Screening the Human Genome for New Mitochondrial and Longevity Regulators

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Alvin Jee Yee Ling

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Advisor: Professor David A. Sinclair Alvin Jee Yee Ling

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Abstract

Over the past two decades, genes and pathways have been discovered that can prolong lifespan and delay the onset of age-related diseases. The vast majority of these longevity genes were discovered in screens conducted in lower organisms such as yeast and worms. Given that similar large-scale genetic screens in mammals would be cost prohibitive, it is likely that many mammalian longevity genes remain to be discovered. One way to circumvent this issue is to conduct the screen in mammalian cells and instead use cellular phenotypes that correlate with lifespan extension as a read-out. Mitochondrial dysfunction has been observed in aging and implicated in many age-related diseases. Conversely, calorie restriction, a dietary regimen found to extend lifespan and delay the onset of age-related diseases, is associated with maintained or increased mitochondrial function. Thus, increased mitochondrial content and function is a candidate phenotype for a cell-based screen for mammalian lifespan-regulating genes.

We have performed a genetic screen of 15,483 human genes for open reading frames (ORFs) that modulate mitochondrial function. A total of 76 ORFs were found and validated to be positive regulators of mitochondrial mass while 18 were negative regulators. The gene products of these ORFs include secreted factors, transcription factors and a variety of predicted polypeptides with unknown function.

One of the hits identified from the screen was GLTSCR2 (glioma tumor suppressor candidate region gene 2), a nucleolar protein that has been found to be involved in apoptosis and the cell cycle. GLTSCR2 was discovered as a positive regulator of mitochondrial mass and its overexpression was found to increase cellular respiration. This upregulation of respiration was found to be mediated by Myc, connecting role of GLTSCR2 in oxidative metabolism discovered in this study to its previously reported role in the cell cycle.

Another hit identified from the screen was Cpne2 (copine 2), a member of the Copine family of proteins. Members of the Copine family are calcium-dependent phospholipid binding proteins and although discovered over a decade ago, the cellular role of Cpne2 remains unknown. Cpne2 was found as a negative regulator of mitochondria and its depletion was found to increase mitochondrial mass and function. This increase in mitochondria was not due to an increase in the transcriptional program of mitochondrial biogenesis or a defect in depolarization-induced mitophagy, suggesting the involvement of another independent pathway.

Collectively, this study describes the discovery and characterization of novel mitochondrial regulators, some of which may be longevity genes and may provide a better understanding of the aging process.

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Chapter 1

Introduction

1.1 Global Population Aging

In 2012, the population aged 65 or over in the United States was estimated to be 43.1 million people – about 14% of the national population. This older population is predicted to almost double to 83.7 million by 2050, with 1 in 5 individuals being aged 65 or over [1]. Termed the greying population, this increase in the older population is a world-wide phenomenon, affecting both developed and developing countries [2].

As the global demographic transitions to one with a larger older population, unique problems have begun to arise. Aging itself is characterized by a gradual and progressive loss of physiological function over time, which ultimately decreases health and increases mortality. In addition, advanced age is a major risk factor for diseases such as cancer, diabetes, heart disease and neurodegenerative diseases and thus the prevalence of such chronic diseases has been increasing [3]. Chronic diseases are estimated to account for more than half of the world's disease by 2030 [3]. As the older population increases, these chronic diseases put a socioeconomic strain by limiting workforce productivity and increasing healthcare spending. Therefore, the study of aging and its applications towards improving health in the later years is of great benefit to both the individual and society.

1.2 Lifespan Extension: Calorie Restriction and Genes

Although the causes of aging still remain unknown, multiple discoveries have affirmed that lifespan extension and the delaying of age-related diseases are achievable [4 – 9]. While a reduction in lifespan can be caused by sufficient dysfunction to any organ system, lifespan extension gives greater insight into the aging process as it must delay various age-related diseases and deterioration in every organ system. As such, means of extending lifespan, and subsequently the blocking lifespan extension, have been widely used to probe the molecular mechanism of aging. The methods of inducing lifespan extension can be classified into one of two categories: environmental interventions, particularly calorie restriction, or genetic manipulations.

Calorie restriction, a dietary regimen of food restriction without causing malnutrition, was first discovered to extend lifespan in rodents about 80 years ago [4]. Subsequent studies have shown it to delay to onset of age-related diseases, suggesting that it was an intervention that could delay the aging process [5]. This impressive lifespan-extending effect of calorie restriction has been found to be conserved as it has been shown to also occur in a diverse range of organisms including yeast, worms, flies and mice [6 – 9]. Calorie restriction has even increased the lifespan Ames dwarf mice, which are already long lived compared to their wild-type littermates [10]. The striking opposing effect of calorie restriction on aging has garnered much interest in determining the molecular pathways responsible for its beneficial effects. This pursuit, combined with research on genetic manipulations that extend lifespan, has helped bolster the case for the involvement of several signaling pathways in the aging process.

Over the past 20 years, it has been found that alterations of a single gene could delay the onset of age-related diseases and prolong lifespan [11]. The discovery of such longevity genes

has enabled the identification of gene networks and signaling pathways involved in aging, most of which have been found to be conserved. Some of these signaling pathways have been found to respond to nutrient availability or energy status and thus are proposed to play a role in the benefits brought about by calorie restriction.

Insulin/IGF-1

Reduced signaling through the insulin/IGF-1 signaling pathway was first discovered to extend lifespan in the nematode *C. elegans* in which mutations in members of this pathway such as *age-1* (homolog of PI3 kinase) and notably *daf-2* (homolog of the insulin receptor) resulted in mutant worms that lived dramatically longer [11, 12]. It was later found that the lifespan extending effects of these mutations required *daf-16* (homolog of the FOXO transcription factors) and that mutations in *daf-2* or *age-1* could not extend lifespan in a *daf-16* mutant background [13]. Similarly, mutations in *Inr* (homolog of the insulin receptor) and in *chico* (homolog of IRS) that reduce insuling/IGF-1 signaling in the fly *D. melanogaster* also extend lifespan [14, 15]. Furthermore, overexpression of dFOXO (the homolog of *daf-16*) in flies increases lifespan. While the longevity benefit is evident in worms and flies, it is more complex in mammals as the lack of insulin or insulin sensitivity results in diabetes. However, when restricted to adipose tissue, the longevity benefits re-emerge as fat specific insulin receptor knockout (FIRKO) mice were found to live longer [16]. In addition to FIRKO mice, heterozygous IGF-1 knockout mice live longer as well [17], supporting the studies from worms and flies. Calorie restriction has been found to reduce plasma insulin levels [18], suggesting that reduced insulin signaling could mediate the beneficial effects of calorie restriction. However, the

involvement of insulin/IGF-1 signaling in calorie restriction in worms and flies is unclear as it has varied based on how the dietary restriction was performed [19, 20].

Sirtuins

The longevity effect of the Sirtuins was first discovered in yeast where extra copies of *Sir2*, a yeast sirtuin homolog, extended the replicative lifespan of budding yeast *S. cerevisiae* [6]. Later, it was discovered that the mechanism through which *Sir2* extended lifespan was by suppressing the formation of extrachromosomal rDNA circles (ERCs) [21]. Lifespan extension was also achieved in *C. elegans* and *D. melanogaster* when their respective Sir2 homologs, *Sir2.1* and *dSir2*, were overexpressed [22, 23]. In mammals, there are seven *Sir2* homologs: SIRT1 – 7. Of these, brain-specific overexpressing SIRT1 (BRASTO) mice lived longer [24] while whole-body overexpression of SIRT6 increased the lifespan of male mice [25]. As NAD^+-dependent protein deacylases or ADP-ribosyltransferases, Sirtuins serve as cellular energy sensors and have been proposed to mediate the beneficial effects of calorie restriction. In yeast and flies, Sirtuins have been found to mediate the lifespan extension effect of calorie restriction [23, 26] while their involvement in *C. elegans* has been found to be dependent on the method of calorie restriction [27, 28].

TOR

Another pathway involved in longevity is the TOR (target of rapamycin) pathway, a nutrient-sensing pathway conserved from yeast to mammals. As a regulator of cell growth and metabolism, TOR signaling is activated in response to amino acids and growth factor stimulation. TOR is a kinase that exists in two complexes, TORC1 and TORC2. Of the two, the

TORC1 pathway is better characterized and encompasses the downstream targets found to modulate lifespan: S6 kinases (S6Ks) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Directly affecting TOR by knocking down *let-363* (TOR homolog) or *daf-15* (homolog of Raptor, a TORC1 component) increased the lifespan of *C. elegans* [29, 30]. In addition, overexpression of *dTsc1* and *dTsc2*, negative regulators of TOR signaling, increased lifespan in the fly *D. melanogaster* [31]. For the TORC1 target S6K, deletion of *Sch9* (homolog of S6K) extended both chronological and replicative lifespans of yeast and female homozygous *S6K1* knockout mice lived longer [32, 33]. Not to be left out, the involvement of 4E-BP in longevity was discovered in flies where overexpression of d4E-BP increased lifespan by increasing mitochondrial activity [34]. In terms of calorie restriction, reduction in TOR signaling in yeast (*Sch9* deletion) and flies (*d4E-BP* overexpression) had not additive effect on lifespan extension [32, 34], suggesting that they participate in the same pathway. In *C. elegans*, the majority studies showed that the longevity effects of calorie restriction and decreased TOR signaling were non-additive [35 – 38].

AMPK

Under conditions of low energy, signaling through the AMP-activated protein kinase (AMPK) pathway is increased to as part of energy homeostatic mechanism. To do so, AMPK phosphorylates a number of targets that results in the upregulation cellular processes that generate ATP and a down-regulation of energy-consuming processes. Its role in longevity was discovered in *C. elegans* where overexpression of *aak-2* (homolog of the AMPKα subunit) extended lifespan [39]. This longevity effect was later found to be due to phosphorylation of *crtc-1* by AMPK [40]. In *D. melanogaster*, adult-specific overexpression of AMPK in either the

fat or the muscle was sufficient to extend lifespan [41]. Furthermore, *lkb1* – an upstream kinase that activates AMPK – was found to extend lifespan from a gain-of-function screen [42]. The involvement of AMPK in calorie restriction has so far only been investigated in *C. elegans*, where its requirement for lifespan extension has been dependent on the calorie restriction protocol [43 – 46].

1.3 Mitochondria in Health and Aging

Mitochondria are organelles best known for their role in generating energy in the form of ATP through the oxidation of nutrients. However, their role in the cell extends beyond energy production and also includes calcium signaling, apoptosis and biosynthesis. Several lines of evidence have suggested that mitochondria play key roles in aging and calorie restriction.

Firstly, gene expression profiling studies across various mouse tissues from have found that the expression of mitochondrial genes decreases with age and this decrease is prevented, to a large extent, by calorie restriction [47]. In fact, decreased expression of mitochondrial genes with age was identified as a common gene expression signature of aging from a meta-analysis involving 27 datasets across various human, mouse and rat tissues [48].

Secondly, mitochondria have been noted to become dysfunctional with age. Mitochondria from aged tissues have been found to produce more reactive oxygen species, have lower membrane potential, possess ultrastructural abnormalities and damaged mitochondrial proteins, lipids and DNA [49 – 52]. Furthermore, mitochondrial dysfunction has also been found to occur in age-related diseases such as diabetes, cancer and neurodegeneration [53]. On the other hand, calorie restriction has been found to attenuate or prevent these age-related changes to the mitochondria [54, 55]. In fact, calorie restriction has been found to increase mitochondrial biogenesis in mice and humans [56, 57]. Taken together, these studies suggest that finding ways to increase mitochondrial function would be beneficial in treating age-related diseases and would provide new insight into longevity pathways.

1.4 Mitochondrial Biogenesis

One way to increase mitochondrial function is to increase mitochondrial biogenesis, the process through which functional mitochondria are made. Mitochondrial biogenesis is a complex process that must be well-coordinated because essential macromolecules required for proper mitochondrial function are encoded in two spatially distinct genomes, one in the nucleus (nDNA) and the other in mitochondria (mtDNA). Between the two genomes, most of the mitochondrial proteins are encoded in nDNA, including proteins involved in mitochondrial transcription and most of the components in the electron transport chain. Although much smaller than the nuclear genome, the mitochondrial genome encodes important macromolecules such as the remaining components of the electron transport chain as well as the mitochondrial tRNAs and rRNAs. Since mitochondrial components are encoded in both the nucleus and the mitochondria itself, mitochondrial biogenesis requires coordinated expression of genes in the mitochondria and the nuclear-encoded mitochondrial genes.

Nuclear transcription factors involved in mitochondrial biogenesis have been identified by their ability to upregulate the expression of nuclear genes encoding components of the electron transport chain. These transcription factors include NRF-1, NRF-2, CREB, YY1 and ERRα [58 – 61]. These nuclear transcription factors are capable of coordinating the transcription in the mitochondria with the nucleus as they upregulate the expression of mitochondrial transcription factors such as mitochondrial transcription factors (TFAM, TFB1M and TFB2M) which are also encoded in the nucleus [62, 63]. In addition to the aforementioned nuclear transcription factors, the PPARs regulate another important subset of nuclear-encoded mitochondrial genes involved in fatty acid oxidation [64]. As mitochondrial gene expression in

the nucleus is regulated by several transcription factors, mitochondrial biogenesis therefore requires a way to coordinate the activities of these different transcription factors.

This coordination is achieved by the PPARγ coactivator 1 (PGC-1) family of transcriptional coactivators which comprise of PGC-1α, PGC-1β and PRC. Binding of PGC-1 coactivators to the nuclear transcription factors that regulate the expression of respiratory nuclear genes activates their transcriptional activity and initiates the transcriptional program of mitochondrial biogenesis [66 – 68]. Furthermore, PGC-1α plays an important role in regulating mitochondrial biogenesis to meet cellular energetic demands as it is capable of integrating multiple upstream cellular signals and relay it to the mitochondrial biogenesis program. These include signals from energy sensors SIRT1 and AMPK as well as hormonal signals via PKA, CaMKK and Akt [69 – 74].

Another nuclear transcription factor that regulates mitochondrial biogenesis is Myc. Although better known for its role in cell cycle progression, overexpression of Myc has been found to increase mitochondrial mass while its depletion results in a reduction in mitochondria [75]. Myc has been discovered to induce the expression of respiratory genes by either binding directly to their promoters to upregulate their expression [75] or by inducing the expression of the mitochondrial biogenesis regulator PGC-1β [76]. This upregulation of mitochondrial biogenesis by Myc has been found to be suppressed by HIF-1α through mechanisms dependent and independent of the PGC1 coactivators [76, 77].

1.5 Mitophagy

Even though oxidative phosphorylation plays a critical role in ATP synthesis and energy production, it also produces reactive oxygen species (ROS) as by-product. While ROS serve physiological roles as signaling molecules, they pose a danger to the cell when in present excess. Due to their unstable and chemically reactive nature, ROS react with cellular components, chemically alter them and ultimately disrupt their functions. As the site of oxidative phosphorylation, mitochondria are thus highly susceptible to damage from ROS. Since mitochondria also play a part in cell death/survival, amongst many other important processes, it is therefore extremely important to remove damaged and dysfunctional mitochondria to prevent cell, and ultimately organ, dysfunction. The elimination of such dysfunctional mitochondria is achieved by mitophagy, the process of selective degradation of mitochondria by autophagy. Mitophagy is thus another process, in addition to mitochondrial biogenesis, that plays a key role in regulating mitochondrial abundance and function.

The best characterized pathway involved in mitophagy is the PINK1/Parkin pathway which degrades depolarized mitochondria [78 – 80]. PINK1 (PTEN-induced putative kinase 1) is a mitochondria-targeted serine/threonine kinase that is continuously synthesized and – in healthy mitochondria – is continuously cleaved by PARL (presenilin-associated rhomboid-like protein) for degradation [81]. In depolarized mitochondria, full-length PINK1 accumulates on the outer mitochondrial membrane where it recruits and activates the ubiquitin E3 ligase Parkin [82 – 84]. At the outer mitochondrial membrane, Parkin ubiquitinates various proteins on the outer mitochondrial membrane involved in mitochondrial fusion (MFN1 and MFN2) and motility (MIRO) to isolate the depolarized mitochondria and prevent them from fusing with the functional mitochondrial network [85 – 88]. The ubiquitination of the outer mitochondrial

proteins also recruits the autophagic machinery to enclose the depolarized mitochondria into autophagasomes – a process dependent on the autophagosome marker LC3 – for lysosomal degradation [89 – 92].

Mitophagy can also occur independently of the PINK1/Parkin pathway. In PINK1/Parkin-independent mitophagy, the autophagic machinery is recruited to mitochondria by direct autophagy receptors. BNIP3, NIX and FUNDC1 all localize to the outer mitochondrial membrane and have LIR (LC3-interacting region) motifs that enable them to interact with LC3 and recruit the autophagic machinery [93 – 95]. All three of these autophagy receptors have been found to regulate hypoxia-induced mitophagy. Under hypoxic conditions, BNIP3 and NIX and induced transcriptionally [96] and while FUNDC1 becomes dephosphorylated, which augments its ability to interact with LC3 [97]. In addition, NIX has been found to be required for eliminating mitochondria during red blood cell maturation [98, 99]. Proteins are not the only autophagy receptors, the inner mitochondrial membrane lipid cardiolipin has also been found to bind to LC3 upon externalization to the outer membrane to recruit the autophagic machinery.

1.6 Context for the Dissertation Work

The vast majority of longevity genes were discovered from simple organisms such as yeast, worms and flies whose short lifespan and genetic manipulability made it feasible to screen for long-lived mutants and identify the genes responsible for their lifespan extension. However, as these longevity genes were mainly identified in simple organisms, it is very likely that important mammalian longevity genes have yet to be identified. Conducting a screen for longevity genes in mammals would require thousands of mice and take a long time, making it cost prohibitive. One way to circumvent the issues of cost and time is to conduct the screen in mammalian cells instead of the actual organism. However, to conduct a screen in cells would require cellular phenotypes that correlate with aging and lifespan extension. Given that mitochondrial dysfunction is consistently observed in aging as well as age-related diseases and that oxidative metabolism is increased with calorie restriction, this suggests that increased mitochondrial function is a strong candidate phenotype to screen cells for longevity genes.

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Chapter 2

hORFeome screen for mitochondrial regulators identifies GLTSCR2/PICT1 as a positive regulator of mitochondrial function

Adapted from:

John C. Yoon[1,2,3,6], Alvin J. Y. Ling[1,4,6], Meltem Isik[1,5], Dong-Young Donna Lee[1,2], Michael J. Steinbaugh[1,5], Laura M. Sack[1,2], Abigail N. Boduch[1,2], T. Keith Blackwell[1,5], David A. Sinclair[1,4], and Stephen J. Elledge[1,2]. (2014). GLTSCR2/PICT1 links mitochondrial stress and Myc signaling. *Proc Natl Acad Sci USA* 111(10): 3781–3786.

[1]Department of Genetics, Harvard Medical School, Boston, MA 02115

[2]Howard Hughes Medical Institute, Division of Genetics, Brigham and Women's Hospital, Boston, MA 02114

[3]Department of Medicine, Massachusetts General Hospital, Boston, MA 02114

[4]Paul F. Glenn Laboratories for the Biological Mechanisms of Aging, Harvard Medical School, Boston, MA 02115

[5]Joslin Diabetes Center, Boston, MA 02115

[6]These authors contributed equally to this work

2.1 Introduction

In the eukaryotic cell, mitochondria generate energy to support cellular life and regulate diverse processes such as apoptosis and calcium signaling. Mitochondrial insufficiency can carry deleterious consequences, including impaired oxidative phosphorylation (OXPHOS) and reduced ATP synthesis, which can culminate in human disease [1, 2]. For example, respiratory chain disorders can be caused by inherited or spontaneous mutations in mitochondrial DNA or nuclear genes that encode respiratory chain subunits. Defects in oxidative phosphorylation may also occur as a secondary effect of mutations in genes encoding mitochondrial proteins involved in other aspects of mitochondrial physiology. Mitochondrial disorders commonly exhibit tissue selectivity and clinical heterogeneity, which may reflect varying bioenergetics thresholds of different cell types, intrinsic complexities of mitochondrial genetics and biochemistry, and environmental influences that introduce further variability. In addition to the primary mitochondrial disorders, mitochondrial dysfunction is implicated in a broad spectrum of age-related diseases such as neurodegeneration, metabolic syndrome, and cancer [1, 2]. That mitochondrial defects feature so prominently in a wide range of disease processes points to the potential utility of targeting this organelle for therapeutic purposes.

One possible approach to compensate for inherited or acquired mitochondrial respiratory defects may be to actively induce mitochondrial OXPHOS capacity. Several recent studies with mouse models of defective cytochrome c-oxidase activity have reported beneficial effects of genetic or pharmacological manipulations that enhance OXPHOS activity [3, 4]. Such manipulations help preserve ATP levels in mutant mouse tissues and in cultured cells from human patients, and have been observed to improve motor function in mice deficient in cytochrome c oxidase genes. Endurance exercise, which has been shown to counteract the

accelerated aging phenotype in the PolG mitochondrial mutator mice, restores mitochondrial abundance and cytochrome c-oxidase activity [5]. These results suggest that partial restoration of mitochondrial function in some disease states may be achievable by harnessing endogenous regulatory pathways controlling mitochondrial biogenesis or activity. It thus becomes worthwhile to ascertain which specific cellular pathways may be experimentally exploited for this purpose.

To address this issue, we carried out an unbiased, large-scale gain-of-function genetic screen to identify genes whose overproduction can enhance mitochondrial abundance or activity. Because traditional cDNA libraries commonly suffer from incomplete clones and skewed gene representation, we used the human ORFeome (hORFeome v5.1), a normalized collection of 15,483 human ORFs in the Gateway cloning system [6, 7]. To enable a high-throughput screening platform, we adapted the ORFeome library for use in a pooled format, allowing simultaneous evaluation of all ORFs, and deployed fluorescent mitochondria- selective probes as reporters of mitochondrial activity or abundance in live cells. We have identified 76 candidate genes that increase the mitochondrial reporter signals upon overexpression and 18 genes that have the opposite effect. These genes encode secreted factors, transcription factors, as well as predicted polypeptides of unknown function, and represent a resource that will facilitate further efforts to understand and manipulate mitochondrial regulatory mechanisms.

2.2 Results

2.2.1 A Genome-Scale Gain-of-Function Screen for Mitochondrial Regulators

Our strategy was to perform a high-throughput flow cytometry-based mitochondrial screen with an expression library containing the human ORFeome collection (Figure 2.2.1). Whereas large-scale RNAi screens have become commonplace in recent years, aided in part by the ease of transfecting siRNA oligonucleotides into most cell types, genome-scale gain-of-function screens have been hampered by the relative difficulty of introducing expression plasmids into mammalian cells at a high efficiency. We transferred the entire human ORFeome collection as a pool into a lentiviral expression vector (pHAGE T-Rex) to ensure efficient gene delivery and stable expression. The use of pooled ORF clones for lentiviral packaging and transduction was compatible with rapid, phenotype-based selection of cell subpopulations that we designed to be a central feature of the screen, as opposed to testing individual ORF clones arrayed in a microwell plate format. The human ORFeome offers superior gene representation relative to traditional genomic cDNA libraries and the viral titers are largely preserved across different ORF sizes ranging from 75 bp to 10.5 kb, with a median ORF size of 1 kb. We selected the viral transduction conditions that would provide an approximately 1,000-fold coverage of each ORF in the cell population being screened. The frequency of multiple integration events was reduced by using a relatively low multiplicity of infection (MOI = 1) at the time of lentiviral transduction and is mitigated by the representation of 1,000 for each given viral construct.

We performed the screen in C2C12 myoblast cells, which are a well-characterized, non-transformed cellular system that has been extensively used for studies of mitochondrial regulation in cultured cells. Following lentiviral transduction, antibiotic selection, and propagation for 3 days, we carried out flow-cytometry– based cell sorting in conjunction with

mitochondria-selective fluorescent reporters to enrich for genes that increase or reduce mitochondrial abundance or activity. Live cells were stained with two different mitochondria-selective probes, nonyl acridine orange (NAO) and MitoTracker deep red (MT-DR), and were then sorted to isolate subpopulations exhibiting concordant changes in the signal intensities of both reporters, thus increasing specificity. These two dyes have previously been found to be sensitive to both mitochondrial abundance and membrane potential [8–10].

We subsequently harvested genomic DNA from the high signal (overlap between the top 5% based on NAO fluorescence and the top 5% based on MT-DR fluorescence) and low signal (bottom 5% in NAO fluorescence and in MT-DR fluorescence) fractions collected by cell sorting and compared each of these fractions to the unsorted fraction by PCR amplifying and labeling the DNA samples with Cy3 and Cy5, and hybridizing competitively to custom microarrays. Following deconvolution of the microarray data, the ratio of the abundance of each ORF in the high signal fraction versus the unsorted fraction was used to identify a set of genes that were consistently enriched by more than 2.5-fold in two independent experiments. These genes represented potential positive regulators of mitochondrial abundance or activity. Likewise, we compared the enrichment in the low signal fraction versus the unsorted fraction to identify potential negative regulators. The candidate genes were then tested individually by lentiviral-mediated stable expression in C2C12 cells, which were assayed directly for NAO and MT-DR signals. We scored candidate genes as confirmed hits if there was more than a 20% increase in either NAO or MT-DR signal relative to cells transduced with the empty control vector. Based on these criteria, we obtained 76 positive mitochondrial regulators and 18 negative regulators.

These confirmed genes are a functionally diverse group. Based on the PANTHER gene ontology system, the majority of these genes were classified as being involved in metabolic

processes, cellular processes, and cellular communication. Represented protein classes included hydrolases, nucleic acid binding proteins, transferases, and transcription factors. Of interest were genes linked to positive regulation of cell communication (HTR2B, PTGS2, BCL10, ZDHHC13, GPC3, and ALS2), calcium ion transport and signaling (CACNA2D1, CASQ1, PLCG2, and PTGS2), and cellular stress response (NFE2L2 and EIF2AK4), each of which may have significant connections to mitochondrial physiology. The composition of the validated gene subset is consistent with the idea that mitochondrial abundance and activity are subject to modulation by a myriad of external effector signals and intracellular signaling cascades converging upon metabolic pathways.

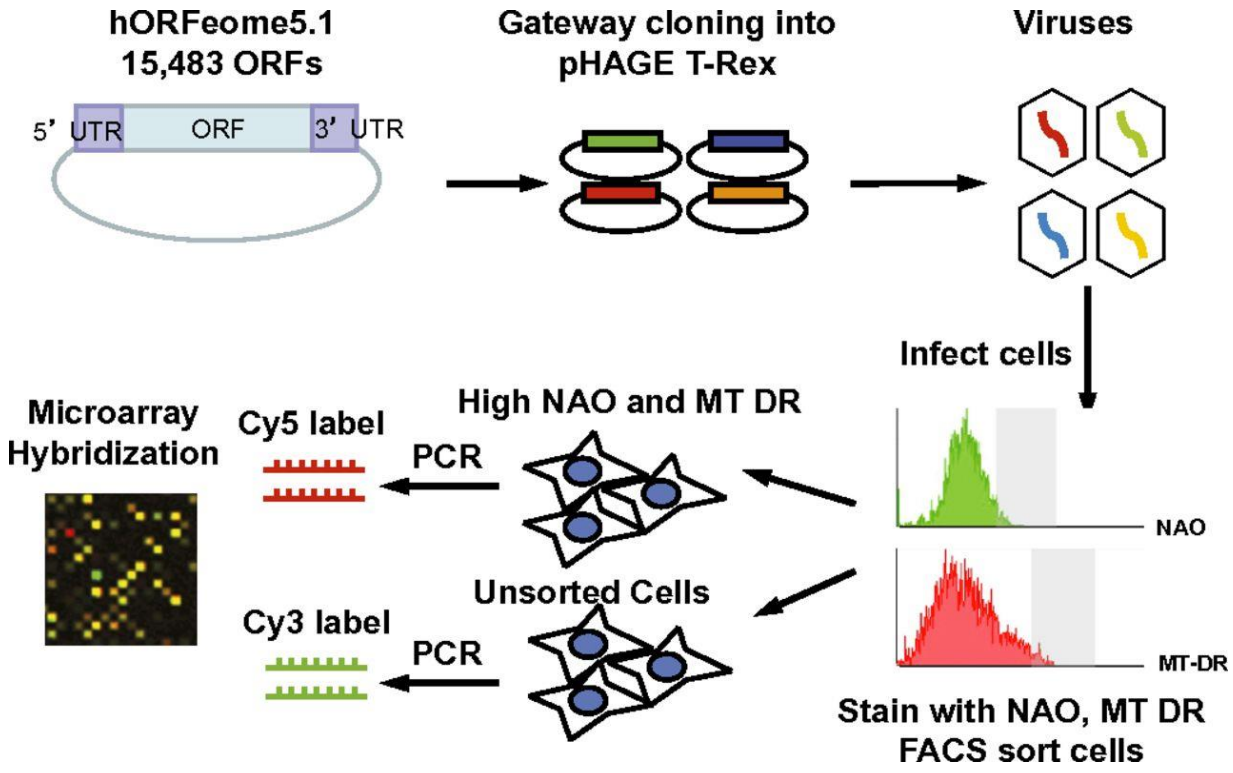

Figure 2.2.1: Schematic diagram of the genome-scale overexpression screen.
The human ORFeome collection was cloned into the lentiviral vector pHAGE T-Rex by Gateway cloning and packaged into viruses. C2C12 mouse myoblast cells were transduced with the viruses, selected, and stained with NAO and MitoTracker deep red dyes for FACS sorting. Microarray hybridization was used to compare the genomic DNA samples collected from high NAO, high MT-DR cells (shown as grayed out areas in the FACS profiles) vs. the unsorted cells. Similarly, the low NAO, low MT-DR cells and the unsorted cells were compared.

2.2.2 Measurements of Mitochondrial Membrane Potential and Assessment of Loss-of-Function Phenotypes

To further examine the effects of these 94 reconfirmed candidates on mitochondrial physiology, we measured gene overexpression-induced changes in mitochondrial membrane potential using the potentiometric dye tetramethylrhoadamine methyl ester (TMRM), which distributes within polarized mitochondria in a Nernstian fashion. The majority of the genes we tested produced only small alterations in mitochondrial membrane potential relative to the control, with less than a 20% change in magnitude (Figures 2.2.2 A and B). We suspect that one reason for this low percentage may be that genes causing much larger changes in mitochondrial membrane potential may have reduced cell growth and viability. Dissipation of mitochondrial membrane potential interferes with ATP production and is often seen in early apoptosis, whereas high mitochondrial membrane potential may excessively increase reactive oxygen species production. In contrast, the NAO and MT-DR signals showed much wider variations (Figures 2.2.2C and D). Whereas nearly all of the confirmed genes showed simultaneous increases or decreases in both NAO and MT-DR signal intensities, as expected from the constraints imposed at the time of cell sorting, the correlation between the two reporters is not strong ($r = 0.10$), suggesting significant differences in the properties of the two reporters (Figure 2.2.2E).

To identify genes that may potentially serve as a control point in mitochondrial regulation, we also assessed loss-of-function phenotypes by small interfering RNA (siRNA)-based gene depletion in conjunction with flow cytometry. We reasoned that a given gene may be a key regulator of mitochondrial function in vivo if its depletion had the opposite effect of its

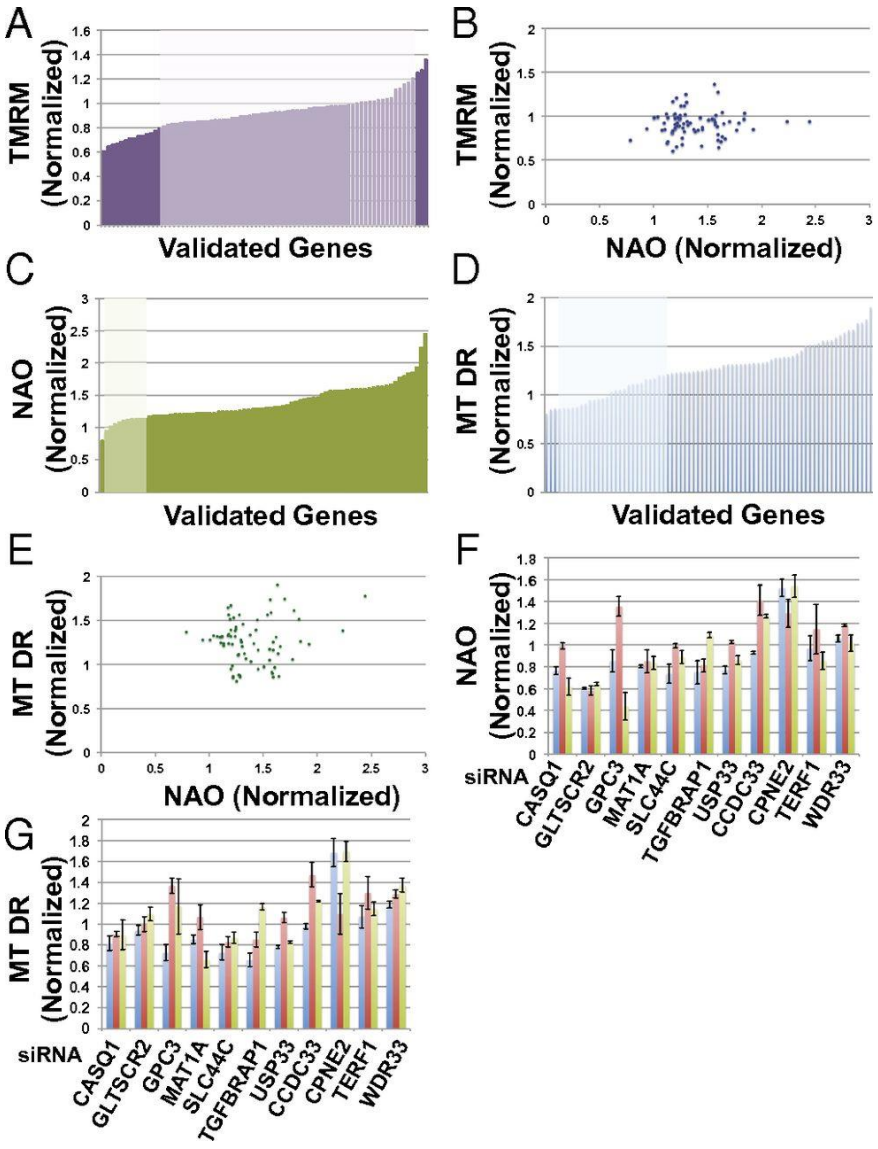

Figure 2.2.2: Further analysis of the confirmed hits from the screen using secondary assays.
(A) Distribution of TMRM signals among the 94 confirmed hits expressed in C2C12 cells. The genes that produce less than a 20% change in the TMRM signal relative to the control cells are colored in a lighter shade. (B) Distribution of the NAO vs. TMRM signals among the 94 confirmed hits. (C) Distribution of NAO signals among the 94 confirmed hits. The genes whose overexpression produce less than a 20% change in the NAO signal relative to the control cells are colored in a lighter shade. (D) Distribution of MitoTracker deep red signals among the 94 confirmed hits. The genes that produce less than a 20% change in the MT-DR signal relative to the control cells are colored in a lighter shade. (E) Distribution of the NAO versus MitoTracker deep red signals among the 94 confirmed hits. (F) NAO signal values for the 11 genes that scored in the mini-RNAi screen in IMR90 cells with at least two of three independent siRNAs. (G) MitoTracker deep red signal values for the 11 genes that scored in the mini-RNAi screen in IMR90 cells with at least two of three independent siRNAs indicated by different colored bars.

overproduction. A mini-RNAi screen targeting the 94 confirmed hits was performed in IMR90 human primary fibroblast cells using siRNAs targeting the corresponding human orthologs, thus also evaluating functional conservation across distinct cell types and species. We considered a gene to score in the mini-RNAi screen if at least two of three independent siRNA oligonucleotides targeting the same gene produced a significant change in the NAO or MT-DR signal, compared with the control siRNA. Eight such genes produced a statistically significant ($P < 0.05$) loss-of-function phenotype that was the opposite of the gain-of-function phenotype noted in the ORFeome screen, and three came close ($0.05 \leq P < 0.1$) (Figures 2.2.2F and G). Seven of these 11 genes (CASQ1, GLTSCR2, GPC3, MAT1A, SLC44A5, TGFBRAP1, and USP33) were predicted to be positive mitochondrial regulators, and the other four (CCDC33, CPNE2, TERF1, and WDR33) negative regulators. This subset of 11 genes included genes involved in calcium-mediated signaling, cell adhesion, transcription, proteolysis, and other cellular processes.

2.2.3 GLTSCR2 Regulates Mitochondrial Respiration

We focused on glioma tumor-suppressor candidate region gene 2 (GLTSCR2, also known as PICT1), because it displayed consistency between the loss- and gain-of-function phenotypes and has previously been implicated in cancer [11]. GLTSCR2 significantly increased both NAO and MT-DR signals when overexpressed in C2C12 cells in the ORFeome screen (Figure 2.2.3A). siRNAs targeting GLTSCR2 decreased the NAO signals in the mini-RNAi screen we subsequently performed in IMR90 cells (Figure 2.2.2F). These results suggested conservation of function across multiple cell types. For a more direct assessment of mitochondrial OXPHOS activity, we examined oxygen consumption rates in intact cells.

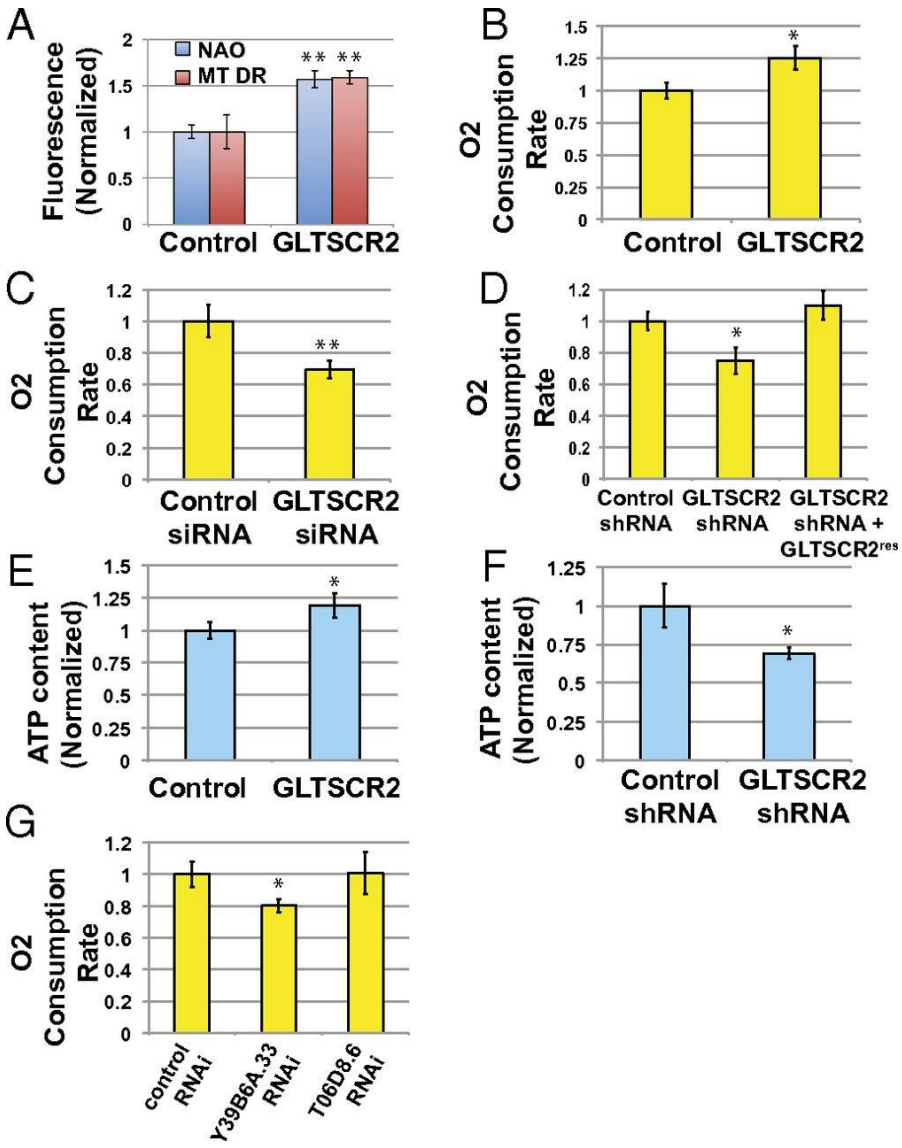

Figure 2.2.3: GLTSCR2 regulates mitochondrial respiration in primary human fibroblasts and in the nematode C. elegans.

(A) Stable expression of GLTSCR2 cDNA in C2C12 cells increases both NAO and MitoTracker Deep Red signals. (B) Stable expression of GLTSCR2 cDNA in IMR90 cells increases oxygen consumption. (C) Depletion of GLTSCR2 in IMR90 cells by siRNA transfection reduces oxygen consumption. Cells were examined 4 d after transfection with the Seahorse XF24 flux analyzer. (D) Depletion of GLTSCR2 in IMR90 cells by stable shRNA expression reduces oxygen consumption, which can be rescued by expressing an RNAi-resistant GLTSCR2 cDNA. (E) Stable expression of GLTSCR2 cDNA in IMR90 cells increases cellular ATP content. (F) Depletion of GLTSCR2 in IMR90 cells by stable shRNA expression reduces cellular ATP content. (G) RNAi inactivation of the GLTSCR2 ortholog Y39B6A.33 in C. elegans reduces oxygen consumption. *P < 0.05 by unpaired t test; **P < 0.01.

Measurements of cellular oxygen consumption in IMR90 fibroblasts stably expressing GLTSCR2 showed a 25% enhanced respiration relative to the control (Figure 2.2.3B). Depletion of GLTSCR2 in IMR90 fibroblasts by siRNA reduced oxygen consumption by 30% (Figure 2.2.3C), and similar results were obtained with shRNA-based depletion (Figure 2.2.3D). This phenotype could be rescued by expressing an shRNA-resistant cDNA (Figure 2.2.3D). The cellular ATP levels appeared to reflect the changes in respiration in IMR90 cells occurring upon stable overexpression of the GLTSCR2 cDNA or depletion by shRNA (Figures 2.2.3E and F).

These data indicate that GLTSCR2 acts to regulate respiration in mammalian cells but whether this function is evolutionarily conserved is unknown. To evaluate a potentially conserved role for GLTSCR2 across species, we assayed oxygen consumption rates of *Caenorhabditis elegans* worms undergoing RNAi inactivation of Y39B6.33, the *C. elegans* ortholog of GLTSCR2, and found lower oxygen consumption rates relative to the control RNAi worms (Figure 2.2.3G). RNAi inactivation of another mitochondrial gene, T06D8.6, did not produce a change of similar magnitude, consistent with previous results [12]. Together, these findings suggest that GLTSCR2 has a physiological role in regulating mitochondrial respiration that is functionally conserved across different species in an in vivo setting.

2.2.4 GLTSCR2 Controls Cellular Proliferation and Respiration via the Transcription Factor Myc

The role of GLTSCR2 in cancer appears to be complex and may depend upon cellular context. GLTSCR2 was initially proposed to be a potential tumor suppressor based on its location on chromosome 19q13.32, which is frequently deleted in human tumors, particularly in gliomas, and its interaction with phosphatase and tensin homolog (PTEN) [13, 14].

Overexpression of GLTSCR2 in glioblastoma cell lines was reported to induce apoptotic cell death (15). However, it was recently suggested that GLTSCR2 promotes oncogenesis based on the observation that the GLTSCR2+/− mice were more resistant to chemically induced skin cancers [11]. GLTSCR2 null ES cells accumulated p53 and underwent apoptosis, and shRNA-mediated depletion of GLTSCR2 in cancer cell lines induced p53 as well [11]. This group also noted that lower expression of the GLTSCR2 mRNA transcript correlated with improved survival in some colorectal and esophageal cancers with intact p53.

To further investigate a possible role for GLTSCR2 in cancer, we examined the effect of overexpressing or depleting GLTSCR2 in IMR90 primary fibroblasts. Unexpectedly, we found that siRNA-mediated depletion of GLTSCR2 significantly reduced p53 levels in IMR90 primary cells, as did stable expression of an shRNA targeting a different region of the GLTSCR2 gene (Figure 2.2.4A). Stable overexpression of GLTSCR2 induced p53 (Figure 2.2.4B). Despite elevated basal levels of p53, the GLTSCR2-overexpressing IMR90 cells proliferated substantially faster than the control cells, implying a potential involvement of a growth-promoting effector protein (Figure 2.2.4C). We thus considered possible downstream target proteins that may impact upon cell proliferation and mitochondrial respiration.

GLTSCR2 was previously shown to physically interact with RPL11 and sequester it in the nucleolus [11]. Others have reported that RPL11 inhibits Myc activity by competing with Myc coactivators at the target gene promoters and also reduces Myc levels by binding and destabilizing the Myc mRNA [16–18]. This suggested that GLTSCR2 may regulate Myc. We observed that overexpression of GLTSCR2 in IMR90 cells induces Myc protein levels, whereas depletion of GLTSCR2 by RNAi reduced Myc levels in these cells (Figures 2.2.4A and B). We also confirmed the interaction between GLTSCR2 and RPL11 (Figure 2.2.4D). Furthermore,

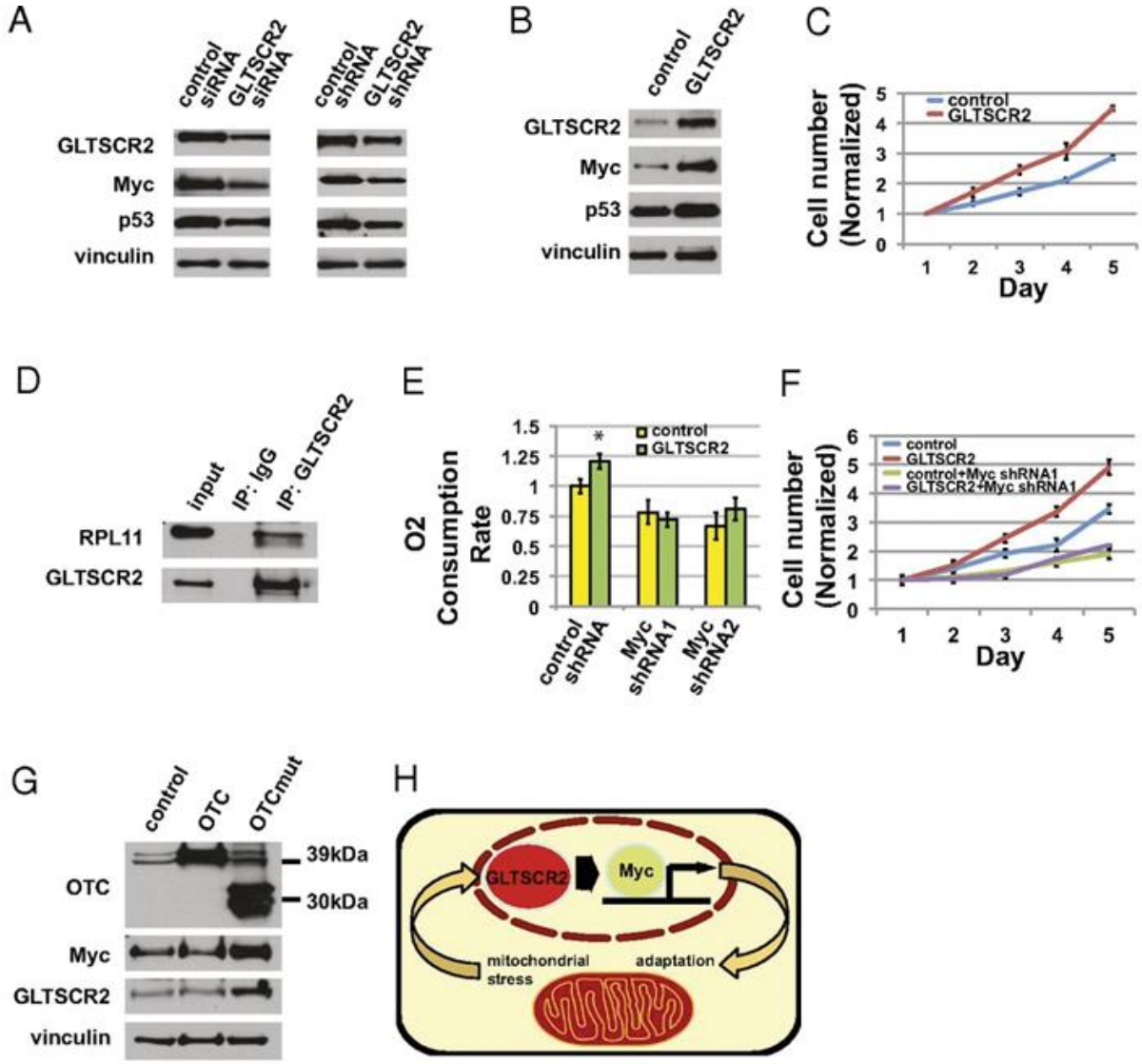

Figure 2.2.4: GLTSCR2 responds to mitochondrial stress and controls cell proliferation and respiration via Myc.

(A) Depletion of GLTSCR2 in IMR90 cells by siRNA transfection (Left) or by stable shRNA expression (Right) induces Myc and p53 levels. Protein samples were isolated 72 h after siRNA transfection. (B) Stable expression of GLTSCR2 in IMR90 cells induces Myc and p53 levels. (C) GLTSCR2 increases cell proliferation in IMR90 cells. Cells were counted in triplicates every day for 5 consecutive days. (D) GLTSCR2 interacts with RPL11. The 293T-Rex cells stably carrying HA-GLTSCR2 under a tetracycline-inducible promoter were treated with doxycycline for 24 h and the lysates were immunoprecipitated with anti-HA antibody or IgG. (E) Depletion of Myc in IMR90 cells by stable shRNA expression eliminates GLTSCR2-mediated increases in oxygen consumption. (F) Depletion of Myc in IMR90 cells by stable shRNA expression abolishes GLTSCR2-mediated increases in cell proliferation. Cells were counted in triplicates every day for 5 consecutive days. (G) Expression of mutant OTC in IMR90 cells induces GLTSCR2 and Myc expression. The wild-type OTC protein runs at 39 kDa, whereas the deletion mutant lacking amino acids 30–114 runs at 30 kDa. (H) Model of mitochondrial stress signaling involving GLTSCR2 and Myc.

depletion of Myc by RNAi abrogated GLTSCR2- mediated effects on cell proliferation and oxygen consumption in IMR90 cells (Figures 2.2.4E and F). Myc activation is known to induce p53 in normal human fibroblasts via p14 alternate reading frame (p14ARF)-independent mechanisms, which may explain the concomitant increase in p53 levels in GLTSCR2-expressing IMR90 cells [19]. These results argue that the induction of Myc is a key downstream event in GLTSCR2 signaling and that GLTSCR2 may act to promote proliferation and possibly oncogenesis through Myc.

2.2.5 GLTSCR2 Is Regulated by Mitochondrial Stress

GLTSCR2 is primarily localized to the nucleolus, where the production of ribosomal subunits must be carefully coordinated with changing cellular needs and external signals [20]. The nucleolus has increasingly become recognized as a sensor and integrator for several forms of cellular stress [21]. We were therefore interested to know if GLTSCR2 could be subject to regulation by stresses emanating from mitochondria. Impaired mitochondrial function is known to activate stress response pathways that signal to the nucleus and set off nuclear changes [22]. An important example of mitochondrial stress signaling is triggered by misfolded mitochondrial proteins, analogous to the unfolded protein response activated in the endoplasmic reticulum in response to proteotoxic stress in that organelle. In mammalian cells, the mitochondrial unfolded protein response has been primarily studied using overexpression of a deletion mutant form of the mitochondrial matrix protein ornithine transcarbamylase (OTC) [23]. We found that GLTSCR2 is induced by overexpression of the mutant OTC with a concomitant induction of Myc (Figure 2.2.4G). This suggests the possibility that GLTSCR2 and Myc may be part of the mitochondrial unfolded protein response.

Together, our data are consistent with the model that mitochondrial stress signaling pathways activate the GLTSCR2–Myc axis by mechanisms yet to be determined, resulting in adaptive responses (Figure 2.2.4H). In this context, it is noteworthy that Myc is known to enhance mitochondrial respiration, and importantly, also to stimulate glycolysis, which can provide an alternative source of energy for cellular needs even when oxidative phosphorylation remains impaired [24].

2.3 Discussion

Mitochondrial function is central to cellular physiology and human health. Understanding how mitochondria are regulated is therefore a major research goal in cell biology. Here we have performed a gain-of-function genetic screen for proteins that alter mitochondrial abundance or function. Using the human ORFeome as a pool of high complexity allowed unbiased, simultaneous screening of tens of millions of lentiviral particles carrying some 15,483 human ORFs in the target cell population. The lentiviral expression system provided highly efficient transduction and long-term gene expression. The strategies for enrichment, based on the fluorescent intensities of mitochondria-selective reporters, and the microarray-based strategies for recovery of the ORF identities produced a set of 94 candidate proteins involved in mitochondrial regulation.

Among the candidate proteins identified in the screen, we focused on the nuclear protein GLTSCR2 and have demonstrated that it controls mitochondrial respiration in primary human cells. Depletion of GLTSCR2 reduces mitochondrial respiration, whereas increasing its levels enhances respiration. The role of GLTSCR2 in regulating oxygen consumption is conserved across evolution as reduction of the GLTSCR2 ortholog in *C. elegans* also lowers oxygen consumption. Thus, GLTSCR2 possesses several of the hallmarks of a physiological regulator of mitochondrial respiration in mammals.

How GLTSCR2 executes its regulation of mitochondria is a key question. Our observations point to Myc as a main link between GLTSCR2 and its effects on cellular respiration and proliferation. Furthermore, the GLTSCR2 and Myc protein levels are regulated by mitochondrial proteotoxic stress. Consistent with our observations, Myc has previously been reported to be up-regulated in response to mitochondrial dysfunction associated with depletion of

mitochondrial DNA in mammalian cell lines [25, 26]. Other studies have suggested that interfering with Myc's activity may contribute to mitochondrial dysfunction in aging. Myc controls mitochondrially encoded OXPHOS genes by directly activating mitochondrial transcription factor A, and a pseudohypoxic state induced during aging may cause a decline in mitochondrial function via disruption of Myc's activity [27].

The induction of Myc in conditions of mitochondrial stress may serve an adaptive function of stimulating glucose uptake and glycolysis to compensate for the impairment of oxidative phosphorylation, while also attempting to restore mitochondrial activity. Previous studies have suggested that GLTSCR2 is induced by genotoxic stressors, such as ionizing radiation and UV radiation, and low-dose actinomycin D, which is thought to cause ribosomal stress by inhibiting rRNA synthesis [28, 29]. It is possible that some of these agents may also impinge upon mitochondrial function and activate pathways leading to the induction of GLTSCR2.

It remains unknown how mitochondrial stress regulates the GLTSCR2 levels. The mitochondrial unfolded protein response has been primarily associated with misfolded mitochondrial proteins, but it is now known that this pathway can be activated by multiple types of mitochondrial stress that perturb the balance of mitochondrial proteins, such as loss of mitochondrial DNA, deficiencies in certain electron transport proteins, or disruption of the mitochondrial import machinery [30]. In the nematode *C. elegans*, the mitochondrial unfolded protein signaling is apparently required for the lifespan-extending effect of electron transport chain defects in that organism [31]. The mitochondrial unfolded protein response in mammalian cells remains poorly understood at a mechanistic level, representing a wealth of opportunities to obtain new insights into this crucial signaling pathway. Overall, much remains to be learned

about the signaling pathways to and from mitochondria, and as the present study illustrates, systematic screens for the identification of regulatory proteins in the mitochondrial signaling networks provide a potentially useful approach.

2.4 Materials and Methods

Constructs and Reagents

Antibodies were purchased from commercial vendors including GLTSCR2 (Abnova), p53 (EMD), Myc (Cell Signaling), RPL11 (Abcam), OTC (Novus), and vinculin (Sigma).

Cell Culture

C2C12 cells (American Type Culture Collection) were maintained in DMEM containing 10% (vol/vol) FCS (Invitrogen). IMR90 cells were maintained in DMEM with 10% FCS in a low-oxygen (3%) incubator.

Lentiviral Library Screening

The human ORFeome collection version 5.1 (hORFeome v5.1) was used for this study. The ORFs were divided into 10 separate subpools and transferred to the pHAGE T-Rex lentiviral expression vector via the LR recombinase reaction. The pHAGE T-Rex vector combines the promoter and the DEST cassette regions of pT-Rex DEST 30 (Life Technologies; 12301-016) and the backbone of the pHAGE-TRE-HA-Puro-DEST vector. The expression vectors containing the ORFs were transfected into 293T cells along with packaging plasmids to produce lentiviral supernatants. The viruses were collected 48 h after transfection, filtered with a 0.45-mm filter, and stored at −80 °C. The titers were determined for each subpool. Fifteen million C2C12 cells were transduced with pooled viruses in the presence of 5 μg/mL polybrene (Sigma) with an average representation of 1,000 cells per ORF at a MOI of 1. The viruses were removed after an overnight incubation. The transduced cell populations were selected with 2 μg/mL of puromycin and propagated until they reached a number of ∼100 million cells. Cells were then

stained with NAO and MitoTracker deep red (Invitrogen) by incubation at 37 °C for 30 min, collected by trypsinization, and sorted by flow cytometry (BD FACSAria II) based on the NAO and MitoTracker signal intensities. The high signal (top 5% in both NAO and MT-DR) and the low signal (bottom 5% in both NAO and MT-DR) fractions were collected and genomic DNA was harvested from these samples as well as the unsorted cells.

Microarray Hybridization

The ORF inserts were amplified from genomic DNA by PCR using Ex Taq HS (Takara), using the forward primer GATCCCTACCGGTGATATCC and the reverse primer TAATACGACTCACTATAGGGAGAC. The PCR products were used as templates for in vitro transcription with a MEGAscript kit (Ambion). The RNA probes were then labeled with Cy3 and Cy5 with a ULS labeling kit (Kreatech), column purified, fragmented at 60°C, and hybridized to custom microarrays synthesized by Agilent. Each ORF was represented by an average of three different probes on the microarray and nearly all ORFs (>95%) were detected. Scanning and feature extraction were performed with an Agilent DNA microarray scanner (G2505C).

Validation Screen

The individual ORFs were shuttled into the pHAGE T-Rex vector using LR Clonase. Viral supernatants were produced as described above and cells were stained with NAO and MitoTracker deep red, and analyzed with a BD LSRII flow cytometer. The NAO signal was collected using the FITC channel at 530 nm, whereas the MitoTracker deep red signal was collected using the APC channel.

Mini siRNA Screen

siRNAs targeting the 94 confirmed hits were cherry picked from the Ambion Silencer Select Human Genome siRNA Library and were reformatted into 96-wells at the Harvard Medical School Institute for Chemistry and Chemical Biology. At least three distinct siRNAs targeted each gene. The siRNAs were transfected into IMR90 cells with Lipofectamine RNAiMax (Invitrogen) at a 15-nM final concentration. After 5 d, cells were stained with NAO and MitoTracker deep red, trypsinized, and analyzed with a BD LSRII flow cytometer.

Western Analysis

Whole cell extracts were prepared by cell lysis, and equal amounts of lysates were resolved on SDS/PAGE, transferred to Immobilon-P membrane (Millipore), and probed with the appropriate antibodies. The proteins were visualized by ECL chemiluminscence (Pierce). Oxygen Consumption

Measurements in Cells and in C. elegans

Real-time measurements of oxygen tension and pH in cultured cells were obtained in 24-well microplates using the XF24 flux analyzer (Seahorse Bioscience). The oxygen consumption rate in each well was calculated from these measurements and normalized to protein content from cell lysates. For the C. elegans studies, synchronized 1-d-old adult worms were fed bacteria expressing dsRNA targeting Y39B6A.33 or T06D8.6, or an RNAi control for 3 d. Culture plates contained 5-fluorodeoxyuridine to prevent egg hatching.Worms were washed off the plates with M9 buffer and pipetted into 24-microplates for oxygen consumption rate measurements with the XF24 flux analyzer. Each well contained 100 worms, with five replicate wells per condition. The

oxygen consumption rate was normalized to protein content from worm lysates. Each experiment was performed twice.

ATP Measurements

Cellular ATP content was determined using ATP Bioluminescence Assay kit HS (Roche) using quadruplicate samples.

Immunoprecipitation of Protein Complexes

293T-Rex cells stably carrying HA-GLTSCR2 under a tetracycline-inducible promoter were treated for 24 h with 2 μg/mL doxycycline, washed with PBS, and lysed in 1× lysis buffer (20mM Tris·HCl at pH 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA). Immunoprecipitation was performed with anti-HA antibody or mouse IgG conjugated to agarose beads (Sigma).

Gene Overexpression and Depletion

For transient gene depletion, nontargeting control siRNA (D-001210-02-05) and GLTSCR2 siRNA were purchased from Dharmacon and transfected into cells with Lipofectamine RNAiMax at a 15-nM concentration. The protein and gene expression were analyzed 3 d following siRNA transfection.

For stable lentiviral expression of cDNAs or shRNAs, viral supernants were prepared by transfecting 293E cells with packaging plasmids as described above. The shRNA hairpin inserts included the following sequences:

Nontargeting control: CCTAAGGTTAAGTCGCCCTCG

GLTSCR2 shRNA: AAGTCCAGAAGAAGTCACTGC

Myc shRNA1: TTGAGGCAGTTTACATTATGG

Myc shRNA2: TTTAAGGATAACTACCTTGGG

Cell Growth Measurement

IMR90 cells were seeded at 75,000 cells per 60-mm dish and counted in triplicates each day for 5 consecutive days using a Z2 Coulter cell counter.

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Chapter 3

Discovery of Cpne2 as a regulator of mitochondria, autophagy and lysosomal biogenesis

3.1 Introduction

Mitochondria are organelles that are involved in a myriad of important cellular processes such as oxidative metabolism, cell signaling, macromolecular biosynthesis and cell survival. Their impact on health is most apparent in mitochondrial diseases – a collection of disorders caused by genetic mutations that result in mitochondrial dysfunction [1]. Mitochondrial diseases also highlight the complex nature of the mitochondria as the affected organs and the clinical manifestations of these diseases are highly diverse. These clinical manifestations include stroke-like episodes in mitochondrial encephalopathy lactic acidosis and strokes (MELAS), vision loss in Leber hereditary optic neuropathy (LHON) and pancreas dysfunction in Pearson Syndrome [2]. Even in the absence of hereditary genetic mutations, mitochondria have been found to become dysfunctional with age [3]. In fact, mitochondrial dysfunction has implicated in many age-related diseases such as heart failure, neurodegeneration, cancer and metabolic syndrome [3]. Given that mitochondria are involved in many diverse inherited and acquired diseases, there has been great interest in finding ways to increase mitochondrial function to treat these diseases.

Indeed, by targeting the mitochondrial biogenesis pathway, compounds that increase mitochondrial oxidative metabolism have successfully been identified. These compounds include PPAR ligands, SIRT1 activators and AMPK agonists [4 – 12]. However, by modulating a specific pathway, target-based drug discovery is limited in scope. In order to perform its many aforementioned functions, mitochondria must respond to a variety of cellular signals in an integrated manner and therefore must be supported by an extensive coordinated regulatory network. Furthermore, as alluded to by the heterogeneous nature of mitochondrial diseases, the regulation and composition of mitochondria vary between different tissues in order to perform tissue-specific functions. This adds an additional layer of complexity for advancing

mitochondrial therapeutics. Thus, in addition to target-based drug discovery, it is important to discover novel mitochondrial regulatory pathways to enable more extensive modulation of the mitochondrial regulatory network and to expand the repertoire of drug targets [13].

In order to discover novel mitochondrial pathways, we carried out a screen to find mitochondrial regulators [14]. From the screen, we identified Copine 2 (Cpne2) as a negative regulator of mitochondrial mass and function. The Copine family of proteins was discovered as calcium-dependent phospholipid binding proteins over a decade ago [15]. However, since then, not much is known about the cellular role of Cpne2. Here, we report that depletion of Cpne2 results in an increase in mitochondrial mass and function. Rather than upregulating the mitochondrial biogenesis pathway, this increase in mitochondria was found to be due to a reduction in the elimination of mitochondria. Yet, there was no defect in depolarization-induced mitophagy as cells with Cpne2 knocked down could still clear their mitochondria upon depolarization. These findings indicate that there is a basal removal of mitochondria and that it is decreased upon Cpne2 knockdown.

While investigating its effects on the mitochondria, we noticed that knockdown of Cpne2 also increases autophagy at both the level of induction and gene expression. Yet to our surprise, upstream energy and nutrient signaling pathways known to induce autophagy were unaltered. Depletion of Cpne2 also results in increased lysosomal biogenesis, a response frequently coordinated with upregulated autophagy. Although the MiT/TFE transcription family members TFEB and TFE3 were discovered to positively regulate this process [16 – 18], we found that they did not mediate in the lysosomal phenotype brought about by Cpne2 knockdown. Thus, our findings point to Cpne2 as a regulator of mitochondria, autophagy and lysosomal biogenesis through novel, uncharacterized mechanisms.

3.2 Results

3.2.1 Cpne2 knockdown increases mitochondrial mass

Cpne2 was identified out of the ORFeome screen for mitochondrial regulators as a negative regulator of mitochondrial mass [14]. Knockdown of Cpne2 using two separate shRNAs in C2C12 myoblasts increased mitochondrial mass compared to the non-targeting hairpin control as measured by the fluorsescence of the mitochondrial probes nonyl-acridine orange (NAO) and MitoTracker Deep Red FM (Figure 3.2.1A). Although C2C12 myoblasts are most often differentiated into myotubes for experimental purposes, Cpne2 knockdown C2C12 myoblasts were unable to differentiate and thus the myoblast form was used for the subsequent experiments. Consistent with the results from fluorescent probes, Cpne2 knockdown cells also had more mtDNA copy number and mitochondrial proteins compared to control cells (Figures 3.2.1B and C). Direct visualization by electron microscopy revealed that the mitochondria in Cpne2 knockdown cells were more numerous and more electron-dense (Figures 3.2.1E and F), indicating that the mitochondria were in a less swollen state and contained more proteins.

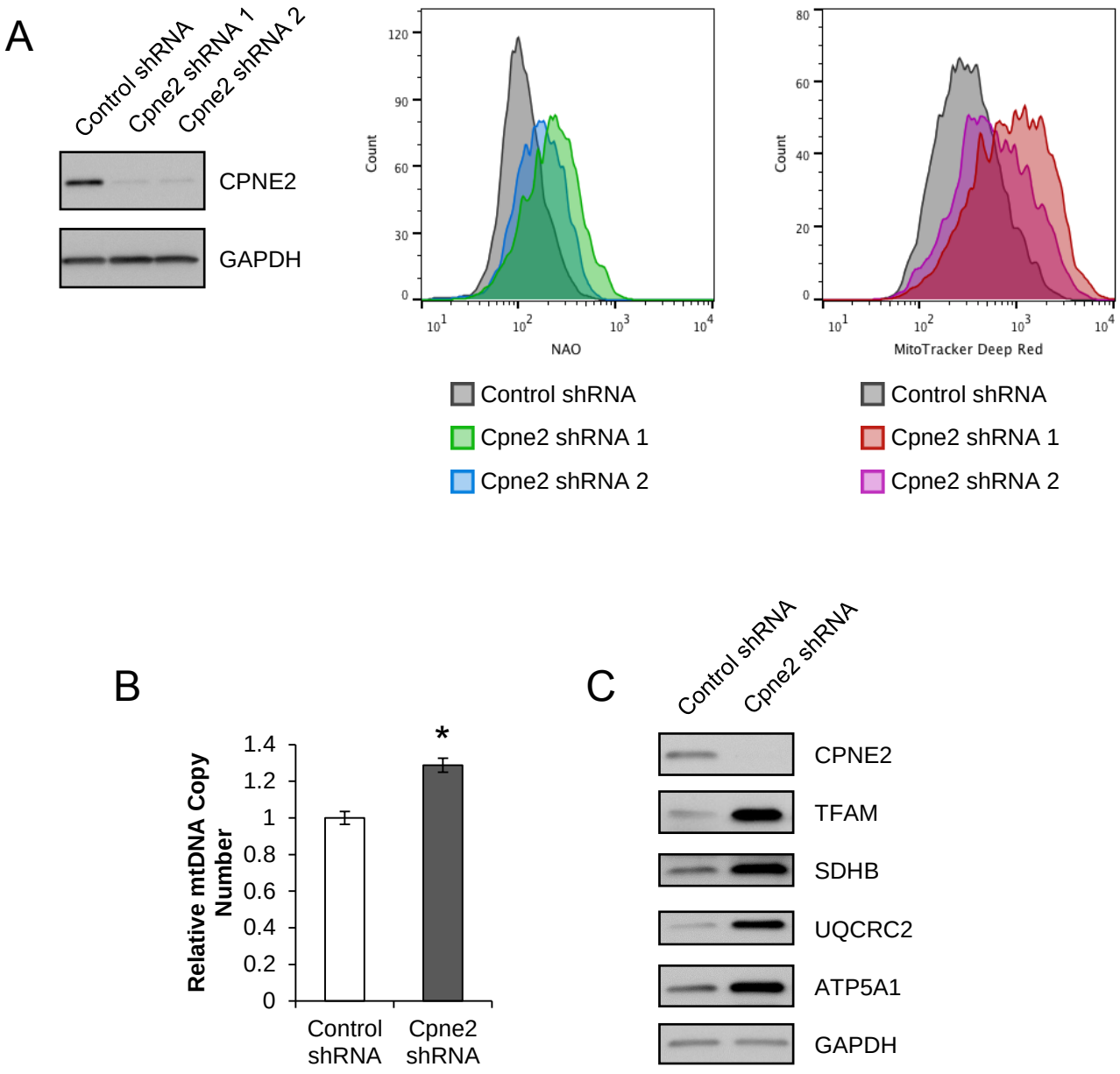

Figure 3.2.1: Cpne2 knockdown increases mitochondrial mass
(A) Stable knockdown of Cpne2 in C2C12 cells increases both NAO and MitoTracker Deep Red fluorescence signals. (B) Stable knockdown of Cpne2 in C2C12 cells increases mtDNA copy number. (C) Stable knockdown of Cpne2 in C2C12 cells increases mitochondrial proteins. Values are expressed as mean ± SEM. * $p<0.05$.

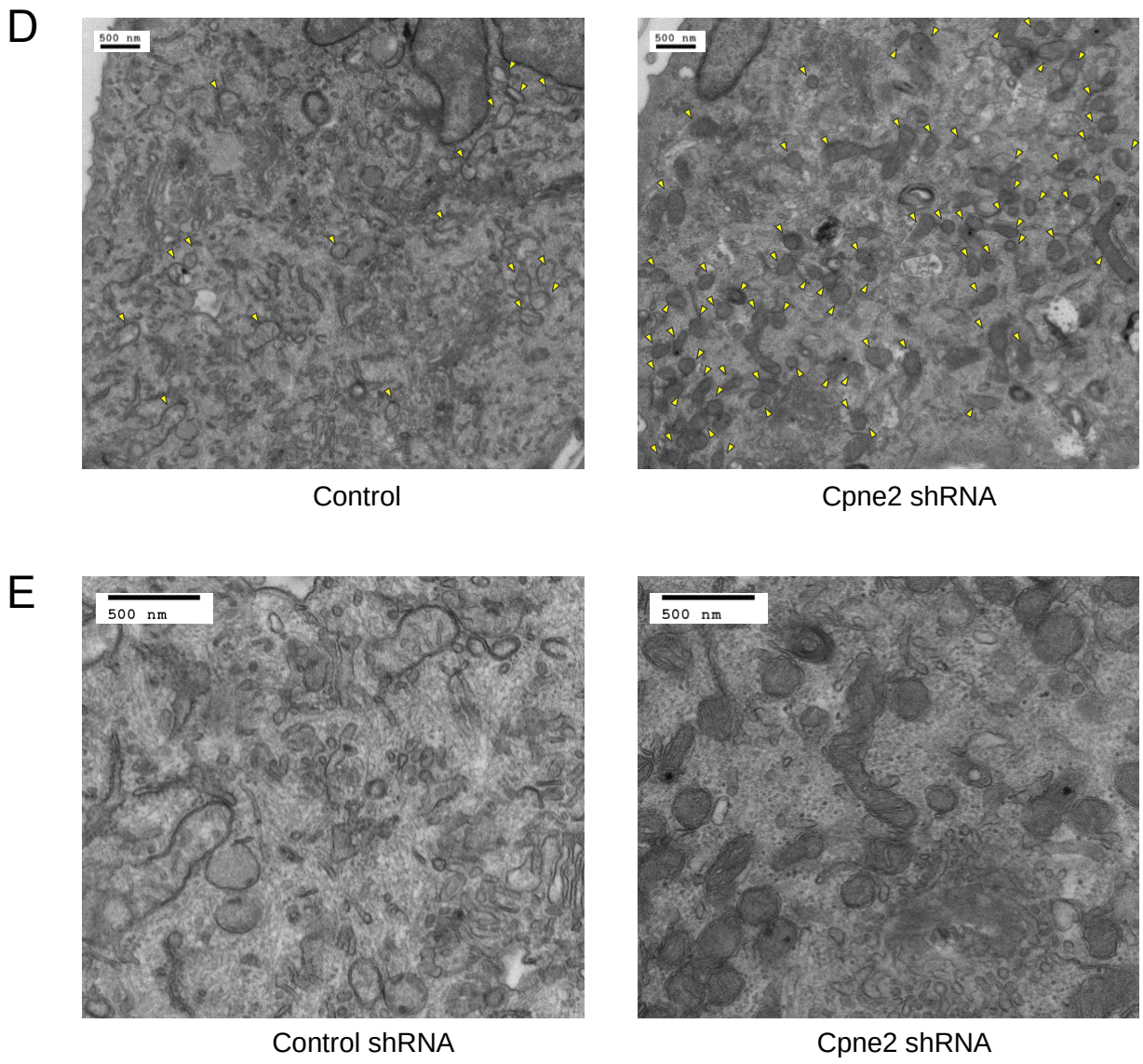

Figure 3.2.1 continued: Cpne2 knockdown increases mitochondrial mass
(D) Electron micrographs of control and Cpne2 knockdown cells at 6800x magnification. Mitochondria are marked by yellow arrows. (E) Ultrastructure of mitochondrial in control and Cpne2 knockdown cells in electron micrographs at 11000x magnification.

3.2.2 Cpne2 knockdown increases mitochondrial function

An increase in mitochondria may be either due to an actual increase in functional mitochondria or a compensatory response to mitochondrial dysfunction. To determine which of the two occurred upon Cpne2 knockdown, we measured oxygen consumption, mitochondrial membrane potential and ATP production, all of which collectively help give a more thorough assessment of oxidative phosphorylation.

In terms of oxygen consumption, cells with Cpne2 knockdown were found to have increased basal oxygen consumption compared to control cells (Figure 3.2.2A). This result indicates that electron transport chain activity is increased upon Cpne2 depletion. This increase in oxygen consumption is not due to an increase in uncoupling because Cpne2 knockdown cells did not have a lower mitochondrial membrane potential ($\Delta\Psi$). Instead, Cpne2 knockdown cells actually had increased $\Delta\Psi$ when compared to control cells (Figure 3.2.2B). While increased $\Delta\Psi$ can be caused by a block in ATP-synthase, this would result in a drop in oxygen consumption, which was not observed in Cpne2 knockdown cells. In fact, mitochondrial ATP production was found to be increased upon knockdown of Cpne2 (Figure 3.2.2C). Collectively, these findings indicate that oxidative phosphorylation is increased with Cpne2 knockdown.

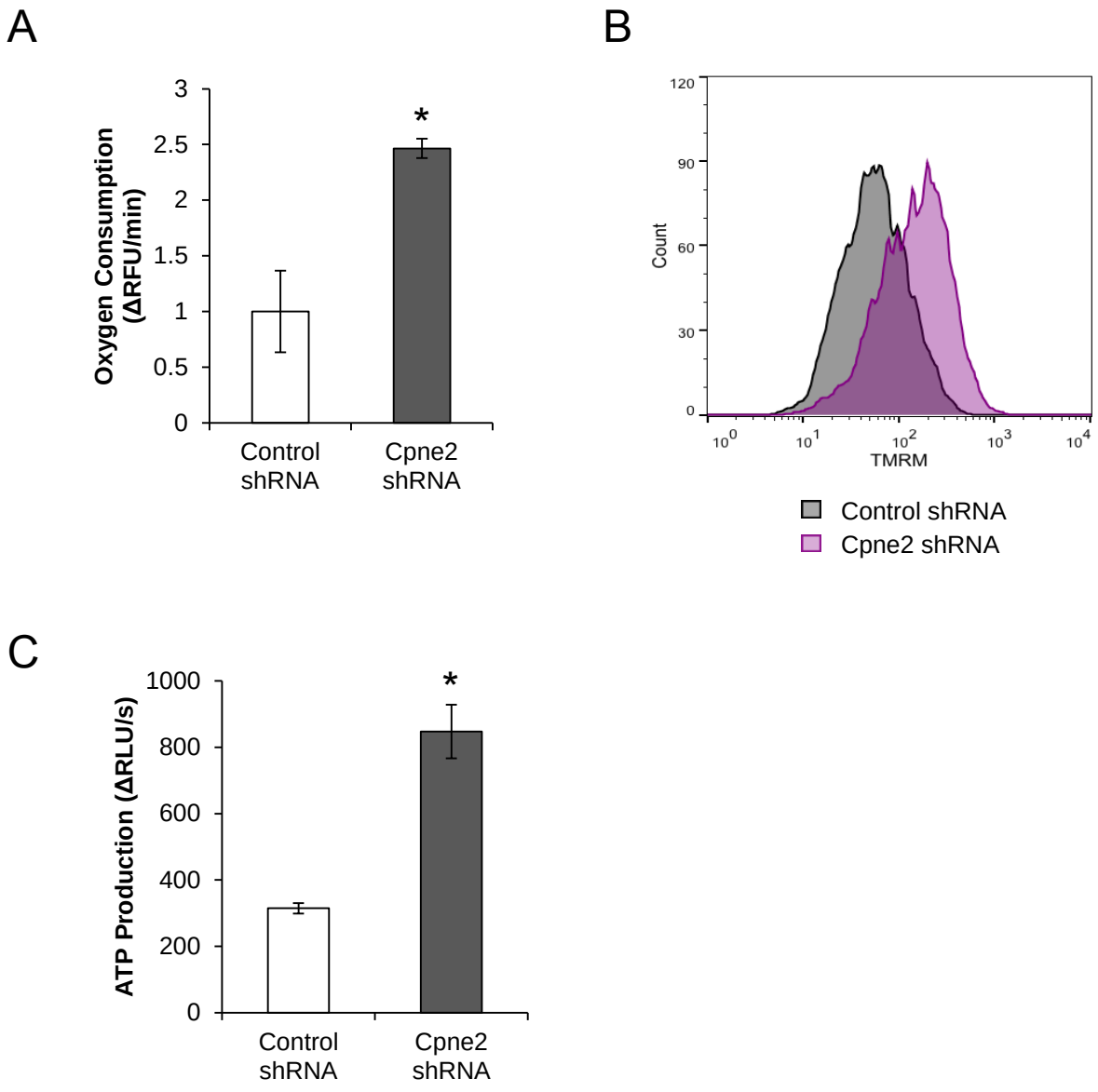

Figure 3.2.2: Cpne2 knockdown increases mitochondrial function

(A) Stable knockdown of Cpne2 increases basal oxygen consumption of digitonin-permeabilized cells. (B) Stable knockdown of Cpne2 increases mitochondrial membrane potential ($\Delta\Psi$) as measured by TMRM fluorescence. (C) Stable knockdown of Cpne2 increases ATP production in digitonin-permeabilized cells. Values are expressed as mean ± SEM. * $p<0.05$.

3.2.3 Knockdown of Cpne2 increases mitochondria independent of the mitochondrial biogenesis

Mitochondrial biogenesis, particularly PGC-1α induced mitochondrial biogenesis, is the most characterized process by which a cell increases functional mitochondria and was therefore the first candidate mechanism that we investigated.

Surprisingly, the protein levels of PGC-1α and NRF-1, nuclear transcriptional regulators of mitochondrial biogenesis [19], which were predicted to be elevated, were instead found to remain unaltered with Cpne2 knockdown (Figure 3.2.3A). Furthermore, it was found that genes whose protein levels were increased upon knockdown of Cpne2 (Figure 3.2.1C) did not exhibit an increase in their mRNA transcript levels (Figure 3.2.3B). To look more directly at transcriptional activity rather than steady-state mRNA transcript levels, the promoter of Tfam (mitochondrial transcription factor A) was cloned and its activity was measured in control and Cpne2 knockdown cells using a dual-luciferase assay system. From this experiment, the promoter activity of Tfam was found to be actually lower in Cpne2 knockdown cells (Figure 3.2.3C). Collectively, these results show that the increase in mitochondria upon Cpne2 knockdown occurs through non-transcriptional mechanisms.

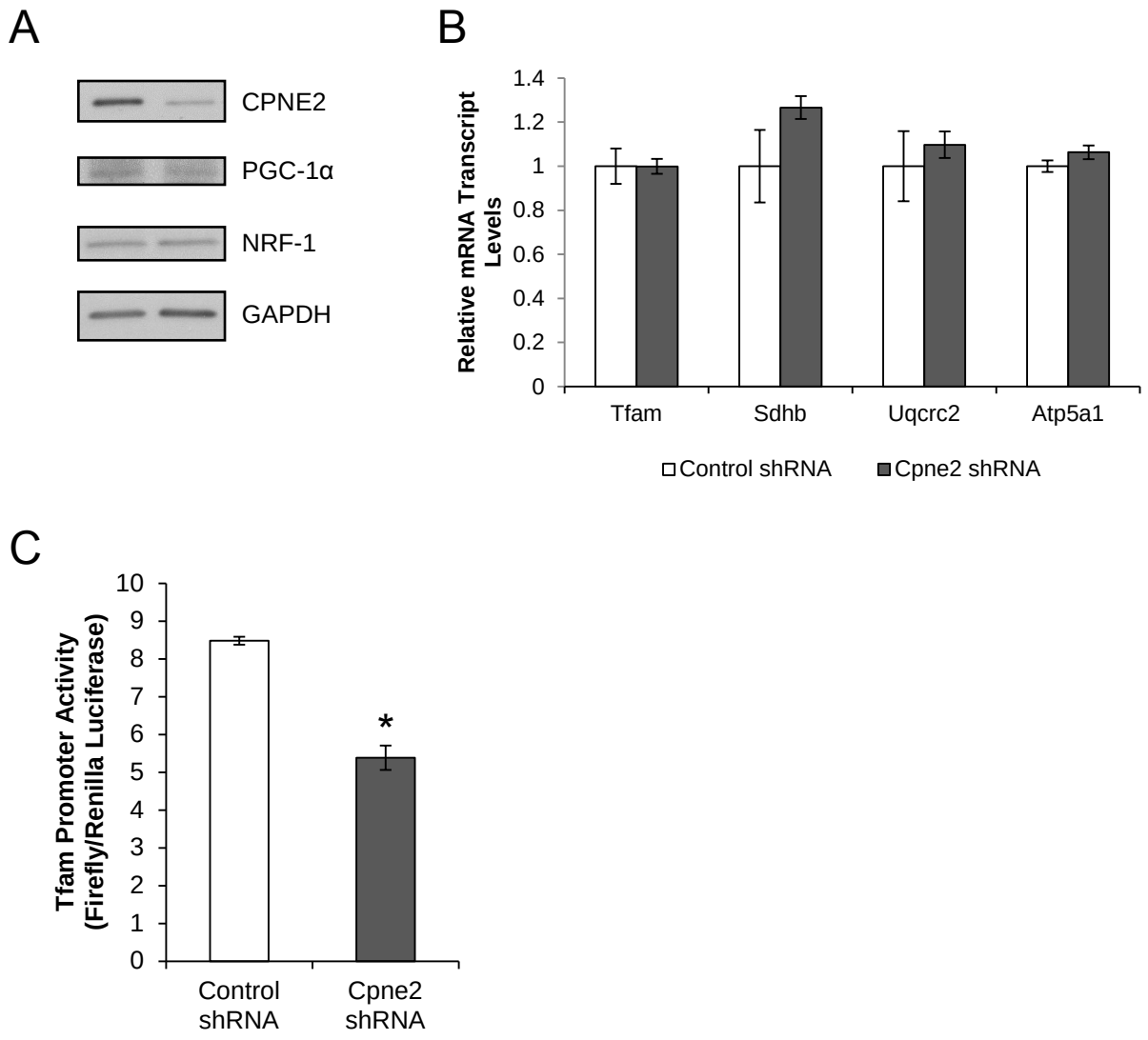

Figure 3.2.3: Knockdown of Cpne2 increases mitochondria independent of the mitochondrial biogenesis
(A) Protein levels of mitochondrial regulators PGC-1α and NRF1 in control or Cpne2 shRNA knockdown C2C12 cells. (B) mRNA transcript levels of mitochondrial genes corresponding to the proteins in Figure 3.2.1A in control or Cpne2 shRNA knockdown C2C12 cells (normalized to 18S rRNA). (C) Tfam promoter activity, determined using a dual-luciferase assay, in control or Cpne2 shRNA knockdown C2C12 cells. * $p<0.05$.

3.2.4 Knockdown of Cpne2 decreases mitochondrial clearance without affecting depolarization-induced mitophagy

Since cells with Cpne2 knocked down did not upregulate the transcriptional mitochondrial biogenesis program, we wanted to determine whether the increase in mitochondrial proteins was due to increased protein stability. To assess whether the stability of the mitochondrial proteins was affected, a cycloheximide chase was carried out on control and Cpne2 knockdown C2C12 cells. This experiment revealed that mitochondrial proteins were more stable in C2C12 cells with Cpne2 knocked down compared to control cells (Figure 3.2.4A).

This increase in mitochondrial protein stability prompted a closer look whether there was a defect in mitophagy, the process through which mitochondria are selectively degraded by autophagy. The PINK1/Parkin pathway is the best studied pathway involved in mitophagy, particularly depolarization-induced mitophagy. In PINK1/Parkin-mediated mitophagy, mitochondrial depolarization leads to an accumulation in full-length PINK1, which recruits Parkin to ubiquitinate proteins in the outer mitochondrial membrane, which then in turn marks the mitochondria to be degraded by autophagy [20 – 22].

To test whether there was a defect in mitophagy, control and Cpne2 knockdown C2C12 cells were treated with the mitochondrial uncoupler FCCP to depolarize the mitochondria and induce mitophagy. Subsequently, the levels of full-length PINK1, the autophagosome marker LC3b-II, and mitochondrial proteins were determined. In both control and Cpne2 knockdown C2C12 cells, FCCP treatment resulted in the accumulation of full-length PINK1, an increase in LC3b-II, and a decrease in the levels of mitochondrial proteins (Figure 3.2.4B), showing that cells with Cpne2 depleted were still capable of depolarization-induced mitophagy. This result was consistent with the finding that defects in depolarization-induced mitophagy causes

mitochondrial dysfunction. This was confirmed with the experiment where knockdown of PINK1 in C2C12 myoblasts decreased the mitochondrial membrane potential (ΔΨ) (Figure 3.2.4C). However, even when PINK1 was depleted, knockdown of Cpne2 was still able to increase ΔΨ (Figure 3.2.4C). Taken together, these results indicate that a defect in depolarization-induced mitophagy would be an unlikely explanation for the observed phenotype.

Surprisingly, Parkin was found to not be expressed in the C2C12 myoblasts (from RNA-Seq data), suggesting that there are other proteins capable of performing the role of Parkin in mitophagy in these cells.

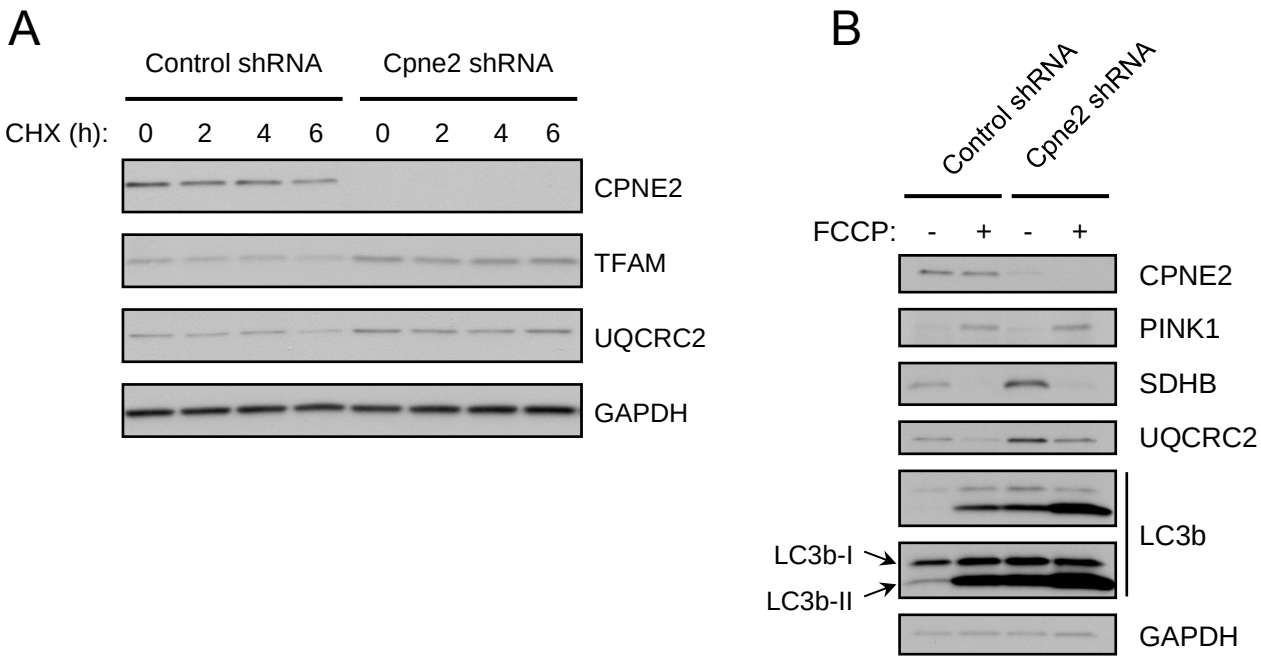

Figure 3.2.4: Knockdown of Cpne2 decreases mitochondrial clearance without affecting depolarization-induced mitophagy

(A) Cpne2 knockdown increases stability of mitochondrial proteins. Cycloheximide (CHX) chase of mitochondrial proteins in control and Cpne2 knockdown cells. Cells were treated with 100 μg/ml of CHX and harvested at the indicated time-points. The levels of mitochondrial proteins were then determined by immunblot. (B) Cells with Cpne2 knockdown do not have defects in depolarization-induced mitophagy. C2C12 cells were treated with 20 nM of the uncoupler FCCP to mdepolarize the mitochondria and were harvested 24h post-treatment.

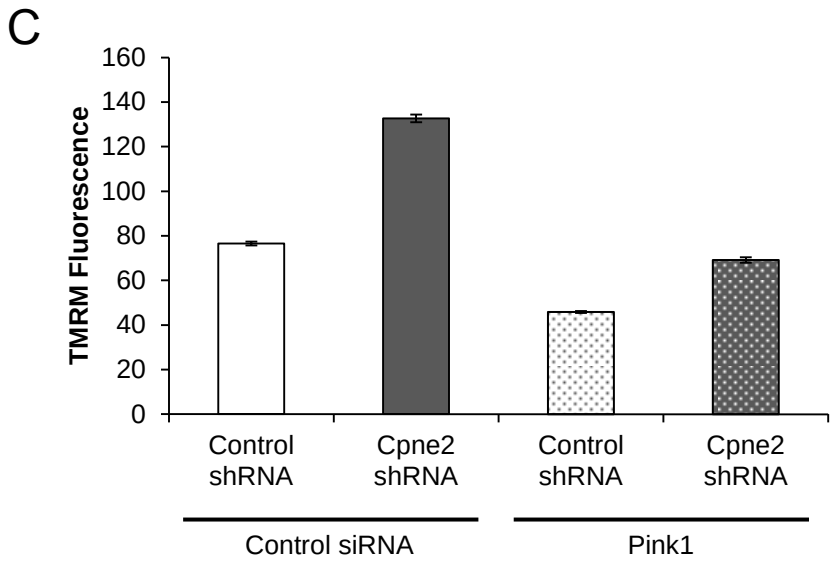

Figure 3.2.4 continued: Knockdown of Cpne2 decreases mitochondrial clearance without affecting depolarization-induced mitophagy

(C) Knockdown Cpne2 still increases mitochondrial membrane potential even in cells with depleted Pink1. Control and Cpne2 knockdown cells were transfected with either control or Pink1 siRNA. Cells were stained with TMRM, trypsinized and analyzed by FACS 48h post-transfection.

3.2.5 Cpne2 knockdown increases autoghagy

Mitochondrial removal by mitophagy involves components of the autophagic machinery and marked by a conversion of LC3b from LC3b-I to LC3b-II. Although C2C12 cells with Cpne2 knocked down can successfully carry out depolarization-induced mitophagy, their levels of LC3-II were noticed to be higher when compared to the control shRNA cells even without FCCP treatment (Figure 3.2.4B). To determine whether this increase in LC3b-II was due to increase autophagosome formation or a defect in autophagosome clearance, control and Cpne2 knockdown cells were treated with the V-type ATPase inhibitor Bafilomycin A1 (Baf-A1) to inhibit autophagosome degradation. Treatment of Baf-A1 caused an increase in LC3b-II levels in both control and Cpne2 knockdown cells (Figure 3.2.5A), indicating that the increase in LC3b-II levels was not due to defective autophagosome degradation but rather increased autophagosome formation.

Autophagy has been found to be regulated by nutrient availability and cellular energy status through nutrient-sensing signaling such as mTOR and eIF2α as well as energy-sensing signaling such as AMPK. Increased AMPK or eIF2α signaling has been found to increase autophagy whereas increased mTOR signaling causes suppression of autophagy [23 – 28]. However, this increase in autophagosome formation did not appear to be due decreased mTOR signaling (as measured by S6K and 4E-BP phosphorylation), increased AMPK signaling (as measured by AMPKα and ACC phosphorylation) or increased eIF2α signaling (as measured by eiF2α phosphorylation) (Figure 3.2.5B).

In addition to increased LC3b-II levels, Cpne2 knockdown cells were also observed to have more LC3b-I compared to control cells. This increase in total LC3b suggested that depletion of Cpne2 increases total LC3b synthesis. We therefore measured the mRNA transcript

levels of autophagy genes in control and Cpne2 knockdown cells by qPCR and found that they were upregulated with Cpne2 depletion (Figure 3.2.5C). Thus, in addition to an increase in conversion from LC3b-I to LC3b-II, knockdown of Cpne2 also increases the expression of autophagy genes, suggesting that depletion of Cpne2 causes a sustained increase in autophagy.

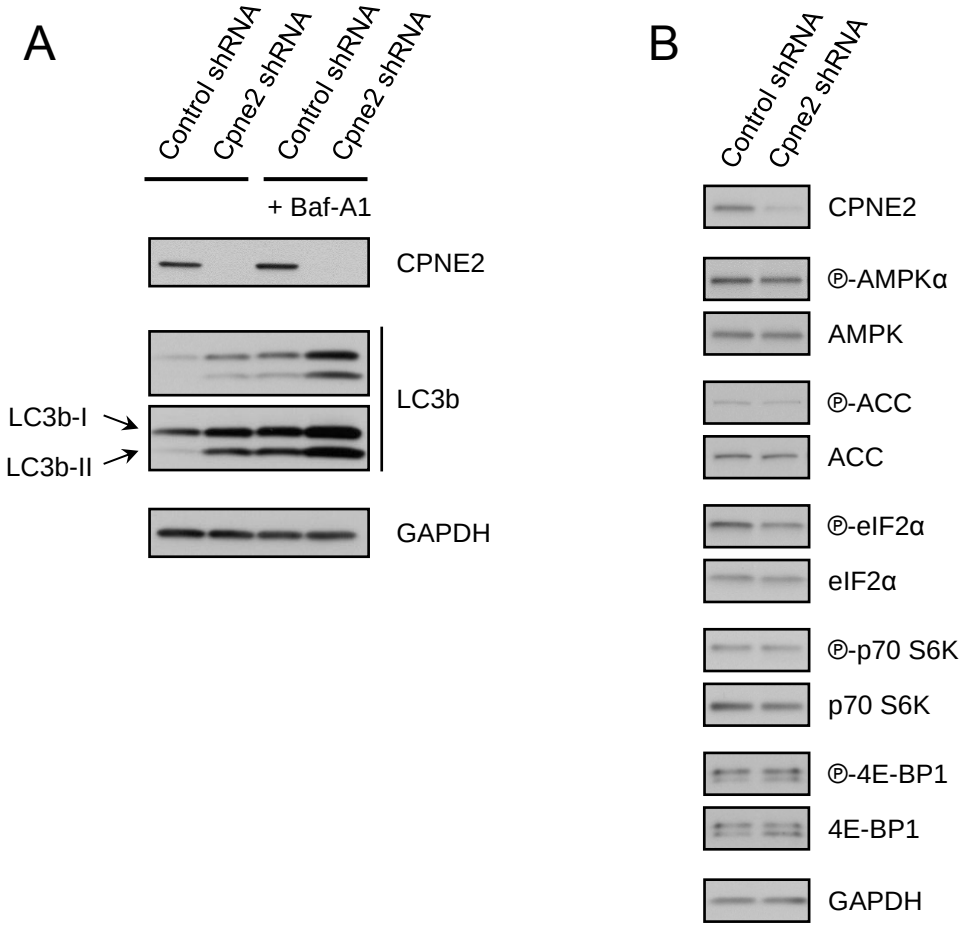

Figure 3.2.5: Cpne2 knockdown increases autoghagy
(A) Cpne2 knockdown increases autophagosome formation. Control and Cpne2 knockdown C2C12 cells were treated with either DMSO of Bafilomycin A1 (400 nM) and harvested after 4h. Levels of LC3b were then determined by immunoblot. (B) Knockdown of Cpne2 does not affect upstream energy and nutrient-sensing pathways. Signaling through the AMPK, mTORC1 and eIF2α was assessed in control and Cpne2 knockdown cells (grown in media containing FBS) by immunoblot.

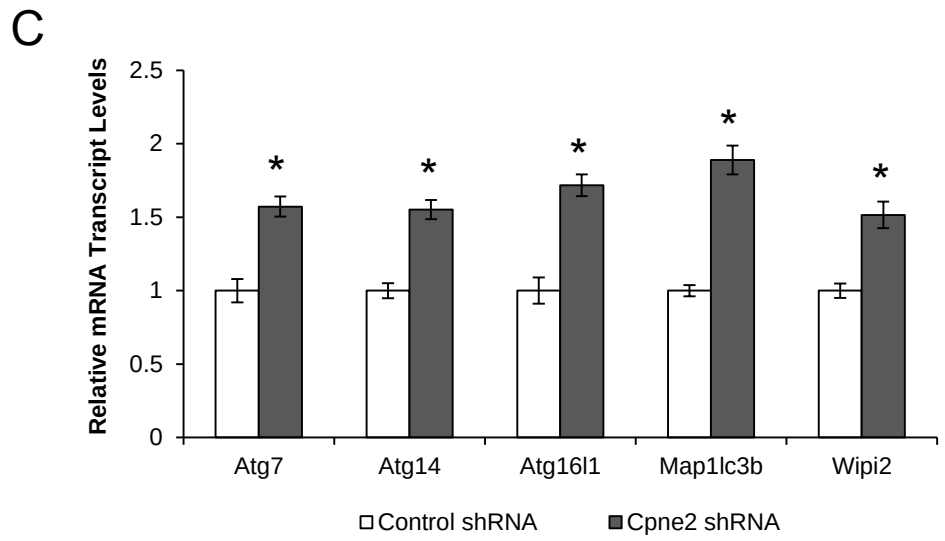

Figure 3.2.5 continued: Cpne2 knockdown increases autoghagy

(C) Knockdown of Cpne2 upregulation of autophagy genes. Cells (grown in media containing FBS) were harvested for RNA extraction and mRNA transcript levels were measured by qPCR. . Values are expressed as mean ± SEM. * $p<0.05$.

3.2.6 Cpne2 knockdown increases lysosomal biogenesis

Since the phenotypes brought about by Cpne2 knockdown seemed to be mediated by mechanisms independent of well-characterized pathways, we performed RNA-Seq on control and Cpne2 knockdown cells to get an overall picture of the pathways and processes that are altered by Cpne2 depletion. Pathway analysis of genes whose transcript levels significantly changed revealed that Cpne2 knockdown upregulated the lysosomal pathway (Appendix Figure A.1A) and this was confirmed by qPCR (Figure 3.2.6A). This is consistent with the observed increase in autophagy since an increase in autophagosome formation must be accompanied by increased lysosome abundance in order for autophagy to proceed efficiently. Consistent with this, Cpne2 knockdown increased levels of the lysosomal protein LAMP1 and the fluorescent signal of the lysosomal probe Lysotracker Red (Figures 3.2.6B and C).

TFEB and TFE3, members of the basic helix-loop-helix leucine zipper transcription factor MiT/TFE family, have been both been found to coordinately upregulate genes involved in lysosomal biogenesis and autophagy [16 – 18]. Of the two, TFE3 was found to be much more highly expressed (from RNA-Seq data) and was therefore the candidate transcription factor that we focused on. Surprisingly, contrary to our prediction, TFE3 knockdown actually increased the levels of LC3b-II and LAMP1 (Figure 3.2.6E). Consistent with this, knockdown of TFE3 did not suppress the increase in Lysotracker fluorescence that occurred upon Cpne2 knockdown (Figure 3.2.6F). A similar result was observed for the Lysotracker fluorescence when TFEB was knocked down (Appendix Figure A.1C). Taken together, this suggests that the increase in lysosomal biogenesis observed with Cpne2 knockdown is mediated through an independent pathway and not by either TFE3 or TFEB.

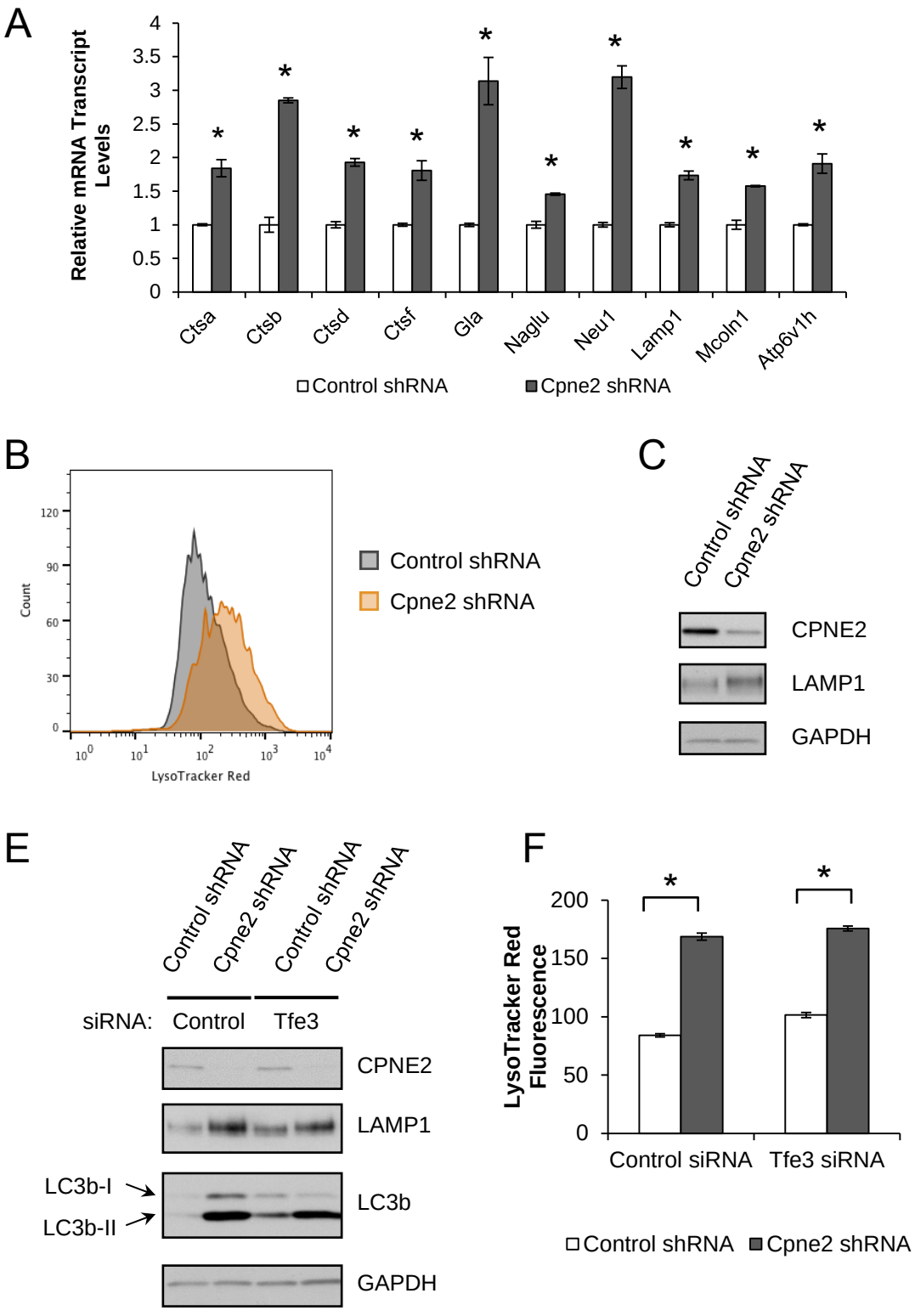

Figure 3.2.6: Cpne2 knockdown increases lysosomal biogenesis

Figure 3.2.6 continued: Cpne2 knockdown increases lysosomal biogenesis

(A) Knockdown of Cpne2 upregulation of lysosome genes. Cells (grown in media containing FBS) were harvested for RNA extraction and mRNA transcript levels were measured by qPCR. . Values are expressed as mean ± SEM. * $p<0.05$. (B) Stable knockdown of Cpne2 in C2C12 cells increases LysoTracker Red fluorescence signal. (C) Cpne2 knockdown increases LAMP1 protein levels. (D) Knockdown Cpne2 still increases LysoTracker Red fluorescence even in cells with depleted of Tfe3. Control and Cpne2 knockdown cells were transfected with either control or Tfe3 siRNA. Cells were stained with LysoTracker Red, trypsinized and analyzed by FACS 48h post-transfection. (E) Knockdown Cpne2 still increases LAMP1 and LC3b-II levels even in cells with depleted of Tfe3. Control and Cpne2 knockdown cells were transfected with either control or Tfe3 siRNA. After 48h, cells were harvested and levels of the proteins LAMP1 and LC3b-II were determined by immunoblot.

3.3 Discussion

As organelles that are involved in vital cellular processes and that become dysfunctional with age and age-related diseases, there has been great interest in understanding the regulation of mitochondria. In this study, we have discovered Cpne2 as a negative regulator of mitochondrial mass and function. Depletion of Cpne2 was first found to increase mitochondrial mass as measured by increased staining if the mitochondrial probes NAO and MitoTracker Deep Red. This increased in mitochondria was confirmed by an increase in mtDNA copy number, mitochondrial proteins and electron microscopy. Although an increase in mitochondrial mass may be the result of a compensatory response to mitochondrial dysfunction, this was found not to be the case with Cpne2 knockdown. Instead, Cpne2 knockdown increased mitochondrial membrane potential, oxygen consumption and ATP production, all of which collectively indicate an increase in mitochondrial function.

Even though the depletion of Cpne2 caused an increase in functional mitochondria, the mechanism by which this is achieved remains elusive. While increasing functional mitochondria has been found to be due to an increase in the transcriptional program of mitochondrial biogenesis [19], this was not observed with Cpne2 knockdown. Furthermore, although the steady-state levels of mitochondrial proteins were increased, the level of their respective mRNA transcript levels remained unaltered. These results all point to a non-transcriptional mechanism that increases mitochondria when Cpne2 is depleted.

On the other hand, the stability of these mitochondria proteins was increased with Cpne2 knockdown, indicating that the observed increase in mitochondria is due to a reduction in the clearance of mitochondria. Therefore a decrease in mitophagy, the process that selectively removes mitochondria by autophagy, was a candidate mechanism. The process of mitophagy has

been best studied in its role in removing depolarized mitochondria through the PINK1/Parkin pathway [20 – 22]. However, when we tested for a potential defect in depolarization-induced mitophagy in Cpne2 knockdown cells by treating them with the uncoupler FCCP, we found that they were still able to remove their depolarized mitochondria. This result indicates that even with Cpne2 depletion, cells can still conduct depolarization-induced mitophagy. This outcome was actually not surprising as a defect in depolarization-induced mitophagy has been found to cause mitochondrial dysfunction [29] rather than the increase in mitochondrial function that was observed in the Cpne2 knockdown cells. Still, other forms of mitophagy exist that remain to be explored, for example in response to hypoxia and iron chelation [30 – 32]. However, a defect in hypoxia or iron chelation-induced mitophagy is predicted to be an unlikely mechanism for the increase in mitochondria seen upon Cpne2 knockdown. This is because these are pathways that serve to also remove dysfunctional mitochondria (sometimes even pre-emptively) and a defect in these pathways would still result in mitochondrial dysfunction.

Although there has been much progress in the study of how mitochondria are removed in mammalian systems, it has been rather narrow in scope. Current research has focused on the induction of mitophagy in response to a stimulus, such as depolarization, hypoxia or iron chelation. However, the discovery of mitochondria-derived vesicles (MDVs) suggests that mitochondria can be removed through processes other than mitophagy [33 – 34]. Furthermore, the events of mitochondrial removal under basal, unstimulated conditions remain to be explored. Considering that Cpne2 knockdown simultaneously increases mitochondrial function and decreases mitochondrial clearance, this raises the idea that under basal conditions, functional mitochondria are continuously being removed. In this case, Cpne2 would be involved in this basal mitochondrial removal and disruption of this elimination pathway by Cpne2 knockdown

would therefore result in an increase in mitochondria that are functional. Whether this basal mitochondrial elimination pathway involves mitophagy or not remains to be investigated.

In addition to increasing mitochondrial content and function, knockdown of Cpne2 also increased autophagy. This upregulation of autophagy was found to occur at two levels: post-translational induction of autophagy and transcriptional upregulation of autophagy genes. While previous studies would attribute this to a state of cellular starvation [35], energy and nutrient signaling pathways were not activated with Cpne2 knockdown. This disjointing between the upstream sensors and the downstream effectors suggests that there is an independent pathway that acts on the downstream effectors to mediate these effects. To address the former, additional experiments must be conducted to determine if components further downstream in autophagy induction, such as beclin-1 or ULK1 [36 – 37], are aberrantly activated. As for the transcriptional upregulation of autophagy, other transcription factors, such as FOXOs and E2Fs, have been found to also promote the transcription of autophagy genes [38 – 39]. E2Fs stand out as a promising candidate since cell cycle regulation and DNA synthesis, processes that are known to be regulated by E2F1 [40 – 41], were found to be significantly downregulated from the RNA-Seq pathway analysis (Appendix Figure A.1B).

Consistent with an increase in autophagy, lysosomal biogenesis was found to be upregulated by Cpne2 knockdown. This coordinated expression of autophagy and lysosomal genes has been observed and many studies have attributed this to the members of the MiT/TFE family of transcription factors, particularly TFEB and TFE3. Yet when we tested for their involvement in mediating the increase in autophagy and lysosomal biogenesis, we found that there knockdowns acted opposite to what was predicted. While this could be attributed to cell-

type specific effects, it also demonstrates that there remains much to be discovered about these processes and transcription factors.

Overall, while increased mitochondrial function and degradation through the autophagy-lysosomal degradation are orchestrated responses to nutrient and growth factor withdrawal, it is becoming clear that these processes can still be coordinately regulated via other pathways. The stimuli and mediators involved in these novel pathways remain to be elucidated and reflects the unexplored research opportunities of these organelles.

3.4 Materials and Methods

Cell culture and compound treatments

C2C12 cells (American Type Culture Collection) were cultured in DMEM (4.5g/L glucose, with glutamine and sodium pyruvate) containing 10% (vol/vol) FBS and 1% Penicillin-Streptomycin. Cycloheximide (R&D) was used at a concentration of 100 μg/ml. FCCP (Sigma) was used at a concentration of 20 nM for 24h to induce mitophagy. Bafilomycin A1 (R&D) was used at a concentration of 400 nM for 4h to inhibit autophagosome degradation.

Gene silencing

For transient gene depletion, siGenome SMARTPool non-targeting control, Pink1 and Tfe3 siRNA were purchased from Dharmacon and transfected into cells with Lipofectamine RNAiMax at a concentration of 20 nM. Cells were harvested 48h post-transfection for analysis

Non-targeting and Cpne2 shRNA lentiviruses were produced by cotransfection of 293T cells with the shRNA plasmids along with the packing plasmids psPAX2 and pMDG.2 using X-tremeGENE HD at a ratio of 3:2 [Transfection reagent (μl): DNA (μg)]. The media of these cells was changed 24h post-transfection and viral supernatants were collected at 48h and 72h. The viral supernatants were pooled and filtered, after which they were added to C2C12 cells with 5 μg/ml of polybrene. C2C12 cells were transduced by spinfection (2000 rpm, 30 min) and selection of resistant cells was carried out 24h later using puromycin (2 μg/ml). The shRNA hairpin inserts included the following sequences:

Non-targeting control: CAACAAGATGAAGAGCACCAA

Cpne2 shRNA 1: CGACCCTTCTTCTCTCCATTA

Cpne2 shRNA 2: GCTCCCTTTGTCAGAGTCAAT

Mitochondrial mass

C2C12 cells were incubated with cell culture media containing nonyl acridine orange (NAO) (10 nM) and MitoTracker Deep Red (10 nM) for 30 min at 37°C. After which, cells were trypsinized and resuspended in probe-free culture media. Fluorescence of NAO and MitoTracker Deep Red were determined by flow cytometry on the FACSCalibur (BD Biosciences) using the 488 nm laser for NAO (measured in the FL-1 channel) and 635 nm laser for MitoTracker Deep Red (measured in the FL-4 channel).

mtDNA copy number

Cells were trypsinized, spun down (1000x *g*, 5 min) and total DNA was extracted with a DNeasy blood and tissue kit (QIAGEN). qPCR from the DNA extracts using the LightCycler 480 system (Roche). mtDNA was amplified using primers specific for the mtDNA-encoded Cytochrome B (*mt-Cytb*) gene and normalized to genomic DNA by amplification of the ribosomal protein s18 (*Rps18*) nuclear gene. The primer sequences used are listed below:

Gene	Directionality	Sequence
Rps18	Forward	TGTGTTAGGGGACTGGTGGACA
	Reverse	CATCACCCACTTACCCCCAAAA
mt-Cytb	Forward	CCCTAGCAATCGTTCACCTC
	Reverse	TGGGTCTCCTAGTATGTCTGG

Protein extraction and immunoblotting

Cells were washed once in PBS, lifted using a cell scraper, spun down (1000x *g*, 5 min) and resuspended in ice-cold lysis buffer (pH 7.4, 0.025M Tris, 0.15M NaCl, 0.001M EDTA, 1% NP40, 5% glycerol) supplemented with protease and phosphatase inhibitors (Roche). The cell suspension was rotating at 4°C for 10 min and spun down (16 000 *g*, 5 min) after which the

supernatant was collected as the cell lysate. Protein concentration in the cell lysates were measured using a Bradford assay (BioRad) with BSA as the protein standard.

For immunoblotting, cell lysate was mixed with SDS sample buffer (supplemented with DTT) and boiled at 95°C for 5 min. For SDS PAGE, a total of 25 μg of protein was run on each well of a 4 – 20% acrylamide gel (BioRad). Proteins were transferred onto a PVDF membrane and blocked using 5% BSA in TBS-T. Membranes were incubated with primary antibody overnight at 4°C and after three TBS-T washes, were incubated in secondary antibody for 1h at room temperature. Membranes were once again washed three times in TBS-T and protein blots were revealed using Amershan ECL (GE Healthcare).

The antibodies used in this study are as follows: ATP5A, UQCRC2, SDHB, NRF-1, PINK1 and LAMP1 from Abcam; TFAM and GAPDH from EMD Millipore; LC3b from Novus Biologicals; PGC-1α from Santa Cruz; total and phospho ACC, AMPK α p70-S6K, eIF2α and 4E-BP1 from Cell Signaling. The Cpne2 antibody was custom-made by Yenzym.

Electron microscopy

Cells were fixed in 1.25% formaldehyde, 2.5 % glutaraldehyde and 0.03% picric acid in 0.1 M Sodium cacodylate buffer, pH 7.4 at room temperature for 1h. The samples were then embedded, sectioned and mounted onto grids. The grids were stained with lead citrate and images were captured on the Tecnai G2 Spirit BioTWIN transmission electron microscope.

Mitochondrial membrane potential

C2C12 cells were incubated with cell culture media containing tetramethylrhodamine, methyl ester (TMRM) (100 nM) for 15 min at 37°C. After which, cells were trypsinized and

resuspended in probe-free culture media. Fluorescence of TMRM was determined by flow cytometry on the FACSCalibur (BD Biosciences) using the 488 nm laser (measured in the FL-2 channel).

Oxygen consumption

Cells trypsinized, spun down and resuspended in assay buffer (150 mM KCl, 25 mM, Tris–HCl, 2mM EDTA, 0.1% BSA, 10-mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) containing 25 μg/ml digitonin to permeabilize the cells. After 1 min of permeabilization, the cells were spun down (800x g, 5 min) at room temperature and resuspended in assay buffer containing an addition of 1 mM pyruvate, 1 mM malate and 0.1 mM ADP. The oxygen sensing probe MitoXpress-Xtra was added to the resuspended cells in the ratio of 2:15 (v:v) and pipetted into a black 96 multiwell plate. Mineral oil was overlayed over each well and the probe fluorescence was measured overtime in a plate reader (340 nm Ex/642 nm Em). Oxygen consumption was calculated as the rate of change in the probe fluorescence.

ATP production

ATP production was conducted as described previously [42]. Cells trypsinized, spun down and resuspended in assay buffer (150 mM KCl, 25 mM, Tris–HCl, 2mM EDTA, 0.1% BSA, 10-mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) containing 25 μg/ml digitonin to permeabilize the cells. After 1 min of permeabilization, the cells were spun down (800x g, 5 min) at room temperature and resuspended in assay buffer containing an addition of 1 mM pyruvate, 1 mM malate, 0.1 mM ADP, 0.15 mM P^1,P^5-di(adenosine) pentaphosphate. Luciferase-luciferin reagent (0.5-M Tris–acetate, pH 7.75, 0.8 mM luciferin, 20 mg/ml luciferase) is added to the resuspended cells in a 1:16 ratio (luciferase-luciferin: cell suspension) to start the reaction.

Luminescence over time was measured in a luminometer and the ATP production was calculated as the rate of change in luminescence.

RNA extraction and gene expression

Cells were washed in PBS, lysed directly and total RNA was extracted using RNeasy kit (QIAGEN). cDNA synthesis was performed using the iScript cDNA synthesis kit (BioRad) and qPCR was performed using the LightCycler 480 system (Roche). The primer sequences used are listed below:

Gene	Directionality	Sequence
Rn18s	Forward	GAGACTCTGGCATGCTAACTAG
	Reverse	GGACATCTAAGGGCATCACAG
Tfam	Forward	CACCCAGATGCAAAACTTTCAG
	Reverse	CTGCTCTTTATACTTGCTCACAG
Atp5a1	Forward	CATTGGTGATGGTATTGCGC
	Reverse	TCCCAAACACGACAACTCC
Uqcrc2	Forward	TTCCAGTGCAGATGTCCAAG
	Reverse	CTGTTGAAGGACGGTAGAAGG
Sdhb	Forward	ACCCCTTCTCTGTCTACCG
	Reverse	AATGCTCGCTTCTCCTTGTAG
Map1lc3b	Forward	ACAAAGAGTGGAAGATGTCCG
	Reverse	CCCCTTGTATCGCTCTATAATCAC
Wipi2	Forward	CATCTGCTCACTAACCACAATTC
	Reverse	CGTCTCCATACTGCCATCAAG
Atg14	Forward	CTGAACGCAAACATCCTTTACC
	Reverse	TCTGCTCGAACTTCAAAGGG
Atg7	Forward	TCTCCTACTCCAATCCCGTG
	Reverse	TGCTCATGTTGAACCCTCTG
Atg16l1	Forward	TGACCCAGAAACTACAAGCAG
	Reverse	AGCTCTTCCTGGTGTTTGATC
Ctsa	Forward	GATGAGTGGTTTGTGGATTCG
	Reverse	TTGATGGTGAGGAAGGTGATG
Ctsb	Forward	AGACCTGCTTACTTGCTGTG
	Reverse	GGAGGGATGGTGTATGGTAAG
Ctsd	Forward	TGACAAGTCCAGCACCTATG

	Reverse	CTCCACCTTGATACCTCTTGC
Ctsf	Forward	TCATCGACCATGCTGTGTTG
	Reverse	CCAGATCCACGGTACAAGTAG
Mcoln1	Forward	TTGACAATAAAGCGCACAGTG
	Reverse	ACCACATCAAACAGAAGCCG
Gla	Forward	CCCTACTCATGTCCAACGATC
	Reverse	CGTTCCCAAACCTCAATGTG
Naglu	Forward	TGACTGGATGGCACTGAATG
	Reverse	CGGTGAAGTACGTATCGATCTC
Neu1	Forward	GCCAAGTTCATCGCCATG
	Reverse	CCCTGTGTCTACATCGTTCAC
Lamp1	Forward	ACAAACCCCACTGTATCCAAG
	Reverse	CATTTGGGCTGATGTTGAACG
Atp6v1h	Forward	CTTTGACTATGCGAAACGAAGC
	Reverse	CCTTCCATCAGTTCTTTCCCC

Promoter Assay

The Tfam promoter (1.44 kb upstream of the Tfam start codon) was amplified from mouse genomic DNA and cloned into the pGL4.15 firefly luciferase vector (Tfam-Luc). The Tfam-Luc vector was mix (1:1, v:v) with the pRL-Tk vector (as a transfection control) and transfected into control and Cpne2 knockdown cells using X-tremeGENE HD at a ratio of 6:2 [Transfection reagent (μl): DNA (μg)]. Cells were harvested 48h post-transfection. Using the dual-luciferase reporter assay system (Promega), cells were lysed and the luminescence of firefly and Renilla luciferase was measured using a luminometer.

RNA-Seq: sample preparation and computational analysis

Cells were washed in PBS, lysed directly and total RNA was extracted using RNeasy Plus kit (QIAGEN). mRNA was extracted by using the Dynabeads mRNA purification kit (Life Technologies) according to the manufacturer's instruction. The cDNA libraries were made using the NEXTflex RNA-Seq Kit (Bioo) and quantified using the Library Quantification Kit from

KAPA Biosystems according to the manufacturer's instruction. Next generation sequencing was performed on the Illmina NextSeq 500.

Differential gene expression was analyzed from the sequencing output using the Tuxedo suite comprising of Tophat, Cufflinks, Cuffmerge and Cuffdiff. Pathway analysis was conducted using DAVID to determine over-represented pathways.

Lysosomal abundance

C2C12 cells were incubated with cell culture media LysoTracker Red DND-99 (500 nM) for 30 min at 37°C. After which, cells were trypsinized and resuspended in probe-free culture media. Fluorescence of TMRM was determined by flow cytometry on the FACSCalibur (BD Biosciences) using the 488 nm laser (measured in the FL-2 channel).

3.5 References

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Chapter 4

Conclusion

4.1 Closing Remarks

The process of aging could be summarized as changes in the body that occur as time progresses. More often, it refers to the changes that take place towards the advanced or later years in life. Under this definition, aging is associated with a decline in the physiological performance of the body and consequently a reduction in productivity [1]. In addition, the body becomes more vulnerable to diseases, both infectious and chronic diseases, with aging [2]. As the world population becomes more and more aged in proportion, these health concerns become increasingly prominent [3, 4] and there has been great research interest in finding ways to improve health in the elderly.

The discovery of genes, termed longevity genes, that can extend lifespan and delay the onset of age-related diseases [5 – 7] has provided much insight into the aging process and help identify important pathways and processes that can be targeted to treat age-related diseases. One of these emerging targets is the mitochondria. Known for its role in energy production via oxidative phosphorylation, mitochondrial function has been found to decline with age and in a multitude of age-related diseases [8 – 12]. Conversely, improving mitochondrial function – through dietary interventions or pharmacologically – has been found to improve health and even lifespan [13, 14]. Since longevity genes have been mostly been discovered in lower organisms, and combined with the involvement of mitochondria in aging, we found this to be a good opportunity to screen for mammalian longevity genes using mitochondria as a surrogate read-out rather than lifespan extension since the latter is still extremely cost prohibitive.

The screen is described in Chapter 2 and identifies GLTSCR2 as a positive regulator of mitochondria. Overexpression of GLTSCR2 was found to increases respiration and this was conserved in the worm *C. elegans*. Although cells with increased proliferation generally exhibit a

shift towards glycolytic metabolism, GLTSCR2 overexpressing cells actually have increased cell proliferation in conjunction with increased mitochondrial function. This led to the identification of Myc as a downstream component that mediated the mitochondrial effects of GLTSCR2 overexpression since Myc has been found to positively regulate both cell proliferation and mitochondrial function. In addition, GLTSCR2 has been found to be induced by mitochondrial stress, suggesting that it may act, together with Myc, as part of the mitochondrial unfolded protein response.

In Chapter 3, we identify Cpne2 as a negative regulator of mitochondria and its depletion by knockdown increased both mitochondrial content and function. Surprisingly, the transcriptional program of mitochondrial biogenesis was not upregulated. We therefore turned our attention towards mitochondrial elimination and found that mitochondrial proteins were stabilized with Cpne2 knockdown. Although this finding might suggest a defect in mitophagy, Cpne2 knockdown cells could still carry out depolarization-induced mitophagy when challenged with an uncoupler. These findings point to the possibility there is a basal elimination of functional mitochondria and this is suppressed when Cpne2 is depleted. In addition, Cpne2 depletion was found to increase autophagy and lysosomal biogenesis. Although these phenotypes, when combined with upregulated mitochondrial function, all indicate a cellular state of nutrient deprivation upstream energy and nutrient sensing signaling pathways were not changed. This suggests that there is another pathway, likely responding to another stimulus, mediates the phenotypes observed with Cpne2 knockdown.

This study demonstrates that it is feasible to perform an overexpression screen to identify mitochondrial regulators and complements the more common RNAi screening platforms to give more extensive coverage. In addition, the work from these two hits re-emphasizes that the

regulation of mitochondria is highly complex. Even among these two hits, the increase in respiration is associated with increased cell proliferation in one case (GLTSCR2) but decreased cell proliferation in the other (Cpne2). In each case, the mitochondria are regulated through different pathways and further studies must be conducted to determine how cells choose one pathway from another even though both increase mitochondrial function. It is also important to note that different pathways that increase mitochondria are likely to produce mitochondria with different properties for specialized purposes. Therefore, in addition to the characterizing the novel regulatory pathways, the mitochondria that are produced must also be studied. An understanding of how to direct the biogenesis or remodeling of mitochondria for a specific function would be useful in ameliorating specific deficiencies in mitochondria and would improve the efficacy of treatments. Also, there remains much to be discovered about how mitochondria are regulated in relation to different stimuli. While most work in this area has focused on nutrients and energy [15 – 17], the effect of other cellular processes on mitochondria (such as iron handling and cell cycle) have begun to be appreciated [18, 19]. This should not come as a surprise, considering that all cellular processes require energy and that most of it is produced by the mitochondria.

Lastly, it is worth remembering that this work is just the beginning of a long journey to find new mammalian longevity genes. While we have identified mitochondrial regulators, it would be interesting to see if they affect organismal health, aging and lifespan. However it is important to note that even though mitochondria have indeed been found to be intimately involved in aging and lifespan extension, they are regulated by many pathways in response to many internal and external stimuli, some of which may not even be related to aging and lifespan extension. Although there is no complete substitute for lifespan extension, the identification of

longevity genes can be further refined by assessing other phenotypes that correlate with lifespan extension, such as stress resistance [20]. Nevertheless, even without lifespan extension, research into the phenotypic changes that occur with age is still valuable and can give clues into the aging process, unveil targets for therapeutics for age-related diseases and discover novel longevity genes.

4.2 References

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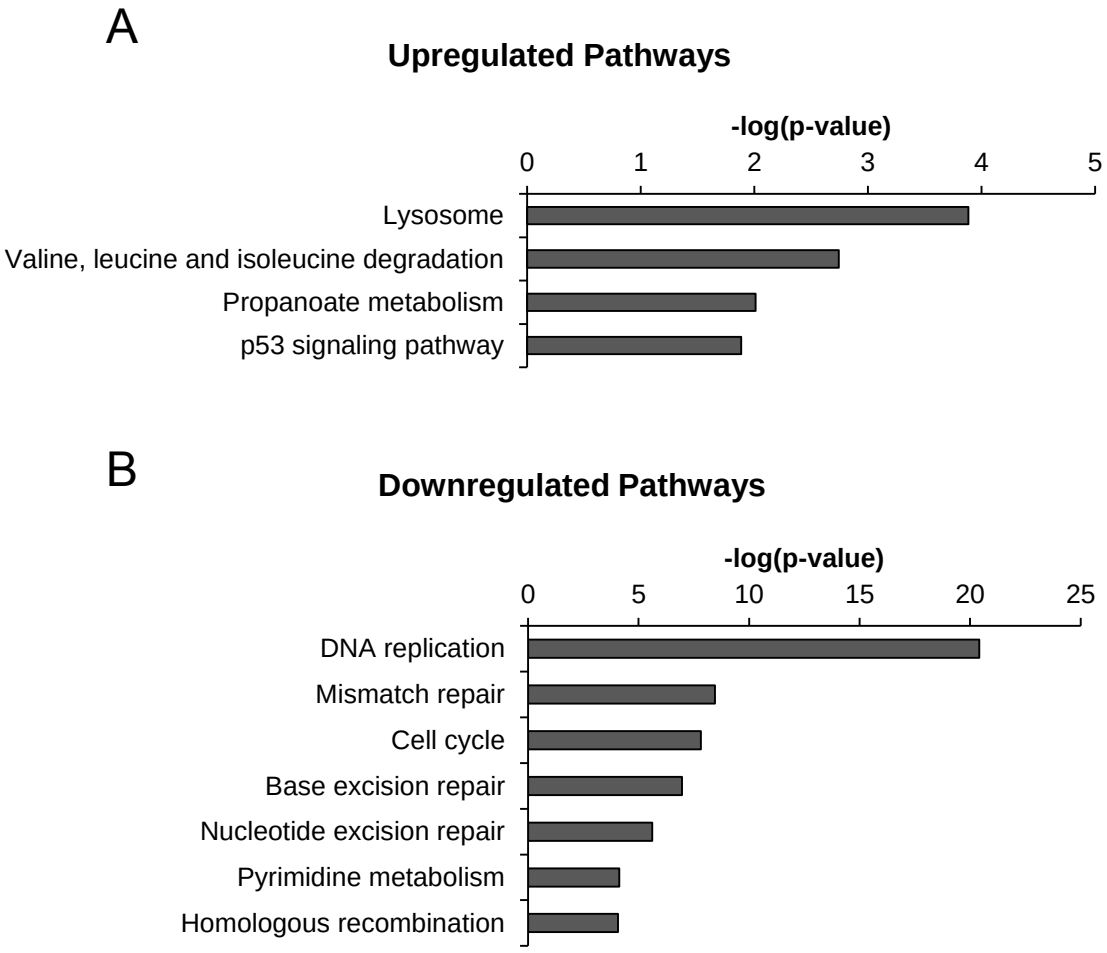
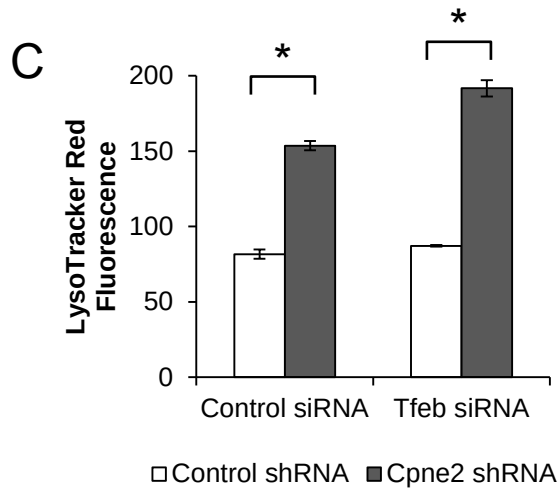
Appendix

A.1 Supplemental Figures



Appendix Figure A.1: Cpne2 knockdown increases lysosomal biogenesis
(A) Significantly upregulated pathways in Cpne2 knockdown cells compared to control as determined by differential gene expression. (B) Significantly downregulated pathways in Cpne2 knockdown cells compared to control as determined by differential gene expression.



Appendix Figure A.1 continued: Cpne2 knockdown increases lysosomal biogenesis
(C) Knockdown Cpne2 still increases LysoTracker Red fluorescence even in cells with depleted of Tfeb. Control and Cpne2 knockdown cells were transfected with either control or Tfeb siRNA. Cells were stained with LysoTracker Red, trypsinized and analyzed by FACS 48h post-transfection.