Obp57c | 1 | 0 | 0 | 0 | 0 |
| FBgn0037416 | Osi9 | 1 | 0 | 0 | 0 | 0 |
| FBgn0262636 | Lin29 | 1 | 0 | 1 | 0 | 0 |
| FBgn0038463 | CG3534 | 1 | 0 | 0 | 0 | 0 |
| FBgn0260451 | CG14042 | 1 | 0 | 0 | 0 | 0 |
| FBgn0004656 | fs(1)h | 1 | 0 | 0 | 0 | 0 |
| FBgn0039808 | CG12071 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051632 | sens-2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0023441 | fus | 1 | 0 | 0 | 0 | 0 |
| FBgn0051637 | CG31637 | 1 | 1 | 0 | 0 | 0 |
| FBgn0031457 | CG3077 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|------------|---|---|---|---|---|
| FBgn0031375 | erm | 1 | 0 | 0 | 0 | 0 |
| FBgn0010350 | CdsA | 1 | 0 | 0 | 0 | 0 |
| FBgn0261239 | Hr39 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040532 | CG8369 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039431 | CG6490 | 1 | 0 | 1 | 0 | 0 |
| FBgn0016794 | dos | 1 | 0 | 0 | 0 | 0 |
| FBgn0003870 | ttk | 1 | 0 | 1 | 0 | 0 |
| FBgn0030668 | CG8128 | 1 | 0 | 0 | 0 | 0 |
| FBgn0008654 | 0 | 1 | 0 | 1 | 0 | 0 |
| FBgn0262737 | mub | 1 | 0 | 1 | 0 | 0 |
| FBgn0033609 | fbl6 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038611 | CG14309 | 1 | 0 | 0 | 0 | 0 |
| FBgn0005633 | fln | 1 | 0 | 0 | 0 | 0 |
| FBgn0003733 | tor | 1 | 0 | 0 | 0 | 0 |
| FBgn0011656 | Mef2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029846 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0004101 | bs | 1 | 0 | 0 | 0 | 0 |
| FBgn0034808 | CG9896 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032023 | CG14274 | 1 | 0 | 0 | 0 | 0 |
| FBgn0030408 | CG11085 | 1 | 0 | 0 | 0 | 0 |
| FBgn0030362 | regucalcin | 1 | 0 | 0 | 0 | 0 |
| FBgn0036819 | dysb | 1 | 0 | 0 | 0 | 0 |
| FBgn0036323 | CG14118 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261648 | salm | 1 | 0 | 0 | 0 | 0 |
| FBgn0036285 | toe | 1 | 0 | 0 | 0 | 0 |
| FBgn0034304 | CG5742 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261274 | Ero1L | 1 | 0 | 0 | 0 | 0 |
| FBgn0038461 | CG3678 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033448 | hebe | 1 | 0 | 0 | 0 | 0 |
| FBgn0259750 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0052105 | CG32105 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000562 | egl | 1 | 0 | 0 | 0 | 0 |
| FBgn0040099 | lectin-28C | 1 | 0 | 0 | 0 | 0 |
| FBgn0260963 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0086372 | lap | 1 | 0 | 0 | 1 | 0 |
| FBgn0051038 | CG31038 | 1 | 0 | 0 | 0 | 0 |
| FBgn0004397 | Vinc | 1 | 0 | 0 | 0 | 0 |
| FBgn0050463 | CG30463 | 1 | 0 | 0 | 0 | 0 |
| FBgn0026313 | X11L | 1 | 0 | 0 | 0 | 0 |
| FBgn0003209 | raw | 1 | 0 | 0 | 0 | 0 |
| FBgn0032513 | CG6565 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032129 | jp | 1 | 1 | 0 | 0 | 0 |
| FBgn0037988 | CG14740 | 1 | 0 | 0 | 0 | 0 |
| FBgn0015014 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0021895 | ytr | 1 | 0 | 0 | 0 | 0 |
| FBgn0039380 | CG5890 | 1 | 0 | 0 | 0 | 0 |
| FBgn0003162 | Pu | 1 | 0 | 0 | 0 | 0 |
| FBgn0035308 | CG15822 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031698 | Ncoa6 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028421 | Kap3 | 1 | 1 | 0 | 0 | 0 |
| FBgn0262740 | Evi5 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | | |
|-------------|---------------|---|---|---|---|---|---|
| FBgn0086910 | | 0 | 1 | 0 | 0 | 0 | 0 |
| FBgn0026616 | alpha-Man-IIb | | 1 | 0 | 0 | 0 | 0 |
| FBgn0034420 | CG10737 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0004370 | Ptp10D | | 1 | 0 | 0 | 0 | 0 |
| FBgn0005677 | dac | | 1 | 0 | 0 | 0 | 0 |
| FBgn0030499 | CG11178 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0259986 | nab | | 1 | 0 | 0 | 0 | 0 |
| FBgn0004381 | Klp68D | | 1 | 0 | 0 | 0 | 0 |
| FBgn0035338 | CG13800 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0036360 | CG10713 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0003507 | srp | | 1 | 1 | 0 | 1 | 0 |
| FBgn0015323 | VAcHT | | 1 | 0 | 0 | 0 | 0 |
| FBgn0259791 | bora | | 1 | 0 | 0 | 0 | 0 |
| FBgn0025360 | Optix | | 1 | 0 | 0 | 0 | 0 |
| FBgn0028997 | nmdyn-D7 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0039187 | CG6454 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0004914 | Hnf4 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0040752 | Prosap | | 1 | 0 | 0 | 0 | 0 |
| FBgn0020309 | crol | | 1 | 0 | 0 | 0 | 0 |
| FBgn0013759 | CASK | | 1 | 0 | 0 | 0 | 0 |
| FBgn0015776 | nrv1 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0085414 | dpr12 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0030997 | CG7990 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0033984 | Lap1 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0043841 | vir-1 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0037241 | CG14646 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0260400 | elav | | 1 | 0 | 0 | 1 | 0 |
| FBgn0015129 | | 0 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051869 | CG31869 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0010303 | hep | | 1 | 0 | 0 | 0 | 0 |
| FBgn0039257 | tnc | | 1 | 0 | 0 | 0 | 0 |
| FBgn0034271 | CG4996 | | 1 | 0 | 0 | 1 | 0 |
| FBgn0034327 | CG14505 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0000567 | Eip74EF | | 1 | 0 | 0 | 0 | 0 |
| FBgn0030778 | CG4678 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0023215 | Mnt | | 1 | 0 | 0 | 0 | 0 |
| FBgn0030004 | CG10958 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0030774 | spherioide | | 1 | 0 | 0 | 0 | 0 |
| FBgn0036544 | sff | | 1 | 0 | 0 | 0 | 0 |
| FBgn0051005 | qlless | | 1 | 0 | 0 | 0 | 0 |
| FBgn0039852 | nyo | | 1 | 0 | 1 | 0 | 0 |
| FBgn0051191 | CG31191 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0035540 | Syx17 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0024238 | Fim | | 1 | 0 | 0 | 0 | 0 |
| FBgn0005640 | Eip63E | | 1 | 0 | 0 | 0 | 0 |
| FBgn0039887 | CG2053 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0052600 | dpr8 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0028872 | CG18095 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0000120 | Arr1 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0052638 | CG32638 | | 1 | 0 | 0 | 0 | 1 |
| FBgn0038787 | CG4360 | | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|------------|---|---|---|---|---|
| FBgn0263097 | Glut4EF | 1 | 0 | 0 | 0 | 0 |
| FBgn0037101 | CG7634 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039132 | AP-1sigma | 1 | 0 | 0 | 0 | 0 |
| FBgn0029893 | CG14442 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261986 | RASSF8 | 1 | 1 | 1 | 0 | 0 |
| FBgn0034057 | CG8314 | 1 | 0 | 0 | 0 | 0 |
| FBgn0262350 | #N/A | 1 | 1 | 0 | 0 | 0 |
| FBgn0004198 | ct | 1 | 0 | 0 | 0 | 1 |
| FBgn0013948 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0052553 | CG32553 | 1 | 0 | 0 | 0 | 0 |
| FBgn0024555 | ffl | 1 | 0 | 0 | 0 | 0 |
| FBgn0033138 | Tsp42Eq | 1 | 0 | 0 | 0 | 0 |
| FBgn0038281 | RpL10Aa | 1 | 0 | 0 | 0 | 0 |
| FBgn0262573 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0262582 | cic | 1 | 0 | 0 | 0 | 0 |
| FBgn0039528 | dsd | 1 | 0 | 0 | 0 | 0 |
| FBgn0036623 | CG4729 | 1 | 0 | 0 | 0 | 0 |
| FBgn0052119 | CG32119 | 1 | 0 | 0 | 0 | 0 |
| FBgn0043070 | MESK2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0014870 | Psi | 1 | 0 | 0 | 0 | 1 |
| FBgn0002283 | I(3)73Ah | 1 | 0 | 0 | 0 | 0 |
| FBgn0003254 | rib | 1 | 0 | 0 | 0 | 0 |
| FBgn0034433 | endoB | 1 | 0 | 0 | 1 | 0 |
| FBgn0004873 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0050392 | CG30392 | 1 | 0 | 0 | 0 | 0 |
| FBgn0087007 | bbg | 1 | 0 | 0 | 0 | 0 |
| FBgn0003060 | CG9757 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032340 | Ge-1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0003149 | Prm | 1 | 0 | 0 | 0 | 0 |
| FBgn0259231 | CCKLR-17D1 | 1 | 1 | 0 | 0 | 0 |
| FBgn0028500 | Rich | 1 | 0 | 0 | 0 | 0 |
| FBgn0024944 | Oamb | 1 | 0 | 0 | 0 | 0 |
| FBgn0020245 | 0 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036927 | CG7433 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028996 | onecut | 1 | 0 | 0 | 0 | 0 |
| FBgn0015229 | glec | 1 | 0 | 0 | 0 | 0 |
| FBgn0052195 | CG32195 | 1 | 0 | 0 | 0 | 0 |
| FBgn0259937 | Nop60B | 1 | 0 | 0 | 0 | 0 |
| FBgn0004569 | aos | 1 | 0 | 0 | 0 | 0 |
| FBgn0037107 | CG7166 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034230 | CG4853 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038658 | CG14292 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036958 | CG17233 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036271 | Pbgs | 1 | 0 | 0 | 0 | 0 |
| FBgn0262353 | CR43051 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029761 | SK | 1 | 1 | 1 | 0 | 1 |
| FBgn0031453 | Bacc | 1 | 0 | 0 | 0 | 0 |
| FBgn0000064 | Ald | 1 | 0 | 0 | 0 | 0 |
| FBgn0035574 | RhoGEF64C | 1 | 0 | 0 | 0 | 0 |
| FBgn0053543 | CG33543 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038296 | CG6752 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|-----------|---|---|---|---|---|
| FBgn0044823 | Spec2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0050424 | CG30424 | 1 | 0 | 0 | 0 | 0 |
| FBgn0086677 | jeb | 1 | 0 | 0 | 0 | 0 |
| FBgn0036483 | CG12316 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085424 | nub | 1 | 0 | 0 | 0 | 0 |
| FBgn0004242 | Syt1 | 1 | 1 | 0 | 0 | 0 |
| FBgn0039911 | CG1909 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029996 | Ubc-E2H | 1 | 0 | 0 | 0 | 0 |
| FBgn0085376 | CG34347 | 1 | 0 | 0 | 0 | 0 |
| FBgn0003715 | 0 | 1 | 0 | 0 | 1 | 0 |
| FBgn0050147 | Hil | 1 | 0 | 0 | 0 | 0 |
| FBgn0038332 | CG6136 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000037 | mAcR | 1 | 0 | 0 | 0 | 0 |
| FBgn0261836 | Msp-300 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037428 | Osi18 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085404 | CG34375 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040395 | Unc-76 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036770 | Prestin | 1 | 0 | 0 | 0 | 0 |
| FBgn0025821 | l-t | 1 | 0 | 0 | 0 | 0 |
| FBgn0028541 | TM95F4 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036814 | CG14073 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031304 | CG4552 | 1 | 0 | 0 | 0 | 0 |
| FBgn0015778 | rin | 1 | 0 | 0 | 0 | 0 |
| FBgn0262729 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0083949 | CG34113 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039078 | CG4374 | 1 | 1 | 1 | 0 | 0 |
| FBgn0035016 | CG4612 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000210 | br | 1 | 0 | 0 | 0 | 0 |
| FBgn0039958 | CG12567 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000541 | E(bx) | 1 | 0 | 0 | 0 | 0 |
| FBgn0000317 | ck | 1 | 0 | 0 | 0 | 0 |
| FBgn0086736 | GckIII | 1 | 0 | 0 | 0 | 0 |
| FBgn0259935 | CG42458 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261560 | Thor | 1 | 0 | 0 | 0 | 0 |
| FBgn0013983 | imd | 1 | 0 | 0 | 0 | 0 |
| FBgn0250910 | Octbeta3R | 1 | 0 | 0 | 0 | 0 |
| FBgn0025186 | ari-2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034399 | CG15083 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085383 | CG34354 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040697 | Teh3 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051536 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0035270 | CG13933 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038388 | CG4287 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033589 | CG13227 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032700 | CG10338 | 1 | 0 | 0 | 1 | 0 |
| FBgn0000547 | ed | 1 | 0 | 0 | 0 | 0 |
| FBgn0010905 | Spn | 1 | 0 | 0 | 1 | 0 |
| FBgn0030617 | CG9095 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000394 | cv | 1 | 0 | 0 | 0 | 0 |
| FBgn0015623 | Cpr | 1 | 0 | 0 | 0 | 0 |
| FBgn0050080 | CG30080 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------------|---|---|---|---|---|
| FBgn0032021 | CG7781 | 1 | 0 | 0 | 1 | 0 |
| FBgn0015919 | caup | 1 | 0 | 0 | 0 | 0 |
| FBgn0038109 | CG11656 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000524 | dx | 1 | 0 | 0 | 1 | 0 |
| FBgn0086378 | Alg-2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0041004 | CG17715 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031981 | CG7466 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029715 | CG11444 | 1 | 0 | 0 | 0 | 0 |
| FBgn0035429 | CG12017 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040334 | Tsp3A | 1 | 0 | 0 | 0 | 0 |
| FBgn0044826 | Pak3 | 1 | 0 | 0 | 0 | 1 |
| FBgn0261244 | inaE | 1 | 0 | 0 | 0 | 0 |
| FBgn0035229 | CG7852 | 1 | 1 | 0 | 1 | 0 |
| FBgn0029861 | CG3815 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038124 | CG14380 | 1 | 0 | 0 | 1 | 0 |
| FBgn0039704 | neo | 1 | 1 | 0 | 0 | 0 |
| FBgn0039411 | dys | 1 | 0 | 0 | 0 | 0 |
| FBgn0038818 | Nep4 | 1 | 0 | 0 | 0 | 0 |
| FBgn0012051 | CalpA | 1 | 0 | 0 | 0 | 0 |
| FBgn0020389 | Papss | 1 | 0 | 0 | 0 | 0 |
| FBgn0022935 | D19A | 1 | 0 | 0 | 0 | 0 |
| FBgn0005671 | Vha55 | 1 | 0 | 0 | 0 | 0 |
| FBgn0023407 | B4 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039161 | CG13606 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261786 | mi | 1 | 0 | 0 | 0 | 0 |
| FBgn0001138 | grn | 1 | 0 | 0 | 0 | 0 |
| FBgn0051140 | CG31140 | 1 | 0 | 1 | 0 | 0 |
| FBgn0263117 | CG34377 | 1 | 0 | 1 | 0 | 0 |
| FBgn0031676 | CG14040 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036760 | CG5567 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033906 | CG8331 | 1 | 0 | 0 | 0 | 0 |
| FBgn0030252 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0030532 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0039634 | alpha-Man-Ib | 1 | 0 | 0 | 1 | 0 |
| FBgn0000577 | en | 1 | 0 | 0 | 0 | 0 |
| FBgn0025724 | beta'Cop | 1 | 0 | 0 | 0 | 0 |
| FBgn0030788 | Sap30 | 1 | 0 | 0 | 0 | 0 |
| FBgn0030680 | CG8944 | 1 | 1 | 1 | 0 | 0 |
| FBgn0003334 | Scm | 1 | 0 | 0 | 0 | 0 |
| FBgn0004197 | Ser | 1 | 0 | 0 | 0 | 0 |
| FBgn0035490 | CG1136 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038304 | CG12241 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038339 | CG6118 | 1 | 0 | 0 | 0 | 0 |
| FBgn0046704 | Liprin-alpha | 1 | 0 | 0 | 0 | 0 |
| FBgn0028875 | nAcRalpha-34E | 1 | 0 | 0 | 0 | 0 |
| FBgn0033677 | CG8321 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031478 | CG8814 | 1 | 0 | 0 | 0 | 0 |
| FBgn0001259 | in | 1 | 0 | 0 | 0 | 0 |
| FBgn0041203 | LIMK1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0052445 | CG32445 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039730 | CG7903 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|-----------|---|---|---|---|---|
| FBgn0015278 | Pi3K68D | 1 | 0 | 0 | 0 | 0 |
| FBgn0030654 | CG15643 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040816 | CG12521 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033608 | CG13220 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000286 | Cf2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029092 | ced-6 | 1 | 0 | 0 | 0 | 1 |
| FBgn0010280 | Taf4 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028371 | jbug | 1 | 0 | 0 | 0 | 0 |
| FBgn0035772 | Sh3beta | 1 | 0 | 0 | 0 | 0 |
| FBgn0029970 | Nek2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037847 | SelR | 1 | 0 | 0 | 0 | 0 |
| FBgn0040271 | Sulf1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0041210 | HDAC4 | 1 | 0 | 0 | 0 | 0 |
| FBgn0030745 | CG4239 | 1 | 0 | 0 | 0 | 0 |
| FBgn0030756 | CG9903 | 1 | 0 | 0 | 0 | 0 |
| FBgn0035253 | CG7971 | 1 | 0 | 0 | 0 | 0 |
| FBgn0067864 | Patj | 1 | 0 | 0 | 0 | 0 |
| FBgn0028999 | nerfin-1 | 1 | 0 | 1 | 0 | 0 |
| FBgn0004436 | UbcD6 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034926 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0015295 | shark | 1 | 0 | 0 | 0 | 0 |
| FBgn0003301 | rut | 1 | 0 | 0 | 0 | 0 |
| FBgn0030930 | GalNAc-T2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0002921 | Atpalpha | 1 | 0 | 0 | 0 | 0 |
| FBgn0001324 | kto | 1 | 0 | 0 | 0 | 0 |
| FBgn0067628 | CG33331 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040230 | dbo | 1 | 0 | 0 | 0 | 1 |
| FBgn0038420 | CG10311 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038435 | Gyc-89Da | 1 | 0 | 0 | 0 | 0 |
| FBgn0030812 | CG8949 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037279 | CG1129 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031760 | Tsp26A | 1 | 0 | 0 | 1 | 0 |
| FBgn0003721 | Tm1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0043536 | Obp57d | 1 | 0 | 0 | 0 | 0 |
| FBgn0040502 | CG8343 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033958 | CG12858 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037993 | dpr15 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032140 | CG13117 | 1 | 0 | 0 | 0 | 0 |
| FBgn0027835 | Dp1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032601 | yellow-b | 1 | 0 | 0 | 0 | 0 |
| FBgn0040343 | CG3713 | 1 | 0 | 0 | 0 | 0 |
| FBgn0259749 | mmy | 1 | 0 | 0 | 0 | 0 |
| FBgn0028704 | Nckx30C | 1 | 1 | 0 | 0 | 0 |
| FBgn0032341 | Reps | 1 | 0 | 1 | 1 | 0 |
| FBgn0259708 | CG42362 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085371 | CG34342 | 1 | 0 | 0 | 0 | 0 |
| FBgn0050446 | Tdc2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033058 | CCHa2r | 1 | 0 | 0 | 0 | 0 |
| FBgn0001297 | kay | 1 | 0 | 0 | 0 | 0 |
| FBgn0000054 | Adf1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085196 | CG34167 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------|---|---|---|---|---|
| FBgn0036761 | MED19 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038460 | CG18622 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033465 | Etf-QO | 1 | 0 | 0 | 0 | 0 |
| FBgn0262716 | Arp3 | 1 | 0 | 0 | 0 | 0 |
| FBgn0260466 | Indy-2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0260470 | SP555 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036008 | CG3408 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037794 | CG6254 | 1 | 0 | 0 | 0 | 0 |
| FBgn0003042 | Pc | 1 | 0 | 0 | 0 | 0 |
| FBgn0026386 | Or47a | 1 | 0 | 0 | 0 | 0 |
| FBgn0004598 | Fur2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0053203 | CG33203 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085432 | pan | 1 | 0 | 0 | 0 | 0 |
| FBgn0030447 | CG2200 | 1 | 0 | 0 | 0 | 0 |
| FBgn0053196 | dp | 1 | 0 | 0 | 0 | 0 |
| FBgn0052150 | CG32150 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033222 | CG12824 | 1 | 0 | 0 | 0 | 0 |
| FBgn0086472 | RpS25 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028582 | lqf | 1 | 0 | 0 | 0 | 0 |
| FBgn0260941 | app | 1 | 0 | 0 | 0 | 0 |
| FBgn0031636 | CG12194 | 1 | 0 | 0 | 0 | 0 |
| FBgn0003028 | ovo | 1 | 1 | 0 | 0 | 0 |
| FBgn0030296 | CG15196 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036334 | CG11267 | 1 | 0 | 0 | 0 | 0 |
| FBgn0011742 | Arp2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0045852 | ham | 1 | 0 | 0 | 0 | 0 |
| FBgn0051342 | CG31342 | 1 | 0 | 0 | 0 | 0 |
| FBgn0010381 | Drs | 1 | 0 | 0 | 0 | 0 |
| FBgn0036488 | CG6878 | 1 | 0 | 0 | 0 | 0 |
| FBgn0042083 | CG3267 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034517 | Cpr57A | 1 | 0 | 0 | 0 | 0 |
| FBgn0030018 | slpr | 1 | 0 | 0 | 0 | 0 |
| FBgn0011300 | babo | 1 | 0 | 0 | 0 | 0 |
| FBgn0030913 | CG6123 | 1 | 1 | 0 | 0 | 0 |
| FBgn0005619 | Hdc | 1 | 0 | 0 | 0 | 0 |
| FBgn0037326 | CG14669 | 1 | 0 | 0 | 0 | 0 |
| FBgn0014343 | mirr | 1 | 0 | 0 | 0 | 0 |
| FBgn0040208 | Kat60 | 1 | 0 | 0 | 0 | 0 |
| FBgn0052702 | CG32702 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031882 | Rab30 | 1 | 0 | 0 | 0 | 0 |
| FBgn0086901 | cv-c | 1 | 0 | 0 | 0 | 0 |
| FBgn0000117 | arm | 1 | 0 | 0 | 0 | 0 |
| FBgn0034957 | CG3121 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031395 | CG10874 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000119 | arr | 1 | 0 | 0 | 0 | 0 |
| FBgn0033382 | Hydr1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0259211 | grh | 1 | 0 | 0 | 0 | 0 |
| FBgn0035383 | CG2107 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034072 | Dg | 1 | 0 | 0 | 0 | 0 |
| FBgn0013733 | shot | 1 | 0 | 0 | 1 | 0 |
| FBgn0261552 | ps | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|-------------|---|---|---|---|---|
| FBgn0004395 | unk | 1 | 0 | 0 | 0 | 0 |
| FBgn0028879 | CG15270 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000546 | EcR | 1 | 0 | 1 | 0 | 0 |
| FBgn0004924 | Top1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0027885 | Aac11 | 1 | 0 | 0 | 0 | 0 |
| FBgn0262511 | Vha44 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036165 | chrB | 1 | 0 | 0 | 0 | 0 |
| FBgn0259142 | 0 | 1 | 0 | 0 | 0 | 0 |
| FBgn0020762 | Atet | 1 | 0 | 0 | 0 | 0 |
| FBgn0026206 | mei-P26 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000273 | Pka-C1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0046247 | CG5938 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000097 | aop | 1 | 0 | 0 | 0 | 0 |
| FBgn0000464 | Lar | 1 | 0 | 0 | 0 | 0 |
| FBgn0040759 | CG13177 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032178 | Spn31A | 1 | 0 | 0 | 0 | 0 |
| FBgn0004666 | sim | 1 | 0 | 0 | 0 | 0 |
| FBgn0039580 | Gfat2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033853 | CG6145 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000028 | acj6 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039776 | PH4alphaEFB | 1 | 0 | 0 | 1 | 0 |
| FBgn0052651 | CG32651 | 1 | 0 | 0 | 0 | 0 |
| FBgn0026084 | cib | 1 | 0 | 0 | 0 | 0 |
| FBgn0000303 | Cha | 1 | 0 | 0 | 0 | 0 |
| FBgn0085434 | NaCP60E | 1 | 0 | 0 | 0 | 0 |
| FBgn0003016 | osp | 1 | 0 | 0 | 0 | 1 |
| FBgn0031950 | Herp | 1 | 0 | 0 | 0 | 0 |
| FBgn0037120 | CG11247 | 1 | 0 | 0 | 0 | 0 |
| FBgn0004647 | N | 1 | 0 | 0 | 0 | 0 |
| FBgn0001291 | Jra | 1 | 0 | 0 | 0 | 0 |
| FBgn0028916 | CG33090 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036688 | Fit2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0262738 | norpA | 1 | 1 | 1 | 0 | 0 |
| FBgn0085385 | CG34356 | 1 | 1 | 0 | 0 | 0 |
| FBgn0038858 | CG5793 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032901 | sky | 1 | 0 | 0 | 0 | 0 |
| FBgn0035388 | CG2162 | 1 | 0 | 0 | 0 | 0 |
| FBgn0026371 | SAK | 1 | 0 | 0 | 0 | 0 |
| FBgn0052579 | CG32579 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031879 | uif | 1 | 0 | 0 | 0 | 0 |
| FBgn0027535 | botv | 1 | 0 | 0 | 0 | 0 |
| FBgn0036485 | FucTA | 1 | 0 | 0 | 0 | 0 |
| FBgn0024244 | drm | 1 | 0 | 0 | 0 | 0 |
| FBgn0031174 | CG1486 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261555 | CG42673 | 1 | 0 | 0 | 0 | 0 |
| FBgn0027594 | drpr | 1 | 0 | 0 | 0 | 0 |
| FBgn0015591 | Ast | 1 | 0 | 0 | 0 | 0 |
| FBgn0031232 | CG11617 | 1 | 0 | 0 | 0 | 0 |
| FBgn0262739 | AGO1 | 1 | 1 | 0 | 0 | 0 |
| FBgn0035987 | CG3689 | 1 | 0 | 0 | 1 | 0 |
| FBgn0035955 | CG5194 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------------|---|---|---|---|---|
| FBgn0033876 | Syng1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0015774 | NetB | 1 | 0 | 0 | 1 | 0 |
| FBgn0262111 | f | 1 | 0 | 0 | 0 | 0 |
| FBgn0035241 | CG12105 | 1 | 0 | 0 | 0 | 0 |
| FBgn0025726 | unc-13 | 1 | 0 | 0 | 0 | 0 |
| FBgn0010473 | tut1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0086359 | Invadolysin | 1 | 0 | 0 | 0 | 0 |
| FBgn0037842 | CG6567 | 1 | 0 | 0 | 0 | 0 |
| FBgn0030985 | Obp18a | 1 | 0 | 0 | 0 | 0 |
| FBgn0031298 | Atg4 | 1 | 0 | 0 | 0 | 0 |
| FBgn0035170 | dpr20 | 1 | 0 | 0 | 0 | 0 |
| FBgn0052085 | CG32085 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037472 | CG10098 | 1 | 0 | 0 | 0 | 0 |
| FBgn0259739 | CG42393 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051313 | CG31313 | 1 | 0 | 0 | 0 | 0 |
| FBgn0001970 | Pgant35A | 1 | 0 | 0 | 0 | 0 |
| FBgn0022770 | Peritrophin-A | 1 | 0 | 0 | 0 | 0 |
| FBgn0037636 | CG9821 | 1 | 1 | 0 | 0 | 0 |
| FBgn0030049 | Trf4-1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0011740 | alpha-Man-II | 1 | 0 | 0 | 1 | 0 |
| FBgn0031770 | CG13995 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034266 | CG4975 | 1 | 0 | 0 | 1 | 0 |
| FBgn0051915 | CG31915 | 1 | 0 | 0 | 0 | 0 |
| FBgn0053556 | form3 | 1 | 0 | 0 | 1 | 0 |
| FBgn0015799 | Rbf | 1 | 0 | 0 | 0 | 0 |
| FBgn0261999 | CG42817 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037622 | CG8202 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038341 | CG14869 | 1 | 1 | 1 | 0 | 0 |
| FBgn0037917 | wkd | 1 | 0 | 0 | 0 | 0 |
| FBgn0031592 | Art2 | 1 | 0 | 0 | 0 | 0 |
| FBgn0011826 | Pp2B-14D | 1 | 0 | 0 | 0 | 0 |
| FBgn0033915 | CG8485 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034425 | CG11906 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036556 | CG5830 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037848 | Tsp86D | 1 | 0 | 0 | 0 | 0 |
| FBgn0052767 | CG32767 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029508 | Tsp42Ea | 1 | 0 | 0 | 1 | 0 |
| FBgn0000253 | Cam | 1 | 0 | 1 | 0 | 0 |
| FBgn0062978 | CG31808 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033638 | CG9005 | 1 | 0 | 0 | 0 | 0 |
| FBgn0263042 | CG43337 | 1 | 0 | 0 | 0 | 0 |
| FBgn0250848 | 26-29-p | 1 | 0 | 0 | 0 | 0 |
| FBgn0037516 | CG11286 | 1 | 0 | 0 | 0 | 0 |
| FBgn0010417 | Taf6 | 1 | 0 | 0 | 0 | 0 |
| FBgn0023423 | slmb | 1 | 0 | 0 | 0 | 0 |
| FBgn0037364 | Rab23 | 1 | 0 | 0 | 0 | 0 |
| FBgn0033279 | CG2291 | 1 | 0 | 0 | 0 | 0 |
| FBgn0050419 | CG30419 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051949 | CG31949 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261387 | CG17528 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032409 | Ced-12 | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------|---|---|---|---|---|
| FBgn0024277 | trio | 1 | 0 | 0 | 0 | 0 |
| FBgn0037130 | Syn1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0035593 | CG4603 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028431 | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0015513 | mbc | 1 | 0 | 0 | 0 | 0 |
| FBgn0002577 | m | 1 | 0 | 0 | 0 | 0 |
| FBgn0032120 | CG33298 | 1 | 0 | 0 | 0 | 1 |
| FBgn0031675 | CG9121 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031310 | CG4764 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038237 | Pde6 | 1 | 0 | 0 | 0 | 0 |
| FBgn0036948 | CG7298 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038893 | CG6353 | 1 | 0 | 0 | 0 | 0 |
| FBgn0024362 | CG11412 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085405 | CG34376 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038880 | SIFR | 1 | 0 | 0 | 0 | 0 |
| FBgn0011582 | Dop1R1 | 1 | 1 | 1 | 1 | 0 |
| FBgn0000448 | Hr46 | 1 | 1 | 0 | 0 | 0 |
| FBgn0033607 | CG9062 | 1 | 0 | 0 | 0 | 0 |
| FBgn0015872 | Drip | 1 | 0 | 0 | 0 | 0 |
| FBgn0022268 | KdelR | 1 | 0 | 0 | 0 | 0 |
| FBgn0262896 | CG43251 | 1 | 0 | 0 | 0 | 0 |
| FBgn0039925 | Kif3C | 1 | 0 | 0 | 0 | 0 |
| FBgn0031260 | Spp | 1 | 0 | 0 | 0 | 0 |
| FBgn0261553 | CG42671 | 1 | 1 | 0 | 0 | 0 |
| FBgn0028476 | CG15817 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031257 | CG4133 | 1 | 0 | 0 | 0 | 1 |
| FBgn0000221 | brn | 1 | 0 | 0 | 0 | 0 |
| FBgn0035529 | CG1319 | 1 | 0 | 0 | 0 | 0 |
| FBgn0029962 | CG1402 | 1 | 0 | 0 | 0 | 0 |
| FBgn0260812 | inaF-D | 1 | 0 | 0 | 0 | 0 |
| FBgn0038652 | CG7720 | 1 | 0 | 0 | 0 | 0 |
| FBgn0051523 | CG31523 | 1 | 0 | 0 | 0 | 0 |
| FBgn0032987 | Rpl21 | 1 | 0 | 0 | 0 | 0 |
| FBgn0031811 | CG13982 | 1 | 0 | 0 | 0 | 0 |
| FBgn0037556 | CG9636 | 1 | 1 | 0 | 0 | 0 |
| FBgn0011676 | Nos | 1 | 0 | 0 | 0 | 0 |
| FBgn0038324 | CG5038 | 1 | 0 | 0 | 0 | 0 |
| FBgn0023000 | mth | 1 | 0 | 0 | 0 | 0 |
| FBgn0026259 | eIF5B | 1 | 0 | 0 | 0 | 0 |
| FBgn0032003 | CG8349 | 1 | 0 | 0 | 0 | 0 |
| FBgn0053516 | dpr3 | 1 | 0 | 0 | 0 | 0 |
| FBgn0052452 | CG32452 | 1 | 0 | 0 | 0 | 0 |
| FBgn0011584 | Trp1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0005278 | Sam-S | 1 | 0 | 0 | 0 | 0 |
| FBgn0024814 | Clc | 1 | 0 | 0 | 0 | 0 |
| FBgn0032763 | CG17568 | 1 | 0 | 0 | 0 | 0 |
| FBgn0026086 | Adar | 1 | 0 | 1 | 0 | 0 |
| FBgn0033693 | CG13175 | 1 | 0 | 0 | 0 | 0 |
| FBgn0263131 | CG43373 | 1 | 0 | 1 | 0 | 0 |
| FBgn0035504 | Teh4 | 1 | 1 | 0 | 0 | 0 |
| FBgn0023091 | dimm | 1 | 0 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|-----------|---|---|---|---|---|
| FBgn0052574 | Twdlalpha | 1 | 0 | 0 | 0 | 0 |
| FBgn0042630 | Sox21b | 1 | 0 | 0 | 0 | 1 |
| FBgn0053555 | #N/A | 0 | 1 | 0 | 0 | 0 |
| FBgn0013753 | Bgb | 0 | 1 | 0 | 0 | 0 |
| FBgn0024234 | gbb | 0 | 1 | 1 | 0 | 0 |
| FBgn0262579 | Ect4 | 0 | 1 | 1 | 0 | 0 |
| FBgn0040388 | boi | 0 | 1 | 1 | 0 | 0 |
| FBgn0023081 | gek | 0 | 1 | 1 | 0 | 0 |
| FBgn0032702 | CG10376 | 0 | 1 | 0 | 0 | 0 |
| FBgn0037336 | CG2519 | 0 | 1 | 1 | 1 | 0 |
| FBgn0032895 | twit | 0 | 1 | 0 | 0 | 0 |
| FBgn0083961 | CG34125 | 0 | 1 | 0 | 0 | 0 |
| FBgn0000490 | dpp | 0 | 1 | 0 | 0 | 0 |
| FBgn0038118 | timeout | 0 | 1 | 0 | 0 | 0 |
| FBgn0036610 | CG13058 | 0 | 1 | 0 | 0 | 0 |
| FBgn0004449 | Ten-m | 0 | 1 | 0 | 0 | 0 |
| FBgn0040682 | CG14664 | 0 | 1 | 0 | 0 | 0 |
| FBgn0263218 | 0 | 0 | 1 | 1 | 0 | 0 |
| FBgn0036402 | CG6650 | 0 | 1 | 0 | 0 | 0 |
| FBgn0038705 | CG11626 | 0 | 1 | 0 | 0 | 0 |
| FBgn0026147 | CG16833 | 0 | 1 | 0 | 0 | 0 |
| FBgn0259210 | prom | 0 | 1 | 0 | 0 | 0 |
| FBgn0085450 | Snoo | 0 | 1 | 0 | 0 | 0 |
| FBgn0028484 | Ack | 0 | 1 | 0 | 0 | 0 |
| FBgn0260499 | qvr | 0 | 1 | 1 | 0 | 0 |
| FBgn0037856 | CG4674 | 0 | 1 | 0 | 0 | 0 |
| FBgn0034158 | CG5522 | 0 | 1 | 0 | 0 | 0 |
| FBgn0034691 | synj | 0 | 1 | 0 | 0 | 0 |
| FBgn0035151 | CG17129 | 0 | 1 | 0 | 0 | 0 |
| FBgn0031816 | CG16947 | 0 | 1 | 0 | 0 | 0 |
| FBgn0085400 | CG34371 | 0 | 1 | 1 | 0 | 0 |
| FBgn0004839 | otk | 0 | 1 | 0 | 0 | 0 |
| FBgn0038545 | CG7713 | 0 | 1 | 0 | 0 | 0 |
| FBgn0027364 | Six4 | 0 | 1 | 0 | 0 | 0 |
| FBgn0024321 | NK7.1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0261456 | hpo | 0 | 1 | 0 | 0 | 0 |
| FBgn0085397 | Fili | 0 | 1 | 0 | 0 | 0 |
| FBgn0260632 | dl | 0 | 1 | 0 | 0 | 0 |
| FBgn0015396 | jumu | 0 | 1 | 0 | 0 | 0 |
| FBgn0086253 | rumi | 0 | 1 | 0 | 0 | 0 |
| FBgn0027546 | CG4766 | 0 | 1 | 0 | 0 | 0 |
| FBgn0037722 | CG8319 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030482 | CG1673 | 0 | 1 | 0 | 0 | 0 |
| FBgn0032374 | CG14931 | 0 | 1 | 0 | 0 | 0 |
| FBgn0259985 | Mppe | 0 | 1 | 0 | 0 | 0 |
| FBgn0038320 | Sra-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0029514 | 312 | 0 | 1 | 0 | 0 | 0 |
| FBgn0011211 | blw | 0 | 1 | 0 | 0 | 0 |
| FBgn0025632 | CG4313 | 0 | 1 | 0 | 0 | 0 |
| FBgn0032485 | CG9426 | 0 | 1 | 0 | 0 | 0 |
| FBgn0004622 | Takr99D | 0 | 1 | 1 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|--------------|---|---|---|---|---|
| FBgn0035510 | Cpr64Aa | 0 | 1 | 0 | 0 | 0 |
| FBgn0035148 | CG3402 | 0 | 1 | 0 | 0 | 0 |
| FBgn0010894 | sinu | 0 | 1 | 0 | 0 | 0 |
| FBgn0003071 | Pfk | 0 | 1 | 0 | 0 | 1 |
| FBgn0037408 | NPFR1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0259244 | CG42342 | 0 | 1 | 0 | 0 | 0 |
| FBgn0035997 | phol | 0 | 1 | 0 | 0 | 0 |
| FBgn0085335 | #N/A | 0 | 1 | 0 | 0 | 0 |
| FBgn0033901 | O-fut1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0031149 | CG1518 | 0 | 1 | 0 | 0 | 0 |
| FBgn0032955 | CG2201 | 0 | 1 | 0 | 0 | 0 |
| FBgn0035359 | CG1143 | 0 | 1 | 0 | 0 | 0 |
| FBgn0027512 | CG10254 | 0 | 1 | 1 | 0 | 0 |
| FBgn0260934 | par-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0010548 | Aldh-III | 0 | 1 | 0 | 0 | 0 |
| FBgn0029878 | Pat1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030309 | CG1572 | 0 | 1 | 0 | 0 | 0 |
| FBgn0051960 | CG31960 | 0 | 1 | 0 | 0 | 0 |
| FBgn0004892 | sob | 0 | 1 | 0 | 1 | 0 |
| FBgn0050035 | Tret1-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0028550 | Atf3 | 0 | 1 | 1 | 0 | 0 |
| FBgn0044028 | Notum | 0 | 1 | 0 | 0 | 0 |
| FBgn0016059 | Sema-1b | 0 | 1 | 1 | 0 | 0 |
| FBgn0031632 | CG15628 | 0 | 1 | 1 | 0 | 0 |
| FBgn0015268 | Nap1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0036564 | #N/A | 0 | 1 | 0 | 0 | 0 |
| FBgn0040777 | CG14767 | 0 | 1 | 0 | 0 | 0 |
| FBgn0034720 | Liprin-gamma | 0 | 1 | 0 | 0 | 0 |
| FBgn0000667 | Actn | 0 | 1 | 0 | 0 | 0 |
| FBgn0036317 | CG10948 | 0 | 1 | 0 | 1 | 0 |
| FBgn0038874 | ETHR | 0 | 1 | 0 | 0 | 0 |
| FBgn0263116 | S-HT1B | 0 | 1 | 0 | 0 | 0 |
| FBgn0028506 | CG4455 | 0 | 1 | 1 | 0 | 0 |
| FBgn0051716 | Cnot4 | 0 | 1 | 0 | 0 | 0 |
| FBgn0032723 | ssp3 | 0 | 1 | 0 | 0 | 0 |
| FBgn0038829 | CG17271 | 0 | 1 | 0 | 1 | 0 |
| FBgn0083991 | CG34155 | 0 | 1 | 0 | 0 | 0 |
| FBgn0039532 | Mtl | 0 | 1 | 0 | 0 | 0 |
| FBgn0030877 | Arp8 | 0 | 1 | 0 | 0 | 0 |
| FBgn0037475 | Fer1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0033652 | ths | 0 | 1 | 0 | 0 | 0 |
| FBgn0033827 | CG17047 | 0 | 1 | 0 | 0 | 0 |
| FBgn0086783 | #N/A | 0 | 1 | 0 | 0 | 0 |
| FBgn0036450 | CG13472 | 0 | 1 | 0 | 0 | 0 |
| FBgn0250862 | CG42237 | 0 | 1 | 0 | 0 | 0 |
| FBgn0037643 | skap | 0 | 1 | 0 | 0 | 0 |
| FBgn0039945 | CG17159 | 0 | 1 | 0 | 0 | 0 |
| FBgn0036518 | RhoGAP71E | 0 | 1 | 0 | 0 | 0 |
| FBgn0032934 | CG8679 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030932 | Ggt-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0040305 | MTF-1 | 0 | 1 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|------------|---|---|---|---|---|
| FBgn0035583 | CG13704 | 0 | 1 | 0 | 0 | 0 |
| FBgn0003206 | Ras64B | 0 | 1 | 0 | 0 | 0 |
| FBgn0013988 | #N/A | 0 | 1 | 0 | 0 | 0 |
| FBgn0036922 | CG14182 | 0 | 1 | 0 | 0 | 0 |
| FBgn0262160 | CG9932 | 0 | 1 | 0 | 0 | 0 |
| FBgn0010389 | htl | 0 | 1 | 0 | 0 | 0 |
| FBgn0003459 | stwl | 0 | 1 | 0 | 0 | 0 |
| FBgn0034472 | CG8517 | 0 | 1 | 0 | 0 | 0 |
| FBgn0027951 | MTA1-like | 0 | 1 | 0 | 0 | 0 |
| FBgn0038595 | CG7142 | 0 | 1 | 1 | 0 | 0 |
| FBgn0053558 | mim | 0 | 1 | 1 | 0 | 0 |
| FBgn0010435 | emp | 0 | 1 | 0 | 0 | 0 |
| FBgn0016792 | dmt | 0 | 1 | 0 | 0 | 0 |
| FBgn0010238 | Lac | 0 | 1 | 0 | 0 | 0 |
| FBgn0260012 | pds5 | 0 | 1 | 0 | 0 | 0 |
| FBgn0261804 | CG42750 | 0 | 1 | 0 | 0 | 0 |
| FBgn0025469 | slv | 0 | 1 | 0 | 0 | 0 |
| FBgn0014930 | CG2846 | 0 | 1 | 0 | 0 | 0 |
| FBgn0052062 | A2bp1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0035424 | CG11505 | 0 | 1 | 0 | 0 | 0 |
| FBgn0039152 | Rootletin | 0 | 1 | 0 | 0 | 0 |
| FBgn0039283 | danr | 0 | 1 | 0 | 0 | 0 |
| FBgn0035914 | CG6282 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030432 | CG4404 | 0 | 1 | 0 | 0 | 1 |
| FBgn0051778 | CG31778 | 0 | 1 | 0 | 0 | 0 |
| FBgn0034135 | Syn2 | 0 | 1 | 0 | 0 | 0 |
| FBgn0037031 | CG11456 | 0 | 1 | 0 | 0 | 0 |
| FBgn0033347 | CG8248 | 0 | 1 | 0 | 0 | 0 |
| FBgn0261859 | CG42788 | 0 | 1 | 0 | 0 | 0 |
| FBgn0024963 | GluClalpha | 0 | 1 | 0 | 0 | 1 |
| FBgn0039249 | CG11168 | 0 | 1 | 0 | 0 | 0 |
| FBgn0035475 | CG10866 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030766 | mth1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0014135 | bnl | 0 | 1 | 0 | 0 | 0 |
| FBgn0050362 | boly | 0 | 1 | 0 | 0 | 0 |
| FBgn0040232 | cmet | 0 | 1 | 0 | 0 | 0 |
| FBgn0037188 | CG7369 | 0 | 1 | 0 | 0 | 0 |
| FBgn0029705 | CG12693 | 0 | 1 | 0 | 0 | 0 |
| FBgn0067102 | GlcT-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0028360 | l(1)G0148 | 0 | 1 | 0 | 0 | 0 |
| FBgn0026430 | Grip84 | 0 | 1 | 0 | 0 | 0 |
| FBgn0034606 | ASPP | 0 | 1 | 0 | 0 | 0 |
| FBgn0263132 | Cht6 | 0 | 1 | 0 | 0 | 0 |
| FBgn0004366 | 0 | 0 | 1 | 0 | 0 | 0 |
| FBgn0011758 | B-H1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0028496 | CG30116 | 0 | 1 | 0 | 0 | 0 |
| FBgn0024732 | Drep-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0040465 | Dip3 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030053 | CG12081 | 0 | 1 | 0 | 0 | 0 |
| FBgn0039454 | CG14247 | 0 | 1 | 0 | 0 | 0 |
| FBgn0031995 | CG8475 | 0 | 1 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|-----------|---|---|---|---|---|
| FBgn0037249 | elF3-S10 | 0 | 1 | 0 | 0 | 0 |
| FBgn0026136 | CklIbeta2 | 0 | 1 | 0 | 0 | 0 |
| FBgn0004648 | svr | 0 | 1 | 0 | 0 | 0 |
| FBgn0002940 | ninaE | 0 | 1 | 0 | 0 | 0 |
| FBgn0036522 | CG7372 | 0 | 1 | 1 | 0 | 0 |
| FBgn0036446 | CG9384 | 0 | 1 | 0 | 1 | 0 |
| FBgn0003210 | rb | 0 | 1 | 0 | 0 | 0 |
| FBgn0032797 | CG10186 | 0 | 1 | 0 | 0 | 0 |
| FBgn0015372 | RabX1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0011725 | twin | 0 | 1 | 0 | 0 | 0 |
| FBgn0022987 | qkr54B | 0 | 1 | 0 | 0 | 0 |
| FBgn0035085 | CG3770 | 0 | 1 | 0 | 0 | 1 |
| FBgn0030961 | CG7058 | 0 | 1 | 0 | 0 | 0 |
| FBgn0054051 | CG34051 | 0 | 1 | 0 | 0 | 0 |
| FBgn0261053 | Cad86C | 0 | 1 | 0 | 0 | 0 |
| FBgn0039335 | Vps33B | 0 | 1 | 0 | 0 | 0 |
| FBgn0031498 | CG17260 | 0 | 1 | 0 | 0 | 0 |
| FBgn0034049 | bdg | 0 | 1 | 0 | 0 | 0 |
| FBgn0016047 | nompA | 0 | 1 | 0 | 0 | 0 |
| FBgn0035372 | CG12093 | 0 | 1 | 0 | 0 | 0 |
| FBgn0032378 | CycY | 0 | 1 | 1 | 1 | 0 |
| FBgn0031001 | CG7884 | 0 | 1 | 0 | 0 | 0 |
| FBgn0039435 | TwrdIP | 0 | 1 | 0 | 0 | 0 |
| FBgn0031195 | CG17600 | 0 | 1 | 0 | 0 | 0 |
| FBgn0085442 | NKAIN | 0 | 1 | 0 | 0 | 0 |
| FBgn0026869 | Thd1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0038720 | CG6231 | 0 | 1 | 0 | 0 | 0 |
| FBgn0035235 | CG7879 | 0 | 1 | 0 | 0 | 0 |
| FBgn0017418 | ari-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0033285 | CG18449 | 0 | 1 | 0 | 0 | 0 |
| FBgn0039970 | CG17508 | 0 | 1 | 0 | 0 | 0 |
| FBgn0024754 | Flo-1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0028683 | spt4 | 0 | 1 | 0 | 0 | 0 |
| FBgn0039816 | CG11317 | 0 | 1 | 0 | 0 | 0 |
| FBgn0037680 | CG8121 | 0 | 1 | 0 | 0 | 0 |
| FBgn0039014 | CG6982 | 0 | 1 | 0 | 0 | 0 |
| FBgn0022355 | Tsf1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0032633 | Lrch | 0 | 1 | 0 | 0 | 0 |
| FBgn0030485 | CG1998 | 0 | 1 | 0 | 0 | 0 |
| FBgn0040505 | Alk | 0 | 1 | 0 | 0 | 0 |
| FBgn0034025 | GalNAc-T1 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030864 | CG8173 | 0 | 1 | 0 | 0 | 0 |
| FBgn0263112 | Mitf | 0 | 1 | 0 | 0 | 0 |
| FBgn0039527 | CG5639 | 0 | 1 | 0 | 0 | 0 |
| FBgn0038139 | PK2-R2 | 0 | 1 | 0 | 0 | 0 |
| FBgn0030544 | CG13403 | 0 | 1 | 0 | 0 | 0 |
| FBgn0000015 | Abd-B | 0 | 1 | 0 | 0 | 0 |
| FBgn0263111 | cac | 0 | 1 | 0 | 0 | 0 |
| FBgn0039229 | Saf-B | 0 | 1 | 0 | 0 | 0 |
| FBgn0045202 | #N/A | 0 | 1 | 0 | 0 | 0 |
| FBgn0003371 | sgg | 0 | 1 | 0 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------|---|---|---|---|---|
| FBgn0005672 | spl | 0 | 1 | 0 | 0 | 0 |
| FBgn0000633 | fas | 0 | 1 | 0 | 0 | 0 |
| FBgn0261085 | Syt12 | 0 | 1 | 0 | 0 | 0 |
| FBgn0038149 | CG9796 | 0 | 1 | 0 | 0 | 0 |
| FBgn0037440 | CG1041 | 0 | 1 | 0 | 0 | 0 |
| FBgn0031637 | CG2950 | 0 | 1 | 0 | 1 | 0 |
| FBgn0003944 | Ubx | 0 | 1 | 0 | 0 | 0 |
| FBgn0250850 | rig | 0 | 1 | 0 | 0 | 0 |
| FBgn0035101 | p130CAS | 0 | 1 | 0 | 0 | 0 |
| FBgn0016641 | PTP-ER | 0 | 0 | 1 | 0 | 1 |
| FBgn0003710 | tipE | 0 | 0 | 1 | 0 | 0 |
| FBgn0001250 | if | 0 | 0 | 1 | 0 | 0 |
| FBgn0086687 | desat1 | 0 | 0 | 1 | 0 | 0 |
| FBgn0038089 | d-cup | 0 | 0 | 1 | 0 | 0 |
| FBgn0035464 | CG12006 | 0 | 0 | 1 | 0 | 0 |
| FBgn0036260 | Rh7 | 0 | 0 | 1 | 0 | 0 |
| FBgn0040009 | CG17490 | 0 | 0 | 1 | 0 | 0 |
| FBgn0261914 | #N/A | 0 | 0 | 1 | 0 | 0 |
| FBgn0036353 | CG10171 | 0 | 0 | 1 | 0 | 0 |
| FBgn0001612 | l(1)dd4 | 0 | 0 | 1 | 0 | 0 |
| FBgn0035285 | CG12025 | 0 | 0 | 1 | 0 | 0 |
| FBgn0261245 | sing | 0 | 0 | 1 | 0 | 0 |
| FBgn0040089 | meso18E | 0 | 0 | 1 | 1 | 0 |
| FBgn0259938 | cwo | 0 | 0 | 1 | 1 | 0 |
| FBgn0030357 | Sclp | 0 | 0 | 1 | 0 | 0 |
| FBgn0262722 | CG43166 | 0 | 0 | 1 | 0 | 0 |
| FBgn0002643 | mam | 0 | 0 | 1 | 0 | 0 |
| FBgn0025743 | mbt | 0 | 0 | 1 | 0 | 0 |
| FBgn0038065 | Snx3 | 0 | 0 | 1 | 0 | 0 |
| FBgn0031885 | Mnn1 | 0 | 0 | 1 | 0 | 0 |
| FBgn0038453 | CG10326 | 0 | 0 | 1 | 0 | 0 |
| FBgn0036202 | CG6024 | 0 | 0 | 1 | 1 | 0 |
| FBgn0029974 | dpr14 | 0 | 0 | 1 | 0 | 0 |
| FBgn0036951 | CG7017 | 0 | 0 | 1 | 0 | 0 |
| FBgn0052177 | Ndfip | 0 | 0 | 1 | 0 | 0 |
| FBgn0037614 | CG8116 | 0 | 0 | 1 | 0 | 0 |
| FBgn0029957 | CG12155 | 0 | 0 | 1 | 0 | 0 |
| FBgn0033155 | Br140 | 0 | 0 | 1 | 0 | 0 |
| FBgn0038243 | CG8066 | 0 | 0 | 1 | 0 | 0 |
| FBgn0261514 | NimA | 0 | 0 | 1 | 0 | 0 |
| FBgn0038890 | CG7956 | 0 | 0 | 1 | 1 | 0 |
| FBgn0053995 | CG33995 | 0 | 0 | 1 | 0 | 0 |
| FBgn0031948 | CG7149 | 0 | 0 | 1 | 0 | 0 |
| FBgn0051262 | CG31262 | 0 | 0 | 1 | 0 | 0 |
| FBgn0260954 | CG42586 | 0 | 0 | 1 | 0 | 0 |
| FBgn0003165 | pum | 0 | 0 | 1 | 0 | 0 |
| FBgn0028537 | CG31775 | 0 | 0 | 1 | 0 | 0 |
| FBgn0039157 | Myo95E | 0 | 0 | 1 | 0 | 0 |
| FBgn0262718 | #N/A | 0 | 0 | 1 | 0 | 0 |
| FBgn0020299 | stumps | 0 | 0 | 1 | 0 | 0 |
| FBgn0032025 | CG7778 | 0 | 0 | 1 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------|---|---|---|---|---|
| FBgn0031434 | insv | 0 | 0 | 1 | 0 | 0 |
| FBgn0034435 | CG9975 | 0 | 0 | 1 | 0 | 0 |
| FBgn0000635 | Fas2 | 0 | 0 | 1 | 0 | 0 |
| FBgn0031397 | CG15385 | 0 | 0 | 1 | 0 | 0 |
| FBgn0031674 | #N/A | 0 | 0 | 1 | 0 | 0 |
| FBgn0052815 | CG32815 | 0 | 0 | 1 | 0 | 0 |
| FBgn0053202 | dpr11 | 0 | 0 | 1 | 0 | 0 |
| FBgn0053207 | pxb | 0 | 0 | 1 | 0 | 0 |
| FBgn0031119 | CG1812 | 0 | 0 | 1 | 0 | 0 |
| FBgn0032281 | CG17107 | 0 | 0 | 1 | 0 | 0 |
| FBgn0023416 | Ac3 | 0 | 0 | 1 | 0 | 0 |
| FBgn0036762 | CG7430 | 0 | 0 | 1 | 0 | 0 |
| FBgn0259225 | #N/A | 0 | 0 | 1 | 0 | 0 |
| FBgn0032476 | CG5439 | 0 | 0 | 1 | 0 | 0 |
| FBgn0030346 | CG11802 | 0 | 0 | 1 | 0 | 0 |
| FBgn0031097 | obst-A | 0 | 0 | 1 | 0 | 0 |
| FBgn0034546 | CG13442 | 0 | 0 | 1 | 0 | 0 |
| FBgn0263102 | psq | 0 | 0 | 1 | 0 | 0 |
| FBgn0013432 | bcn92 | 0 | 0 | 1 | 0 | 0 |
| FBgn0085446 | CG34417 | 0 | 0 | 1 | 1 | 0 |
| FBgn0259209 | Mlp60A | 0 | 0 | 1 | 0 | 0 |
| FBgn0000363 | #N/A | 0 | 0 | 1 | 0 | 0 |
| FBgn0035245 | GC | 0 | 0 | 1 | 0 | 0 |
| FBgn0034286 | dpr13 | 0 | 0 | 1 | 0 | 0 |
| FBgn0033426 | CG1814 | 0 | 0 | 1 | 0 | 0 |
| FBgn0259234 | Camta | 0 | 0 | 1 | 0 | 0 |
| FBgn0034763 | RYBP | 0 | 0 | 1 | 0 | 0 |
| FBgn0020513 | ade5 | 0 | 0 | 1 | 0 | 0 |
| FBgn0011225 | jar | 0 | 0 | 1 | 0 | 0 |
| FBgn0004456 | mew | 0 | 0 | 1 | 0 | 0 |
| FBgn0085470 | lmgB | 0 | 0 | 1 | 0 | 0 |
| FBgn0262734 | Rbp2 | 0 | 0 | 1 | 0 | 1 |
| FBgn0032297 | CG17124 | 0 | 0 | 1 | 0 | 0 |
| FBgn0261477 | slim | 0 | 0 | 1 | 0 | 0 |
| FBgn0262742 | Fas1 | 0 | 0 | 1 | 0 | 0 |
| FBgn0000395 | cv-2 | 0 | 0 | 1 | 0 | 1 |
| FBgn0039069 | CG6763 | 0 | 0 | 1 | 0 | 0 |
| FBgn0262871 | lute | 0 | 0 | 1 | 0 | 0 |
| FBgn0014340 | mof | 0 | 0 | 1 | 0 | 0 |
| FBgn0034262 | swi2 | 0 | 0 | 1 | 0 | 0 |
| FBgn0027508 | Tnks | 0 | 0 | 1 | 0 | 0 |
| FBgn0033524 | Cyp49a1 | 0 | 0 | 1 | 0 | 0 |
| FBgn0004228 | mex1 | 0 | 0 | 1 | 0 | 0 |
| FBgn0045770 | S-Lap3 | 0 | 0 | 1 | 0 | 0 |
| FBgn0039927 | CG11155 | 0 | 0 | 1 | 0 | 0 |
| FBgn0053056 | CG33056 | 0 | 0 | 1 | 0 | 0 |
| FBgn0024941 | RSG7 | 0 | 0 | 1 | 0 | 0 |
| FBgn0035998 | CG3437 | 0 | 0 | 1 | 0 | 0 |
| FBgn0010313 | corto | 0 | 0 | 1 | 0 | 0 |
| FBgn0015402 | ksr | 0 | 0 | 1 | 0 | 0 |
| FBgn0259699 | CG42353 | 0 | 0 | 1 | 0 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------------|---|---|---|---|---|
| FBgn0034978 | CG3257 | 0 | 0 | 1 | 0 | 0 |
| FBgn0086779 | step | 0 | 0 | 1 | 0 | 0 |
| FBgn0001247 | Ide | 0 | 0 | 1 | 0 | 0 |
| FBgn0037138 | P5CDh1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0001169 | H | 0 | 0 | 0 | 1 | 0 |
| FBgn0003093 | Pkc98E | 0 | 0 | 0 | 1 | 0 |
| FBgn0032587 | CG5953 | 0 | 0 | 0 | 1 | 0 |
| FBgn0259100 | #N/A | 0 | 0 | 0 | 1 | 0 |
| FBgn0020278 | loco | 0 | 0 | 0 | 1 | 0 |
| FBgn0022764 | Sin3A | 0 | 0 | 0 | 1 | 0 |
| FBgn0039113 | CG10217 | 0 | 0 | 0 | 1 | 0 |
| FBgn0262473 | Tl | 0 | 0 | 0 | 1 | 0 |
| FBgn0026189 | prominin-like | 0 | 0 | 0 | 1 | 0 |
| FBgn0020907 | Scp2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0003861 | trp | 0 | 0 | 0 | 1 | 0 |
| FBgn0043364 | cbt | 0 | 0 | 0 | 1 | 0 |
| FBgn0038295 | Gyc88E | 0 | 0 | 0 | 1 | 0 |
| FBgn0046874 | Pif1B | 0 | 0 | 0 | 1 | 0 |
| FBgn0015371 | chn | 0 | 0 | 0 | 1 | 0 |
| FBgn0050280 | CG30280 | 0 | 0 | 0 | 1 | 0 |
| FBgn0040636 | CG13255 | 0 | 0 | 0 | 1 | 0 |
| FBgn0042180 | CG18870 | 0 | 0 | 0 | 1 | 0 |
| FBgn0036677 | CG13023 | 0 | 0 | 0 | 1 | 0 |
| FBgn0037659 | Kdm2 | 0 | 0 | 0 | 1 | 1 |
| FBgn0028407 | Drep-3 | 0 | 0 | 0 | 1 | 0 |
| FBgn0035078 | Tpc2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0034267 | CG4984 | 0 | 0 | 0 | 1 | 0 |
| FBgn0020236 | ATPCL | 0 | 0 | 0 | 1 | 0 |
| FBgn0053156 | CG33156 | 0 | 0 | 0 | 1 | 0 |
| FBgn0034300 | CG5098 | 0 | 0 | 0 | 1 | 0 |
| FBgn0035145 | MED14 | 0 | 0 | 0 | 1 | 0 |
| FBgn0015777 | nrv2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0004607 | zfh2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033321 | CG8738 | 0 | 0 | 0 | 1 | 0 |
| FBgn0086346 | ALIX | 0 | 0 | 0 | 1 | 0 |
| FBgn0261673 | nemy | 0 | 0 | 0 | 1 | 0 |
| FBgn0043799 | CG31381 | 0 | 0 | 0 | 1 | 0 |
| FBgn0004875 | enc | 0 | 0 | 0 | 1 | 0 |
| FBgn0026320 | Tom | 0 | 0 | 0 | 1 | 0 |
| FBgn0002931 | net | 0 | 0 | 0 | 1 | 0 |
| FBgn0086365 | Orct2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0030758 | CanA-14F | 0 | 0 | 0 | 1 | 1 |
| FBgn0003041 | pbl | 0 | 0 | 0 | 1 | 0 |
| FBgn0250851 | CG33981 | 0 | 0 | 0 | 1 | 0 |
| FBgn0000588 | esc | 0 | 0 | 0 | 1 | 0 |
| FBgn0040281 | Aplip1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0040319 | Gclc | 0 | 0 | 0 | 1 | 0 |
| FBgn0036273 | CG10426 | 0 | 0 | 0 | 1 | 0 |
| FBgn0032796 | CG10188 | 0 | 0 | 0 | 1 | 0 |
| FBgn0001941 | ifc | 0 | 0 | 0 | 1 | 0 |
| FBgn0032312 | CG14071 | 0 | 0 | 0 | 1 | 1 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|------------|---|---|---|---|---|
| FBgn0034583 | CG10527 | 0 | 0 | 0 | 1 | 0 |
| FBgn0262614 | pyd | 0 | 0 | 0 | 1 | 0 |
| FBgn0261015 | Pif1A | 0 | 0 | 0 | 1 | 0 |
| FBgn0050423 | CG30423 | 0 | 0 | 0 | 1 | 0 |
| FBgn0034491 | Hsl | 0 | 0 | 0 | 1 | 0 |
| FBgn0011746 | ana | 0 | 0 | 0 | 1 | 0 |
| FBgn0028406 | Drep-4 | 0 | 0 | 0 | 1 | 0 |
| FBgn0020930 | Dgkepsilon | 0 | 0 | 0 | 1 | 0 |
| FBgn0037235 | CG1103 | 0 | 0 | 0 | 1 | 0 |
| FBgn0030033 | CG1387 | 0 | 0 | 0 | 1 | 0 |
| FBgn0085313 | CG34284 | 0 | 0 | 0 | 1 | 0 |
| FBgn0030327 | FucT6 | 0 | 0 | 0 | 1 | 0 |
| FBgn0013305 | Nmda1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0037521 | CG2993 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033679 | CG8888 | 0 | 0 | 0 | 1 | 0 |
| FBgn0027570 | Nep2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033391 | CG8026 | 0 | 0 | 0 | 1 | 0 |
| FBgn0036732 | Oatp74D | 0 | 0 | 0 | 1 | 1 |
| FBgn0010453 | Wnt4 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033686 | Hen1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0017397 | #N/A | 0 | 0 | 0 | 1 | 0 |
| FBgn0024320 | Npc1a | 0 | 0 | 0 | 1 | 0 |
| FBgn0039644 | CG11897 | 0 | 0 | 0 | 1 | 0 |
| FBgn0037015 | cmpy | 0 | 0 | 0 | 1 | 0 |
| FBgn0037552 | CG7800 | 0 | 0 | 0 | 1 | 0 |
| FBgn0015793 | Rab19 | 0 | 0 | 0 | 1 | 0 |
| FBgn0039596 | CG10000 | 0 | 0 | 0 | 1 | 0 |
| FBgn0039632 | Cul-5 | 0 | 0 | 0 | 1 | 0 |
| FBgn0004611 | Plc21C | 0 | 0 | 0 | 1 | 0 |
| FBgn0033627 | CG13204 | 0 | 0 | 0 | 1 | 0 |
| FBgn0086676 | spin | 0 | 0 | 0 | 1 | 0 |
| FBgn0040079 | pkaap | 0 | 0 | 0 | 1 | 0 |
| FBgn0035239 | CG18170 | 0 | 0 | 0 | 1 | 0 |
| FBgn0086708 | stv | 0 | 0 | 0 | 1 | 0 |
| FBgn0000414 | Dab | 0 | 0 | 0 | 1 | 0 |
| FBgn0039467 | CG14253 | 0 | 0 | 0 | 1 | 0 |
| FBgn0030590 | CG9518 | 0 | 0 | 0 | 1 | 0 |
| FBgn0261625 | CG42708 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033569 | CG12942 | 0 | 0 | 0 | 1 | 0 |
| FBgn0036789 | AICR2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0051217 | modSP | 0 | 0 | 0 | 1 | 0 |
| FBgn0036290 | CG10638 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033483 | egr | 0 | 0 | 0 | 1 | 0 |
| FBgn0032514 | CG9302 | 0 | 0 | 0 | 1 | 0 |
| FBgn0039056 | CenB1A | 0 | 0 | 0 | 1 | 0 |
| FBgn0000346 | comt | 0 | 0 | 0 | 1 | 0 |
| FBgn0029729 | CG12682 | 0 | 0 | 0 | 1 | 0 |
| FBgn0038088 | CG10126 | 0 | 0 | 0 | 1 | 0 |
| FBgn0011829 | Ret | 0 | 0 | 0 | 1 | 0 |
| FBgn0000542 | ec | 0 | 0 | 0 | 1 | 0 |
| FBgn0038826 | Syp | 0 | 0 | 0 | 1 | 0 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|-----------|---|---|---|---|---|
| FBgn0028509 | CenG1A | 0 | 0 | 0 | 1 | 0 |
| FBgn0033856 | CG13334 | 0 | 0 | 0 | 1 | 0 |
| FBgn0261526 | NT1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0032123 | Oatp30B | 0 | 0 | 0 | 1 | 0 |
| FBgn0038721 | CG16718 | 0 | 0 | 0 | 1 | 0 |
| FBgn0038693 | unc79 | 0 | 0 | 0 | 1 | 0 |
| FBgn0017551 | Rca1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0028525 | c(2)M | 0 | 0 | 0 | 1 | 0 |
| FBgn0011764 | Dsp1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0031857 | CG11321 | 0 | 0 | 0 | 1 | 0 |
| FBgn0035678 | CG10469 | 0 | 0 | 0 | 1 | 0 |
| FBgn0001087 | g | 0 | 0 | 0 | 1 | 0 |
| FBgn0000636 | Fas3 | 0 | 0 | 0 | 1 | 0 |
| FBgn0020240 | #N/A | 0 | 0 | 0 | 1 | 0 |
| FBgn0041147 | ida | 0 | 0 | 0 | 1 | 0 |
| FBgn0038619 | CG7685 | 0 | 0 | 0 | 1 | 0 |
| FBgn0034027 | CG8187 | 0 | 0 | 0 | 1 | 0 |
| FBgn0039831 | CG12054 | 0 | 0 | 0 | 1 | 0 |
| FBgn0259822 | Ca-beta | 0 | 0 | 0 | 1 | 0 |
| FBgn0051760 | CG31760 | 0 | 0 | 0 | 1 | 0 |
| FBgn0016754 | sba | 0 | 0 | 0 | 1 | 0 |
| FBgn0033473 | CG12128 | 0 | 0 | 0 | 1 | 0 |
| FBgn0050118 | CG30118 | 0 | 0 | 0 | 1 | 0 |
| FBgn0003638 | su(w[a]) | 0 | 0 | 0 | 1 | 0 |
| FBgn0011259 | Sema-1a | 0 | 0 | 0 | 1 | 0 |
| FBgn0014859 | Hr38 | 0 | 0 | 0 | 1 | 0 |
| FBgn0025682 | scf | 0 | 0 | 0 | 1 | 0 |
| FBgn0053129 | CG33129 | 0 | 0 | 0 | 1 | 0 |
| FBgn0015609 | CadN | 0 | 0 | 0 | 1 | 0 |
| FBgn0032200 | CG5676 | 0 | 0 | 0 | 1 | 0 |
| FBgn0028433 | Ggamma30A | 0 | 0 | 0 | 1 | 0 |
| FBgn0032817 | CG10631 | 0 | 0 | 0 | 1 | 0 |
| FBgn0003732 | Top2 | 0 | 0 | 0 | 1 | 0 |
| FBgn0034731 | CG10384 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033375 | CG8078 | 0 | 0 | 0 | 1 | 0 |
| FBgn0035157 | CG13894 | 0 | 0 | 0 | 1 | 0 |
| FBgn0040376 | CG13759 | 0 | 0 | 0 | 1 | 0 |
| FBgn0036843 | CG6812 | 0 | 0 | 0 | 1 | 0 |
| FBgn0004373 | fwd | 0 | 0 | 0 | 1 | 0 |
| FBgn0041096 | rols | 0 | 0 | 0 | 1 | 0 |
| FBgn0011837 | Tis11 | 0 | 0 | 0 | 1 | 0 |
| FBgn0034570 | CG10543 | 0 | 0 | 0 | 1 | 0 |
| FBgn0010399 | Nmdar1 | 0 | 0 | 0 | 1 | 0 |
| FBgn0003513 | ss | 0 | 0 | 0 | 1 | 0 |
| FBgn0035830 | CG8209 | 0 | 0 | 0 | 1 | 0 |
| FBgn0030912 | CG6023 | 0 | 0 | 0 | 1 | 0 |
| FBgn0261090 | Sytbeta | 0 | 0 | 0 | 1 | 0 |
| FBgn0033739 | Dyb | 0 | 0 | 0 | 1 | 0 |
| FBgn0028400 | Syt4 | 0 | 0 | 0 | 0 | 1 |
| FBgn0011818 | oaf | 0 | 0 | 0 | 0 | 1 |
| FBgn0004587 | B52 | 0 | 0 | 0 | 0 | 1 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|-----------------|---|---|---|---|---|
| FBgn0004865 | Eip78C | 0 | 0 | 0 | 0 | 1 |
| FBgn0037796 | CG12814 | 0 | 0 | 0 | 0 | 1 |
| FBgn0261602 | RpL8 | 0 | 0 | 0 | 0 | 1 |
| FBgn0038391 | GATAe | 0 | 0 | 0 | 0 | 1 |
| FBgn0038498 | beat-IIa | 0 | 0 | 0 | 0 | 1 |
| FBgn0034447 | CG7744 | 0 | 0 | 0 | 0 | 1 |
| FBgn0012034 | AcCoAS | 0 | 0 | 0 | 0 | 1 |
| FBgn0029762 | NAAT1 | 0 | 0 | 0 | 0 | 1 |
| FBgn0029807 | CG3108 | 0 | 0 | 0 | 0 | 1 |
| FBgn0020238 | 14-3-3epsilon | 0 | 0 | 0 | 0 | 1 |
| FBgn0085384 | CG34355 | 0 | 0 | 0 | 0 | 1 |
| FBgn0260430 | CG42525 | 0 | 0 | 0 | 0 | 1 |
| FBgn0001253 | ImpE1 | 0 | 0 | 0 | 0 | 1 |
| FBgn0261837 | pre-mod(mdg4)-T | 0 | 0 | 0 | 0 | 1 |
| FBgn0030503 | Tango2 | 0 | 0 | 0 | 0 | 1 |
| FBgn0019990 | Gcn2 | 0 | 0 | 0 | 0 | 1 |
| FBgn0015831 | RtnI2 | 0 | 0 | 0 | 0 | 1 |
| FBgn0085436 | Not1 | 0 | 0 | 0 | 0 | 1 |
| FBgn0039851 | mey | 0 | 0 | 0 | 0 | 1 |
| FBgn0031724 | CG18266 | 0 | 0 | 0 | 0 | 1 |
| FBgn0034307 | CG10914 | 0 | 0 | 0 | 0 | 1 |
| FBgn0013325 | RpL11 | 0 | 0 | 0 | 0 | 1 |
| FBgn0263197 | Patronin | 0 | 0 | 0 | 0 | 1 |
| FBgn0085429 | #N/A | 0 | 0 | 0 | 0 | 1 |
| FBgn0041605 | cpx | 0 | 0 | 0 | 0 | 1 |
| FBgn0036259 | CG9760 | 0 | 0 | 0 | 0 | 1 |
| FBgn0030328 | Amun | 0 | 0 | 0 | 0 | 1 |
| FBgn0085248 | CG34219 | 0 | 0 | 0 | 0 | 1 |
| FBgn0035056 | spz6 | 0 | 0 | 0 | 0 | 1 |
| FBgn0034795 | MED23 | 0 | 0 | 0 | 0 | 1 |
| FBgn0033597 | Cpr47Ea | 0 | 0 | 0 | 0 | 1 |
| FBgn0030367 | Cyp311a1 | 0 | 0 | 0 | 0 | 1 |
| FBgn0036940 | obst-J | 0 | 0 | 0 | 0 | 1 |
| FBgn0011760 | ctp | 0 | 0 | 0 | 0 | 1 |
| FBgn0016976 | stnA | 0 | 0 | 0 | 0 | 1 |
| FBgn0261838 | pre-mod(mdg4)-Z | 0 | 0 | 0 | 0 | 1 |
| FBgn0025631 | moody | 0 | 0 | 0 | 0 | 1 |
| FBgn0263255 | #N/A | 0 | 0 | 0 | 0 | 1 |
| FBgn0051882 | CG31882 | 0 | 0 | 0 | 0 | 1 |
| FBgn0027836 | Dgp-1 | 0 | 0 | 0 | 0 | 1 |
| FBgn0036381 | CG8745 | 0 | 0 | 0 | 0 | 1 |
| FBgn0013984 | InR | 0 | 0 | 0 | 0 | 1 |
| FBgn0038225 | soti | 0 | 0 | 0 | 0 | 1 |
| FBgn0020258 | ppk | 0 | 0 | 0 | 0 | 1 |
| FBgn0087035 | AGO2 | 0 | 0 | 0 | 0 | 1 |
| FBgn0001108 | Gl | 0 | 0 | 0 | 0 | 1 |
| FBgn0262975 | cnc | 0 | 0 | 0 | 0 | 1 |
| FBgn0039538 | CG12883 | 0 | 0 | 0 | 0 | 1 |
| FBgn0085387 | shakB | 0 | 0 | 0 | 0 | 1 |
| FBgn0011666 | msi | 0 | 0 | 0 | 0 | 1 |
| FBgn0037414 | Osi7 | 0 | 0 | 0 | 0 | 1 |

Figure S2.3 (Continued)

| | | | | | | |
|-------------|---------|---|---|---|---|---|
| FBgn0052369 | CG32369 | 0 | 0 | 0 | 0 | 1 |
| FBgn0039024 | CG4721 | 0 | 0 | 0 | 0 | 1 |
| FBgn0016975 | stnB | 0 | 0 | 0 | 0 | 1 |
| FBgn0036786 | skl | 0 | 0 | 0 | 0 | 1 |
| FBgn0027490 | D12 | 0 | 0 | 0 | 0 | 1 |
| FBgn0033497 | CG12912 | 0 | 0 | 0 | 0 | 1 |
| FBgn0039697 | CG7834 | 0 | 0 | 0 | 0 | 1 |
| FBgn0003651 | svp | 0 | 0 | 0 | 0 | 1 |
| FBgn0036566 | ClC-c | 0 | 0 | 0 | 0 | 1 |
| FBgn0040087 | p115 | 0 | 0 | 0 | 0 | 1 |
| FBgn0032286 | CG7300 | 0 | 0 | 0 | 0 | 1 |
| FBgn0259203 | CG42307 | 0 | 0 | 0 | 0 | 1 |
| FBgn0035246 | CG13928 | 0 | 0 | 0 | 0 | 1 |
| FBgn0033088 | PGAP3 | 0 | 0 | 0 | 0 | 1 |
| FBgn0259246 | brp | 0 | 0 | 0 | 0 | 1 |
| FBgn0037135 | CG7414 | 0 | 0 | 0 | 0 | 1 |
| FBgn0037853 | CG14696 | 0 | 0 | 0 | 0 | 1 |
| FBgn0027932 | Akap200 | 0 | 0 | 0 | 0 | 1 |
| FBgn0020372 | TM4SF | 0 | 0 | 0 | 0 | 1 |
| FBgn0026058 | OdsH | 0 | 0 | 0 | 0 | 1 |

Target gene predictions were obtained using DIANA micro-T cds for all differentially expressed canonical mature microRNAs.(3)

Figure S2.4 PATHER pathway analysis for genes targeted by regulated miRNAs

| Pathway | Drosophila REFLIST | Target Genes | Target Genes (expected) | over/under | FDR Adj P-value |
|---|--------------------|--------------|-------------------------|------------|-----------------|
| Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway | 42 | 12 | | 3.38 + | 3.67E-02 |
| Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway | 23 | 8 | | 1.85 + | 1.18E-01 |
| Inflammation mediated by chemokine and cytokine signaling pathway | 59 | 12 | | 4.75 + | 6.40E-01 |
| Thyrotropin-releasing hormone receptor signaling pathway | 24 | 7 | | 1.93 + | 6.63E-01 |
| Wnt signaling pathway | 126 | 20 | | 10.15 + | 6.88E-01 |
| Huntington disease | 95 | 16 | | 7.65 + | 9.52E-01 |
| Alzheimer disease-amyloid secretase pathway | 32 | 4 | | 2.58 + | 1.00E+00 |
| Alpha adrenergic receptor signaling pathway | 8 | 2 | | 0.64 + | 1.00E+00 |
| Adrenaline and noradrenaline biosynthesis | 17 | 2 | | 1.37 + | 1.00E+00 |
| Nicotine pharmacodynamics pathway | 20 | 3 | | 1.61 + | 1.00E+00 |
| Toll pathway_drosophila | 27 | 3 | | 2.18 + | 1.00E+00 |
| SCW signaling pathway | 21 | 1 | | 1.69 - | 1.00E+00 |
| MYO signaling pathway | 10 | 1 | | 0.81 + | 1.00E+00 |
| GBB signaling pathway | 13 | 1 | | 1.05 - | 1.00E+00 |
| DPP signaling pathway | 22 | 2 | | 1.77 + | 1.00E+00 |
| DPP-SCW signaling pathway | 24 | 2 | | 1.93 + | 1.00E+00 |
| BMP signaling pathway-drosophila | 30 | 4 | | 2.42 + | 1.00E+00 |
| Xanthine and guanine salvage pathway | 3 | 0 | | 0.24 - | 1.00E+00 |
| Activinbetasignaling pathway | 11 | 1 | | 0.89 + | 1.00E+00 |
| Vitamin B6 metabolism | 5 | 0 | | 0.4 - | 1.00E+00 |
| Valine biosynthesis | 1 | 1 | | 0.08 + | 1.00E+00 |
| Tyrosine biosynthesis | 3 | 0 | | 0.24 - | 1.00E+00 |
| Tryptophan biosynthesis | 1 | 0 | | 0.08 - | 1.00E+00 |
| Triacylglycerol metabolism | 1 | 1 | | 0.08 + | 1.00E+00 |
| Thiamine metabolism | 3 | 1 | | 0.24 + | 1.00E+00 |
| ALP23B signaling pathway | 10 | 1 | | 0.81 + | 1.00E+00 |
| Synaptic vesicle trafficking | 12 | 4 | | 0.97 + | 1.00E+00 |
| GABA-B receptor II signaling | 9 | 0 | | 0.73 - | 1.00E+00 |
| Endogenous cannabinoid signaling | 1 | 0 | | 0.08 - | 1.00E+00 |
| Sulfate assimilation | 1 | 1 | | 0.08 + | 1.00E+00 |
| Succinate to propionate conversion | 1 | 1 | | 0.08 + | 1.00E+00 |
| Serine glycine biosynthesis | 4 | 0 | | 0.32 - | 1.00E+00 |
| Salvage pyrimidine ribonucleotides | 16 | 3 | | 1.29 + | 1.00E+00 |
| Salvage pyrimidine deoxyribonucleotides | 5 | 2 | | 0.4 + | 1.00E+00 |
| Sadenosyl methionine biosynthesis | 1 | 1 | | 0.08 + | 1.00E+00 |
| Pyruvate metabolism | 25 | 3 | | 2.01 + | 1.00E+00 |
| Pyrimidine Metabolism | 14 | 2 | | 1.13 + | 1.00E+00 |
| Pyridoxal phosphate salvage pathway | 4 | 0 | | 0.32 - | 1.00E+00 |
| Purine metabolism | 13 | 0 | | 1.05 - | 1.00E+00 |
| Proline biosynthesis | 2 | 0 | | 0.16 - | 1.00E+00 |
| Phenylethylamine degradation | 3 | 0 | | 0.24 - | 1.00E+00 |
| Phenylalanine biosynthesis | 2 | 0 | | 0.16 - | 1.00E+00 |
| Pentose phosphate pathway | 7 | 0 | | 0.56 - | 1.00E+00 |
| p38 MAPK pathway | 12 | 2 | | 0.97 + | 1.00E+00 |
| Opioid proopiomelanocortin pathway | 10 | 0 | | 0.81 - | 1.00E+00 |
| Opioid prodynorphin pathway | 10 | 0 | | 0.81 - | 1.00E+00 |
| Opioid proenkephalin pathway | 9 | 0 | | 0.73 - | 1.00E+00 |
| Nicotine degradation | 36 | 0 | | 2.9 - | 1.00E+00 |
| Enkephalin release | 14 | 1 | | 1.13 - | 1.00E+00 |
| Dopamine receptor mediated signaling pathway | 34 | 5 | | 2.74 + | 1.00E+00 |
| Angiotensin II-stimulated signaling through G proteins and beta-arrestin | 5 | 0 | | 0.4 - | 1.00E+00 |
| PLP biosynthesis | 4 | 0 | | 0.32 - | 1.00E+00 |
| Ornithine degradation | 3 | 0 | | 0.24 - | 1.00E+00 |
| O-antigen biosynthesis | 2 | 1 | | 0.16 + | 1.00E+00 |
| N-acetylglucosamine metabolism | 4 | 1 | | 0.32 + | 1.00E+00 |
| Methylmalonyl pathway | 1 | 1 | | 0.08 + | 1.00E+00 |
| Methylcitrate cycle | 4 | 0 | | 0.32 - | 1.00E+00 |
| Methionine biosynthesis | 3 | 0 | | 0.24 - | 1.00E+00 |
| Mannose metabolism | 5 | 2 | | 0.4 + | 1.00E+00 |
| Lipoate biosynthesis | 5 | 0 | | 0.4 - | 1.00E+00 |
| Ubiquitin proteasome pathway | 65 | 2 | | 5.24 - | 1.00E+00 |
| Leucine biosynthesis | 1 | 1 | | 0.08 + | 1.00E+00 |
| Isoleucine biosynthesis | 2 | 1 | | 0.16 + | 1.00E+00 |
| Heme biosynthesis | 14 | 1 | | 1.13 - | 1.00E+00 |
| Glutamine glutamate conversion | 4 | 0 | | 0.32 - | 1.00E+00 |
| Fructose galactose metabolism | 13 | 2 | | 1.05 + | 1.00E+00 |
| Formyltetrahydroformate biosynthesis | 9 | 0 | | 0.73 - | 1.00E+00 |
| Folate biosynthesis | 6 | 1 | | 0.48 + | 1.00E+00 |
| Flavin biosynthesis | 2 | 1 | | 0.16 + | 1.00E+00 |
| De novo pyrimidine ribonucleotides biosynthesis | 9 | 1 | | 0.73 + | 1.00E+00 |
| p53 pathway | 41 | 6 | | 3.3 + | 1.00E+00 |
| mRNA splicing | 4 | 0 | | 0.32 - | 1.00E+00 |
| VEGF signaling pathway | 32 | 4 | | 2.58 + | 1.00E+00 |
| Transcription regulation by bZIP transcription factor | 43 | 4 | | 3.46 + | 1.00E+00 |
| Toll receptor signaling pathway | 17 | 2 | | 1.37 + | 1.00E+00 |
| T cell activation | 21 | 5 | | 1.69 + | 1.00E+00 |
| p53 pathway feedback loops 2 | 27 | 4 | | 2.18 + | 1.00E+00 |
| TGF-beta signaling pathway | 44 | 7 | | 3.54 + | 1.00E+00 |
| p53 pathway by glucose deprivation | 11 | 0 | | 0.89 - | 1.00E+00 |
| TCA cycle | 22 | 1 | | 1.77 - | 1.00E+00 |
| Vitamin D metabolism and pathway | 5 | 2 | | 0.4 + | 1.00E+00 |
| Vasopressin synthesis | 9 | 0 | | 0.73 - | 1.00E+00 |
| Ras Pathway | 31 | 6 | | 2.5 + | 1.00E+00 |
| De novo pyrimidine deoxyribonucleotide biosynthesis | 11 | 1 | | 0.89 + | 1.00E+00 |

Figure S2.4 (Continued)

| | | | | |
|--|----|----|--------|----------|
| P53 pathway feedback loops 1 | 1 | 1 | 0.08 + | 1.00E+00 |
| De novo purine biosynthesis | 20 | 1 | 1.61 - | 1.00E+00 |
| Oxytocin receptor mediated signaling pathway | 21 | 3 | 1.69 + | 1.00E+00 |
| Cysteine biosynthesis | 1 | 0 | 0.08 - | 1.00E+00 |
| Coenzyme A biosynthesis | 4 | 0 | 0.32 - | 1.00E+00 |
| Carnitine metabolism | 2 | 0 | 0.16 - | 1.00E+00 |
| Carnitine and CoA metabolism | 2 | 0 | 0.16 - | 1.00E+00 |
| Asparagine and aspartate biosynthesis | 4 | 0 | 0.32 - | 1.00E+00 |
| Parkinson disease | 80 | 4 | 6.44 - | 1.00E+00 |
| PI3 kinase pathway | 21 | 4 | 1.69 + | 1.00E+00 |
| PDGF signaling pathway | 56 | 8 | 4.51 + | 1.00E+00 |
| Oxidative stress response | 16 | 0 | 1.29 - | 1.00E+00 |
| Notch signaling pathway | 27 | 2 | 2.18 - | 1.00E+00 |
| Nicotinic acetylcholine receptor signaling pathway | 46 | 8 | 3.71 + | 1.00E+00 |
| Muscarinic acetylcholine receptor 2 and 4 signaling pathway | 25 | 3 | 2.01 + | 1.00E+00 |
| Muscarinic acetylcholine receptor 1 and 3 signaling pathway | 29 | 5 | 2.34 + | 1.00E+00 |
| Histamine synthesis | 5 | 2 | 0.4 + | 1.00E+00 |
| Metabotropic glutamate receptor group I pathway | 13 | 3 | 1.05 + | 1.00E+00 |
| Histamine H2 receptor mediated signaling pathway | 12 | 1 | 0.97 + | 1.00E+00 |
| Metabotropic glutamate receptor group II pathway | 21 | 3 | 1.69 + | 1.00E+00 |
| Histamine H1 receptor mediated signaling pathway | 16 | 3 | 1.29 + | 1.00E+00 |
| Gamma-aminobutyric acid synthesis | 5 | 1 | 0.4 + | 1.00E+00 |
| Ascorbate degradation | 6 | 1 | 0.48 + | 1.00E+00 |
| Arginine biosynthesis | 4 | 0 | 0.32 - | 1.00E+00 |
| Androgen/estrogene/progesterone biosynthesis | 30 | 0 | 2.42 - | 1.00E+00 |
| Corticotropin releasing factor receptor signaling pathway | 12 | 0 | 0.97 - | 1.00E+00 |
| Aminobutyrate degradation | 2 | 1 | 0.16 + | 1.00E+00 |
| Allantoin degradation | 4 | 0 | 0.32 - | 1.00E+00 |
| Alanine biosynthesis | 1 | 1 | 0.08 + | 1.00E+00 |
| Adenine and hypoxanthine salvage pathway | 8 | 0 | 0.64 - | 1.00E+00 |
| Acetate utilization | 2 | 1 | 0.16 + | 1.00E+00 |
| ATP synthesis | 8 | 1 | 0.64 + | 1.00E+00 |
| Metabotropic glutamate receptor group III pathway | 34 | 5 | 2.74 + | 1.00E+00 |
| Ionotropic glutamate receptor pathway | 30 | 7 | 2.42 + | 1.00E+00 |
| Interleukin signaling pathway | 16 | 4 | 1.29 + | 1.00E+00 |
| Interferon-gamma signaling pathway | 11 | 0 | 0.89 - | 1.00E+00 |
| Integrin signalling pathway | 71 | 11 | 5.72 + | 1.00E+00 |
| Beta3 adrenergic receptor signaling pathway | 10 | 0 | 0.81 - | 1.00E+00 |
| Insulin/IGF pathway-protein kinase B signaling cascade | 16 | 3 | 1.29 + | 1.00E+00 |
| Gonadotropin releasing hormone receptor pathway | 69 | 11 | 5.56 + | 1.00E+00 |
| Beta2 adrenergic receptor signaling pathway | 19 | 1 | 1.53 - | 1.00E+00 |
| Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade | 15 | 3 | 1.21 + | 1.00E+00 |
| Beta1 adrenergic receptor signaling pathway | 19 | 1 | 1.53 - | 1.00E+00 |
| 5HT4 type receptor mediated signaling pathway | 12 | 0 | 0.97 - | 1.00E+00 |
| Hypoxia response via HIF activation | 20 | 2 | 1.61 + | 1.00E+00 |
| 5HT3 type receptor mediated signaling pathway | 7 | 0 | 0.56 - | 1.00E+00 |
| 5HT2 type receptor mediated signaling pathway | 29 | 4 | 2.34 + | 1.00E+00 |
| 5HT1 type receptor mediated signaling pathway | 18 | 1 | 1.45 - | 1.00E+00 |
| 5-Hydroxytryptamine degradation | 6 | 2 | 0.48 + | 1.00E+00 |
| 5-Hydroxytryptamine biosynthesis | 8 | 2 | 0.64 + | 1.00E+00 |
| Heterotrimeric G-protein signaling pathway-rod outer segment phototransduction | 16 | 5 | 1.29 + | 1.00E+00 |
| Hedgehog signaling pathway | 22 | 2 | 1.77 + | 1.00E+00 |
| Glycolysis | 23 | 2 | 1.85 + | 1.00E+00 |
| General transcription regulation | 32 | 3 | 2.58 + | 1.00E+00 |
| General transcription by RNA polymerase I | 12 | 0 | 0.97 - | 1.00E+00 |
| FGF signaling pathway | 59 | 7 | 4.75 + | 1.00E+00 |
| FAS signaling pathway | 14 | 2 | 1.13 + | 1.00E+00 |
| Endothelin signaling pathway | 62 | 9 | 4.99 + | 1.00E+00 |
| EGF receptor signaling pathway | 63 | 9 | 5.08 + | 1.00E+00 |
| DNA replication | 20 | 2 | 1.61 + | 1.00E+00 |
| Cytoskeletal regulation by Rho GTPase | 44 | 4 | 3.54 + | 1.00E+00 |
| Circadian clock system | 6 | 0 | 0.48 - | 1.00E+00 |
| Cholesterol biosynthesis | 8 | 1 | 0.64 + | 1.00E+00 |
| Cell cycle | 18 | 0 | 1.45 - | 1.00E+00 |
| Cadherin signaling pathway | 51 | 7 | 4.11 + | 1.00E+00 |
| Blood coagulation | 1 | 0 | 0.08 - | 1.00E+00 |
| B cell activation | 12 | 4 | 0.97 + | 1.00E+00 |
| Axon guidance mediated by netrin | 11 | 2 | 0.89 + | 1.00E+00 |
| Axon guidance mediated by Slit/Robo | 10 | 2 | 0.81 + | 1.00E+00 |
| Axon guidance mediated by semaphorins | 7 | 2 | 0.56 + | 1.00E+00 |
| Apoptosis signaling pathway | 47 | 7 | 3.79 + | 1.00E+00 |
| Angiogenesis | 63 | 10 | 5.08 + | 1.00E+00 |
| Alzheimer disease-presenilin pathway | 40 | 7 | 3.22 + | 1.00E+00 |

The PANTHER gene ontology annotation database was used to identify pathways overrepresented in the set of genes targeted by differentially expressed microRNAs.(4) This analysis identifies the “Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway” as the only pathway overrepresented.

Figure S2.5 PATHER GO analysis for genes targeted by regulated miRNAs

| Molecular Function | Drosophila REFLIST | Target Genes | Target Genes (expected) | over/under | FDR Adj P-value |
|---|--------------------|--------------|-------------------------|------------|-----------------|
| transcription factor activity | 956 | 140 | | 77.01 + | 1.62E-09 |
| transcription regulator activity | 956 | 140 | | 77.01 + | 1.62E-09 |
| DNA binding | 1114 | 153 | | 89.74 + | 1.63E-08 |
| binding | 3360 | 354 | | 270.67 + | 1.45E-06 |
| receptor activity | 774 | 110 | | 62.35 + | 1.59E-06 |
| protein binding | 1369 | 161 | | 110.28 + | 1.42E-04 |
| structural constituent of cytoskeleton | 485 | 71 | | 39.07 + | 2.64E-04 |
| small GTPase regulator activity | 197 | 37 | | 15.87 + | 5.08E-04 |
| Unclassified | 6361 | 439 | | 512.42 - | 8.07E-04 |
| voltage-gated sodium channel activity | 12 | 7 | | 0.97 + | 9.93E-03 |
| kinase activity | 355 | 51 | | 28.6 + | 1.18E-02 |
| cytoskeletal protein binding | 148 | 27 | | 11.92 + | 1.62E-02 |
| oxidoreductase activity | 714 | 32 | | 57.52 - | 1.92E-02 |
| actin binding | 96 | 20 | | 7.73 + | 2.30E-02 |
| guanyl-nucleotide exchange factor activity | 67 | 16 | | 5.4 + | 2.31E-02 |
| voltage-gated calcium channel activity | 15 | 7 | | 1.21 + | 3.85E-02 |
| protein kinase activity | 230 | 35 | | 18.53 + | 5.44E-02 |
| ligand-dependent nuclear receptor activity | 22 | 8 | | 1.77 + | 7.46E-02 |
| nucleic acid binding | 2127 | 209 | | 171.34 + | 1.94E-01 |
| helicase activity | 99 | 1 | | 7.98 - | 4.50E-01 |
| motor activity | 68 | 13 | | 5.48 + | 6.27E-01 |
| enzyme regulator activity | 565 | 64 | | 45.51 + | 7.03E-01 |
| RNA helicase activity | 66 | 0 | | 5.32 - | 7.22E-01 |
| receptor binding | 443 | 52 | | 35.69 + | 7.98E-01 |
| cation transmembrane transporter activity | 285 | 29 | | 22.96 + | 1.00E+00 |
| G-protein coupled receptor activity | 241 | 25 | | 19.41 + | 1.00E+00 |
| glucosidase activity | 11 | 2 | | 0.89 + | 1.00E+00 |
| transaminase activity | 17 | 4 | | 1.37 + | 1.00E+00 |
| galactosidase activity | 5 | 0 | | 0.4 - | 1.00E+00 |
| lipid binding | 3 | 0 | | 0.24 - | 1.00E+00 |
| DNA ligase activity | 4 | 0 | | 0.32 - | 1.00E+00 |
| cytokine receptor activity | 7 | 2 | | 0.56 + | 1.00E+00 |
| extracellular matrix structural constituent | 29 | 4 | | 2.34 + | 1.00E+00 |
| exoribonuclease activity | 25 | 2 | | 2.01 - | 1.00E+00 |
| ATPase activity, coupled to transmembrane movement of substances | 86 | 4 | | 6.93 - | 1.00E+00 |
| tumor necrosis factor receptor binding | 1 | 1 | | 0.08 + | 1.00E+00 |
| growth factor activity | 44 | 6 | | 3.54 + | 1.00E+00 |
| phosphoric diester hydrolase activity | 26 | 4 | | 2.09 + | 1.00E+00 |
| transferase activity | 1021 | 99 | | 82.25 + | 1.00E+00 |
| exodeoxyribonuclease activity | 12 | 0 | | 0.97 - | 1.00E+00 |
| phosphoprotein phosphatase activity | 103 | 15 | | 8.3 + | 1.00E+00 |
| endoribonuclease activity | 46 | 1 | | 3.71 - | 1.00E+00 |
| endodeoxyribonuclease activity | 33 | 3 | | 2.66 + | 1.00E+00 |
| DNA strand annealing activity | 5 | 0 | | 0.4 - | 1.00E+00 |
| ligand-gated ion channel activity | 94 | 7 | | 7.57 - | 1.00E+00 |
| calcium-dependent phospholipid binding | 60 | 3 | | 4.83 - | 1.00E+00 |
| non-membrane spanning protein tyrosine kinase activity | 51 | 7 | | 4.11 + | 1.00E+00 |
| transmembrane receptor protein tyrosine kinase activity | 35 | 7 | | 2.82 + | 1.00E+00 |
| hydrogen ion transmembrane transporter activity | 53 | 4 | | 4.27 - | 1.00E+00 |
| nuclease activity | 172 | 15 | | 13.86 + | 1.00E+00 |
| glutamate receptor activity | 51 | 3 | | 4.11 - | 1.00E+00 |
| transmembrane receptor protein serine/threonine kinase activity | 23 | 2 | | 1.85 + | 1.00E+00 |
| lipase activity | 112 | 4 | | 9.02 - | 1.00E+00 |
| acetylcholine receptor activity | 24 | 2 | | 1.93 + | 1.00E+00 |
| cysteine-type endopeptidase inhibitor activity | 5 | 2 | | 0.4 + | 1.00E+00 |
| serine-type endopeptidase inhibitor activity | 62 | 4 | | 4.99 - | 1.00E+00 |
| GABA receptor activity | 24 | 2 | | 1.93 + | 1.00E+00 |
| hydrogen ion transporting ATP synthase activity, rotational mechanism | 18 | 3 | | 1.45 + | 1.00E+00 |
| RNA splicing factor activity, transesterification mechanism | 176 | 18 | | 14.18 + | 1.00E+00 |
| DNA polymerase processivity factor activity | 3 | 0 | | 0.24 - | 1.00E+00 |
| ligase activity | 367 | 22 | | 29.56 - | 1.00E+00 |
| enzyme inhibitor activity | 185 | 11 | | 14.9 - | 1.00E+00 |
| transmembrane transporter activity | 696 | 56 | | 56.07 - | 1.00E+00 |
| deaminase activity | 26 | 3 | | 2.09 + | 1.00E+00 |
| SNAP receptor activity | 22 | 1 | | 1.77 - | 1.00E+00 |
| centromeric DNA binding | 14 | 2 | | 1.13 + | 1.00E+00 |
| enzyme activator activity | 72 | 5 | | 5.8 - | 1.00E+00 |
| cytokine activity | 12 | 2 | | 0.97 + | 1.00E+00 |

Figure S2.5 (Continued)

| | | | | |
|--|------|-----|----------|----------|
| catalytic activity | 3980 | 302 | 320.62 - | 1.00E+00 |
| transmembrane receptor protein kinase activity | 49 | 8 | 3.95 + | 1.00E+00 |
| calmodulin binding | 155 | 15 | 12.49 + | 1.00E+00 |
| intramolecular transferase activity | 8 | 0 | 0.64 - | 1.00E+00 |
| lipid transporter activity | 30 | 5 | 2.42 + | 1.00E+00 |
| metallopeptidase activity | 181 | 14 | 14.58 - | 1.00E+00 |
| serine-type peptidase activity | 349 | 16 | 28.11 - | 1.00E+00 |
| cysteine-type peptidase activity | 59 | 4 | 4.75 - | 1.00E+00 |
| peptidase activity | 627 | 34 | 50.51 - | 1.00E+00 |
| ubiquitin-protein ligase activity | 139 | 9 | 11.2 - | 1.00E+00 |
| phosphorylase activity | 9 | 0 | 0.73 - | 1.00E+00 |
| pyrophosphatase activity | 11 | 0 | 0.89 - | 1.00E+00 |
| microtubule motor activity | 47 | 5 | 3.79 + | 1.00E+00 |
| calcium ion binding | 243 | 30 | 19.58 + | 1.00E+00 |
| racemase and epimerase activity | 75 | 1 | 6.04 - | 1.00E+00 |
| isomerase activity | 164 | 6 | 13.21 - | 1.00E+00 |
| intermediate filament binding | 1 | 0 | 0.08 - | 1.00E+00 |
| deacetylase activity | 20 | 2 | 1.61 + | 1.00E+00 |
| phosphatase inhibitor activity | 14 | 2 | 1.13 + | 1.00E+00 |
| phosphatase activator activity | 3 | 0 | 0.24 - | 1.00E+00 |
| kinase inhibitor activity | 63 | 1 | 5.08 - | 1.00E+00 |
| cation channel activity | 57 | 11 | 4.59 + | 1.00E+00 |
| acyltransferase activity | 152 | 12 | 12.24 - | 1.00E+00 |
| kinase activator activity | 45 | 4 | 3.63 + | 1.00E+00 |
| aspartic-type endopeptidase activity | 16 | 1 | 1.29 - | 1.00E+00 |
| phosphatase regulator activity | 35 | 2 | 2.82 - | 1.00E+00 |
| kinase regulator activity | 156 | 10 | 12.57 - | 1.00E+00 |
| microtubule binding | 51 | 7 | 4.11 + | 1.00E+00 |
| nucleotide phosphatase activity | 39 | 1 | 3.14 - | 1.00E+00 |
| carbohydrate phosphatase activity | 6 | 0 | 0.48 - | 1.00E+00 |
| amino acid kinase activity | 6 | 0 | 0.48 - | 1.00E+00 |
| nucleotide kinase activity | 48 | 5 | 3.87 + | 1.00E+00 |
| phospholipase activity | 22 | 3 | 1.77 + | 1.00E+00 |
| RNA methyltransferase activity | 20 | 0 | 1.61 - | 1.00E+00 |
| carbohydrate kinase activity | 41 | 2 | 3.3 - | 1.00E+00 |
| anion channel activity | 37 | 4 | 2.98 + | 1.00E+00 |
| protein disulfide isomerase activity | 28 | 1 | 2.26 - | 1.00E+00 |
| guanylate cyclase activity | 26 | 5 | 2.09 + | 1.00E+00 |
| hydro-lyase activity | 47 | 2 | 3.79 - | 1.00E+00 |
| carboxy-lyase activity | 31 | 3 | 2.5 + | 1.00E+00 |
| hydrolase activity, hydrolyzing N-glycosyl compounds | 49 | 3 | 3.95 - | 1.00E+00 |
| aminoacyl-tRNA ligase activity | 36 | 0 | 2.9 - | 1.00E+00 |
| voltage-gated potassium channel activity | 37 | 1 | 2.98 - | 1.00E+00 |
| methyltransferase activity | 104 | 3 | 8.38 - | 1.00E+00 |
| phosphatase activity | 158 | 16 | 12.73 + | 1.00E+00 |
| amino acid transmembrane transporter activity | 106 | 6 | 8.54 - | 1.00E+00 |
| voltage-gated ion channel activity | 60 | 11 | 4.83 + | 1.00E+00 |
| gap junction channel activity | 8 | 1 | 0.64 + | 1.00E+00 |
| translation release factor activity | 6 | 0 | 0.48 - | 1.00E+00 |
| translation elongation factor activity | 64 | 2 | 5.16 - | 1.00E+00 |
| translation initiation factor activity | 74 | 8 | 5.96 + | 1.00E+00 |
| lyase activity | 162 | 17 | 13.05 + | 1.00E+00 |
| adenylate cyclase activity | 41 | 8 | 3.3 + | 1.00E+00 |
| hydrolase activity, acting on ester bonds | 483 | 41 | 38.91 + | 1.00E+00 |
| hydrolase activity | 1724 | 121 | 138.88 - | 1.00E+00 |
| transketolase activity | 4 | 0 | 0.32 - | 1.00E+00 |
| transaldolase activity | 1 | 0 | 0.08 - | 1.00E+00 |
| translation regulator activity | 126 | 8 | 10.15 - | 1.00E+00 |
| peroxidase activity | 25 | 0 | 2.01 - | 1.00E+00 |
| structural molecule activity | 873 | 90 | 70.33 + | 1.00E+00 |
| DNA-directed RNA polymerase activity | 44 | 3 | 3.54 - | 1.00E+00 |
| structural constituent of ribosome | 194 | 9 | 15.63 - | 1.00E+00 |
| DNA primase activity | 2 | 0 | 0.16 - | 1.00E+00 |
| tumor necrosis factor receptor activity | 1 | 0 | 0.08 - | 1.00E+00 |
| single-stranded DNA binding | 61 | 7 | 4.91 + | 1.00E+00 |
| nucleotidyltransferase activity | 63 | 5 | 5.08 - | 1.00E+00 |
| double-stranded DNA binding | 15 | 1 | 1.21 - | 1.00E+00 |
| poly(A) RNA binding | 58 | 8 | 4.67 + | 1.00E+00 |
| GTPase activity | 115 | 8 | 9.26 - | 1.00E+00 |

Figure S2.5 (Continued)

| | | | | |
|--|-----|----|---------|----------|
| mRNA binding | 207 | 21 | 16.68 + | 1.00E+00 |
| hydrolase activity, hydrolyzing O-glycosyl compounds | 32 | 2 | 2.58 - | 1.00E+00 |
| DNA-directed DNA polymerase activity | 28 | 1 | 2.26 - | 1.00E+00 |
| transforming growth factor beta receptor activity | 5 | 1 | 0.4 + | 1.00E+00 |
| RNA binding | 345 | 31 | 27.79 + | 1.00E+00 |
| neuropeptide hormone activity | 1 | 0 | 0.08 - | 1.00E+00 |
| DNA replication origin binding | 35 | 5 | 2.82 + | 1.00E+00 |
| DNA-methyltransferase activity | 19 | 2 | 1.53 + | 1.00E+00 |
| damaged DNA binding | 38 | 1 | 3.06 - | 1.00E+00 |
| chromatin binding | 130 | 17 | 10.47 + | 1.00E+00 |
| carbohydrate transmembrane transporter activity | 87 | 6 | 7.01 - | 1.00E+00 |
| acetyltransferase activity | 81 | 8 | 6.53 + | 1.00E+00 |
| ion channel activity | 256 | 27 | 20.62 + | 1.00E+00 |
| translation factor activity, nucleic acid binding | 149 | 10 | 12 - | 1.00E+00 |
| transporter activity | 730 | 61 | 58.81 + | 1.00E+00 |
| DNA topoisomerase activity | 3 | 2 | 0.24 + | 1.00E+00 |
| antioxidant activity | 29 | 0 | 2.34 - | 1.00E+00 |
| DNA photolyase activity | 2 | 0 | 0.16 - | 1.00E+00 |
| hormone activity | 14 | 0 | 1.13 - | 1.00E+00 |
| transcription cofactor activity | 129 | 14 | 10.39 + | 1.00E+00 |
| structural constituent of myelin sheath | 2 | 0 | 0.16 - | 1.00E+00 |
| DNA helicase activity | 49 | 1 | 3.95 - | 1.00E+00 |
| amylase activity | 16 | 0 | 1.29 - | 1.00E+00 |
| peptidase inhibitor activity | 93 | 6 | 7.49 - | 1.00E+00 |
| transferase activity, transferring glycosyl groups | 176 | 16 | 14.18 + | 1.00E+00 |
| cyclic nucleotide-gated ion channel activity | 9 | 0 | 0.73 - | 1.00E+00 |

Figure S2.5 (Continued)

| PANTHER Protein Class | Drosophila REFLIST | Target Genes | Target Genes (expected) | over/under | FDR Adj P-value |
|---|--------------------|--------------|-------------------------|------------|-----------------|
| transcription factor | 956 | 140 | 77.01 + | | 1.95E-09 |
| actin family cytoskeletal protein | 220 | 47 | 17.72 + | | 7.17E-07 |
| cell adhesion molecule | 222 | 46 | 17.88 + | | 2.57E-06 |
| receptor | 773 | 109 | 62.27 + | | 3.37E-06 |
| Unclassified | 5944 | 391 | 478.83 - | | 8.81E-06 |
| zinc finger transcription factor | 312 | 53 | 25.13 + | | 1.08E-04 |
| G-protein modulator | 203 | 39 | 16.35 + | | 1.98E-04 |
| cytoskeletal protein | 485 | 71 | 39.07 + | | 3.18E-04 |
| voltage-gated sodium channel | 12 | 7 | 0.97 + | | 1.20E-02 |
| sodium channel | 12 | 7 | 0.97 + | | 1.20E-02 |
| kinase | 355 | 51 | 28.6 + | | 1.42E-02 |
| oxidoreductase | 712 | 32 | 57.36 - | | 2.51E-02 |
| non-motor actin binding protein | 96 | 20 | 7.73 + | | 2.77E-02 |
| guanyl-nucleotide exchange factor | 67 | 16 | 5.4 + | | 2.79E-02 |
| calcium channel | 15 | 7 | 1.21 + | | 4.65E-02 |
| voltage-gated calcium channel | 15 | 7 | 1.21 + | | 4.65E-02 |
| homeobox transcription factor | 108 | 21 | 8.7 + | | 4.80E-02 |
| helix-turn-helix transcription factor | 108 | 21 | 8.7 + | | 4.80E-02 |
| dehydrogenase | 261 | 7 | 21.03 - | | 6.37E-02 |
| protein kinase | 230 | 35 | 18.53 + | | 6.57E-02 |
| actin binding motor protein | 21 | 8 | 1.69 + | | 6.66E-02 |
| immunoglobulin superfamily cell adhesion molecule | 59 | 14 | 4.75 + | | 7.46E-02 |
| nuclear hormone receptor | 22 | 8 | 1.77 + | | 9.01E-02 |
| membrane-bound signaling molecule | 100 | 19 | 8.06 + | | 1.20E-01 |
| cytokine receptor | 36 | 10 | 2.9 + | | 1.51E-01 |
| cell junction protein | 50 | 12 | 4.03 + | | 1.70E-01 |
| enzyme modulator | 729 | 83 | 58.73 + | | 2.19E-01 |
| membrane trafficking regulatory protein | 59 | 13 | 4.75 + | | 2.28E-01 |
| extracellular matrix linker protein | 33 | 9 | 2.66 + | | 3.06E-01 |
| immunoglobulin receptor superfamily | 29 | 8 | 2.34 + | | 5.05E-01 |
| helicase | 99 | 1 | 7.98 - | | 5.43E-01 |
| RNA helicase | 66 | 0 | 5.32 - | | 8.72E-01 |
| mitochondrial carrier protein | 66 | 3 | 5.32 - | | 1.00E+00 |
| microtubule family cytoskeletal protein | 175 | 16 | 14.1 + | | 1.00E+00 |
| microtubule binding motor protein | 47 | 5 | 3.79 + | | 1.00E+00 |
| methyltransferase | 104 | 3 | 8.38 - | | 1.00E+00 |
| metalloprotease | 181 | 14 | 14.58 - | | 1.00E+00 |
| membrane traffic protein | 193 | 26 | 15.55 + | | 1.00E+00 |
| major histocompatibility complex antigen | 5 | 1 | 0.4 + | | 1.00E+00 |
| mRNA splicing factor | 176 | 18 | 14.18 + | | 1.00E+00 |
| mRNA processing factor | 207 | 21 | 16.68 + | | 1.00E+00 |
| mRNA polyadenylation factor | 58 | 8 | 4.67 + | | 1.00E+00 |
| lyase | 163 | 17 | 13.13 + | | 1.00E+00 |
| lipase | 112 | 4 | 9.02 - | | 1.00E+00 |
| ligase | 367 | 22 | 29.56 - | | 1.00E+00 |
| ligand-gated ion channel | 94 | 7 | 7.57 - | | 1.00E+00 |
| kinase modulator | 156 | 10 | 12.57 - | | 1.00E+00 |
| kinase inhibitor | 63 | 1 | 5.08 - | | 1.00E+00 |
| kinase activator | 45 | 4 | 3.63 + | | 1.00E+00 |
| isomerase | 158 | 5 | 12.73 - | | 1.00E+00 |
| ionotropic glutamate receptor | 51 | 3 | 4.11 - | | 1.00E+00 |
| ion channel | 234 | 25 | 18.85 + | | 1.00E+00 |
| intracellular calcium-sensing protein | 155 | 15 | 12.49 + | | 1.00E+00 |
| exoribonuclease | 25 | 2 | 2.01 - | | 1.00E+00 |
| intermediate filament binding protein | 1 | 0 | 0.08 - | | 1.00E+00 |
| exodeoxyribonuclease | 12 | 0 | 0.97 - | | 1.00E+00 |
| esterase | 108 | 2 | 8.7 - | | 1.00E+00 |
| epimerase/racemase | 75 | 1 | 6.04 - | | 1.00E+00 |
| endoribonuclease | 45 | 1 | 3.63 - | | 1.00E+00 |
| endodeoxyribonuclease | 34 | 3 | 2.74 + | | 1.00E+00 |
| dehydratase | 32 | 2 | 2.58 - | | 1.00E+00 |

Figure S2.5 (Continued)

| | | | | |
|---|------|-----|----------|----------|
| defense/immunity protein | 172 | 22 | 13.86 + | 1.00E+00 |
| intermediate filament | 3 | 0 | 0.24 - | 1.00E+00 |
| hydroxylase | 40 | 3 | 3.22 - | 1.00E+00 |
| hydrolase | 1489 | 102 | 119.95 - | 1.00E+00 |
| decarboxylase | 32 | 3 | 2.58 + | 1.00E+00 |
| hydratase | 19 | 0 | 1.53 - | 1.00E+00 |
| deaminase | 26 | 3 | 2.09 + | 1.00E+00 |
| deacetylase | 20 | 2 | 1.61 + | 1.00E+00 |
| damaged DNA-binding protein | 38 | 1 | 3.06 - | 1.00E+00 |
| cytokine | 12 | 2 | 0.97 + | 1.00E+00 |
| cysteine protease inhibitor | 5 | 2 | 0.4 + | 1.00E+00 |
| cysteine protease | 59 | 4 | 4.75 - | 1.00E+00 |
| cyclic nucleotide-gated ion channel | 9 | 0 | 0.73 - | 1.00E+00 |
| histone | 13 | 1 | 1.05 - | 1.00E+00 |
| heterotrimeric G-protein | 15 | 1 | 1.21 - | 1.00E+00 |
| guanylate cyclase | 26 | 5 | 2.09 + | 1.00E+00 |
| growth factor | 44 | 6 | 3.54 + | 1.00E+00 |
| glycosyltransferase | 167 | 16 | 13.45 + | 1.00E+00 |
| cyclase | 43 | 8 | 3.46 + | 1.00E+00 |
| glycosidase | 49 | 3 | 3.95 - | 1.00E+00 |
| complement component | 13 | 3 | 1.05 + | 1.00E+00 |
| chromatin/chromatin-binding protein | 130 | 17 | 10.47 + | 1.00E+00 |
| chaperonin | 31 | 1 | 2.5 - | 1.00E+00 |
| chaperone | 156 | 5 | 12.57 - | 1.00E+00 |
| centromere DNA-binding protein | 14 | 2 | 1.13 + | 1.00E+00 |
| glucosidase | 11 | 2 | 0.89 + | 1.00E+00 |
| gap junction | 8 | 1 | 0.64 + | 1.00E+00 |
| galactosidase | 5 | 0 | 0.4 - | 1.00E+00 |
| extracellular matrix structural protein | 29 | 4 | 2.34 + | 1.00E+00 |
| extracellular matrix protein | 189 | 24 | 15.23 + | 1.00E+00 |
| extracellular matrix glycoprotein | 48 | 4 | 3.87 + | 1.00E+00 |
| cation transporter | 229 | 18 | 18.45 - | 1.00E+00 |
| carbohydrate transporter | 87 | 6 | 7.01 - | 1.00E+00 |
| carbohydrate phosphatase | 6 | 0 | 0.48 - | 1.00E+00 |
| carbohydrate kinase | 41 | 2 | 3.3 - | 1.00E+00 |
| calmodulin | 155 | 15 | 12.49 + | 1.00E+00 |
| calcium-binding protein | 237 | 27 | 19.09 + | 1.00E+00 |
| cadherin | 13 | 3 | 1.05 + | 1.00E+00 |
| basic leucine zipper transcription factor | 6 | 3 | 0.48 + | 1.00E+00 |
| basic helix-loop-helix transcription factor | 61 | 9 | 4.91 + | 1.00E+00 |
| aspartic protease | 16 | 1 | 1.29 - | 1.00E+00 |
| apolipoprotein | 30 | 5 | 2.42 + | 1.00E+00 |
| antibacterial response protein | 54 | 3 | 4.35 - | 1.00E+00 |
| annexin | 60 | 3 | 4.83 - | 1.00E+00 |
| anion channel | 37 | 4 | 2.98 + | 1.00E+00 |
| voltage-gated potassium channel | 37 | 1 | 2.98 - | 1.00E+00 |
| amylase | 16 | 0 | 1.29 - | 1.00E+00 |
| voltage-gated ion channel | 60 | 11 | 4.83 + | 1.00E+00 |
| aminoacyl-tRNA synthetase | 36 | 0 | 2.9 - | 1.00E+00 |
| amino acid transporter | 106 | 6 | 8.54 - | 1.00E+00 |
| amino acid kinase | 6 | 0 | 0.48 - | 1.00E+00 |
| aldolase | 4 | 1 | 0.32 + | 1.00E+00 |
| adenylate cyclase | 41 | 8 | 3.3 + | 1.00E+00 |
| acyltransferase | 99 | 7 | 7.98 - | 1.00E+00 |
| viral protein | 1 | 1 | 0.08 + | 1.00E+00 |
| vesicle coat protein | 26 | 5 | 2.09 + | 1.00E+00 |
| ubiquitin-protein ligase | 139 | 9 | 11.2 - | 1.00E+00 |
| tyrosine protein kinase receptor | 35 | 7 | 2.82 + | 1.00E+00 |
| actin and actin related protein | 16 | 3 | 1.29 + | 1.00E+00 |
| acetyltransferase | 81 | 8 | 6.53 + | 1.00E+00 |
| acetylcholine receptor | 24 | 2 | 1.93 + | 1.00E+00 |
| replication origin binding protein | 23 | 3 | 1.85 + | 1.00E+00 |

Figure S2.5 (Continued)

| | | | | |
|---|-----|----|---------|----------|
| tumor necrosis factor receptor | 1 | 0 | 0.08 - | 1.00E+00 |
| reductase | 231 | 14 | 18.61 - | 1.00E+00 |
| TGF-beta receptor | 5 | 1 | 0.4 + | 1.00E+00 |
| SNARE protein | 22 | 1 | 1.77 - | 1.00E+00 |
| pyrophosphatase | 11 | 0 | 0.89 - | 1.00E+00 |
| RNA methyltransferase | 20 | 0 | 1.61 - | 1.00E+00 |
| protein phosphatase | 104 | 15 | 8.38 + | 1.00E+00 |
| protein kinase receptor | 49 | 8 | 3.95 + | 1.00E+00 |
| RNA binding protein | 808 | 51 | 65.09 - | 1.00E+00 |
| protease inhibitor | 93 | 6 | 7.49 - | 1.00E+00 |
| protease | 627 | 34 | 50.51 - | 1.00E+00 |
| tumor necrosis factor family member | 1 | 1 | 0.08 + | 1.00E+00 |
| tubulin | 12 | 0 | 0.97 - | 1.00E+00 |
| transporter | 857 | 66 | 69.04 - | 1.00E+00 |
| transmembrane receptor regulatory/adaptor protein | 39 | 4 | 3.14 + | 1.00E+00 |
| translation release factor | 6 | 0 | 0.48 - | 1.00E+00 |
| translation initiation factor | 74 | 8 | 5.96 + | 1.00E+00 |
| translation factor | 128 | 10 | 10.31 - | 1.00E+00 |
| KRAB box transcription factor | 114 | 18 | 9.18 + | 1.00E+00 |
| translation elongation factor | 64 | 2 | 5.16 - | 1.00E+00 |
| Hsp90 family chaperone | 3 | 0 | 0.24 - | 1.00E+00 |
| transketolase | 4 | 0 | 0.32 - | 1.00E+00 |
| Hsp70 family chaperone | 12 | 0 | 0.97 - | 1.00E+00 |
| primase | 2 | 0 | 0.16 - | 1.00E+00 |
| transferase | 983 | 96 | 79.19 + | 1.00E+00 |
| potassium channel | 37 | 1 | 2.98 - | 1.00E+00 |
| phosphorylase | 9 | 0 | 0.73 - | 1.00E+00 |
| phospholipase | 22 | 3 | 1.77 + | 1.00E+00 |
| HMG box transcription factor | 31 | 3 | 2.5 + | 1.00E+00 |
| GABA receptor | 24 | 2 | 1.93 + | 1.00E+00 |
| phosphodiesterase | 26 | 4 | 2.09 + | 1.00E+00 |
| phosphatase modulator | 35 | 2 | 2.82 - | 1.00E+00 |
| G-protein coupled receptor | 241 | 25 | 19.41 + | 1.00E+00 |
| phosphatase inhibitor | 14 | 2 | 1.13 + | 1.00E+00 |
| G-protein | 115 | 8 | 9.26 - | 1.00E+00 |
| phosphatase activator | 3 | 0 | 0.24 - | 1.00E+00 |
| phosphatase | 221 | 21 | 17.8 + | 1.00E+00 |
| peroxidase | 25 | 0 | 2.01 - | 1.00E+00 |
| transfer/carrier protein | 294 | 18 | 23.68 - | 1.00E+00 |
| transcription cofactor | 129 | 14 | 10.39 + | 1.00E+00 |
| transaminase | 17 | 4 | 1.37 + | 1.00E+00 |
| transaldolase | 1 | 0 | 0.08 - | 1.00E+00 |
| tight junction | 5 | 1 | 0.4 + | 1.00E+00 |
| DNA-directed RNA polymerase | 42 | 3 | 3.38 - | 1.00E+00 |
| surfactant | 11 | 1 | 0.89 + | 1.00E+00 |
| structural protein | 196 | 14 | 15.79 - | 1.00E+00 |
| DNA-directed DNA polymerase | 28 | 1 | 2.26 - | 1.00E+00 |
| storage protein | 47 | 1 | 3.79 - | 1.00E+00 |
| peptide hormone | 14 | 0 | 1.13 - | 1.00E+00 |
| DNA topoisomerase | 3 | 2 | 0.24 + | 1.00E+00 |
| DNA strand-pairing protein | 5 | 0 | 0.4 - | 1.00E+00 |
| oxygenase | 151 | 4 | 12.16 - | 1.00E+00 |
| DNA polymerase processivity factor | 3 | 0 | 0.24 - | 1.00E+00 |
| DNA photolyase | 2 | 0 | 0.16 - | 1.00E+00 |
| oxidase | 122 | 5 | 9.83 - | 1.00E+00 |
| DNA methyltransferase | 19 | 2 | 1.53 + | 1.00E+00 |
| nucleotidyltransferase | 63 | 5 | 5.08 - | 1.00E+00 |
| DNA ligase | 4 | 0 | 0.32 - | 1.00E+00 |
| DNA helicase | 49 | 1 | 3.95 - | 1.00E+00 |
| nucleotide phosphatase | 39 | 1 | 3.14 - | 1.00E+00 |
| nucleotide kinase | 48 | 5 | 3.87 + | 1.00E+00 |
| DNA glycosylase | 4 | 1 | 0.32 + | 1.00E+00 |

Figure S2.5 (Continued)

| | | | | |
|--|------|-----|----------|----------|
| nucleic acid binding | 1761 | 162 | 141.86 + | 1.00E+00 |
| nuclease | 172 | 15 | 13.86 + | 1.00E+00 |
| small GTPase | 68 | 4 | 5.48 - | 1.00E+00 |
| signaling molecule | 442 | 51 | 35.61 + | 1.00E+00 |
| serine/threonine protein kinase receptor | 23 | 2 | 1.85 + | 1.00E+00 |
| serine protease inhibitor | 62 | 4 | 4.99 - | 1.00E+00 |
| serine protease | 349 | 16 | 28.11 - | 1.00E+00 |
| ribosomal protein | 194 | 9 | 15.63 - | 1.00E+00 |
| DNA binding protein | 624 | 62 | 50.27 + | 1.00E+00 |
| ribonucleoprotein | 79 | 13 | 6.36 + | 1.00E+00 |
| non-receptor tyrosine protein kinase | 51 | 7 | 4.11 + | 1.00E+00 |
| CREB transcription factor | 6 | 3 | 0.48 + | 1.00E+00 |
| non-receptor serine/threonine protein kinase | 147 | 18 | 11.84 + | 1.00E+00 |
| non-motor microtubule binding protein | 51 | 7 | 4.11 + | 1.00E+00 |
| ATP-binding cassette (ABC) transporter | 87 | 4 | 7.01 - | 1.00E+00 |
| ATP synthase | 53 | 4 | 4.27 - | 1.00E+00 |
| neuropeptide | 1 | 0 | 0.08 - | 1.00E+00 |
| myelin protein | 2 | 0 | 0.16 - | 1.00E+00 |
| mutase | 8 | 0 | 0.64 - | 1.00E+00 |

The PANTHER gene ontology annotation database was used to identify protein class and molecular function annotations overrepresented in the set of genes targeted by differentially expressed microRNAs.(4)

Figure S2.6. Diana MicroT target predictions for genes with “Neurological systems process” GO annotation

| FlyBase ID | Symbol | Name | dme-miR-312-3p | dme-miR-314-3p | dme-miR-956-3p | dme-miR-958-3p | dme-miR-958-5p |
|-------------|---------------|---|----------------|----------------|----------------|----------------|----------------|
| FBgn0004396 | CrebA | Cyclic-AMP response element binding protein A | 1 | 0 | 0 | 0 | 0 |
| FBgn0038439 | Cad89D | Cadherin 89D | 1 | 0 | 0 | 0 | 0 |
| FBgn0029835 | CG5921 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0017549 | 0 | | 0 | 1 | 1 | 0 | 0 |
| FBgn0039530 | Tusp | Tusp | 1 | 0 | 0 | 0 | 0 |
| FBgn0086475 | sec3 | sec3 | 1 | 0 | 0 | 0 | 0 |
| FBgn0034509 | Obp57c | Odorant-binding protein 57c | 1 | 0 | 0 | 0 | 0 |
| FBgn0039431 | CG6490 | | 0 | 1 | 0 | 1 | 0 |
| FBgn0003870 | ttk | tramtrack | 1 | 0 | 1 | 0 | 0 |
| FBgn0262737 | mub | mushroom-body expressed | 1 | 0 | 1 | 0 | 0 |
| FBgn0029846 | #N/A | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0259750 | #N/A | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0039380 | CG5890 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0035308 | CG15822 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0015323 | VAcHT | VAcHT | 1 | 0 | 0 | 0 | 0 |
| FBgn0039187 | CG6454 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0004914 | Hnf4 | Hepatocyte nuclear factor 4 | 1 | 0 | 0 | 0 | 0 |
| FBgn0013759 | CASK | CASK ortholog | 1 | 0 | 0 | 0 | 0 |
| FBgn0085414 | dpr12 | dpr12 | 1 | 0 | 0 | 0 | 0 |
| FBgn0015129 | 0 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0035540 | Syx17 | Syntaxin 17 | 1 | 0 | 0 | 0 | 0 |
| FBgn0052600 | dpr8 | dpr8 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028872 | CG18095 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0000120 | Arr1 | Arrestin 1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0262350 | #N/A | #N/A | 1 | 1 | 0 | 0 | 0 |
| FBgn0033138 | Tsp42Eq | Tetraspanin 42Eq | 1 | 0 | 0 | 0 | 0 |
| FBgn0039528 | dsd | distracted | 1 | 0 | 0 | 0 | 0 |
| FBgn0014870 | Psi | P-element somatic inhibitor | 1 | 0 | 0 | 0 | 1 |
| FBgn0034433 | endoB | endophilin B | 1 | 0 | 0 | 1 | 0 |
| FBgn0087007 | bbg | big bang | 1 | 0 | 0 | 0 | 0 |
| FBgn0003149 | Prm | Paramyosin | 1 | 0 | 0 | 0 | 0 |
| FBgn0259231 | CCKLR-17D1 | CCK-like receptor at 17D1 | 1 | 1 | 0 | 0 | 0 |
| FBgn0024944 | Oamb | Octopamine receptor in mushroom bodies | 1 | 0 | 0 | 0 | 0 |
| FBgn0036927 | CG7433 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0004242 | Syt1 | Synaptotagmin 1 | 1 | 1 | 0 | 0 | 0 |
| FBgn0039911 | CG1909 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0000037 | mAcR | muscarinic Acetylcholine Receptor | 1 | 0 | 0 | 0 | 0 |
| FBgn0261836 | Msp-300 | Muscle-specific protein 300 | 1 | 0 | 0 | 0 | 0 |
| FBgn0040395 | Unc-76 | Unc-76 | 1 | 0 | 0 | 0 | 0 |
| FBgn0000210 | br | broad | 1 | 0 | 0 | 0 | 0 |
| FBgn0000317 | ck | crinkled | 1 | 0 | 0 | 0 | 0 |
| FBgn0250910 | Octbeta3R | Octopamine beta3 receptor | 1 | 0 | 0 | 0 | 0 |
| FBgn0085383 | CG34354 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0031981 | CG7466 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0040334 | Tsp3A | Tetraspanin 3A | 1 | 0 | 0 | 0 | 0 |
| FBgn0038339 | CG6118 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0028875 | nAcRalpha-34E | nicotinic Acetylcholine Receptor alpha 34E | 1 | 0 | 0 | 0 | 0 |
| FBgn0028371 | jbug | jitterbug | 1 | 0 | 0 | 0 | 0 |
| FBgn0015295 | shark | SH2 ankyrin repeat kinase | 1 | 0 | 0 | 0 | 0 |
| FBgn0031760 | Tsp26A | Tetraspanin 26A | 1 | 0 | 0 | 1 | 0 |
| FBgn0037993 | dpr15 | dpr15 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028704 | Nckx30C | Nckx30C | 1 | 1 | 0 | 0 | 0 |
| FBgn0032341 | Reps | | 0 | 1 | 0 | 1 | 1 |
| FBgn0033058 | CCHa2r | CCHamide-2 receptor | 1 | 0 | 0 | 0 | 0 |
| FBgn0001297 | kay | kayak | 1 | 0 | 0 | 0 | 0 |
| FBgn0026386 | Or47a | Odorant receptor 47a | 1 | 0 | 0 | 0 | 0 |
| FBgn0028582 | lqf | liquid facets | 1 | 0 | 0 | 0 | 0 |
| FBgn0037326 | CG14669 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0040208 | Kat60 | Katanin 60 | 1 | 0 | 0 | 0 | 0 |
| FBgn0052702 | CG32702 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0013733 | shot | short stop | 1 | 0 | 0 | 1 | 0 |
| FBgn0261552 | ps | pasilla | 1 | 0 | 0 | 0 | 0 |
| FBgn0000273 | Pka-C1 | cAMP-dependent protein kinase 1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0085434 | NaCP60E | Na channel protein 60E | 1 | 0 | 0 | 0 | 0 |
| FBgn0033876 | SyngR | Synaptogyrin | 1 | 0 | 0 | 0 | 0 |
| FBgn0015774 | NetB | Netrin-B | 1 | 0 | 0 | 1 | 0 |
| FBgn0262111 | f | forked | 1 | 0 | 0 | 0 | 0 |
| FBgn0010473 | tutl | turtle | 1 | 0 | 0 | 0 | 0 |
| FBgn0035170 | dpr20 | dpr20 | 1 | 0 | 0 | 0 | 0 |
| FBgn0261999 | CG42817 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0037848 | Tsp86D | Tetraspanin 86D | 1 | 0 | 0 | 0 | 0 |
| FBgn0029508 | Tsp42Ea | Tetraspanin 42Ea | 1 | 0 | 0 | 1 | 0 |
| FBgn0261387 | CG17528 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0024277 | trio | trio | 1 | 0 | 0 | 0 | 0 |
| FBgn0037130 | Syn1 | Syntrophin-like 1 | 1 | 0 | 0 | 0 | 0 |
| FBgn0028431 | #N/A | #N/A | 1 | 0 | 0 | 0 | 0 |
| FBgn0038237 | Pde6 | Phosphodiesterase 6 | 1 | 0 | 0 | 0 | 0 |

Figure S2.6 (Continued)

| | | | | | | | | |
|-------------|------------|---|---|---|---|---|---|---|
| FBgn0085405 | CG34376 | | 0 | 1 | 0 | 0 | 0 | 0 |
| FBgn0038880 | SIFR | SIFamide receptor | | 1 | 0 | 0 | 0 | 0 |
| FBgn0011582 | Dop1R1 | Dopamine 1-like receptor 1 | | 1 | 1 | 1 | 1 | 0 |
| FBgn0000448 | Hr46 | Hormone receptor-like in 46 | | 1 | 1 | 0 | 0 | 0 |
| FBgn0023000 | mth | methuselah | | 1 | 0 | 0 | 0 | 0 |
| FBgn0053516 | dpr3 | dpr3 | | 1 | 0 | 0 | 0 | 0 |
| FBgn0026086 | Adar | Adenosine deaminase acting on RNA | | 1 | 0 | 1 | 0 | 0 |
| FBgn0023091 | dimm | dimmed | | 1 | 0 | 0 | 0 | 0 |
| FBgn0053555 | #N/A | #N/A | | 0 | 1 | 0 | 0 | 0 |
| FBgn0040388 | boi | brother of ihog | | 0 | 1 | 1 | 0 | 0 |
| FBgn0038118 | timeout | timeout | | 0 | 1 | 0 | 0 | 0 |
| FBgn0263218 | 0 | | 0 | 0 | 1 | 1 | 0 | 0 |
| FBgn0034691 | synj | synaptotagmin | | 0 | 1 | 0 | 0 | 0 |
| FBgn0085397 | Fili | Fish-lips | | 0 | 1 | 0 | 0 | 0 |
| FBgn0025632 | CG4313 | | 0 | 0 | 1 | 0 | 0 | 0 |
| FBgn0004622 | Takr99D | Tachykinin-like receptor at 99D | | 0 | 1 | 1 | 0 | 0 |
| FBgn0037408 | NPFR1 | neuropeptide F receptor | | 0 | 1 | 0 | 0 | 0 |
| FBgn0028550 | Atf3 | Activating transcription factor 3 | | 0 | 1 | 1 | 0 | 0 |
| FBgn0016059 | Sema-1b | Sema-1b | | 0 | 1 | 1 | 0 | 0 |
| FBgn0000667 | Actn | alpha actinin | | 0 | 1 | 0 | 0 | 0 |
| FBgn0086783 | #N/A | #N/A | | 0 | 1 | 0 | 0 | 0 |
| FBgn0003206 | Ras64B | Ras oncogene at 64B | | 0 | 1 | 0 | 0 | 0 |
| FBgn0034135 | Syn2 | Syntrophin-like 2 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0024963 | GluClalpha | GluClalpha | | 0 | 1 | 0 | 0 | 1 |
| FBgn0030766 | mth1 | methuselah-like 1 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0002940 | ninaE | neither inactivation nor afterpotential E | | 0 | 1 | 0 | 0 | 0 |
| FBgn0261053 | Cad86C | Cadherin 86C | | 0 | 1 | 0 | 0 | 0 |
| FBgn0039335 | Vps33B | Vacuolar protein sorting 33B | | 0 | 1 | 0 | 0 | 0 |
| FBgn0034049 | bdg | bedraggled | | 0 | 1 | 0 | 0 | 0 |
| FBgn0038720 | CG6231 | | 0 | 0 | 1 | 0 | 0 | 0 |
| FBgn0038139 | PK2-R2 | Pyrokinin 2 receptor 2 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0000633 | fas | faint sausage | | 0 | 1 | 0 | 0 | 0 |
| FBgn0261085 | Syt12 | Synaptotagmin 12 | | 0 | 1 | 0 | 0 | 0 |
| FBgn0038089 | d-cup | davis-cup | | 0 | 0 | 1 | 0 | 0 |
| FBgn0036260 | Rh7 | Rhodopsin 7 | | 0 | 0 | 1 | 0 | 0 |
| FBgn0029974 | dpr14 | dpr14 | | 0 | 0 | 1 | 0 | 0 |
| FBgn0038890 | CG7956 | | 0 | 0 | 0 | 1 | 1 | 0 |
| FBgn0039157 | Myo95E | Myosin 95E | | 0 | 0 | 1 | 0 | 0 |
| FBgn0000635 | Fas2 | Fasciclin 2 | | 0 | 0 | 1 | 0 | 0 |
| FBgn0053202 | dpr11 | dpr11 | | 0 | 0 | 1 | 0 | 0 |
| FBgn0031119 | CG1812 | | 0 | 0 | 0 | 1 | 0 | 0 |
| FBgn0259225 | #N/A | #N/A | | 0 | 0 | 1 | 0 | 0 |
| FBgn0263102 | psq | pipsqueak | | 0 | 0 | 1 | 0 | 0 |
| FBgn0085446 | CG34417 | | 0 | 0 | 0 | 1 | 1 | 0 |
| FBgn0034286 | dpr13 | dpr13 | | 0 | 0 | 1 | 0 | 0 |
| FBgn0011225 | jar | jaguar | | 0 | 0 | 1 | 0 | 0 |
| FBgn0262742 | Fas1 | Fasciclin 1 | | 0 | 0 | 1 | 0 | 0 |
| FBgn0039069 | CG6763 | | 0 | 0 | 0 | 1 | 0 | 0 |
| FBgn0039927 | CG11155 | | 0 | 0 | 0 | 1 | 0 | 0 |
| FBgn0003861 | trp | transient receptor potential | | 0 | 0 | 0 | 1 | 0 |
| FBgn0002931 | net | net | | 0 | 0 | 0 | 1 | 0 |
| FBgn0086365 | Orct2 | Organic cation transporter 2 | | 0 | 0 | 0 | 1 | 0 |
| FBgn0036273 | CG10426 | | 0 | 0 | 0 | 0 | 1 | 0 |
| FBgn0033679 | CG8888 | | 0 | 0 | 0 | 0 | 1 | 0 |
| FBgn0039644 | CG11897 | | 0 | 0 | 0 | 0 | 1 | 0 |
| FBgn0037552 | CG7800 | | 0 | 0 | 0 | 0 | 1 | 0 |
| FBgn0036789 | AICR2 | allatostatin C receptor 2 | | 0 | 0 | 0 | 1 | 0 |
| FBgn0000346 | comt | comatose | | 0 | 0 | 0 | 1 | 0 |
| FBgn0038826 | Syp | Syncrip | | 0 | 0 | 0 | 1 | 0 |
| FBgn0259822 | Ca-beta | Ca2+-channel-protein-beta-subunit | | 0 | 0 | 0 | 1 | 0 |
| FBgn0011259 | Sema-1a | Sema-1a | | 0 | 0 | 0 | 1 | 0 |
| FBgn0015609 | CadN | Cadherin-N | | 0 | 0 | 0 | 1 | 0 |
| FBgn0028433 | Ggamma30A | G protein gamma30A | | 0 | 0 | 0 | 1 | 0 |
| FBgn0010399 | Nmdar1 | NMDA receptor 1 | | 0 | 0 | 0 | 1 | 0 |
| FBgn0261090 | Sybeta | Synaptotagmin beta | | 0 | 0 | 0 | 1 | 0 |
| FBgn0033739 | Dyb | Dystrobrevin-like | | 0 | 0 | 0 | 1 | 0 |
| FBgn0028400 | Syt4 | Synaptotagmin 4 | | 0 | 0 | 0 | 0 | 1 |
| FBgn0029762 | NAAT1 | Nutrient Amino Acid Transporter 1 | | 0 | 0 | 0 | 0 | 1 |
| FBgn0085429 | #N/A | #N/A | | 0 | 0 | 0 | 0 | 1 |
| FBgn0041605 | cpx | complexin | | 0 | 0 | 0 | 0 | 1 |
| FBgn0025631 | moody | moody | | 0 | 0 | 0 | 0 | 1 |
| FBgn0036381 | CG8745 | | 0 | 0 | 0 | 0 | 0 | 1 |
| FBgn0020258 | ppk | pickpocket | | 0 | 0 | 0 | 0 | 1 |
| FBgn0011666 | msi | musashi | | 0 | 0 | 0 | 0 | 1 |
| FBgn0003651 | svp | seven up | | 0 | 0 | 0 | 0 | 1 |
| FBgn0259246 | brp | bruchpilot | | 0 | 0 | 0 | 0 | 1 |

The PANTHER gene ontology annotation database was used to identify biological process gene ontology annotations overrepresented in the set of genes targeted by differentially expressed microRNAs.(4) This analysis identifies the “Neurological systems process” as an overrepresented annotation. Due to our interest in identifying target genes of differentially expressed microRNAs that are involved in memory formation, this term was used to refine the results of our PANTHER analysis.

Figure S3.1. High exon coverage transcripts

| HECT | Symbol | plus_reads | plus_coverage | minus_reads | minus_coverage | plus_minus_read_ratio | intron |
|-------------|-----------|------------|---------------|-------------|----------------|-----------------------|--------|
| FBtr0070040 | CG32230 | 1626 | 0.9817768 | 4 | 0.1321184 | 406.5 | 2 |
| FBtr0070041 | CG32230 | 1884 | 1 | 3 | 0.1134565 | 628 | 3 |
| FBtr0070156 | RpL36 | 3539 | 0.95 | 31 | 0.2982759 | 114.1612903 | 2 |
| FBtr0070159 | CG13364 | 272 | 0.9895833 | 11 | 0.203125 | 24.72727273 | 1 |
| FBtr0070179 | CG14629 | 1363 | 0.9774368 | 29 | 0.2057762 | 47 | 0 |
| FBtr0070381 | CG3835 | 2175 | 0.9769065 | 59 | 0.1804511 | 36.86440678 | 4 |
| FBtr0070386 | CG3621 | 1338 | 0.9764706 | 26 | 0.1058824 | 51.46153846 | 1 |
| FBtr0070611 | VhaAC39-1 | 1636 | 0.9526726 | 47 | 0.185412 | 34.80851064 | 2 |
| FBtr0070708 | l(1)G0334 | 7994 | 0.9817981 | 39 | 0.1395477 | 204.974359 | 4 |
| FBtr0070709 | l(1)G0334 | 7595 | 0.9820036 | 36 | 0.1421716 | 210.9722222 | 5 |
| FBtr0070801 | RpL35 | 3145 | 0.9983444 | 39 | 0.2996689 | 80.64102564 | 2 |
| FBtr0070822 | Act5C | 7981 | 0.9743433 | 32 | 0.1954795 | 249.40625 | 1 |
| FBtr0070907 | kdn | 24324 | 0.9911802 | 53 | 0.2225955 | 458.9433962 | 2 |
| FBtr0070909 | Marf | 3642 | 0.9686684 | 81 | 0.2106179 | 44.96296296 | 7 |
| FBtr0070910 | Marf | 3836 | 0.9634146 | 84 | 0.2103658 | 45.66666667 | 7 |
| FBtr0070915 | RpL7A | 6487 | 0.9802073 | 51 | 0.2196041 | 127.1960784 | 5 |
| FBtr0070916 | RpL7A | 6530 | 0.9725023 | 41 | 0.2227314 | 159.2682927 | 4 |
| FBtr0070924 | CG3446 | 1443 | 0.9654812 | 37 | 0.3483264 | 39 | 2 |
| FBtr0070933 | Ubi-p5E | 13130 | 0.9796239 | 99 | 0.2032393 | 132.6262626 | 1 |
| FBtr0070953 | l(1)G0255 | 6464 | 0.9894552 | 24 | 0.0849443 | 269.3333333 | 4 |
| FBtr0070954 | l(1)G0255 | 6137 | 0.9611536 | 32 | 0.0971159 | 191.78125 | 4 |
| FBtr0071094 | RpS14a | 3075 | 0.9875583 | 31 | 0.3499222 | 99.19354839 | 2 |
| FBtr0071123 | CG2233 | 7862 | 0.9810811 | 27 | 0.1405405 | 291.1851852 | 4 |
| FBtr0071135 | RpS6 | 3815 | 0.9927235 | 19 | 0.1528067 | 200.7894737 | 2 |
| FBtr0071140 | CG18624 | 1919 | 0.9876289 | 6 | 0.1237113 | 319.8333333 | 1 |
| FBtr0071180 | ND75 | 9497 | 0.9938295 | 53 | 0.1542615 | 179.1886792 | 5 |
| FBtr0071181 | ND75 | 9341 | 0.98663 | 54 | 0.1631931 | 172.9814815 | 4 |
| FBtr0071345 | His.3B | 1720 | 0.9562764 | 96 | 0.2165021 | 17.91666667 | 2 |
| FBtr0071360 | RpS28b | 3529 | 0.9750548 | 11 | 0.2072937 | 320.8181818 | 1 |
| FBtr0071361 | Hex-A | 4594 | 0.9534236 | 66 | 0.1926752 | 69.60606061 | 0 |
| FBtr0071362 | Hex-A | 4594 | 0.9534236 | 66 | 0.1926752 | 69.60606061 | 0 |
| FBtr0071419 | Yp1 | 38518 | 0.9961783 | 67 | 0.3025478 | 574.8955224 | 1 |
| FBtr0071424 | Yp2 | 29336 | 0.9967804 | 173 | 0.3251771 | 169.5722543 | 1 |
| FBtr0071438 | l(1)G0230 | 2825 | 0.9924925 | 17 | 0.2117117 | 166.1764706 | 3 |
| FBtr0071444 | CG17841 | 1594 | 0.9546729 | 37 | 0.1565421 | 43.08108108 | 3 |
| FBtr0071449 | Neb-cGP | 2638 | 0.9926829 | 15 | 0.204878 | 175.8666667 | 2 |
| FBtr0071498 | Atg8a | 3126 | 0.9636664 | 85 | 0.4459125 | 36.77647059 | 2 |
| FBtr0071519 | Act57B | 10459 | 0.9831982 | 97 | 0.1755504 | 107.8247423 | 1 |
| FBtr0071537 | Treh | 3529 | 0.961929 | 135 | 0.2407542 | 26.14074074 | 5 |
| FBtr0071540 | Treh | 3503 | 0.9596031 | 151 | 0.2625797 | 23.1986755 | 5 |
| FBtr0071592 | RpL29 | 1164 | 0.9908537 | 10 | 0.222561 | 116.4 | 3 |
| FBtr0071593 | RpL29 | 1183 | 1 | 10 | 0.1931217 | 118.3 | 2 |
| FBtr0071599 | CG9485 | 5031 | 0.9508163 | 257 | 0.2367347 | 19.57587549 | 7 |
| FBtr0071601 | CG9485 | 5038 | 0.9555146 | 261 | 0.2443624 | 19.30268199 | 7 |
| FBtr0071663 | CG10320 | 1925 | 0.9533169 | 5 | 0.1228501 | 385 | 1 |
| FBtr0071784 | Swim | 2512 | 0.951148 | 45 | 0.2188569 | 55.82222222 | 3 |
| FBtr0071785 | Swim | 2783 | 0.9647533 | 42 | 0.2019134 | 66.26190476 | 3 |
| FBtr0071813 | Gp150 | 5968 | 0.973983 | 110 | 0.1903974 | 54.25454545 | 5 |
| FBtr0071814 | Gp150 | 5972 | 0.9668703 | 111 | 0.1926692 | 53.8018018 | 5 |
| FBtr0071815 | Gp150 | 5958 | 0.9616533 | 118 | 0.1986223 | 50.49152542 | 4 |
| FBtr0071855 | RpS16 | 4156 | 0.9740933 | 14 | 0.1830743 | 296.8571429 | 4 |
| FBtr0071883 | blw | 40834 | 0.9982571 | 93 | 0.1350763 | 439.0752688 | 3 |
| FBtr0071897 | RpL23 | 2136 | 0.9797794 | 9 | 0.1727941 | 237.3333333 | 3 |
| FBtr0071935 | RpS24 | 2130 | 0.9860465 | 8 | 0.1534884 | 266.25 | 1 |
| FBtr0072030 | CG30415 | 3047 | 0.9741518 | 32 | 0.2875606 | 95.21875 | 2 |
| FBtr0072031 | CG30415 | 2769 | 0.9695586 | 35 | 0.3105023 | 79.11428571 | 1 |
| FBtr0072061 | levy | 4834 | 0.9712838 | 13 | 0.1334459 | 371.8461538 | 1 |
| FBtr0072121 | CG3906 | 1351 | 0.9941107 | 16 | 0.147232 | 84.4375 | 1 |
| FBtr0072141 | Tal | 1221 | 0.9604672 | 27 | 0.1132075 | 45.22222222 | 2 |
| FBtr0072164 | PebIII | 2149 | 0.9698997 | 66 | 0.4481605 | 32.56060606 | 1 |
| FBtr0072172 | eIF-5A | 2933 | 0.9870634 | 76 | 0.1746442 | 38.59210526 | 4 |
| FBtr0072173 | eIF-5A | 3338 | 0.98713 | 76 | 0.1737452 | 43.92105263 | 3 |
| FBtr0072175 | RpL12 | 3428 | 0.9633758 | 19 | 0.2085987 | 180.4210526 | 4 |
| FBtr0072176 | RpL12 | 3283 | 0.9595016 | 19 | 0.2040498 | 172.7894737 | 3 |
| FBtr0072185 | RpL39 | 3991 | 0.9895105 | 8 | 0.3286713 | 498.875 | 2 |
| FBtr0072188 | tsr | 924 | 0.9571984 | 15 | 0.1258106 | 61.6 | 3 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|------------|-------|-----------|-----|-----------|-------------|------|
| FBtr0072211 | Ca-P60A | 29667 | 0.99694 | 111 | 0.1704406 | 267.2702703 | 8 |
| FBtr0072212 | Ca-P60A | 30267 | 0.9921781 | 114 | 0.1729844 | 265.5 | 8 |
| FBtr0072213 | Ca-P60A | 29377 | 0.9872856 | 111 | 0.1646954 | 264.6576577 | 8 |
| FBtr0072214 | Ca-P60A | 29338 | 0.9905344 | 120 | 0.1822901 | 244.4833333 | 8 |
| FBtr0072216 | Ca-P60A | 32431 | 0.9610364 | 187 | 0.1729982 | 173.4278075 | 8 |
| FBtr0072217 | Ca-P60A | 29509 | 0.9814073 | 122 | 0.1893593 | 241.8770492 | 8 |
| FBtr0072339 | CG4692 | 2522 | 0.9744526 | 16 | 0.2372263 | 157.625 | 2 |
| FBtr0072340 | CG4692 | 2087 | 0.9965096 | 17 | 0.2547993 | 122.7647059 | 1 |
| FBtr0072405 | RpL19 | 3874 | 0.9697352 | 25 | 0.2055486 | 154.96 | 2 |
| FBtr0072406 | RpL19 | 3873 | 0.9960053 | 21 | 0.2050599 | 184.4285714 | 2 |
| FBtr0072436 | GstE12 | 2121 | 0.9875 | 67 | 0.3840909 | 31.65671642 | 2 |
| FBtr0072676 | mtacp1 | 2236 | 0.9716216 | 30 | 0.1594595 | 74.53333333 | 3 |
| FBtr0072677 | mtacp1 | 1924 | 0.9824 | 18 | 0.1408 | 106.8888889 | 3 |
| FBtr0072805 | RpL23A | 4643 | 0.9872047 | 34 | 0.1486221 | 136.5588235 | 2 |
| FBtr0072848 | sls | 33663 | 0.9726844 | 655 | 0.1607494 | 51.39389313 | 13 |
| FBtr0072924 | RpL8 | 3834 | 0.993988 | 18 | 0.1342685 | 213 | 2 |
| FBtr0073040 | Hsp83 | 3694 | 0.9767784 | 112 | 0.237007 | 32.98214286 | 1 |
| FBtr0073097 | RpL28 | 2101 | 0.9798903 | 14 | 0.2522852 | 150.0714286 | 3 |
| FBtr0073113 | CG12079 | 3047 | 0.9979508 | 26 | 0.2858607 | 117.1923077 | 2 |
| FBtr0073151 | Scsalpha | 3048 | 0.9858012 | 44 | 0.2082488 | 69.27272727 | 3 |
| FBtr0073295 | DOR | 5948 | 0.9727391 | 146 | 0.1389009 | 40.73972603 | 4 |
| FBtr0073296 | DOR | 5018 | 0.9737883 | 145 | 0.1716123 | 34.60689655 | 4 |
| FBtr0073421 | sesB | 40973 | 0.998164 | 84 | 0.2331701 | 487.7738095 | 3 |
| FBtr0073423 | sesB | 42688 | 0.9969512 | 86 | 0.2567073 | 496.372093 | 3 |
| FBtr0073439 | CG15201 | 517 | 0.9672801 | 14 | 0.200409 | 36.92857143 | 2 |
| FBtr0073452 | CG11752 | 620 | 0.9697581 | 48 | 0.578629 | 12.91666667 | 0 |
| FBtr0073495 | Gs2 | 5689 | 0.9758375 | 243 | 0.4387699 | 23.41152263 | 4 |
| FBtr0073496 | Gs2 | 5432 | 0.9756772 | 243 | 0.4416805 | 22.35390947 | 4 |
| FBtr0073539 | CG1561 | 3321 | 0.9884259 | 86 | 0.2106481 | 38.61627907 | 1 |
| FBtr0073576 | regucalcin | 1753 | 0.9537205 | 42 | 0.2994555 | 41.73809524 | 2 |
| FBtr0073763 | JafraC1 | 1042 | 0.9657258 | 34 | 0.3487903 | 30.64705882 | 1 |
| FBtr0073792 | RpS15Aa | 3996 | 0.9548105 | 19 | 0.3119534 | 210.3157895 | 2 |
| FBtr0073793 | RpS15Aa | 3956 | 0.983631 | 18 | 0.2946429 | 219.7777778 | 2 |
| FBtr0073794 | RpS15Aa | 3987 | 0.9510204 | 24 | 0.3102041 | 166.125 | 1 |
| FBtr0073795 | RpS15Aa | 3955 | 0.9876733 | 18 | 0.3050847 | 219.7222222 | 1 |
| FBtr0073821 | Yp3 | 30772 | 1 | 43 | 0.2619208 | 715.627907 | 2 |
| FBtr0073851 | up | 12349 | 0.9881735 | 17 | 0.130749 | 726.4117647 | 9 |
| FBtr0073852 | up | 12331 | 0.987984 | 16 | 0.1228304 | 770.6875 | 8 |
| FBtr0073960 | CG5548 | 832 | 0.9897959 | 6 | 0.1292517 | 138.6666667 | 1 |
| FBtr0073979 | CG9512 | 3289 | 0.9733967 | 56 | 0.1857482 | 58.73214286 | 2 |
| FBtr0074112 | Gapdh2 | 8218 | 0.9646569 | 76 | 0.4026334 | 108.1315789 | 1 |
| FBtr0074113 | #N/A | 8214 | 0.9855272 | 86 | 0.4162647 | 95.51162791 | #N/A |
| FBtr0074151 | CG9172 | 2462 | 0.9955899 | 19 | 0.1102536 | 129.5789474 | 1 |
| FBtr0074152 | CG9172 | 2435 | 0.9754738 | 15 | 0.1125975 | 162.3333333 | 0 |
| FBtr0074193 | CG8952 | 775 | 0.9645233 | 17 | 0.1618625 | 45.58823529 | 1 |
| FBtr0074311 | RpS19a | 3023 | 0.9637795 | 32 | 0.1874016 | 94.46875 | 2 |
| FBtr0074312 | RpS19a | 3032 | 0.9649924 | 32 | 0.1811263 | 94.75 | 2 |
| FBtr0074387 | CG5010 | 1985 | 0.9510416 | 25 | 0.2270833 | 79.4 | 1 |
| FBtr0074406 | RpS5a | 3785 | 0.9964913 | 14 | 0.1532164 | 270.3571429 | 4 |
| FBtr0074520 | wupA | 5112 | 0.987931 | 298 | 0.1689655 | 17.15436242 | 7 |
| FBtr0074521 | wupA | 5091 | 0.987931 | 299 | 0.1818966 | 17.02675585 | 7 |
| FBtr0074522 | wupA | 5621 | 0.987931 | 298 | 0.1689655 | 18.86241611 | 7 |
| FBtr0074523 | wupA | 5067 | 0.9672414 | 298 | 0.1689655 | 17.0033557 | 7 |
| FBtr0074524 | wupA | 5990 | 0.9895756 | 300 | 0.1571109 | 19.96666667 | 8 |
| FBtr0074559 | Tsf1 | 4201 | 0.9890329 | 191 | 0.3933201 | 21.9947644 | 4 |
| FBtr0074626 | CG15043 | 3586 | 0.9971057 | 15 | 0.1837916 | 239.0666667 | 1 |
| FBtr0074712 | CG12203 | 1572 | 0.9909209 | 23 | 0.151751 | 68.34782609 | 2 |
| FBtr0074731 | RpS10b | 2616 | 0.974428 | 17 | 0.243607 | 153.8823529 | 2 |
| FBtr0074732 | RpS10b | 2571 | 0.9903846 | 16 | 0.2266483 | 160.6875 | 2 |
| FBtr0074780 | l(1)G0156 | 11205 | 0.9887387 | 114 | 0.545045 | 98.28947368 | 4 |
| FBtr0074815 | CoVib | 4839 | 0.9850948 | 32 | 0.3699187 | 151.21875 | 1 |
| FBtr0074816 | CoVib | 4911 | 0.9970194 | 30 | 0.3457526 | 163.7 | 1 |
| FBtr0074817 | CoVib | 4912 | 0.9971265 | 36 | 0.3548851 | 136.4444444 | 1 |
| FBtr0074910 | fln | 7306 | 0.9908814 | 28 | 0.331307 | 260.9285714 | 3 |
| FBtr0075014 | Gbs-76A | 5241 | 0.9517086 | 75 | 0.1440271 | 69.88 | 2 |
| FBtr0075043 | CG18135 | 3877 | 0.9580958 | 44 | 0.0885015 | 88.11363636 | 1 |
| FBtr0075048 | CG3819 | 2082 | 0.9593679 | 27 | 0.1392024 | 77.11111111 | 1 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|-----------|-------|-----------|-----|-----------|-------------|----|
| FBtr0075058 | Cat | 3558 | 0.9613648 | 98 | 0.2112393 | 36.30612245 | 2 |
| FBtr0075066 | RpL26 | 3148 | 0.9922839 | 22 | 0.2283951 | 143.0909091 | 1 |
| FBtr0075069 | CG6839 | 13708 | 0.9879245 | 24 | 0.0935849 | 571.1666667 | 1 |
| FBtr0075157 | CG5506 | 1027 | 0.9591528 | 30 | 0.1694402 | 34.23333333 | 1 |
| FBtr0075217 | CG7630 | 2175 | 0.9976019 | 19 | 0.1870504 | 114.4736842 | 3 |
| FBtr0075220 | CG7603 | 1744 | 0.9645669 | 19 | 0.2191601 | 91.78947368 | 1 |
| FBtr0075263 | Nc73EF | 17298 | 0.96095 | 131 | 0.1461521 | 132.0458015 | 10 |
| FBtr0075264 | Nc73EF | 16723 | 0.9829637 | 117 | 0.1446728 | 142.9316239 | 10 |
| FBtr0075266 | Nc73EF | 17469 | 0.9668798 | 142 | 0.1617177 | 123.0211268 | 10 |
| FBtr0075267 | Nc73EF | 16574 | 0.9938055 | 116 | 0.1400485 | 142.8793103 | 10 |
| FBtr0075268 | Nc73EF | 16841 | 0.9937602 | 117 | 0.1451438 | 143.9401709 | 10 |
| FBtr0075269 | Nc73EF | 16599 | 0.9933137 | 122 | 0.1473656 | 136.057377 | 10 |
| FBtr0075341 | CG9674 | 5378 | 0.9612254 | 164 | 0.1087761 | 32.79268293 | 14 |
| FBtr0075343 | CG9674 | 2532 | 0.9736414 | 24 | 0.109014 | 105.5 | 5 |
| FBtr0075369 | Nplp3 | 1648 | 1 | 16 | 0.2379182 | 103 | 1 |
| FBtr0075375 | retinin | 2068 | 0.9862155 | 6 | 0.1165414 | 344.6666667 | 1 |
| FBtr0075426 | Pdh | 2852 | 0.9989418 | 16 | 0.1216931 | 178.25 | 2 |
| FBtr0075427 | Pdh | 2822 | 0.9989384 | 16 | 0.1220807 | 176.375 | 2 |
| FBtr0075492 | Pgm | 3408 | 0.9945741 | 167 | 0.4910472 | 20.40718563 | 3 |
| FBtr0075648 | Pdi | 2882 | 0.9807692 | 42 | 0.1591346 | 68.61904762 | 1 |
| FBtr0075718 | Mpcp | 4939 | 0.9894118 | 103 | 0.3088235 | 47.95145631 | 3 |
| FBtr0075719 | Mpcp | 4759 | 0.9641435 | 103 | 0.2988048 | 46.2038835 | 3 |
| FBtr0075839 | Nplp2 | 3977 | 0.9933628 | 7 | 0.1172566 | 568.1428571 | 2 |
| FBtr0075878 | RpS12 | 2682 | 0.9722675 | 23 | 0.1843393 | 116.6086957 | 3 |
| FBtr0075884 | RpS4 | 5093 | 0.9508197 | 22 | 0.1639344 | 231.5 | 5 |
| FBtr0076003 | Est-6 | 2126 | 0.9655374 | 18 | 0.1063084 | 118.1111111 | 1 |
| FBtr0076032 | RpL10Ab | 5586 | 0.984 | 23 | 0.2106667 | 242.8695652 | 2 |
| FBtr0076044 | CG14125 | 7036 | 0.9976162 | 35 | 0.1418355 | 201.0285714 | 1 |
| FBtr0076119 | Muc68D | 57282 | 0.9920866 | 234 | 0.2132445 | 244.7948718 | 1 |
| FBtr0076229 | Sod | 785 | 0.9687075 | 28 | 0.1306122 | 28.03571429 | 1 |
| FBtr0076389 | CG18180 | 8019 | 0.9988901 | 38 | 0.3540511 | 211.0263158 | 0 |
| FBtr0076405 | ATPsyn-b | 9323 | 0.9934641 | 25 | 0.1677756 | 372.92 | 2 |
| FBtr0076423 | RpS9 | 6078 | 0.9904632 | 14 | 0.2411444 | 434.1428571 | 3 |
| FBtr0076425 | RpS9 | 5932 | 0.9957507 | 14 | 0.2507082 | 423.7142857 | 2 |
| FBtr0076479 | RpS17 | 2463 | 0.9738318 | 20 | 0.3046729 | 123.15 | 3 |
| FBtr0076501 | UGP | 4635 | 0.9557564 | 147 | 0.2630828 | 31.53061224 | 8 |
| FBtr0076530 | CG13315 | 3412 | 0.9935588 | 19 | 0.3494364 | 179.5789474 | 0 |
| FBtr0076545 | Argk | 14341 | 0.9993417 | 34 | 0.2139565 | 421.7941176 | 1 |
| FBtr0076593 | Prm | 10554 | 0.9795138 | 96 | 0.1761814 | 109.9375 | 8 |
| FBtr0076594 | Prm | 9653 | 0.983881 | 95 | 0.1949783 | 101.6105263 | 8 |
| FBtr0076595 | Prm | 8759 | 0.9641838 | 92 | 0.1464342 | 95.20652174 | 4 |
| FBtr0076596 | Prm | 7858 | 0.9669118 | 91 | 0.1639706 | 86.35164835 | 4 |
| FBtr0076599 | Arr2 | 4567 | 0.989011 | 63 | 0.1215255 | 72.49206349 | 2 |
| FBtr0076633 | RpL14 | 3617 | 0.9838449 | 12 | 0.1260097 | 301.4166667 | 3 |
| FBtr0076667 | ldh | 4251 | 0.9845297 | 21 | 0.1280941 | 202.4285714 | 1 |
| FBtr0076668 | ldh | 4249 | 0.9708384 | 22 | 0.1348724 | 193.1363636 | 1 |
| FBtr0076808 | CG12262 | 1587 | 0.9589322 | 141 | 0.5509925 | 11.25531915 | 2 |
| FBtr0076892 | RpL18 | 2458 | 0.9903315 | 49 | 0.3812155 | 50.16326531 | 3 |
| FBtr0077038 | yip7 | 8467 | 0.9955752 | 53 | 0.449115 | 159.754717 | 1 |
| FBtr0077040 | Jon65Aiv | 15113 | 0.9856195 | 19 | 0.1570797 | 795.4210526 | 1 |
| FBtr0077041 | Jon65Aiii | 9824 | 0.9977143 | 6 | 0.0857143 | 1637.333333 | 1 |
| FBtr0077131 | Msr-110 | 5422 | 0.961058 | 90 | 0.213446 | 60.24444444 | 6 |
| FBtr0077132 | Msr-110 | 5413 | 0.9606679 | 90 | 0.2155844 | 60.14444444 | 5 |
| FBtr0077144 | CG4769 | 10269 | 0.9953977 | 43 | 0.2629849 | 238.8139535 | 5 |
| FBtr0077304 | Obp19d | 3990 | 0.9968102 | 15 | 0.2982456 | 266 | 3 |
| FBtr0077431 | Tps1 | 4855 | 0.9859589 | 150 | 0.2828767 | 32.36666667 | 4 |
| FBtr0077452 | RpL27A | 3508 | 0.9503425 | 6 | 0.0839041 | 584.6666667 | 3 |
| FBtr0077470 | RpL40 | 3272 | 0.9693356 | 50 | 0.4514481 | 65.44 | 1 |
| FBtr0077520 | Pdsw | 2439 | 0.992163 | 11 | 0.2507837 | 221.7272727 | 3 |
| FBtr0077521 | Pdsw | 2313 | 0.9658915 | 13 | 0.2372093 | 177.9230769 | 2 |
| FBtr0077524 | Thor | 1785 | 0.9735099 | 153 | 0.3536424 | 11.66666667 | 1 |
| FBtr0077550 | CG16704 | 176 | 0.9791667 | 15 | 0.1428571 | 11.73333333 | 1 |
| FBtr0077617 | CG12400 | 1770 | 0.9781659 | 21 | 0.2008734 | 84.28571429 | 2 |
| FBtr0077621 | RpS21 | 2207 | 0.9693094 | 1 | 0.0383632 | 2207 | 3 |
| FBtr0077659 | CG3523 | 16629 | 0.9845743 | 336 | 0.1816284 | 49.49107143 | 5 |
| FBtr0077739 | Pgk | 6363 | 0.9979811 | 60 | 0.2469717 | 106.05 | 2 |
| FBtr0077828 | GlyP | 14988 | 0.9977735 | 126 | 0.2490458 | 118.952381 | 3 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|-----------------|-------|-----------|-----|-----------|-------------|----|
| FBtr0077867 | Got2 | 2065 | 0.9542857 | 53 | 0.2101587 | 38.96226415 | 4 |
| FBtr0077909 | Eno | 18551 | 0.995439 | 80 | 0.2035348 | 231.8875 | 1 |
| FBtr0077915 | RFeSP | 3922 | 0.9882491 | 20 | 0.226792 | 196.1 | 2 |
| FBtr0078056 | RpLP1 | 3120 | 0.9950083 | 27 | 0.3760399 | 115.5555556 | 1 |
| FBtr0078118 | CG11455 | 934 | 0.976 | 86 | 0.592 | 10.86046512 | 1 |
| FBtr0078154 | CG3164 | 2968 | 0.9521238 | 55 | 0.1691865 | 53.96363636 | 7 |
| FBtr0078382 | CoVIII | 1608 | 0.997537 | 3 | 0.0369458 | 536 | 1 |
| FBtr0078477 | CG7470 | 3669 | 0.9745467 | 74 | 0.1666667 | 49.58108108 | 11 |
| FBtr0078481 | RpLP0 | 5509 | 0.9925681 | 77 | 0.1907514 | 71.54545455 | 2 |
| FBtr0078643 | CG1213 | 1460 | 0.9591141 | 51 | 0.1607042 | 28.62745098 | 3 |
| FBtr0078650 | Rm62 | 2195 | 0.95321 | 170 | 0.20856 | 12.91176471 | 5 |
| FBtr0078655 | Obp83b | 204 | 0.954717 | 18 | 0.154717 | 11.33333333 | 2 |
| FBtr0078662 | CG2017 | 1791 | 0.9553528 | 63 | 0.2568411 | 28.42857143 | 3 |
| FBtr0078700 | Vha26 | 3261 | 0.972363 | 63 | 0.2851221 | 51.76190476 | 4 |
| FBtr0078701 | Vha26 | 3263 | 0.9616571 | 64 | 0.2794183 | 50.984375 | 3 |
| FBtr0078705 | RpL13A | 4157 | 0.9750692 | 18 | 0.131579 | 230.9444444 | 2 |
| FBtr0078745 | exba | 2446 | 0.9708798 | 32 | 0.185254 | 76.4375 | 7 |
| FBtr0078769 | RpL35A | 2161 | 0.9565217 | 12 | 0.1723027 | 180.0833333 | 4 |
| FBtr0078822 | CG12163 | 1695 | 0.9585547 | 37 | 0.1859724 | 45.81081081 | 4 |
| FBtr0078908 | CG14645 | 3367 | 1 | 16 | 0.2518337 | 210.4375 | 0 |
| FBtr0078968 | Gel | 2959 | 0.954177 | 50 | 0.1240747 | 59.18 | 7 |
| FBtr0078973 | Gel | 2908 | 0.9540598 | 50 | 0.1253561 | 58.16 | 7 |
| FBtr0079001 | Cg25C | 3926 | 0.9625555 | 145 | 0.1167702 | 27.07586207 | 8 |
| FBtr0079003 | Cg25C | 4050 | 0.9541667 | 153 | 0.1205 | 26.47058824 | 8 |
| FBtr0079016 | RpL37A | 2420 | 0.9706458 | 22 | 0.2896282 | 110 | 3 |
| FBtr0079025 | CG8680 | 612 | 0.9779412 | 13 | 0.2224265 | 47.07692308 | 2 |
| FBtr0079031 | elF-3p40 | 1135 | 0.9729272 | 60 | 0.4373943 | 18.91666667 | 2 |
| FBtr0079053 | Trip1 | 843 | 0.9660153 | 37 | 0.2166525 | 22.78378378 | 2 |
| FBtr0079055 | Jon25Biii | 4541 | 0.9920815 | 10 | 0.1131222 | 454.1 | 0 |
| FBtr0079056 | Jon25Biii | 3748 | 0.9975816 | 27 | 0.2756953 | 138.8148148 | 0 |
| FBtr0079072 | cype | 2504 | 0.9947644 | 7 | 0.2486911 | 357.7142857 | 2 |
| FBtr0079147 | Gpdh | 13269 | 0.9963213 | 40 | 0.202943 | 331.725 | 6 |
| FBtr0079175 | elF-4a | 4855 | 0.9668835 | 45 | 0.1738616 | 107.8888889 | 4 |
| FBtr0079176 | elF-4a | 4835 | 0.9819704 | 44 | 0.1790084 | 109.8863636 | 5 |
| FBtr0079177 | elF-4a | 5106 | 0.9593936 | 52 | 0.1781267 | 98.19230769 | 4 |
| FBtr0079178 | elF-4a | 5087 | 0.9710816 | 51 | 0.1810295 | 99.74509804 | 5 |
| FBtr0079216 | CG9140 | 7771 | 0.9843457 | 74 | 0.208516 | 105.0135135 | 3 |
| FBtr0079217 | slmo | 2959 | 0.9681677 | 32 | 0.2088509 | 92.46875 | 0 |
| FBtr0079218 | slmo | 2919 | 0.9923011 | 30 | 0.2044483 | 97.3 | 1 |
| FBtr0079288 | CoVb | 3464 | 0.9561043 | 13 | 0.223594 | 266.4615385 | 2 |
| FBtr0079445 | CG5261 | 9196 | 0.9503836 | 260 | 0.513555 | 35.36923077 | 7 |
| FBtr0079500 | Acp1 | 2812 | 0.9587629 | 13 | 0.1890034 | 216.3076923 | 1 |
| FBtr0079546 | RpL36A | 1703 | 0.9737418 | 17 | 0.2997812 | 100.1764706 | 2 |
| FBtr0079565 | Rack1 | 7357 | 0.9891501 | 25 | 0.198915 | 294.28 | 2 |
| FBtr0079701 | Bace | 9452 | 0.9975 | 40 | 0.2025 | 236.3 | 0 |
| FBtr0079713 | Peritrophin-15a | 430 | 0.9556786 | 7 | 0.1468144 | 61.42857143 | 1 |
| FBtr0079724 | RpS13 | 3204 | 0.974359 | 45 | 0.0705128 | 71.2 | 2 |
| FBtr0079796 | Ggamma30A | 987 | 0.9793916 | 21 | 0.1511286 | 47 | 2 |
| FBtr0079888 | RpL13 | 1580 | 0.969697 | 21 | 0.1414141 | 75.23809524 | 3 |
| FBtr0079905 | RpS2 | 3156 | 0.9781659 | 30 | 0.1877729 | 105.2 | 1 |
| FBtr0079946 | RpL7 | 3968 | 0.9776286 | 40 | 0.3344519 | 99.2 | 2 |
| FBtr0080016 | RpS27A | 4308 | 0.9932432 | 13 | 0.2179054 | 331.3846154 | 2 |
| FBtr0080050 | Mdh1 | 2248 | 0.9732528 | 38 | 0.2847282 | 59.15789474 | 1 |
| FBtr0080127 | CG17108 | 3194 | 0.9871134 | 52 | 0.1176976 | 61.42307692 | 0 |
| FBtr0080167 | I(2)06225 | 4007 | 1 | 54 | 0.2231237 | 74.2037037 | 1 |
| FBtr0080182 | porin | 5235 | 0.9881495 | 84 | 0.1203282 | 62.32142857 | 3 |
| FBtr0080242 | CG31705 | 1877 | 0.9596808 | 76 | 0.1768165 | 24.69736842 | 2 |
| FBtr0080306 | CG6770 | 3798 | 1 | 352 | 0.4384106 | 10.78977273 | 0 |
| FBtr0080418 | Vha68-2 | 5312 | 0.9786806 | 54 | 0.1604988 | 98.37037037 | 4 |
| FBtr0080419 | Vha68-2 | 5114 | 0.9768669 | 51 | 0.1558442 | 100.2745098 | 4 |
| FBtr0080524 | RpL24 | 2929 | 0.9926199 | 10 | 0.1156212 | 292.9 | 2 |
| FBtr0080542 | b | 1469 | 0.9545903 | 35 | 0.171767 | 41.97142857 | 2 |
| FBtr0080603 | CG15293 | 1436 | 0.9656616 | 36 | 0.1432161 | 39.88888889 | 1 |
| FBtr0080771 | I(2)35Di | 1837 | 0.9766764 | 3 | 0.0218659 | 612.3333333 | 2 |
| FBtr0080889 | Cyt-c-p | 7633 | 0.9632893 | 24 | 0.2980911 | 318.0416667 | 1 |
| FBtr0080895 | Mhc | 88213 | 0.9823036 | 177 | 0.1843131 | 498.3785311 | 17 |
| FBtr0080896 | Mhc | 89458 | 0.9896521 | 181 | 0.1889622 | 494.2430939 | 17 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|-------------|-------|-----------|-----|-----------|-------------|----|
| FBtr0080897 | Mhc | 91781 | 0.9977505 | 175 | 0.1894121 | 524.4628571 | 17 |
| FBtr0080898 | Mhc | 88274 | 0.9865027 | 179 | 0.1888122 | 493.150838 | 17 |
| FBtr0080899 | Mhc | 87217 | 0.9815537 | 179 | 0.1865627 | 487.2458101 | 17 |
| FBtr0080900 | Mhc | 89519 | 0.9938512 | 183 | 0.1934613 | 489.1748634 | 17 |
| FBtr0080901 | Mhc | 89409 | 0.9890522 | 181 | 0.1889622 | 493.9723757 | 17 |
| FBtr0080902 | Mhc | 88462 | 0.9889022 | 183 | 0.1912118 | 483.3989071 | 17 |
| FBtr0080903 | Mhc | 88523 | 0.9931014 | 185 | 0.1957109 | 478.5027027 | 17 |
| FBtr0080905 | Mhc | 95833 | 0.9974906 | 194 | 0.193364 | 493.9845361 | 18 |
| FBtr0080906 | Mhc | 96643 | 0.9979079 | 202 | 0.1910739 | 478.4306931 | 18 |
| FBtr0080907 | Mhc | 94532 | 0.9979079 | 194 | 0.1910739 | 487.2783505 | 18 |
| FBtr0081030 | Arr1 | 2742 | 0.9902439 | 34 | 0.138676 | 80.64705882 | 3 |
| FBtr0081089 | RpS26 | 4286 | 0.959375 | 15 | 0.1640625 | 285.7333333 | 1 |
| FBtr0081090 | RpS26 | 4218 | 0.9595646 | 15 | 0.163297 | 281.2 | 1 |
| FBtr0081122 | CG10570 | 1477 | 0.9620563 | 24 | 0.2031824 | 61.54166667 | 1 |
| FBtr0081253 | ref(2)P | 2178 | 0.9615225 | 107 | 0.3582954 | 20.35514019 | 2 |
| FBtr0081300 | ColV | 7858 | 0.9890561 | 10 | 0.1450068 | 785.8 | 2 |
| FBtr0081301 | ColV | 7739 | 0.9874372 | 10 | 0.1331658 | 773.9 | 2 |
| FBtr0081473 | Acon | 35918 | 0.9960245 | 75 | 0.1420311 | 478.9066667 | 3 |
| FBtr0081639 | alphaTub84B | 3898 | 0.9942923 | 105 | 0.239726 | 37.12380952 | 1 |
| FBtr0081855 | CG9603 | 1026 | 0.9693053 | 18 | 0.1550889 | 57 | 2 |
| FBtr0081882 | CG8036 | 2331 | 0.957047 | 59 | 0.1583893 | 39.50847458 | 2 |
| FBtr0081920 | CG8369 | 734 | 1 | 24 | 0.4524362 | 30.58333333 | 2 |
| FBtr0081930 | CG9836 | 2636 | 0.9675393 | 79 | 0.5078534 | 33.36708861 | 2 |
| FBtr0081978 | VhaM8.9 | 1612 | 0.9856948 | 55 | 0.1968665 | 29.30909091 | 2 |
| FBtr0082103 | Crc | 1515 | 0.9853147 | 55 | 0.3370629 | 27.54545455 | 3 |
| FBtr0082136 | RpS29 | 2566 | 0.9820717 | 7 | 0.2131474 | 366.5714286 | 2 |
| FBtr0082158 | MtnA | 1956 | 0.9817629 | 11 | 0.3434651 | 177.8181818 | 1 |
| FBtr0082344 | Tctp | 3538 | 0.9893823 | 127 | 0.5463321 | 27.85826772 | 0 |
| FBtr0082346 | RpL3 | 7376 | 0.9985518 | 96 | 0.2273715 | 76.83333333 | 5 |
| FBtr0082358 | CG5214 | 6478 | 0.9825803 | 55 | 0.1431682 | 117.7818182 | 6 |
| FBtr0082370 | RpS25 | 3064 | 0.9864078 | 4 | 0.1417476 | 766 | 2 |
| FBtr0082372 | SdhC | 1814 | 0.9756098 | 37 | 0.3597561 | 49.02702703 | 1 |
| FBtr0082474 | CoVa | 6551 | 0.9714693 | 16 | 0.2524964 | 409.4375 | 0 |
| FBtr0082534 | Lk6 | 8956 | 0.9625113 | 138 | 0.1458898 | 64.89855072 | 5 |
| FBtr0082535 | Lk6 | 7368 | 0.9640613 | 137 | 0.1437547 | 53.7810219 | 5 |
| FBtr0082598 | Cyp9f2 | 2759 | 0.9664391 | 24 | 0.1353811 | 114.9583333 | 3 |
| FBtr0082607 | GstD1 | 1179 | 0.9805353 | 63 | 0.3990268 | 18.71428571 | 1 |
| FBtr0082626 | desat1 | 5441 | 0.9789636 | 56 | 0.1682914 | 97.16071429 | 4 |
| FBtr0082627 | desat1 | 5924 | 0.9782071 | 62 | 0.1817732 | 95.5483871 | 4 |
| FBtr0082628 | desat1 | 5436 | 0.9596115 | 55 | 0.1610429 | 98.83636364 | 4 |
| FBtr0082629 | desat1 | 5436 | 0.9539939 | 57 | 0.1673408 | 95.36842105 | 4 |
| FBtr0082630 | desat1 | 5454 | 0.9839711 | 55 | 0.1628749 | 99.16363636 | 4 |
| FBtr0082670 | Vha55 | 6063 | 0.9628949 | 107 | 0.1667891 | 56.6635514 | 3 |
| FBtr0082671 | Vha55 | 5863 | 0.9714071 | 108 | 0.1745673 | 54.28703704 | 2 |
| FBtr0082931 | CG3321 | 2347 | 0.9981584 | 47 | 0.3333333 | 49.93617021 | 1 |
| FBtr0082962 | His4r | 1416 | 0.9618056 | 11 | 0.1475694 | 128.7272727 | 2 |
| FBtr0083030 | Mf | 6715 | 0.9901599 | 17 | 0.196802 | 395 | 5 |
| FBtr0083032 | Mf | 4618 | 0.9550971 | 34 | 0.3009709 | 135.8235294 | 4 |
| FBtr0083035 | Gly5 | 5721 | 0.977885 | 57 | 0.2054684 | 100.3684211 | 4 |
| FBtr0083037 | Gly5 | 5577 | 0.9705882 | 58 | 0.2023994 | 96.15517241 | 4 |
| FBtr0083055 | Hsc70-4 | 6370 | 0.9798599 | 111 | 0.2535026 | 57.38738739 | 1 |
| FBtr0083056 | Hsc70-4 | 6381 | 0.9826198 | 111 | 0.245443 | 57.48648649 | 0 |
| FBtr0083057 | Hsc70-4 | 7552 | 0.9923986 | 114 | 0.2592905 | 66.24561404 | 1 |
| FBtr0083058 | Hsc70-4 | 6370 | 0.9815789 | 111 | 0.2539474 | 57.38738739 | 1 |
| FBtr0083059 | Hsc70-4 | 6376 | 0.952381 | 118 | 0.2589286 | 54.03389831 | 1 |
| FBtr0083060 | Hsc70-4 | 6373 | 0.9781958 | 111 | 0.2475417 | 57.41441441 | 1 |
| FBtr0083063 | Oscp | 9690 | 0.9987531 | 12 | 0.1658354 | 807.5 | 2 |
| FBtr0083078 | Tm2 | 5949 | 0.978013 | 41 | 0.1807818 | 145.097561 | 3 |
| FBtr0083143 | Act88F | 23367 | 0.9981191 | 28 | 0.1510972 | 834.5357143 | 2 |
| FBtr0083154 | CG18522 | 5049 | 0.9694891 | 147 | 0.1289026 | 34.34693878 | 5 |
| FBtr0083164 | CG5399 | 3018 | 0.9514964 | 18 | 0.1960784 | 167.6666667 | 2 |
| FBtr0083191 | ND23 | 2242 | 0.995116 | 23 | 0.2918193 | 97.47826087 | 2 |
| FBtr0083563 | Mdh2 | 9976 | 0.988764 | 59 | 0.3116105 | 169.0847458 | 3 |
| FBtr0083712 | NP15.6 | 2517 | 0.9909639 | 80 | 0.561747 | 31.4625 | 0 |
| FBtr0083727 | ATPsyn-d | 3960 | 0.9717115 | 40 | 0.2446959 | 99 | 1 |
| FBtr0083728 | ATPsyn-d | 4816 | 0.9919137 | 39 | 0.212938 | 123.4871795 | 1 |
| FBtr0083804 | Vha13 | 1290 | 0.9743955 | 124 | 0.1337127 | 10.40322581 | 2 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|---------------|-------|-----------|-----|-----------|-------------|------|
| FBtr0083857 | ninaE | 8376 | 0.9874765 | 42 | 0.1540388 | 199.4285714 | 4 |
| FBtr0083890 | MtnB | 466 | 0.978125 | 2 | 0.1 | 233 | 1 |
| FBtr0083935 | CG4000 | 1583 | 0.989 | 154 | 0.685 | 10.27922078 | 2 |
| FBtr0083964 | RpS20 | 2783 | 0.964539 | 9 | 0.212766 | 309.2222222 | 3 |
| FBtr0083969 | RpS30 | 2894 | 0.9923518 | 10 | 0.1357553 | 289.4 | 2 |
| FBtr0083970 | RpS30 | 2865 | 0.9881657 | 10 | 0.1400394 | 286.5 | 1 |
| FBtr0083991 | CG17273 | 1438 | 0.9523321 | 63 | 0.2004101 | 22.82539683 | 4 |
| FBtr0084153 | fit | 1220 | 0.9585688 | 13 | 0.2278719 | 93.84615385 | 0 |
| FBtr0084172 | ND42 | 3917 | 0.9905729 | 32 | 0.173314 | 122.40625 | 2 |
| FBtr0084190 | CG6439 | 7361 | 0.9903278 | 47 | 0.2294465 | 156.6170213 | 5 |
| FBtr0084213 | PyK | 15855 | 0.9878665 | 57 | 0.1501517 | 278.1578947 | 3 |
| FBtr0084214 | PyK | 16668 | 0.995396 | 37 | 0.160221 | 450.4864865 | 3 |
| FBtr0084255 | Pebl1 | 1468 | 0.9643963 | 24 | 0.3854489 | 61.16666667 | 0 |
| FBtr0084410 | RpS3 | 4118 | 0.9634956 | 21 | 0.2488938 | 196.0952381 | 1 |
| FBtr0084432 | ATPsyn-Cf6 | 3142 | 0.9923955 | 12 | 0.256654 | 261.8333333 | 2 |
| FBtr0084466 | CG10219 | 2083 | 0.9709172 | 48 | 0.3948546 | 43.39583333 | 3 |
| FBtr0084847 | tobi | 1990 | 0.9724334 | 44 | 0.1074144 | 45.22727273 | 1 |
| FBtr0084879 | CG5107 | 1540 | 0.9590288 | 17 | 0.1487102 | 90.58823529 | 1 |
| FBtr0084892 | Npl4 | 8261 | 0.9800724 | 37 | 0.1644022 | 223.2702703 | 7 |
| FBtr0084893 | Npl4 | 8401 | 0.9932261 | 99 | 0.1943268 | 84.85858586 | 7 |
| FBtr0084901 | CG5028 | 6088 | 0.9808102 | 38 | 0.1911869 | 160.2105263 | 5 |
| FBtr0084932 | RpL27 | 3501 | 0.9944134 | 34 | 0.4283054 | 102.9705882 | 1 |
| FBtr0084994 | Ald | 27217 | 0.9946889 | 98 | 0.2207891 | 277.7244898 | 3 |
| FBtr0084995 | Ald | 18622 | 0.9984301 | 18 | 0.1562009 | 1034.555556 | 3 |
| FBtr0085001 | Ald | 15594 | 0.9535947 | 46 | 0.2013072 | 339 | 3 |
| FBtr0085077 | CG6295 | 8517 | 1 | 44 | 0.3098075 | 193.5681818 | 1 |
| FBtr0085094 | BM-40-SPARC | 1356 | 0.9702537 | 103 | 0.2992126 | 13.16504854 | 2 |
| FBtr0085153 | CG17192 | 3699 | 0.9838403 | 16 | 0.1568441 | 231.1875 | 1 |
| FBtr0085195 | Mlc1 | 5957 | 0.9920635 | 27 | 0.2414966 | 220.6296296 | 4 |
| FBtr0085196 | Mlc1 | 5999 | 0.9935414 | 27 | 0.2292788 | 222.1851852 | 5 |
| FBtr0085248 | RpL4 | 5149 | 0.9914286 | 42 | 0.1385714 | 122.5952381 | 3 |
| FBtr0085366 | CG11876 | 6207 | 0.9725086 | 37 | 0.1250859 | 167.7567568 | 5 |
| FBtr0085369 | CG11876 | 6060 | 0.994228 | 34 | 0.1183261 | 178.2352941 | 4 |
| FBtr0085384 | Pglym78 | 4101 | 0.971831 | 56 | 0.3961267 | 73.23214286 | 2 |
| FBtr0085392 | Ef1gamma | 4000 | 0.9804849 | 127 | 0.3264341 | 31.49606299 | 3 |
| FBtr0085393 | Ef1gamma | 3992 | 0.9778684 | 126 | 0.3127548 | 31.68253968 | 2 |
| FBtr0085463 | Obp99c | 1411 | 0.9982609 | 24 | 0.4295652 | 58.79166667 | 1 |
| FBtr0085502 | Jon99Cii | 9600 | 0.995221 | 47 | 0.3990442 | 204.2553191 | 0 |
| FBtr0085511 | Jon99Ci | 1969 | 0.9735391 | 14 | 0.1532525 | 140.6428571 | 1 |
| FBtr0085512 | Jon99Ci | 9934 | 0.9883991 | 47 | 0.387471 | 211.3617021 | 0 |
| FBtr0085535 | CG7834 | 1112 | 0.9671814 | 30 | 0.2905405 | 37.06666667 | 2 |
| FBtr0085536 | CG7834 | 1090 | 0.9929221 | 27 | 0.2780586 | 40.37037037 | 1 |
| FBtr0085539 | ATPsyn-gamma | 8442 | 0.9935185 | 24 | 0.1935185 | 351.75 | 1 |
| FBtr0085541 | ATPsyn-gamma | 9289 | 0.9939966 | 26 | 0.1921098 | 357.2692308 | 0 |
| FBtr0085583 | Tpi | 3056 | 0.9820144 | 38 | 0.1906475 | 80.42105263 | 2 |
| FBtr0085592 | RpL32 | 2588 | 0.9961832 | 8 | 0.1908397 | 323.5 | 2 |
| FBtr0085603 | CG7920 | 7356 | 0.9513089 | 40 | 0.2643979 | 183.9 | 3 |
| FBtr0085632 | Fer1HCH | 5983 | 0.9920705 | 33 | 0.2986784 | 181.3030303 | 3 |
| FBtr0085633 | Fer1HCH | 6337 | 0.9926651 | 37 | 0.2974735 | 171.2702703 | 3 |
| FBtr0085634 | Fer1HCH | 6331 | 0.9924179 | 33 | 0.2855939 | 191.8484848 | 3 |
| FBtr0085635 | Fer1HCH | 5493 | 0.9607351 | 40 | 0.3116124 | 137.325 | 2 |
| FBtr0085713 | Sap-r | 7726 | 0.9927326 | 72 | 0.1241279 | 107.3055556 | 6 |
| FBtr0085714 | Sap-r | 7377 | 0.9725291 | 58 | 0.1125709 | 127.1896552 | 5 |
| FBtr0085745 | I(3)O3670 | 1773 | 0.9851064 | 47 | 0.1361702 | 37.72340426 | 2 |
| FBtr0085772 | CG1746 | 32177 | 0.9728978 | 36 | 0.2154274 | 893.8055556 | 3 |
| FBtr0085774 | CG1746 | 31139 | 0.9977728 | 36 | 0.2301411 | 864.9722222 | 2 |
| FBtr0085800 | CycG | 4916 | 0.9869281 | 46 | 0.1877996 | 106.8695652 | 5 |
| FBtr0085803 | CycG | 5110 | 0.9549945 | 54 | 0.1822173 | 94.62962963 | 5 |
| FBtr0085804 | RpL6 | 4310 | 0.9846154 | 28 | 0.132967 | 153.9285714 | 2 |
| FBtr0085805 | RpL6 | 5175 | 0.9865564 | 29 | 0.1416753 | 178.4482759 | 1 |
| FBtr0085864 | #N/A | 2842 | 0.9716088 | 53 | 0.3911672 | 53.62264151 | #N/A |
| FBtr0085892 | His1:CG31617 | 2467 | 0.9933185 | 133 | 0.6636971 | 18.54887218 | 0 |
| FBtr0085893 | His2A:CG31618 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0085896 | Lamp1 | 1871 | 0.9545168 | 40 | 0.1718256 | 46.775 | 3 |
| FBtr0085911 | Ef2b | 18697 | 0.9920606 | 100 | 0.1613136 | 186.97 | 4 |
| FBtr0085961 | RpL21 | 3681 | 0.9723127 | 13 | 0.237785 | 283.1538462 | 1 |
| FBtr0086150 | Vha16-1 | 14292 | 0.9911754 | 128 | 0.2499118 | 111.65625 | 3 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|-------------|-------|-----------|-----|-----------|-------------|------|
| FPtr0086151 | Vha16-1 | 14366 | 0.9907076 | 117 | 0.2323088 | 122.7863248 | 3 |
| FPtr0086152 | Vha16-1 | 14392 | 0.9661188 | 122 | 0.2358269 | 117.9672131 | 2 |
| FPtr0086153 | Vha16-1 | 14367 | 0.9861506 | 117 | 0.2308239 | 122.7948718 | 3 |
| FPtr0086156 | SdhB | 3794 | 0.9835931 | 159 | 0.3281378 | 23.86163522 | 2 |
| FPtr0086216 | CG18067 | 1515 | 0.9913043 | 9 | 0.1428571 | 168.3333333 | 1 |
| FPtr0086273 | RpS18 | 2644 | 0.9771615 | 11 | 0.2120718 | 240.3636364 | 3 |
| FPtr0086303 | CG9090 | 9676 | 0.9928161 | 64 | 0.2104885 | 151.1875 | 3 |
| FPtr0086477 | Obp56d | 658 | 0.9630873 | 10 | 0.135906 | 65.8 | 1 |
| FPtr0086533 | RpL11 | 4012 | 0.9798271 | 16 | 0.2463977 | 250.75 | 4 |
| FPtr0086536 | betaTub56D | 1692 | 0.9795412 | 33 | 0.1680099 | 51.27272727 | 1 |
| FPtr0086552 | SdhA | 12473 | 0.9805195 | 158 | 0.2456073 | 78.94303797 | 4 |
| FPtr0086553 | SdhA | 12548 | 0.973849 | 162 | 0.2526703 | 77.45679012 | 3 |
| FPtr0086554 | SdhA | 12471 | 0.9832317 | 156 | 0.238186 | 79.94230769 | 4 |
| FPtr0086585 | CG10737 | 3732 | 0.9538513 | 83 | 0.1503984 | 44.96385542 | 14 |
| FPtr0086701 | Pepck | 4137 | 0.9574126 | 49 | 0.1361993 | 84.42857143 | 1 |
| FPtr0086727 | CG15068 | 428 | 0.973545 | 9 | 0.2380952 | 47.55555556 | 1 |
| FPtr0086903 | CG6484 | 1867 | 0.9703844 | 54 | 0.2173913 | 34.57407407 | 1 |
| FPtr0086905 | CG14482 | 974 | 1 | 34 | 0.5910653 | 28.64705882 | 1 |
| FPtr0086985 | CG11400 | 1176 | 1 | 9 | 0.1364942 | 130.6666667 | 0 |
| FPtr0087004 | Amy-p | 34555 | 0.9993742 | 131 | 0.3667084 | 263.778626 | 0 |
| FPtr0087005 | Gst51 | 8792 | 0.9935806 | 65 | 0.2232525 | 135.2615385 | 4 |
| FPtr0087006 | Gst51 | 9478 | 0.9920635 | 87 | 0.2261905 | 108.9425287 | 4 |
| FPtr0087105 | RpLP2 | 2738 | 0.9732888 | 22 | 0.3722872 | 124.4545455 | 0 |
| FPtr0087307 | Gpo-1 | 9572 | 0.9871795 | 64 | 0.188172 | 149.5625 | 7 |
| FPtr0087308 | Gpo-1 | 10454 | 0.9895708 | 67 | 0.1945447 | 156.0298507 | 7 |
| FPtr0087309 | Gpo-1 | 10178 | 0.9894934 | 97 | 0.2213884 | 104.9278351 | 7 |
| FPtr0087335 | Vha36-1 | 1569 | 0.9713494 | 75 | 0.2939002 | 20.92 | 0 |
| FPtr0087440 | CG12859 | 757 | 0.9790795 | 17 | 0.3535565 | 44.52941176 | 1 |
| FPtr0087494 | CG30197 | 442 | 0.98125 | 9 | 0.2020833 | 49.11111111 | 3 |
| FPtr0087560 | Arc1 | 4322 | 0.9940878 | 137 | 0.1870777 | 31.54744526 | 0 |
| FPtr0087591 | #N/A | 3885 | 0.9725752 | 39 | 0.2160535 | 99.61538462 | #N/A |
| FPtr0087592 | Cp1 | 3970 | 0.9793609 | 39 | 0.2150466 | 101.7948718 | 3 |
| FPtr0087654 | IM10 | 2277 | 0.9637362 | 170 | 0.7208791 | 13.39411765 | 3 |
| FPtr0087656 | CG33470 | 2548 | 0.9663503 | 224 | 0.7385677 | 11.375 | 3 |
| FPtr0087732 | AGBE | 2721 | 0.9741379 | 143 | 0.2228448 | 19.02797203 | 3 |
| FPtr0087746 | CG4716 | 1325 | 0.9795918 | 25 | 0.3437991 | 53 | 1 |
| FPtr0087747 | CG4716 | 1607 | 0.9768519 | 30 | 0.3217593 | 53.56666667 | 0 |
| FPtr0087783 | bic | 1235 | 0.9837728 | 57 | 0.1673428 | 21.66666667 | 0 |
| FPtr0087796 | CG13324 | 2464 | 0.9958071 | 13 | 0.197065 | 189.5384615 | 0 |
| FPtr0087854 | CG12374 | 28681 | 0.9986348 | 33 | 0.2006826 | 869.1212121 | 4 |
| FPtr0087861 | ox | 2459 | 0.9749216 | 96 | 0.2664577 | 25.61458333 | 1 |
| FPtr0088013 | Oda | 8409 | 0.9626007 | 115 | 0.3124281 | 73.12173913 | 2 |
| FPtr0088014 | Oda | 8495 | 0.9710445 | 124 | 0.3293692 | 68.50806452 | 2 |
| FPtr0088035 | Ef1alpha48D | 16194 | 0.9980286 | 43 | 0.1971415 | 376.6046512 | 1 |
| FPtr0088122 | betaTry | 14113 | 1 | 34 | 0.2506234 | 415.0882353 | 0 |
| FPtr0088123 | CG30031 | 7960 | 0.9864365 | 28 | 0.2552404 | 284.2857143 | 0 |
| FPtr0088124 | deltaTry | 7948 | 0.9975309 | 28 | 0.2555556 | 283.8571429 | 0 |
| FPtr0088158 | CG30025 | 8061 | 1 | 28 | 0.2555556 | 287.8928571 | 0 |
| FPtr0088159 | gammaTry | 7960 | 0.9876543 | 28 | 0.2555556 | 284.2857143 | 0 |
| FPtr0088160 | epsilonTry | 4628 | 0.9903615 | 100 | 0.5795181 | 46.28 | 0 |
| FPtr0088161 | alphaTry | 15415 | 0.9872093 | 57 | 0.3476744 | 270.4385965 | 0 |
| FPtr0088397 | CoVilc | 2544 | 0.9928741 | 16 | 0.1995249 | 159 | 2 |
| FPtr0088413 | 14-3-3zeta | 2981 | 0.9566457 | 18 | 0.1158015 | 165.6111111 | 6 |
| FPtr0088421 | Pfk | 6439 | 0.9611042 | 34 | 0.1282936 | 189.3823529 | 7 |
| FPtr0088422 | Pfk | 7143 | 0.9723489 | 42 | 0.1342645 | 170.0714286 | 7 |
| FPtr0088525 | RpL31 | 2324 | 0.96139 | 19 | 0.2316602 | 122.3157895 | 2 |
| FPtr0088527 | RpL31 | 2133 | 0.9632653 | 16 | 0.2142857 | 133.3125 | 2 |
| FPtr0088587 | VhaAC45 | 2521 | 0.9597574 | 27 | 0.1576626 | 93.37037037 | 4 |
| FPtr0088679 | Pgi | 10047 | 0.9562624 | 102 | 0.245328 | 98.5 | 4 |
| FPtr0088709 | PGRP-SC2 | 602 | 0.9513513 | 19 | 0.3873874 | 31.68421053 | 0 |
| FPtr0088759 | Mal-A1 | 5869 | 0.9956141 | 61 | 0.2001096 | 96.21311475 | 2 |
| FPtr0088816 | Obp44a | 1180 | 0.9984472 | 75 | 0.5854037 | 15.73333333 | 1 |
| FPtr0088872 | ACC | 7852 | 0.9529412 | 320 | 0.2840154 | 24.5375 | 12 |
| FPtr0088898 | cathD | 1569 | 0.9577364 | 49 | 0.3517192 | 32.02040816 | 1 |
| FPtr0089055 | Cyp9b2 | 1256 | 0.95021 | 35 | 0.1391722 | 35.88571429 | 3 |
| FPtr0089105 | CG1970 | 4745 | 0.9802924 | 38 | 0.2403052 | 124.8684211 | 5 |
| FPtr0089175 | Rp53A | 6772 | 0.9934066 | 22 | 0.2021978 | 307.8181818 | 1 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|---------------|-------|-----------|-----|-----------|-------------|------|
| FBtr0089176 | Rp53A | 6196 | 0.9929742 | 23 | 0.234192 | 269.3913043 | 2 |
| FBtr0089186 | ATPsyn-beta | 32325 | 0.9976304 | 42 | 0.2233412 | 769.6428571 | 2 |
| FBtr0089187 | ATPsyn-beta | 32131 | 0.9827685 | 45 | 0.2257323 | 714.0222222 | 1 |
| FBtr0089188 | Rfabg | 51078 | 0.9973753 | 426 | 0.2149315 | 119.9014085 | 7 |
| FBtr0089324 | Lsp2 | 5761 | 0.9973958 | 76 | 0.2829861 | 75.80263158 | 0 |
| FBtr0089329 | Inos | 2042 | 0.9738167 | 28 | 0.1540785 | 72.92857143 | 4 |
| FBtr0089422 | Rp57 | 4606 | 0.9835841 | 19 | 0.24487 | 242.4210526 | 3 |
| FBtr0089497 | Gdh | 5822 | 0.9833024 | 238 | 0.3167903 | 24.46218487 | 4 |
| FBtr0089498 | Gdh | 5855 | 0.9835991 | 241 | 0.3248292 | 24.29460581 | 5 |
| FBtr0089510 | Atpalpha | 12121 | 0.9666115 | 113 | 0.200883 | 107.2654867 | 9 |
| FBtr0089511 | Atpalpha | 13940 | 0.9541762 | 142 | 0.1972506 | 98.16901408 | 8 |
| FBtr0089516 | Atpalpha | 13827 | 0.9508436 | 144 | 0.1980837 | 96.02083333 | 8 |
| FBtr0089517 | AnxB9 | 1409 | 0.9664537 | 56 | 0.2036741 | 25.16071429 | 4 |
| FBtr0089518 | AnxB9 | 1482 | 0.9755068 | 117 | 0.2407095 | 12.66666667 | 4 |
| FBtr0089562 | Zasp66 | 2115 | 0.9587459 | 16 | 0.1443894 | 132.1875 | 7 |
| FBtr0089563 | Zasp66 | 3921 | 0.9883314 | 42 | 0.1616103 | 93.35714286 | 8 |
| FBtr0089566 | Zasp66 | 1958 | 0.9526316 | 21 | 0.1421053 | 93.23809524 | 6 |
| FBtr0089568 | Zasp66 | 3365 | 0.9844804 | 38 | 0.159919 | 88.55263158 | 6 |
| FBtr0089630 | CG10910 | 3621 | 0.959204 | 16 | 0.0661692 | 226.3125 | 2 |
| FBtr0089746 | #N/A | 29763 | 0.991453 | 102 | 0.2064777 | 291.7941176 | #N/A |
| FBtr0089747 | Mlc2 | 34396 | 0.9916885 | 101 | 0.1942257 | 340.5544554 | 2 |
| FBtr0089766 | Cyp6d5 | 3138 | 0.9798781 | 26 | 0.1353659 | 120.6923077 | 4 |
| FBtr0089793 | bt | 73623 | 0.9779481 | 632 | 0.1362561 | 116.4920886 | 40 |
| FBtr0089959 | Tm1 | 7786 | 0.97981 | 28 | 0.1538005 | 278.0714286 | 9 |
| FBtr0089967 | Tm1 | 7028 | 0.9855769 | 23 | 0.1105769 | 305.5652174 | 9 |
| FBtr0091464 | #N/A | 35778 | 0.9879518 | 584 | 0.1843691 | 61.26369863 | #N/A |
| FBtr0091805 | His1:CG33801 | 2431 | 0.9755011 | 135 | 0.6614699 | 18.00740741 | 0 |
| FBtr0091808 | His1:CG33804 | 2442 | 0.9888641 | 131 | 0.6536748 | 18.64122137 | 0 |
| FBtr0091811 | His1:CG33807 | 2420 | 0.9832962 | 136 | 0.6815145 | 17.79411765 | 0 |
| FBtr0091812 | His2A:CG33808 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091814 | His1:CG33810 | 2451 | 0.9933185 | 134 | 0.6837416 | 18.29104478 | 0 |
| FBtr0091817 | His1:CG33813 | 2467 | 0.9933185 | 133 | 0.6636971 | 18.54887218 | 0 |
| FBtr0091818 | His2A:CG33814 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091820 | His1:CG33816 | 2471 | 0.9933185 | 133 | 0.6636971 | 18.57894737 | 0 |
| FBtr0091821 | His2A:CG33817 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091823 | His1:CG33819 | 2451 | 0.9933185 | 134 | 0.6837416 | 18.29104478 | 0 |
| FBtr0091824 | His2A:CG33820 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091826 | His1:CG33822 | 2451 | 0.9933185 | 134 | 0.6837416 | 18.29104478 | 0 |
| FBtr0091827 | His2A:CG33823 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091829 | His1:CG33825 | 2451 | 0.9933185 | 134 | 0.6837416 | 18.29104478 | 0 |
| FBtr0091830 | His2A:CG33826 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091832 | His1:CG33828 | 2451 | 0.9933185 | 134 | 0.6837416 | 18.29104478 | 0 |
| FBtr0091833 | His2A:CG33829 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091835 | His1:CG33831 | 2451 | 0.9933185 | 134 | 0.6837416 | 18.29104478 | 0 |
| FBtr0091836 | His2A:CG33832 | 3195 | 0.9525483 | 230 | 0.8242531 | 13.89130435 | 0 |
| FBtr0091838 | His1:CG33834 | 2194 | 0.986637 | 136 | 0.6815145 | 16.13235294 | 0 |
| FBtr0091841 | His1:CG33837 | 2469 | 0.9933185 | 135 | 0.6614699 | 18.28888889 | 0 |
| FBtr0091844 | His1:CG33840 | 2469 | 0.9933185 | 135 | 0.6614699 | 18.28888889 | 0 |
| FBtr0091847 | His1:CG33843 | 2469 | 0.9933185 | 135 | 0.6614699 | 18.28888889 | 0 |
| FBtr0091850 | His1:CG33846 | 2469 | 0.9933185 | 135 | 0.6614699 | 18.28888889 | 0 |
| FBtr0091853 | His1:CG33849 | 2469 | 0.9933185 | 135 | 0.6614699 | 18.28888889 | 0 |
| FBtr0091856 | His1:CG33852 | 2449 | 0.9933185 | 136 | 0.6815145 | 18.00735294 | 0 |
| FBtr0091859 | His1:CG33855 | 2283 | 0.9743875 | 134 | 0.6837416 | 17.03731343 | 0 |
| FBtr0091862 | His1:CG33858 | 2283 | 0.9743875 | 134 | 0.6837416 | 17.03731343 | 0 |
| FBtr0091865 | His1:CG33861 | 2303 | 0.9743875 | 133 | 0.6636971 | 17.31578947 | 0 |
| FBtr0091868 | His1:CG33864 | 2469 | 0.9933185 | 135 | 0.6614699 | 18.28888889 | 0 |
| FBtr0100164 | RplL3 | 1580 | 0.9922481 | 21 | 0.1447028 | 75.23809524 | 3 |
| FBtr0100182 | 14-3-zeta | 1929 | 0.9691992 | 11 | 0.1273101 | 175.3636364 | 6 |
| FBtr0100183 | 14-3-zeta | 3064 | 0.9629207 | 18 | 0.1158015 | 170.2222222 | 6 |
| FBtr0100231 | RplL41 | 2926 | 1 | 10 | 0.3450479 | 292.6 | 2 |
| FBtr0100289 | ref(2)P | 2126 | 0.9725596 | 104 | 0.3738192 | 20.44230769 | 2 |
| FBtr0100321 | fabp | 2700 | 0.9635854 | 16 | 0.1694678 | 168.75 | 2 |
| FBtr0100379 | CG3164 | 3007 | 0.9521648 | 57 | 0.1714385 | 52.75438596 | 6 |
| FBtr0100387 | Zasp52 | 5646 | 0.9663098 | 107 | 0.1807305 | 52.76635514 | 14 |
| FBtr0100388 | Zasp52 | 15206 | 0.9735778 | 264 | 0.2280938 | 57.59848485 | 14 |
| FBtr0100432 | Jon25Bi | 7481 | 0.9861432 | 31 | 0.2898383 | 241.3225806 | 0 |
| FBtr0100479 | Gapdh1 | 17086 | 1 | 143 | 0.4038911 | 119.4825175 | 0 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|------------|--------|-----------|-----|-----------|-------------|------|
| FPtr0100482 | Pglym78 | 4072 | 0.9823875 | 52 | 0.3806262 | 78.30769231 | 2 |
| FPtr0100483 | Pglym78 | 4060 | 0.9890329 | 51 | 0.3668993 | 79.60784314 | 2 |
| FPtr0100485 | GlyP | 14067 | 0.9958492 | 91 | 0.2161882 | 154.5824176 | 3 |
| FPtr0100541 | RpS13 | 3204 | 1 | 45 | 0.0723684 | 71.2 | 2 |
| FPtr0100561 | up | 12347 | 0.9876118 | 16 | 0.1266346 | 771.6875 | 7 |
| FPtr0100563 | up | 12512 | 0.9881501 | 17 | 0.1310072 | 736 | 9 |
| FPtr0100589 | Adh | 22148 | 1 | 19 | 0.1596639 | 1165.684211 | 3 |
| FPtr0100590 | Adh | 20800 | 0.9990157 | 20 | 0.1584646 | 1040 | 2 |
| FPtr0100594 | Adh | 22100 | 0.9975904 | 49 | 0.1726908 | 451.0204082 | 3 |
| FPtr0100620 | Prx5 | 1217 | 0.9986358 | 28 | 0.2755798 | 43.46428571 | 2 |
| FPtr0100662 | Act5C | 8527 | 0.964032 | 44 | 0.19627 | 193.7954545 | 1 |
| FPtr0100663 | Act5C | 8437 | 0.9756224 | 39 | 0.1950208 | 216.3333333 | 1 |
| FPtr0100857 | mt:ND2 | 11253 | 0.9941521 | 904 | 0.837232 | 12.44800885 | 0 |
| FPtr0100861 | mt:Col | 136896 | 0.9993489 | 928 | 0.4726562 | 147.5172414 | 0 |
| FPtr0100863 | mt:Coll | 39466 | 1 | 65 | 0.3886463 | 607.1692308 | 0 |
| FPtr0100866 | mt:ATPase8 | 984 | 1 | 6 | 0.2777778 | 164 | 0 |
| FPtr0100867 | mt:ATPase6 | 33448 | 1 | 24 | 0.2918518 | 1393.666667 | 0 |
| FPtr0100868 | mt:Coll1 | 46090 | 1 | 47 | 0.4195184 | 980.6382979 | 0 |
| FPtr0100870 | mt:ND3 | 5115 | 1 | 11 | 0.3474576 | 465 | 0 |
| FPtr0100877 | mt:ND5 | 51780 | 0.9994203 | 85 | 0.2243478 | 609.1764706 | 0 |
| FPtr0100879 | mt:ND4 | 30649 | 0.9932886 | 68 | 0.2818792 | 450.7205882 | 0 |
| FPtr0100880 | mt:ND4L | 3569 | 1 | 355 | 0.7491409 | 10.05352113 | 0 |
| FPtr0100884 | mt:Cyt-b | 50188 | 0.9991205 | 639 | 0.4564644 | 78.54147105 | 0 |
| FPtr0100886 | mt:ND1 | 22282 | 1 | 52 | 0.371672 | 428.5 | 0 |
| FPtr0110844 | noe | 4150 | 0.9800664 | 28 | 0.166113 | 148.2142857 | 0 |
| FPtr0110845 | noe | 4436 | 0.9790419 | 30 | 0.1656687 | 147.8666667 | 0 |
| FPtr0110872 | l(2)O6225 | 4453 | 0.95 | 52 | 0.19 | 85.63461538 | 2 |
| FPtr0111048 | Zasp52 | 5890 | 0.9694809 | 120 | 0.1782658 | 49.08333333 | 15 |
| FPtr0111120 | RpL38 | 2441 | 0.9850374 | 3 | 0.1022444 | 813.6666667 | 1 |
| FPtr0111129 | RpL5 | 6389 | 0.9592215 | 11 | 0.1167748 | 580.8181818 | 3 |
| FPtr0111132 | RpL5 | 6397 | 0.9863281 | 10 | 0.1064453 | 639.7 | 4 |
| FPtr0112363 | CG34172 | 957 | 0.9932886 | 3 | 0.1744967 | 319 | 2 |
| FPtr0112413 | CG34220 | 14777 | 1 | 202 | 0.5171952 | 73.15346535 | 1 |
| FPtr0112526 | CG34324 | 3965 | 0.9827236 | 84 | 0.324187 | 47.20238095 | 1 |
| FPtr0112532 | CG34330 | 491 | 0.9677419 | 16 | 0.2225806 | 30.6875 | 0 |
| FPtr0112791 | Argk | 13323 | 0.9530745 | 38 | 0.2195254 | 350.6052632 | 2 |
| FPtr0112860 | Nc73EF | 17011 | 0.9851043 | 157 | 0.1730387 | 108.3503185 | 11 |
| FPtr0113101 | CG10320 | 1952 | 0.9776675 | 5 | 0.1240695 | 390.4 | 2 |
| FPtr0113140 | DOR | 4168 | 0.95383 | 138 | 0.1448059 | 30.20289855 | 4 |
| FPtr0113265 | CG5778 | 1245 | 0.9599156 | 33 | 0.3565401 | 37.72727273 | 2 |
| FPtr0113290 | CG5028 | 6856 | 0.9962686 | 73 | 0.2288557 | 93.91780822 | 5 |
| FPtr0113360 | CG30118 | 2433 | 0.9554962 | 82 | 0.3119715 | 29.67073171 | 9 |
| FPtr0113464 | Unc-89 | 36514 | 0.9902313 | 577 | 0.1791966 | 63.28249567 | 37 |
| FPtr0113742 | RpL15 | 5777 | 0.9915374 | 7 | 0.1184767 | 825.2857143 | 2 |
| FPtr0114472 | #N/A | 3013 | 0.9590214 | 81 | 0.2629969 | 37.19753086 | #N/A |
| FPtr0114473 | #N/A | 1654 | 0.9529873 | 31 | 0.2272282 | 53.35483871 | #N/A |
| FPtr0114536 | Pdh | 2838 | 0.998913 | 16 | 0.125 | 177.375 | 2 |
| FPtr0114537 | Pdh | 2806 | 0.9967141 | 16 | 0.1259584 | 175.375 | 2 |
| FPtr0114548 | ldh | 4279 | 0.9634009 | 24 | 0.1345721 | 178.2916667 | 2 |
| FPtr0273322 | Mal-A6 | 3626 | 0.9861702 | 42 | 0.1718085 | 86.33333333 | 2 |
| FPtr0273393 | CG3214 | 2640 | 0.9644013 | 22 | 0.1666667 | 120 | 4 |
| FPtr0273398 | pst | 3592 | 0.9533516 | 98 | 0.24853 | 36.65306122 | 6 |
| FPtr0273399 | pst | 3571 | 0.9725696 | 87 | 0.2303348 | 41.04597701 | 5 |
| FPtr0290272 | CG7203 | 2096 | 0.9729345 | 37 | 0.3005698 | 56.64864865 | 1 |
| FPtr0290312 | CG10737 | 3753 | 0.9553366 | 63 | 0.1484401 | 59.57142857 | 14 |
| FPtr0290316 | CG10737 | 3285 | 0.9543164 | 63 | 0.1518307 | 52.14285714 | 14 |
| FPtr0299696 | Mlp60A | 934 | 0.98627 | 8 | 0.173913 | 116.75 | 2 |
| FPtr0299697 | Mlp60A | 4509 | 0.9961109 | 34 | 0.1696646 | 132.6176471 | 8 |
| FPtr0299869 | RpL10 | 3788 | 0.9884319 | 15 | 0.1928021 | 252.5333333 | 4 |
| FPtr0299870 | RpL10 | 3674 | 0.9885932 | 16 | 0.2091255 | 229.625 | 4 |
| FPtr0300283 | CG7461 | 2304 | 0.9732938 | 115 | 0.2799209 | 20.03478261 | 0 |
| FPtr0300395 | CG9485 | 5018 | 0.9536751 | 257 | 0.2388306 | 19.52529183 | 7 |
| FPtr0300425 | Acon | 36665 | 0.9986154 | 94 | 0.1481481 | 390.0531915 | 3 |
| FPtr0300635 | CG42502 | 1477 | 0.9620563 | 24 | 0.2031824 | 61.54166667 | 1 |
| FPtr0300680 | CG10320 | 1956 | 0.9779412 | 5 | 0.122549 | 391.2 | 2 |
| FPtr0300730 | Npl4 | 8195 | 0.9797048 | 34 | 0.1526753 | 241.0294118 | 7 |
| FPtr0300828 | RpS15Aa | 4154 | 0.9719764 | 18 | 0.2920354 | 230.7777778 | 2 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|-----------|-------|-----------|-----|-----------|-------------|------|
| FPtr0300899 | CG18624 | 1383 | 0.9854015 | 4 | 0.1094891 | 345.75 | 1 |
| FPtr0301036 | NDUFA8 | 1804 | 0.9511834 | 35 | 0.1967456 | 51.54285714 | 4 |
| FPtr0301154 | Zasp66 | 3764 | 0.9853837 | 47 | 0.1607795 | 80.08510638 | 7 |
| FPtr0301156 | Zasp66 | 3484 | 0.98713 | 39 | 0.1570142 | 89.33333333 | 7 |
| FPtr0301340 | bt | 74280 | 0.9745942 | 648 | 0.1384218 | 114.6296296 | 45 |
| FPtr0301561 | #N/A | 6065 | 0.9718593 | 10 | 0.1095477 | 606.5 | #N/A |
| FPtr0301661 | Vha55 | 5472 | 0.9916992 | 95 | 0.168457 | 57.6 | 3 |
| FPtr0301708 | Men-b | 2952 | 0.9531981 | 48 | 0.124025 | 61.5 | 6 |
| FPtr0301783 | Ucrh | 1369 | 0.9903846 | 4 | 0.1706731 | 342.25 | 2 |
| FPtr0301784 | Ucrh | 1311 | 0.9700461 | 4 | 0.1635945 | 327.75 | 2 |
| FPtr0301827 | Mhc | 90292 | 0.9893095 | 183 | 0.1896065 | 493.3989071 | 18 |
| FPtr0301828 | Mhc | 88034 | 0.9663398 | 178 | 0.1820577 | 494.5730337 | 17 |
| FPtr0301829 | Mhc | 92615 | 0.9977719 | 175 | 0.1876114 | 529.2285714 | 18 |
| FPtr0301919 | up | 12218 | 0.9876118 | 10 | 0.1114935 | 1221.8 | 7 |
| FPtr0301920 | up | 12220 | 0.9881735 | 11 | 0.1162943 | 1110.909091 | 9 |
| FPtr0301921 | up | 12202 | 0.987984 | 10 | 0.1081442 | 1220.2 | 8 |
| FPtr0301922 | up | 12533 | 0.9881501 | 24 | 0.1514154 | 522.2083333 | 10 |
| FPtr0301923 | up | 12503 | 0.9879599 | 23 | 0.1438127 | 543.6086957 | 9 |
| FPtr0301959 | Tm1 | 7082 | 0.9864326 | 24 | 0.1237031 | 295.0833333 | 9 |
| FPtr0301960 | Tm1 | 5894 | 0.9864326 | 21 | 0.1125299 | 280.6666667 | 9 |
| FPtr0302301 | RpL28 | 2098 | 0.9711539 | 16 | 0.2692308 | 131.125 | 3 |
| FPtr0302442 | Ef1gamma | 3984 | 0.9799941 | 128 | 0.3345133 | 31.125 | 3 |
| FPtr0302527 | CG33346 | 2265 | 0.9762774 | 102 | 0.1322993 | 22.20588235 | 2 |
| FPtr0302586 | RpL3 | 7082 | 0.9567536 | 98 | 0.2049763 | 72.26530612 | 5 |
| FPtr0302854 | Phae2 | 1055 | 0.9593679 | 9 | 0.0541761 | 117.2222222 | 0 |
| FPtr0303048 | Actn | 9280 | 0.9779189 | 170 | 0.2683001 | 54.58823529 | 9 |
| FPtr0303096 | Scsalpha | 2564 | 0.9864 | 40 | 0.2208 | 64.1 | 3 |
| FPtr0303099 | CG33470 | 2276 | 0.9690266 | 170 | 0.7256637 | 13.38823529 | 3 |
| FPtr0303859 | #N/A | 3416 | 0.9590588 | 100 | 0.2983529 | 34.16 | #N/A |
| FPtr0303860 | CG42837 | 3416 | 0.9563585 | 101 | 0.3054904 | 33.82178218 | 2 |
| FPtr0304129 | MtnE | 613 | 0.9710982 | 12 | 0.2427746 | 51.08333333 | 1 |
| FPtr0304693 | RpS27 | 2466 | 0.9665211 | 15 | 0.1644833 | 164.4 | 2 |
| FPtr0304812 | CG43078 | 12957 | 0.9571664 | 402 | 0.2184043 | 32.23134328 | 4 |
| FPtr0304813 | CG43078 | 15347 | 0.9517544 | 431 | 0.2011773 | 35.60788863 | 8 |
| FPtr0304814 | CG43078 | 15347 | 0.9647667 | 419 | 0.1976912 | 36.62768496 | 6 |
| FPtr0304815 | CG43078 | 15370 | 0.9534187 | 428 | 0.199239 | 35.91121495 | 7 |
| FPtr0305122 | CG1746 | 29406 | 0.9688889 | 31 | 0.2340741 | 948.5806452 | 2 |
| FPtr0305260 | porin | 6182 | 0.9645902 | 95 | 0.1416393 | 65.07368421 | 3 |
| FPtr0305551 | Vha68-2 | 4825 | 0.9648118 | 49 | 0.150982 | 98.46938776 | 4 |
| FPtr0305669 | RpL29 | 1059 | 0.990099 | 10 | 0.2409241 | 105.9 | 2 |
| FPtr0305977 | cp309 | 13928 | 0.9612514 | 195 | 0.1473106 | 71.42564103 | 10 |
| FPtr0305979 | cp309 | 13954 | 0.952391 | 203 | 0.1484095 | 68.73891626 | 12 |
| FPtr0306086 | CG4769 | 8884 | 1 | 22 | 0.2087336 | 403.8181818 | 5 |
| FPtr0306237 | CG1746 | 16621 | 0.9958449 | 18 | 0.1786704 | 923.3888889 | 2 |
| FPtr0306630 | Fhos | 7439 | 0.9557669 | 198 | 0.163704 | 37.57070707 | 16 |
| FPtr0306632 | Fhos | 9453 | 0.9732957 | 288 | 0.1778073 | 32.82291667 | 16 |
| FPtr0306639 | Prm | 4364 | 0.9705015 | 51 | 0.1545723 | 85.56862745 | 2 |
| FPtr0306657 | Ald | 15587 | 0.9534732 | 46 | 0.2018349 | 338.8478261 | 3 |
| FPtr0307034 | cype | 2667 | 0.977387 | 7 | 0.2386935 | 381 | 2 |
| FPtr0307035 | cype | 2567 | 0.9573991 | 10 | 0.3071749 | 256.7 | 2 |
| FPtr0307492 | Mhc | 87996 | 0.9782768 | 169 | 0.1781888 | 520.6863905 | 17 |
| FPtr0307493 | Mhc | 88737 | 0.9880078 | 173 | 0.1875281 | 512.9306358 | 17 |
| FPtr0307494 | Mhc | 88656 | 0.9799297 | 170 | 0.1800469 | 521.5058824 | 17 |
| FPtr0307495 | Mhc | 89942 | 0.9959221 | 185 | 0.1910588 | 486.172973 | 16 |
| FPtr0307496 | Mhc | 90167 | 0.9898477 | 184 | 0.1892676 | 490.0380435 | 18 |
| FPtr0307904 | l(1)G0156 | 14167 | 0.9950715 | 168 | 0.4938393 | 84.32738095 | 4 |
| FPtr0308034 | DOR | 4222 | 0.9752353 | 141 | 0.1515602 | 29.94326241 | 4 |
| FPtr0308188 | CG34172 | 942 | 0.9932203 | 3 | 0.1762712 | 314 | 2 |
| FPtr0308192 | RpS10b | 2583 | 0.9712042 | 16 | 0.2159686 | 161.4375 | 1 |
| FPtr0308233 | up | 12232 | 0.9887719 | 10 | 0.1136842 | 1223.2 | 7 |
| FPtr0308234 | up | 12532 | 0.9864197 | 24 | 0.1425926 | 522.1666667 | 9 |
| FPtr0308235 | wupA | 5744 | 0.9966997 | 265 | 0.1254125 | 21.6754717 | 9 |
| FPtr0308236 | wupA | 4821 | 0.9727891 | 263 | 0.133139 | 18.33079848 | 8 |
| FPtr0308237 | wupA | 4866 | 0.9865255 | 263 | 0.1318576 | 18.50190114 | 8 |
| FPtr0308333 | RpL15 | 5780 | 0.9911168 | 7 | 0.106599 | 825.7142857 | 2 |
| FPtr0308334 | RpL15 | 5683 | 0.9899135 | 7 | 0.1210375 | 811.8571429 | 2 |
| FPtr0308595 | Mpcp | 4758 | 0.9599097 | 103 | 0.2964427 | 46.19417476 | 3 |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|----------|-------|-----------|------|-----------|-------------|------|
| FBtr0308683 | CoVib | 4486 | 0.9820359 | 30 | 0.3473054 | 149.5333333 | 1 |
| FBtr0310086 | Zasp52 | 6099 | 0.9608732 | 133 | 0.2035724 | 45.85714286 | 15 |
| FBtr0310087 | Zasp52 | 10009 | 0.9661795 | 184 | 0.2194155 | 54.39673913 | 13 |
| FBtr0310088 | Zasp52 | 9280 | 0.972396 | 168 | 0.2037956 | 55.23809524 | 14 |
| FBtr0310136 | up | 12370 | 0.9881735 | 24 | 0.151117 | 515.4166667 | 10 |
| FBtr0310137 | up | 12358 | 0.9881501 | 18 | 0.1408822 | 686.5555556 | 9 |
| FBtr0310138 | up | 12340 | 0.9879599 | 17 | 0.1331104 | 725.8823529 | 8 |
| FBtr0310274 | Rp58 | 3243 | 0.9862259 | 21 | 0.369146 | 154.4285714 | 3 |
| FBtr0310496 | #N/A | 2718 | 0.9709454 | 143 | 0.2241977 | 19.00699301 | #N/A |
| FBtr0310535 | CG14125 | 6349 | 0.9860465 | 35 | 0.1383721 | 181.4 | 1 |
| FBtr0310658 | Actn | 9154 | 0.9655684 | 208 | 0.2760463 | 44.00961538 | 9 |
| FBtr0310659 | Actn | 9047 | 0.9632946 | 209 | 0.2820054 | 43.28708134 | 9 |
| FBtr0310661 | Ald | 16541 | 0.9647059 | 91 | 0.2773994 | 181.7692308 | 3 |
| FBtr0329909 | Zasp52 | 2182 | 0.9572083 | 86 | 0.195619 | 25.37209302 | 9 |
| FBtr0329911 | Zasp52 | 14451 | 0.9783468 | 183 | 0.2181129 | 78.96721311 | 6 |
| FBtr0329912 | Zasp52 | 5456 | 0.9540306 | 112 | 0.2038641 | 48.71428571 | 14 |
| FBtr0329913 | Zasp52 | 5855 | 0.9568346 | 120 | 0.2086331 | 48.79166667 | 14 |
| FBtr0329914 | Zasp52 | 5351 | 0.9530298 | 102 | 0.1954105 | 52.46078431 | 13 |
| FBtr0329915 | Zasp52 | 5758 | 0.952748 | 111 | 0.2060992 | 51.87387387 | 13 |
| FBtr0329916 | Zasp52 | 6289 | 0.9710889 | 128 | 0.1837341 | 49.1328125 | 15 |
| FBtr0330021 | CG43733 | 987 | 0.9793916 | 21 | 0.1511286 | 47 | 2 |
| FBtr0330025 | Men | 4000 | 0.9839255 | 85 | 0.1489002 | 47.05882353 | 2 |
| FBtr0330403 | RpL37a | 1452 | 0.952381 | 12 | 0.1445578 | 121 | 2 |
| FBtr0330640 | CG11455 | 647 | 0.9592326 | 34 | 0.529976 | 19.02941176 | 2 |
| FBtr0330682 | Rp52 | 3053 | 0.9512987 | 26 | 0.1525974 | 117.4230769 | 1 |
| FBtr0331361 | RpL27 | 3498 | 0.9944853 | 32 | 0.3841912 | 109.3125 | 1 |
| FBtr0331425 | Gbs-76A | 5299 | 0.9550056 | 86 | 0.1484814 | 61.61627907 | 2 |
| FBtr0331557 | CG4169 | 12681 | 0.9993494 | 85 | 0.2940794 | 149.1882353 | 3 |
| FBtr0331564 | CG6020 | 6703 | 0.9865139 | 22 | 0.1078894 | 304.6818182 | 3 |
| FBtr0331650 | CG34172 | 949 | 0.970297 | 3 | 0.1716172 | 316.3333333 | 2 |
| FBtr0331810 | CG7430 | 7566 | 0.9847609 | 37 | 0.1529164 | 204.4864865 | 3 |
| FBtr0331864 | mtacp1 | 2127 | 0.985115 | 29 | 0.1393775 | 73.34482759 | 4 |
| FBtr0331936 | CG7712 | 1750 | 0.9542484 | 12 | 0.2663399 | 145.8333333 | 2 |
| FBtr0332029 | porin | 6352 | 0.9790916 | 90 | 0.1319394 | 70.57777778 | 3 |
| FBtr0332168 | Fer2LCH | 4462 | 0.9945005 | 66 | 0.1594867 | 67.60606061 | 2 |
| FBtr0332169 | Fer2LCH | 4412 | 0.9847943 | 66 | 0.1556351 | 66.84848485 | 3 |
| FBtr0332370 | nrv1 | 2459 | 0.9626168 | 89 | 0.2637591 | 27.62921348 | 3 |
| FBtr0332500 | Pif1A | 6397 | 0.954645 | 201 | 0.1494653 | 31.82587065 | 13 |
| FBtr0332503 | Pif1B | 6397 | 0.954645 | 201 | 0.1494653 | 31.82587065 | 13 |
| FBtr0332526 | CG5261 | 10910 | 0.9870067 | 269 | 0.5096247 | 40.55762082 | 6 |
| FBtr0332527 | CG5261 | 11054 | 0.9873001 | 269 | 0.4981185 | 41.0929368 | 6 |
| FBtr0332528 | CG5261 | 10980 | 0.9874243 | 271 | 0.5030275 | 40.51660517 | 6 |
| FBtr0332529 | CG5261 | 11156 | 0.9876993 | 271 | 0.4920273 | 41.16605166 | 6 |
| FBtr0332597 | Aldh | 7141 | 0.9510526 | 104 | 0.2484211 | 68.66346154 | 3 |
| FBtr0332618 | Gapdh2 | 8216 | 0.9912854 | 75 | 0.4139434 | 109.5466667 | 1 |
| FBtr0332651 | CG18135 | 3822 | 0.9587961 | 51 | 0.1010391 | 74.94117647 | 0 |
| FBtr0332683 | sls | 50787 | 0.9546723 | 1130 | 0.1481796 | 44.94424779 | 30 |
| FBtr0332723 | fln | 7315 | 0.9592834 | 61 | 0.3501629 | 119.9180328 | 3 |
| FBtr0332731 | pst | 3571 | 0.9767055 | 87 | 0.2375208 | 41.04597701 | 6 |
| FBtr0332820 | CG43897 | 9017 | 0.9590457 | 192 | 0.2357853 | 46.96354167 | 5 |
| FBtr0332822 | CG43897 | 9406 | 0.9753484 | 166 | 0.2340121 | 56.6626506 | 6 |
| FBtr0332824 | CG43897 | 9582 | 0.9662958 | 173 | 0.2392016 | 55.38728324 | 6 |
| FBtr0332825 | CG43897 | 9408 | 0.9753748 | 166 | 0.2337616 | 56.6746988 | 6 |
| FBtr0332826 | CG43897 | 10405 | 0.9850863 | 138 | 0.2103611 | 75.39855072 | 4 |
| FBtr0332832 | CG43897 | 12530 | 0.9537507 | 218 | 0.2408347 | 57.47706422 | 5 |
| FBtr0332833 | CG43897 | 10114 | 0.9557158 | 190 | 0.2468246 | 53.23157895 | 6 |
| FBtr0332834 | CG43897 | 11595 | 0.9900955 | 150 | 0.2164839 | 77.3 | 4 |
| FBtr0332835 | CG43897 | 11717 | 0.9516524 | 187 | 0.2392901 | 62.65775401 | 5 |
| FBtr0332947 | Ef1beta | 1683 | 0.9938195 | 19 | 0.1248455 | 88.57894737 | 1 |
| FBtr0332966 | Ef2b | 18935 | 0.9930338 | 103 | 0.1609195 | 183.8349515 | 4 |
| FBtr0333111 | CG43078 | 15006 | 0.9502226 | 431 | 0.2015695 | 34.81670534 | 7 |
| FBtr0333112 | CG43078 | 15342 | 0.9578292 | 425 | 0.2022789 | 36.09882353 | 7 |
| FBtr0333113 | CG43078 | 14975 | 0.9597819 | 422 | 0.2041187 | 35.48578199 | 6 |
| FBtr0333126 | AcCoA5 | 2339 | 0.9634011 | 38 | 0.1393715 | 61.55263158 | 5 |
| FBtr0333142 | Ubi-p63E | 33591 | 0.9809451 | 75 | 0.2747713 | 447.88 | 1 |
| FBtr0333276 | GlyS | 5548 | 0.9729074 | 54 | 0.1985443 | 102.7407407 | 4 |
| FBtr0333310 | #N/A | 7824 | 0.9811193 | 27 | 0.1402562 | 289.7777778 | #N/A |

Figure S3.1 (Continued)

| | | | | | | | |
|-------------|------------|-------|-----------|-----|-----------|-------------|------|
| FPtr0333311 | #N/A | 7836 | 0.9811321 | 27 | 0.1401617 | 290.2222222 | #N/A |
| FPtr0333370 | RpL18 | 2424 | 0.9695767 | 49 | 0.3796296 | 49.46938776 | 2 |
| FPtr0333383 | Est-6 | 2155 | 0.9569951 | 18 | 0.0990746 | 119.7222222 | 1 |
| FPtr0333551 | Gst51 | 8702 | 0.9819089 | 73 | 0.2295696 | 119.2054795 | 4 |
| FPtr0333676 | CG1970 | 4728 | 0.9665404 | 42 | 0.2493687 | 112.5714286 | 6 |
| FPtr0333709 | RpL26 | 3106 | 0.9601227 | 22 | 0.2269939 | 141.1818182 | 1 |
| FPtr0333710 | CG3819 | 2096 | 0.9528832 | 30 | 0.1462729 | 69.86666667 | 1 |
| FPtr0333777 | ATPsyn-Cf6 | 3306 | 0.9522184 | 15 | 0.2849829 | 220.4 | 2 |
| FPtr0333801 | Actn | 9353 | 0.96843 | 225 | 0.2753128 | 41.56888889 | 9 |
| FPtr0333802 | Actn | 9149 | 0.9655377 | 208 | 0.2762923 | 43.98557692 | 9 |
| FPtr0333918 | Tm1 | 6241 | 0.9864326 | 21 | 0.1117318 | 297.1904762 | 9 |
| FPtr0333942 | cp309 | 13923 | 0.9554935 | 200 | 0.1509353 | 69.615 | 12 |
| FPtr0333943 | cp309 | 13943 | 0.954643 | 195 | 0.1438988 | 71.5025641 | 11 |
| FPtr0333944 | cp309 | 13784 | 0.9500276 | 194 | 0.1424159 | 71.05154639 | 11 |
| FPtr0334029 | His4r | 1449 | 0.954792 | 11 | 0.1537071 | 131.7272727 | 2 |
| FPtr0334094 | l(3)neo18 | 3770 | 0.9971989 | 16 | 0.2240896 | 235.625 | 3 |
| FPtr0334327 | skap | 9376 | 0.9921094 | 208 | 0.1662283 | 45.07692308 | 6 |
| FPtr0334328 | skap | 9372 | 0.9937304 | 258 | 0.1781609 | 36.3255814 | 7 |
| FPtr0334482 | bt | 72917 | 0.9812534 | 604 | 0.1356031 | 120.7235099 | 34 |
| FPtr0334483 | bt | 74339 | 0.9778902 | 637 | 0.1355186 | 116.7017268 | 43 |
| FPtr0334627 | Rfabg | 51256 | 0.962839 | 486 | 0.2212047 | 105.4650206 | 7 |
| FPtr0334706 | CG7920 | 7351 | 0.9966499 | 40 | 0.2819654 | 183.775 | 3 |
| FPtr0334842 | RFeSP | 4541 | 0.9874777 | 27 | 0.2576029 | 168.1851852 | 2 |
| FPtr0334843 | RFeSP | 4456 | 0.989 | 22 | 0.221 | 202.5454545 | 2 |
| FPtr0334890 | CoVa | 6495 | 1 | 16 | 0.2787402 | 405.9375 | 1 |
| FPtr0335194 | ND42 | 3896 | 0.9845397 | 32 | 0.167955 | 121.75 | 2 |
| FPtr0335199 | CG6455 | 3490 | 0.9580574 | 42 | 0.1449595 | 83.0952381 | 7 |
| FPtr0335200 | CG6455 | 3318 | 0.9753087 | 38 | 0.1393867 | 87.31578947 | 8 |
| FPtr0335387 | CG3523 | 15992 | 0.9769514 | 313 | 0.1746465 | 51.09265176 | 5 |
| FPtr0335523 | #N/A | 24 | 0.972973 | 1 | 0.1351351 | 24 | #N/A |
| FPtr0336613 | Vha68-2 | 4836 | 0.9652385 | 56 | 0.172595 | 86.35714286 | 4 |
| FPtr0336614 | Vha68-2 | 5317 | 0.9723101 | 54 | 0.1578323 | 98.46296296 | 4 |
| FPtr0336620 | Ggamma30A | 919 | 0.9764429 | 21 | 0.1813899 | 43.76190476 | 1 |
| FPtr0336621 | CG43733 | 919 | 0.9764429 | 21 | 0.1813899 | 43.76190476 | 1 |
| FPtr0336648 | Strn-Mlck | 38968 | 0.9995338 | 208 | 0.1459207 | 187.3461538 | 7 |
| FPtr0336668 | l(1)G0156 | 11220 | 0.9921147 | 114 | 0.5290322 | 98.42105263 | 5 |
| FPtr0336707 | Gel | 2761 | 0.9547389 | 48 | 0.1245648 | 57.52083333 | 6 |
| FPtr0336895 | wupA | 5070 | 0.9844694 | 298 | 0.1691113 | 17.01342282 | 7 |
| FPtr0336896 | wupA | 5081 | 0.9599359 | 298 | 0.1570513 | 17.05033557 | 8 |
| FPtr0336897 | wupA | 5456 | 0.982925 | 300 | 0.1566444 | 18.18666667 | 8 |
| FPtr0337053 | Hsc70-4 | 6381 | 0.9811644 | 121 | 0.265839 | 52.73553719 | 1 |

We identified a set of transcripts featuring a high degree of read coverage on the sense strand. We designate transcripts with $\geq 95\%$ sense strand coverage and a $\geq 10:1$ sense:antisense read count ratio as high exon coverage transcripts or “HECTS”.

Figure S3.2. Differential expression analysis for HECTs

| HECT | gene | logCPM | logFC_LTM | PValue_LTM | FDR_LTM | logFC_ODOR | PValue_ODOR | FDR_ODOR | logFC_SHOCK | PValue_SHOCK | FDR_SHOCK |
|-------------|-----------------|-------------|--------------|-------------|-------------|--------------|-------------|-------------|--------------|--------------|-------------|
| Fbtr0079701 | Bace | 10.32945118 | 3.620403329 | 5.10463E-21 | 3.97651E-18 | 2.627876557 | 5.48605E-09 | 4.27364E-06 | 4.256190358 | 4.80278E-20 | 3.74136E-17 |
| Fbtr0079056 | Jon25Biii | 8.030294733 | 3.255188346 | 1.14846E-15 | 4.47324E-13 | 0.730056526 | 0.141481456 | 0.999941333 | 2.860706811 | 2.27747E-09 | 1.36473E-07 |
| Fbtr0087854 | CG12374 | 12.32874927 | 2.632599256 | 1.86599E-12 | 4.84535E-10 | 1.793674285 | 8.07969E-05 | 0.004495768 | 3.46124417 | 9.85298E-14 | 8.83774E-11 |
| Fbtr0084879 | CG5107 | 7.574347614 | 2.770736306 | 5.7296E-12 | 1.11584E-09 | 1.872696981 | 7.58423E-05 | 0.004495768 | 2.516582747 | 7.14526E-08 | 3.34673E-06 |
| Fbtr0079713 | Peritrophin-15a | 5.564630877 | 3.184910136 | 9.76473E-12 | 1.52134E-09 | -2.828751042 | 0.005930072 | 0.159293992 | 3.722063479 | 5.03955E-11 | 5.6083E-09 |
| Fbtr0088759 | Mal-A1 | 9.576363078 | 2.49725059 | 1.92667E-11 | 2.50147E-09 | 1.30508617 | 0.003020341 | 0.084030208 | 1.778216004 | 4.00874E-05 | 0.001040936 |
| Fbtr0085153 | CG17192 | 9.049134117 | 2.421718422 | 1.6136E-10 | 1.79571E-13 | 1.823846913 | 0.000117905 | 0.006123195 | 3.53598536 | 1.81256E-13 | 4.70662E-11 |
| Fbtr0088160 | epsilonTry | 9.074715894 | 2.3786969 | 2.06579E-10 | 2.01157E-08 | 1.514901585 | 0.000773528 | 0.027389927 | 3.008822871 | 3.34098E-11 | 4.33771E-09 |
| Fbtr0084255 | Pepp1 | 6.569260228 | 2.783496261 | 4.15451E-10 | 3.59596E-08 | 1.359470141 | 0.008774301 | 0.207126692 | 3.317037426 | 9.68563E-12 | 1.88628E-09 |
| Fbtr0088161 | alphaTry | 11.37923134 | 2.200453451 | 1.84589E-09 | 1.43795E-07 | 1.495293128 | 0.000824308 | 0.027918957 | 2.328897845 | 2.48044E-07 | 0.01698E-05 |
| Fbtr0273322 | Mal-A6 | 8.858642627 | 2.210382803 | 3.52842E-09 | 2.49876E-07 | 1.078762684 | 0.01670177 | 0.342386277 | 1.667997 | 0.000164 | 0.003193909 |
| Fbtr0085511 | Jon99Ci | 7.686652768 | 2.254264065 | 4.42556E-09 | 2.87292E-07 | 1.452602696 | 0.002510356 | 0.072168754 | 2.566291122 | 7.30353E-08 | 3.34673E-06 |
| Fbtr0085077 | CG6295 | 10.34175254 | 2.154961193 | 5.65506E-09 | 3.38868E-07 | 1.429221296 | 0.001646783 | 0.049340137 | 2.713551567 | 3.45109E-09 | 1.92028E-07 |
| Fbtr0088159 | gammaTry | 10.37877016 | 2.077196184 | 1.32651E-08 | 6.40925E-07 | 1.558935995 | 0.0005304 | 0.020659085 | 2.219592464 | 1.00115E-06 | 3.39085E-05 |
| Fbtr0088123 | CG30031 | 10.37877016 | 2.077196184 | 1.32651E-08 | 6.40925E-07 | 1.558935995 | 0.0005304 | 0.020659085 | 2.219592464 | 1.00115E-06 | 3.39085E-05 |
| Fbtr0088124 | deltaTry | 10.37775752 | 2.078544342 | 1.30416E-08 | 6.40925E-07 | 1.559365748 | 0.000527513 | 0.020659085 | 2.219592464 | 1.04827E-06 | 3.4025E-05 |
| Fbtr0088158 | CG30025 | 10.39229208 | 2.073891997 | 1.39868E-08 | 6.40925E-07 | 1.543676239 | 0.000598689 | 0.022208494 | 2.202167473 | 1.21165E-06 | 3.77549E-05 |
| Fbtr0088122 | betaTry | 11.23906148 | 2.086481279 | 1.9047E-08 | 8.2431E-07 | 1.231449573 | 0.006658721 | 0.172904794 | 2.243423803 | 8.99562E-07 | 3.33695E-05 |
| Fbtr0100432 | Jon25Bi | 9.555398818 | 2.142621737 | 2.1031E-08 | 8.62273E-07 | 0.798803592 | 0.086051987 | 1 | 2.235062405 | 1.34979E-06 | 4.04419E-05 |
| Fbtr0079055 | Jon25Bii | 8.549135672 | 2.121878509 | 2.80383E-08 | 1.09209E-06 | 0.695959055 | 0.139273592 | 1 | 2.602572253 | 1.83901E-08 | 5.5057E-07 |
| Fbtr0085502 | Jon99Ciii | 10.73726917 | 1.932145563 | 2.05872E-07 | 7.63686E-06 | 1.173166125 | 0.010840075 | 0.241269102 | 2.818108392 | 1.31334E-09 | 9.92976E-08 |
| Fbtr0085512 | Jon99Cii | 10.77237955 | 1.923091035 | 2.31057E-07 | 8.18152E-06 | 1.173969413 | 0.01067131 | 0.241269102 | 2.805768841 | 1.52962E-09 | 9.92976E-08 |
| Fbtr0077040 | Jon65Aiv | 10.91616028 | 1.933304498 | 3.18892E-07 | 1.08007E-05 | 1.072585444 | 0.022321045 | 0.434702345 | 2.343115621 | 4.81161E-07 | 1.87412E-05 |
| Fbtr0084847 | tobi | 7.931481431 | 1.891585114 | 4.29184E-07 | 1.39306E-05 | 1.577051723 | 0.00100083 | 0.031185873 | 2.140044451 | 4.001E-06 | 0.00015436 |
| Fbtr0088709 | PGRP-SC2 | 6.543769478 | 1.931778074 | 4.02038E-06 | 0.000125275 | 0.476463408 | 0.366910813 | 1 | 2.974932082 | 1.52108E-09 | 9.92976E-08 |
| Fbtr0086903 | CG6484 | 7.778491615 | 1.759001276 | 5.73808E-06 | 0.000171922 | 0.249965303 | 0.598308307 | 1 | 1.77656859 | 8.06352E-05 | 0.001847495 |
| Fbtr0077041 | Jon65Aiii | 10.43650287 | 1.663028194 | 8.83111E-06 | 0.000254794 | 0.870321993 | 0.061064576 | 1 | 2.093795711 | 5.06365E-06 | 0.000140878 |
| Fbtr0083890 | MtnB | 6.043141561 | 2.051155162 | 1.13597E-05 | 0.000316042 | -0.730190928 | 0.21782834 | 1 | 3.260458429 | 1.98409E-10 | 1.71734E-08 |
| Fbtr0077038 | yip7 | 10.01245674 | 1.395532491 | 0.000173332 | 0.004656055 | 0.589596543 | 0.207885645 | 1 | 1.810158875 | 7.63079E-05 | 0.001801328 |
| Fbtr0085162 | CG5399 | 8.631121752 | 1.384259124 | 0.000180857 | 0.004696244 | 0.395145988 | 0.392573563 | 1 | 1.716239777 | 0.000151494 | 0.003105621 |
| Fbtr0083154 | CG18522 | 9.494026791 | 1.303176167 | 0.000250548 | 0.00629602 | 0.667675858 | 0.135180446 | 1 | 1.984479453 | 7.49882E-06 | 0.00201434 |
| Fbtr0112532 | CG34330 | 6.020163889 | 1.472056411 | 0.000270501 | 0.006585005 | 0.266218186 | 0.623685063 | 1 | 1.556127745 | 0.002180709 | 0.027848726 |
| Fbtr0076389 | CG31810 | 10.31882834 | 1.308776985 | 0.000448068 | 0.010577118 | 0.566555434 | 0.22861722 | 1 | 1.877131479 | 4.54348E-05 | 0.001106054 |
| Fbtr0087004 | Amy-p | 13.05950632 | 1.272139613 | 0.000727538 | 0.016669185 | 0.682910777 | 0.139192125 | 1 | 1.647468922 | 0.000369173 | 0.006390797 |
| Fbtr0087796 | CG13324 | 8.342301808 | 1.223203793 | 0.001961573 | 0.043659011 | 0.077403556 | 0.875206117 | 1 | 1.098904698 | 0.026008872 | 0.082980761 |
| Fbtr0087560 | Arc1 | 9.282015437 | 1.104206037 | 0.002288095 | 0.04951184 | 0.021126078 | 0.961626383 | 1 | 1.404052983 | 0.001161555 | 0.017072666 |
| Fbtr0089055 | Cyp9b2 | 6.545634276 | 1.109452617 | 0.004225708 | 0.08662710 | 0.325423255 | 0.517957391 | 1 | 1.554252395 | 0.001288123 | 0.018582371 |
| Fbtr0112526 | CG34324 | 9.043304547 | 1.008168282 | 0.004544997 | 0.090783396 | -0.694504974 | 0.131340981 | 1 | 1.274904887 | 0.00425866 | 0.036059737 |
| Fbtr0033710 | CG3819 | 7.859239329 | 1.115912627 | 0.00533871 | 0.101435494 | 0.65839357 | 0.258870484 | 1 | 1.528135503 | 0.001488994 | 0.020712974 |
| Fbtr0075048 | CG3819 | 7.847583235 | 1.116425977 | 0.005322113 | 0.101435494 | 0.579179008 | 0.247833109 | 1 | 1.523884415 | 0.001531361 | 0.020928587 |
| Fbtr0075069 | CG6839 | 11.27568614 | 1.013622693 | 0.007713066 | 0.143059015 | 0.355078492 | 0.448469104 | 1 | 1.6289801618 | 0.006326514 | 0.04378475 |
| Fbtr0302854 | Pha2 | 7.207076511 | 0.984864328 | 0.008547324 | 0.151326486 | 0.322287705 | 0.500460694 | 1 | 1.436502489 | 0.002117064 | 0.027848726 |
| Fbtr0336613 | Vha68-2 | 9.106794464 | 0.930963537 | 0.009933009 | 0.168212807 | 0.088179555 | 0.84506902 | 1 | 1.163137873 | 0.00895607 | 0.049254962 |
| Fbtr0305511 | Vha68-2 | 9.102613499 | 0.928261345 | 0.010148911 | 0.168212807 | 0.090586006 | 0.840893386 | 1 | 1.155680997 | 0.009401323 | 0.049254962 |
| Fbtr0075878 | RpS12 | 8.411205496 | 0.907930168 | 0.01184735 | 0.182636759 | 0.022334229 | 0.960761551 | 1 | 1.473960406 | 0.000931891 | 0.013960451 |
| Fbtr0076633 | RpL14 | 8.741912177 | 0.893166169 | 0.012581385 | 0.182636759 | 0.385245365 | 0.386386843 | 1 | 1.241227115 | 0.004884126 | 0.037684862 |
| Fbtr0114548 | ldh | 9.224094114 | 0.813626845 | 0.019572776 | 0.217817035 | 0.031204164 | 0.94555062 | 1 | 1.570161586 | 0.000438522 | 0.007268275 |
| Fbtr0086150 | Vha16-1 | 11.04022178 | 0.828599993 | 0.01952864 | 0.217817035 | 0.171678667 | 0.695863746 | 1 | 1.185643082 | 0.006570929 | 0.044531937 |
| Fbtr0086153 | Vha16-1 | 11.04033763 | 0.830036766 | 0.019290492 | 0.217817035 | 0.178917367 | 0.683763099 | 1 | 1.184324301 | 0.006644002 | 0.044531937 |
| Fbtr0086151 | Vha16-1 | 11.04031351 | 0.829781695 | 0.019329058 | 0.217817035 | 0.178806336 | 0.683952611 | 1 | 1.184211391 | 0.006649917 | 0.044531937 |
| Fbtr0086152 | Vha16-1 | 11.04183638 | 0.830132208 | 0.019286414 | 0.217817035 | 0.178904727 | 0.683787316 | 1 | 1.183377699 | 0.006688365 | 0.044531937 |
| Fbtr0076667 | ldh | 9.216929939 | 0.806834948 | 0.020626858 | 0.22011401 | 0.036139698 | 0.937008706 | 1 | 1.56609233 | 0.000455171 | 0.007315077 |
| Fbtr0076668 | ldh | 9.21590163 | 0.807940858 | 0.020457157 | 0.22011401 | 0.030552996 | 0.946685139 | 1 | 1.564785899 | 0.000460127 | 0.007315077 |
| Fbtr0332168 | Fer2LCH | 8.956971975 | 0.80793668 | 0.023231345 | 0.244556999 | -0.098785913 | 0.825378754 | 1 | 1.648843783 | 0.000176371 | 0.003351042 |
| Fbtr0332169 | Fer2LCH | 8.940482159 | 0.806242014 | 0.023547886 | 0.244584039 | -0.097900584 | 0.826965434 | 1 | 1.635841995 | 0.000198879 | 0.00368873 |
| Fbtr0082598 | Cyp9f2 | 8.477754821 | 0.793937728 | 0.025546703 | 0.261853701 | -0.18897801 | 0.678282028 | 1 | 1.17636306 | 0.008494598 | 0.049254962 |
| Fbtr0074522 | wupA | 12.75902557 | -0.916608422 | 0.026355719 | 0.266637726 | -2.178521708 | 2.6027E-05 | 0.0020275 | -1.526213605 | 0.009457341 | 0.049254962 |
| Fbtr0087105 | RpLP2 | 8.33060914 | 0.797024305 | 0.027330524 | 0.269690273 | 0.30527903 | 0.502691904 | 1 | 1.169028319 | 0.009359349 | 0.049254962 |
| Fbtr0336895 | wupA | 12.74721845 | -0.913008776 | 0.028055415 | 0.273189601 | -2.219842495 | 1.90447E-05 | 0.001984789 | -1.554792496 | 0.008244331 | 0.049254962 |
| Fbtr0336896 | wupA | 12.74740543 | -0.910359269 | 0.028504777 | 0.27413853 | -2.221365629 | 1.88093E-05 | 0.001984789 | -1.55485324 | 0.008229491 | 0.049254962 |
| Fbtr0074520 | wupA | 12.74800803 | -0.907632336 | 0.028936139 | 0.274893318 | -2.219146662 | 1.89515E-05 | 0.001984789 | -1.552782038 | 0.008292469 | 0.049254962 |
| Fbtr0074523 | wupA | 12.74855193 | -0.903021804 | 0.030146161 | 0.282938062 | -2.207226332 | 2.03829E-05 | 0.001984789 | -1.556004836 | 0.008241849 | 0.049254962 |
| Fbtr0074521 | wupA | 12.74790439 | -0.898654364 | | | | | | | | |

Figure S3.2 (Continued)

| | | | | | | | | | | | |
|-------------|-------------|-------------|--------------|-------------|-------------|--------------|-------------|---|--------------|-------------|-------------|
| F8tr030833 | RpL15 | 9.632780748 | 0.650786705 | 0.064014714 | 0.429891914 | 0.222341669 | 0.614990453 | 1 | 1.276070587 | 0.003428411 | 0.031893276 |
| F8tr0113742 | RpL15 | 9.632935551 | 0.653600951 | 0.062979408 | 0.429891914 | 0.224059241 | 0.61232408 | 1 | 1.270493121 | 0.003575302 | 0.032766587 |
| F8tr0308334 | RpL15 | 9.612914294 | 0.641648673 | 0.068100444 | 0.447014087 | 0.193493233 | 0.661548461 | 1 | 1.2605478 | 0.003816 | 0.034168555 |
| F8tr0100231 | RpL41 | 8.810529585 | 0.625740953 | 0.074631282 | 0.46552379 | 0.389596203 | 0.386163465 | 1 | 1.276296508 | 0.004483067 | 0.036955418 |
| F8tr0333126 | AcCoAS | 8.237294871 | 0.637023606 | 0.075481687 | 0.46552379 | 0.363198994 | 0.439190702 | 1 | 1.299388103 | 0.004875643 | 0.037684862 |
| F8tr0085632 | Fer1HCH | 9.636065348 | 0.608584452 | 0.083435622 | 0.488694357 | -0.005864938 | 0.989619235 | 1 | 1.683514547 | 0.000155657 | 0.003109157 |
| F8tr0083804 | Vha13 | 7.810319644 | 0.59884192 | 0.097928062 | 0.524579142 | 0.429998278 | 0.35714062 | 1 | 1.296963804 | 0.004244654 | 0.036059737 |
| F8tr0071592 | RpL29 | 6.982023717 | 0.59424972 | 0.114325377 | 0.524579142 | 0.141874012 | 0.768817312 | 1 | 1.271065499 | 0.006351318 | 0.04378475 |
| F8tr0088527 | RpL31 | 8.131072589 | 0.562134836 | 0.117845122 | 0.524579142 | 0.172084479 | 0.704280201 | 1 | 1.186686095 | 0.007890406 | 0.049254962 |
| F8tr0078056 | RpL15 | 8.66191231 | 0.578612542 | 0.104357473 | 0.524579142 | 0.011927222 | 0.978937952 | 1 | 1.164095751 | 0.009017605 | 0.049254962 |
| F8tr0086273 | RpS18 | 8.366453665 | 0.578718947 | 0.101535686 | 0.524579142 | 0.129007531 | 0.778943214 | 1 | 1.151859224 | 0.010541377 | 0.049254962 |
| F8tr0088525 | RpL31 | 8.234348248 | 0.53375937 | 0.135304799 | 0.550529429 | 0.245896694 | 0.587860134 | 1 | 1.16657019 | 0.009193423 | 0.049254962 |
| F8tr0071135 | RpS6 | 8.812824087 | 0.493182982 | 0.159383449 | 0.600555098 | 0.144536022 | 0.74812387 | 1 | 1.238980342 | 0.006215074 | 0.04378475 |
| F8tr0072188 | tsr | 6.846540883 | 0.518061565 | 0.17585316 | 0.626133231 | 0.471741403 | 0.327679035 | 1 | 1.463171199 | 0.002176436 | 0.027848726 |
| F8tr0088035 | Ef1alpha48D | 11.31582917 | 0.467785091 | 0.176997993 | 0.626133231 | 0.145307202 | 0.74266572 | 1 | 1.153153587 | 0.008886954 | 0.049254962 |
| F8tr0072173 | elF-5A | 8.912274596 | 0.44606917 | 0.204259137 | 0.691816816 | 0.216390996 | 0.6309181 | 1 | 1.178778822 | 0.007820974 | 0.049254962 |
| F8tr0100861 | mt:Col | 16.22051494 | -0.403953351 | 0.252343223 | 0.773918783 | -0.143659718 | 0.738600544 | 1 | -1.241999097 | 0.005062325 | 0.038286907 |
| F8tr0100483 | Pgym78 | 8.265840791 | -0.396305872 | 0.274774643 | 0.77405815 | -0.200768114 | 0.650953735 | 1 | -1.337356046 | 0.004541398 | 0.036955418 |
| F8tr0100482 | Pgym78 | 8.269949184 | -0.393938269 | 0.277622039 | 0.77405815 | -0.203902664 | 0.645912267 | 1 | -1.336999475 | 0.004554198 | 0.036955418 |
| F8tr0076593 | Prm | 9.879726395 | -0.396032796 | 0.262596273 | 0.77405815 | -0.66429827 | 0.133375299 | 1 | -1.211294651 | 0.007395432 | 0.048412114 |
| F8tr0070801 | RpL35 | 8.651293996 | 0.399132016 | 0.259911014 | 0.77405815 | 0.195951202 | 0.661553738 | 1 | 1.150598385 | 0.010019652 | 0.049254962 |
| F8tr0085384 | Pgym78 | 8.279576611 | -0.389529929 | 0.282702686 | 0.780941107 | -0.196951966 | 0.657128843 | 1 | -1.308225777 | 0.005399607 | 0.039994089 |
| F8tr0332820 | CG43897 | 10.33689599 | -0.353898038 | 0.312790228 | 0.81221196 | 0.228403387 | 0.600942174 | 1 | 1.126205826 | 0.010180822 | 0.049254962 |
| F8tr0076594 | Prm | 9.738528635 | -0.348451167 | 0.324917326 | 0.829870811 | -0.647706876 | 0.144027359 | 1 | -1.157569761 | 0.010512918 | 0.049254962 |
| F8tr0334482 | bt | 12.45641559 | -0.328833613 | 0.346669343 | 0.849916937 | -0.076283274 | 0.860292223 | 1 | -1.157079268 | 0.009760232 | 0.049254962 |
| F8tr0089793 | bt | 12.47298657 | -0.328252619 | 0.347362797 | 0.849916937 | -0.079124103 | 0.851588323 | 1 | -1.148905522 | 0.01026816 | 0.049254962 |
| F8tr0089746 | #N/A | 11.66477126 | -0.32661288 | 0.35866263 | 0.854428711 | -0.161620778 | 0.710431153 | 1 | -1.699705952 | 0.00027634 | 0.00506258 |
| F8tr0113290 | CG5028 | 8.953459839 | -0.334976817 | 0.356476998 | 0.854428711 | -0.175629669 | 0.688743096 | 1 | -1.298649095 | 0.004769379 | 0.037684862 |
| F8tr0334483 | bt | 12.48287089 | -0.321628869 | 0.357174345 | 0.854428711 | -0.075609154 | 0.861550334 | 1 | -1.14710665 | 0.010389275 | 0.049254962 |
| F8tr0301340 | bt | 12.48261721 | -0.320888863 | 0.358267741 | 0.854428711 | -0.077314779 | 0.858467247 | 1 | -1.146941532 | 0.010399625 | 0.049254962 |
| F8tr0333918 | Tm1 | 9.129795573 | -0.292712696 | 0.407871274 | 0.903507976 | -0.525096132 | 0.240025505 | 1 | -1.236266225 | 0.006963648 | 0.04597188 |
| F8tr0301960 | Tm1 | 9.070789879 | -0.29500091 | 0.404799559 | 0.903507976 | -0.59547606 | 0.210937079 | 1 | -1.193218151 | 0.009082119 | 0.049254962 |
| F8tr0089747 | Mlc2 | 11.92959086 | -0.282132573 | 0.425773521 | 0.911202122 | -0.101368056 | 0.814653312 | 1 | -1.674187193 | 0.00032878 | 0.00508209 |
| F8tr0304129 | MtnE | 6.35837712 | 0.3439116959 | 0.437446849 | 0.912907327 | -0.301432604 | 0.570790075 | 1 | 1.253530796 | 1.34737E-07 | 5.83112E-06 |
| F8tr0089566 | Zasp66 | 7.409028894 | -0.285610891 | 0.438179199 | 0.912907327 | -0.077984487 | 0.86383126 | 1 | -1.368721159 | 0.004838746 | 0.037684862 |
| F8tr0307492 | Mhc | 13.06673328 | -0.244375869 | 0.486091162 | 0.915850486 | -0.306083127 | 0.484940105 | 1 | -1.353007199 | 0.003065694 | 0.031477757 |
| F8tr0307495 | Mhc | 13.08371155 | -0.245836439 | 0.483537626 | 0.915850486 | -0.30814459 | 0.482056293 | 1 | -1.34987613 | 0.003126702 | 0.031477757 |
| F8tr0307496 | Mhc | 13.09304573 | -0.247653001 | 0.480375988 | 0.915850486 | -0.309623773 | 0.479923296 | 1 | -1.348726642 | 0.003152877 | 0.031477757 |
| F8tr0301827 | Mhc | 13.09116984 | -0.244867853 | 0.485341231 | 0.915850486 | -0.308395513 | 0.481751304 | 1 | -1.348845301 | 0.00315553 | 0.031477757 |
| F8tr0080896 | Mhc | 13.08279688 | -0.246038838 | 0.483167333 | 0.915850486 | -0.309095642 | 0.480693532 | 1 | -1.347925447 | 0.003166034 | 0.031477757 |
| F8tr0080895 | Mhc | 13.06875776 | -0.244764728 | 0.485404578 | 0.915850486 | -0.306608108 | 0.484210074 | 1 | -1.348120068 | 0.003166146 | 0.031477757 |
| F8tr0080901 | Mhc | 13.08201556 | -0.247573292 | 0.480428488 | 0.915850486 | -0.309971254 | 0.479363714 | 1 | -1.347172048 | 0.003177043 | 0.031477757 |
| F8tr0080902 | Mhc | 13.06443362 | -0.239641549 | 0.494613785 | 0.915850486 | -0.299899043 | 0.493732749 | 1 | -1.347658218 | 0.003178655 | 0.031477757 |
| F8tr0080899 | Mhc | 13.05021397 | -0.238288068 | 0.497028269 | 0.915850486 | -0.297303511 | 0.497456435 | 1 | -1.347859689 | 0.00317881 | 0.031477757 |
| F8tr0307493 | Mhc | 13.07839279 | -0.250264624 | 0.475764163 | 0.915850486 | -0.3072163 | 0.483363099 | 1 | -1.346485455 | 0.003197893 | 0.031477757 |
| F8tr0080900 | Mhc | 13.0835511 | -0.248521696 | 0.478740285 | 0.915850486 | -0.310637564 | 0.478449192 | 1 | -1.346045252 | 0.003200345 | 0.031477757 |
| F8tr0080898 | Mhc | 13.06951931 | -0.247269841 | 0.480931192 | 0.915850486 | -0.308160716 | 0.481941695 | 1 | -1.346223786 | 0.003200749 | 0.031477757 |
| F8tr0301828 | Mhc | 13.06768918 | -0.244690978 | 0.485530151 | 0.915850486 | -0.305578598 | 0.485659228 | 1 | -1.345885012 | 0.003211468 | 0.031477757 |
| F8tr0080903 | Mhc | 13.06519742 | -0.24216213 | 0.490063034 | 0.915850486 | -0.301474568 | 0.491407359 | 1 | -1.34574732 | 0.003213604 | 0.031477757 |
| F8tr0307494 | Mhc | 13.06572596 | -0.236338386 | 0.500837365 | 0.915850486 | -0.294179769 | 0.50217685 | 1 | -1.346138671 | 0.003232632 | 0.031477757 |
| F8tr0080907 | Mhc | 13.13336714 | -0.237879213 | 0.498383892 | 0.915850486 | -0.293211625 | 0.503825629 | 1 | -1.342079917 | 0.003330633 | 0.031893276 |
| F8tr0301829 | Mhc | 13.11204079 | -0.24022667 | 0.49404088 | 0.915850486 | -0.295884298 | 0.499844384 | 1 | -1.337109317 | 0.00342776 | 0.031893276 |
| F8tr0080897 | Mhc | 13.10379567 | -0.241377489 | 0.491885517 | 0.915850486 | -0.296566113 | 0.498789394 | 1 | -1.336188321 | 0.00343907 | 0.031893276 |
| F8tr0089967 | Tm1 | 9.292274613 | -0.239418305 | 0.49724263 | 0.915850486 | -0.516248329 | 0.246423656 | 1 | -1.183229062 | 0.009484203 | 0.049254962 |
| F8tr0082158 | MtnA | 9.216561836 | 0.262046542 | 0.515638327 | 0.923347903 | -0.336238006 | 0.47457409 | 1 | 1.898711031 | 4.39015E-05 | 0.001103202 |
| F8tr0080905 | Mhc | 13.14242867 | -0.227860678 | 0.516809725 | 0.923347903 | -0.277640006 | 0.526642588 | 1 | -1.349024027 | 0.003210859 | 0.031477757 |
| F8tr0084901 | CG5028 | 8.68797227 | -0.238616102 | 0.511531227 | 0.923347903 | -0.224542797 | 0.612231139 | 1 | -1.286982764 | 0.005624378 | 0.040094751 |
| F8tr0301959 | Tm1 | 9.30108322 | -0.23282025 | 0.509109144 | 0.923347903 | -0.506225627 | 0.255445976 | 1 | -1.18141516 | 0.009601266 | 0.049254962 |
| F8tr0080906 | Mhc | 13.14367919 | -0.221590464 | 0.528328561 | 0.931882701 | -0.2743475 | 0.53155027 | 1 | -1.351343369 | 0.003165865 | 0.031477757 |
| F8tr0080167 | l(2)06225 | 11.00621224 | 0.201585384 | 0.592495476 | 0.936987748 | -0.018713753 | 0.965373381 | 1 | 1.3045750402 | 1.52801E-11 | 2.38064E-09 |
| F8tr0074910 | fln | 9.093107016 | 0.205253396 | 0.58783586 | 0.936987748 | 0.418882319 | 0.363308236 | 1 | -1.686222144 | 0.000860214 | 0.01313935 |
| F8tr0083030 | Mf | 9.347502964 | -0.219717136 | 0.544708685 | 0.936987748 | -0.170449194 | 0.696720071 | 1 | -1.429261798 | 0.001777759 | 0.023877145 |
| F8tr0337053 | Hsc70-4 | 9.925609164 | 0.209758762 | 0.548732352 | 0.936987748 | -0.056991918 | 0.900690215 | 1 | 1.175358624 | 0.009214622 | 0.049254962 |
| F8tr0083059 | Hsc70-4 | 9.923502261 | 0.210795056 | 0.546574147 | 0.936987748 | -0.068555974 | 0.880721584 | 1 | 1.175589365 | 0.009222994 | 0.049254962 |
| F8tr0083055 | Hsc70-4 | 9.920834788 | 0.210900111 | 0.546541794 | 0.936987748 | -0.067169491 | 0.883089428 | 1 | 1.173986133 | 0.009314814 | 0.049254962 |
| F8tr0083058 | Hsc70-4 | 9.920834788 | 0.210900111 | 0.546541794 | 0.936987748 | -0.067169491 | 0.883089428 | 1 | 1.173986133 | 0.009314814 | 0.049254962 |
| F8tr0083060 | Hsc70-4 | 9.921259102 | 0.209814279 | 0.548611271 | 0.936987748 | -0.067575823 | 0.88239267 | 1 | 1.173578302 | 0.009343053 | 0.049254962 |
| F8tr0083056 | Hsc70-4 | 9.923511694 | 0.207509173 | 0.552963012 | 0.936987748 | -0.073034714 | 0.872959303 | 1 | 1.170280961 | 0.009523847 | 0.049254962 |
| F8tr0301923 | up | 10.09954103 | -0.191989741 | 0.58551652 | 0.936987748 | -0.282763456 | 0.517364173 | 1 | -1.167693782 | 0.01025091 | 0.049254962 |
| F8tr0100563 | up | 10.10029312 | -0.197031381 | 0.575760428 | 0.936987748 | -0.277121688 | 0.525685579 | 1 | -1.162703776 | 0.01054371 | 0.049254962 |
| | | | | | | | | | | | |

Figure S3.2 (Continued)

| | | | | | | | | | | | |
|-------------|-----------|-------------|--------------|-------------|-------------|--------------|-------------|-------------|--------------|-------------|-------------|
| F8tr0301921 | up | 9.998410587 | -0.159988685 | 0.649693406 | 0.966646976 | -0.256558725 | 0.556776555 | 1 | -1.176060015 | 0.00997931 | 0.049254962 |
| F8tr0081030 | Arr1 | 8.841354281 | -0.166546183 | 0.647075148 | 0.966646976 | -1.603857052 | 0.000883754 | 0.028685175 | 1.04136945 | 0.020567972 | 0.074523023 |
| F8tr0308233 | up | 10.02173147 | -0.153714549 | 0.662333491 | 0.97167192 | -0.236124634 | 0.588756348 | 1 | -1.192758946 | 0.009032142 | 0.049254962 |
| F8tr0301919 | up | 9.999304201 | -0.154419966 | 0.661100958 | 0.97167192 | -0.260255455 | 0.551157269 | 1 | -1.171969895 | 0.010240526 | 0.049254962 |
| F8tr0110872 | [(2)06225 | 11.0336913 | 0.142849674 | 0.702837599 | 0.98094734 | 0.01260226 | 0.976795762 | 1 | 2.945746235 | 6.34634E-11 | 6.17975E-09 |
| F8tr0310535 | CG14125 | 9.868059748 | 0.148564747 | 0.70732818 | 0.98094734 | -1.917352437 | 0.000253803 | 0.012145604 | 0.07016217 | 0.883687629 | 0.902218431 |
| F8tr0076044 | CG14125 | 10.03070582 | 0.152791457 | 0.700194653 | 0.98094734 | -1.918761182 | 0.000265052 | 0.012145604 | 0.063817408 | 0.89445325 | 0.909460874 |
| F8tr0083032 | MF | 8.869792383 | -0.129824566 | 0.716546989 | 0.987947088 | -0.095975174 | 0.827417436 | 1 | -1.170240535 | 0.010490947 | 0.049254962 |
| F8tr0083143 | Act88F | 10.978493 | -0.088103911 | 0.805263962 | 0.988849725 | -0.019130924 | 0.964745059 | 1 | -1.676224604 | 0.000396055 | 0.006707102 |
| F8tr0089630 | CG10910 | 9.123099807 | 0.132453313 | 0.732534499 | 0.988849725 | 0.156314552 | 0.745269671 | 1 | 1.418434392 | 0.003217256 | 0.031477757 |
| F8tr0077144 | CG4769 | 9.530115143 | -0.071435232 | 0.843580737 | 0.988849725 | -0.083898738 | 0.846166644 | 1 | -1.326179463 | 0.003704073 | 0.033552007 |
| F8tr0085196 | Mlc1 | 8.533053555 | 0.030101108 | 0.935355787 | 0.988849725 | 0.004233593 | 0.992301353 | 1 | -1.377245253 | 0.003896117 | 0.034489491 |
| F8tr0306086 | CG4769 | 9.322618119 | -0.058425019 | 0.872125978 | 0.988849725 | -0.08287207 | 0.848038119 | 1 | -1.319070164 | 0.003941215 | 0.034496701 |
| F8tr0086303 | CG9090 | 9.400383022 | 0.020173457 | 0.955344381 | 0.988849725 | 0.271627734 | 0.532484276 | 1 | -1.335031974 | 0.004110634 | 0.03557982 |
| F8tr0305122 | CG1746 | 11.85370627 | 0.043738975 | 0.904060587 | 0.988849725 | 0.294452816 | 0.502440044 | 1 | -1.314506351 | 0.005022785 | 0.038286907 |
| F8tr0084994 | Ald | 11.4081457 | -0.109129656 | 0.760274063 | 0.988849725 | 0.066973948 | 0.877262223 | 1 | -1.278846606 | 0.005160008 | 0.038650441 |
| F8tr0085774 | CG1746 | 11.92184796 | 0.056988485 | 0.875188218 | 0.988849725 | 0.292604975 | 0.505591702 | 1 | -1.301851839 | 0.005442071 | 0.039994089 |
| F8tr0085772 | CG1746 | 11.95873861 | 0.059521726 | 0.869768377 | 0.988849725 | 0.292349908 | 0.506268268 | 1 | -1.287833313 | 0.005974527 | 0.043094044 |
| F8tr0083857 | ninaE | 10.70726439 | 0.06356164 | 0.86259996 | 0.988849725 | -1.218166634 | 0.007095975 | 0.178314979 | 1.165292171 | 0.007550181 | 0.048608188 |
| F8tr0331936 | CG7712 | 7.125968058 | -0.01853489 | 0.960315173 | 0.988849725 | 0.258812423 | 0.56899981 | 1 | -1.326557605 | 0.007505124 | 0.048608188 |
| F8tr0306237 | CG1746 | 10.94662408 | 0.061426473 | 0.866807949 | 0.988849725 | 0.161805148 | 0.720658561 | 1 | -1.230839361 | 0.009006215 | 0.049254962 |
| F8tr0085195 | Mlc1 | 8.517058684 | 0.002702449 | 0.994225799 | 0.999524566 | 0.020428117 | 0.963633435 | 1 | -1.419099573 | 0.002997198 | 0.031477757 |
| F8tr0084892 | Npl4 | 9.245821212 | -0.000683439 | 0.998895946 | 0.999524566 | 0.516593856 | 0.229382463 | 1 | -1.247732438 | 0.006098485 | 0.043584589 |
| F8tr0300730 | Npl4 | 9.234933824 | 0.002310775 | 0.99457009 | 0.999524566 | 0.517095432 | 0.22901997 | 1 | -1.24201829 | 0.006322166 | 0.04378475 |

Differential expression analysis for HECTs was conducted using the edgeR Bioconductor software package.(2)

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