Exploring the role of four Kazald paralogs in Axolotl limb regeneration

Exploring the role of four Kazald paralogs in Axolotl limb regeneration

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Abstract

This study aimed to establish the foundation for understanding a broader inquiry into whether other Kazald paralogs function as compensatory mechanisms for the *Kazald2* gene, recognized for its upregulated in blastema and potential critical role in the regeneration process (Bryant et al., 2017). *Kazald2* morpholino morphants exhibited a noticeable delay in the regeneration process; however, researchers observed that knocking out the *Kazald2* gene resulted in regeneration comparatively normal regeneration, suggesting the hypothesis of Gene Compensation among the Kazald paralogs. Gene compensation proposes that the other members of the Kazald paralogs may step in to fulfill the functions of *Kazald2* when it is absent, thereby facilitating the regeneration process to proceed without significant impairment.

The CRISPR-Cas9 system was employed to edit the genome of Kazald paralogs in axolotl embryos by inducing Double-Stranded Breaks. Gel electrophoresis analysis confirmed successful targeting of gene sequences, as evidenced by the presence of two distinct bands upon T7 digestion. This promising outcome suggests the feasibility of generating valuable Kazald mutants for Genetic Compensation research. However, it is important to acknowledge the potential challenge of mosaicism associated with CRISPR. Furthermore, while the Hybridization Chain Reaction experiment successfully executed the protocol, unfortunately, the newly designed probes did not yield the anticipated. This underscores the necessity for meticulous optimization of experimental factors such as probe concentration, hybridization conditions, and detection methods. Addressing these

challenges is pivotal to enhancing the robustness and reliability of the experimental approach for future research applications.

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Chapter I.

Introduction

All living organisms have limited capability to regenerate. The human body has an astounding ability to heal itself, but this marvelous feature has its limits. Humans can regenerate the liver, and we can heal cuts and wounds, but our ability to regrow entire organs or limbs is extremely limited. The enduring story of Prometheus's liver regeneration following its consumption by an eagle serves as a testament to humanity's longstanding fascination with the concept of regeneration. It would be extraordinary if humans had the same extensive regenerative capabilities as animals such as lizards, axolotl, zebrafish, etc. Scientists are studying these animals to better understand the mechanisms involved in regeneration and to explore potential ways to enhance the human body's regenerative capabilities.

Regeneration

The process of regeneration is complex, involving multiple genetic and cellular mechanisms that we do not currently fully understand. Using research model organisms such as zebrafish, planaria, newts, axolotl, and mice, and beyond, scientists have found molecular regulators involved in these regenerative processes (Chen and Poss, 2017, James et. al, 2004, Bryant et al., 2017). While some regenerative genes and signaling pathways have been identified, much more research is needed to fully comprehend these processes and develop effective regenerative therapies for humans (James et. al, 2004, Bryant et al., 2017, Sader et al., 2019).

The increasing knowledge of stem cells, and the advent of biomedical engineering tools such as clustered regularly interspaced short palindromic repeats (CRISPR), and genome sequencing have recently advanced the field of regenerative biology. The primary method for studying regeneration involves surgically amputating specific body parts in an animal model and observing their subsequent growth. In some species, body parts can be completely regenerated with apparent perfection, while in others, regenerated body parts do not fully recapitulate key aspects of the original structure. For example, salamanders stand out for their exceptional ability to fully regenerate lost limbs throughout their lifespan. In contrast, frogs display limited limb regeneration, with tadpoles exhibiting robust regeneration of immature limbs that mature into adult limbs, while adult frogs generally lack substantial regenerative capacity. Mice and humans have restricted regenerative abilities, primarily limited to digit tip regeneration (Wang et. al, 2020).

After employing amputation techniques to elucidate the mechanisms of regeneration in various animal models, researchers recognized the necessity for more refined methodologies to dissect the intricate genetic underpinnings of this process. Conventional approaches, while informative, often lacked the precision required to selectively manipulate individual genes implicated in regeneration. This limitation prompted the adoption of advanced gene-editing technologies, prominently CRISPR-Cas9.

Leveraging the unparalleled precision of CRISPR-Cas9, researchers gained the capability to precisely target and modify specific genes of interest. CRISPR has been used to control any specific gene’s activity by making changes to the Cas-9 protein,

allowing researchers to turn the gene on or off as needed (Jiang and Doudna, 2017). They also found that using this technique was highly effective in knocking out genes with fewer harmful side effects on the cells or the animal model compared to conventional gene-editing methods (Fei et al., 2014). This breakthrough not only facilitated the elucidation of the genetic pathways governing regeneration but also provided insights into the regulatory networks orchestrating tissue repair and regrowth. It is important to note, however, that this research solely uses the mutation capabilities of CRISPR.

Why Investigate Limb Regeneration?

Limb amputation is a common occurrence, currently totaling over one million globally per year, translating to approximately one occurrence every 30 seconds (Access Prosthetics, 2019). At present, 2.1 million people in the United States are coping with limb loss, with this number projected to double by 2050, with an anticipated daily rate of 300-500 amputations (Access Prosthetics, 2019). Although sophisticated prosthetics can vastly improve a patient's quality of life, the ability to regrow amputated limbs would be even better. Regenerative medicine's goal has been to enhance the human body's natural healing ability and utilize innovative treatments and methodologies, such as stem cell therapy and tissue engineering, aimed at repairing, regenerating, or substituting damaged tissues and organs within the body.

Animal species, such as the axolotl, can regenerate their limbs naturally. Researchers are investigating these animals due to the similarities in limb structures they share with human legs. The goal of these studies is to understand the molecular and cellular mechanisms involved in limb regeneration and to develop new therapies for

humans. Currently, there are no approved medicines or therapies for limb regeneration in humans. So, learning more about how axolotls regrow body parts could help improve how we treat injuries and diseases in people through regenerative medicine.

Animal Model

A prime animal model for regeneration and the model used in this research is the axolotl, *Ambystoma mexicanum,* Mexican salamander. Axolotls are an excellent research model for studying limb regeneration not only because of their superpower of regenerating complex body structures such as limbs, heart, and other organs but also because they share homologous structures with human legs making them desirable for regeneration studies in vertebrates (Voss, 2021). Despite this, both human and axolotl legs trace their origins back to a shared ancestor. Invertebrate models, in contrast, have a wide variety of limb structures that are different from those of vertebrates, diminishing their suitability as regenerative models for limb regeneration studies.

Despite their large physical size compared to other animal models such as mice, and. zebrafish, axolotls are easily maintained in laboratory settings; However, their growth and sexual maturation is slower than other animals. Their sexual maturity time—while still almost one year—is less than other salamanders, making them an ideal model for experimental studies, among salamanders. Axolotl eggs are large enough that they are easy to manipulate and observe which makes them well-suited for experiments that involve manipulating embryos or studying early development.

The axolotl has a large genome of ~32Gb whose first-pass full genome sequence has been published and partially annotated (Keinath et al., 2017). The axolotl is the first salamander to have its entire genome sequenced (Schloissnig et al., 2021). This has

allowed researchers to identify genes and regulatory elements involved in regeneration (Tanaka et al., 2018). However, despite the sequencing and assembly of the axolotl genome, their genome remains poorly annotated, and repetitive sequences make some scaffolds difficult to place, making it difficult to study their large-scale genome structure and function. Although its fragmented genome presents a formidable barrier to genetic analysis, the extensive history of axolotl research available can provide a solid foundation for ongoing studies of regeneration and tissue repair. As a result, continuous endeavors are underway within the scientific community to improve the quality of the genome assembly, aiming to deepen our comprehension of the genetic mechanisms underlying regeneration in axolotls (Smith et al., 2019).

Axolotls have been extensively studied in laboratories for over two centuries, and as a result, we have gained a wealth of knowledge about their remarkable regenerative capabilities. By studying how axolotls regenerate their limbs, researchers can gain important insights into how similar processes might be induced in humans to promote tissue repair and regeneration of those who have lost limbs due to injury or disease.

Axolotl Regeneration

Researchers initially relied on macroscopic observations characterized by anatomy and morphology to study regeneration in amphibians (Stocum, 2017). Scientists were performing experimental manipulations by cutting amphibian legs long before microscopic techniques such as histology- the study of tissues by sectioning, staining, and analyzing, existed. As histological techniques improved, scientists were able to further examine the tissue architecture and cellular morphology involved in regeneration.

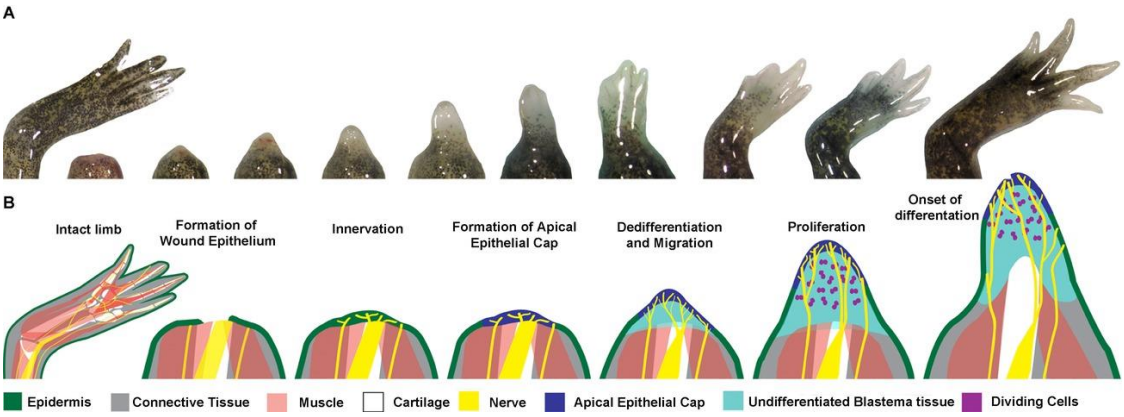

Figure 1: Axolotl limb blastema development

Figure 1(McCusker et al., 2015).above helps us best understand the phenotypic process of how an axolotl limb regenerates after amputation. The left side shows the intact limb while the consecutive picture to the right shows the various stages of regeneration over time, including blastema formation (McCusker et al., 2015). Axolotl regeneration follows distal transformation meaning that they regrow only those parts of the body located beyond the site of amputation (Stocum, 2017). As time goes on, blastema cells begin to differentiate into specialized cells with various parts of the new limb starting to form gradually, starting from the bottom, and moving upwards (McCusker et al.,2015).

In an axolotl, when the limb is amputated, a remarkable sequence of events unfolds. First, a blood clot forms at the cut site to seal the wound and prevent excessive bleeding. Following this, the skin begins to grow over the wound, providing a protective barrier against pathogens. Concurrently, a body-wide proliferation response is triggered promoting the rapid proliferation of cells necessary for tissue regeneration (Payzin-Dorgu et. al, 2023)Subsequently. cells migrate to the wound site from surrounding tissues and undergo dedifferentiation, preparing them for their role in regeneration. This molecular progression is still a puzzle to the scientific community. The next step is where the regeneration process diverges between axolotl and mammals. Cells surrounding the site of injury migrate to the cut site where they multiply eventually forming a visible bud

forming a blastema which resembles the axolotl limb bud structurally. While mammals also form blastema-like structures, unlike many injuries that result in complete regeneration in the axolotl, this usually leads to scar formation (Duygu et al., 2018). The blastema is a structure composed of multi-potent cells transiently formed at the site of injury which eventually gives rise to the major mesenchymal structures of the limb such as bones, muscles, and connective tissues (Seifert and Muneoka, 2017). Many diverse organisms including regenerative animal models such as planaria, flatworms, zebrafish, and salamanders like axolotl, utilize the blastema for regeneration. Yet, it remains uncertain whether these blastemas share a common ancestral history (Tajer, B. et. al, 2023)

Blastema

A blastema is an accumulation of cells in the regenerating limb bud that is capable of regrowth and differentiating into different organs and body parts. Extensive research over the years has provided a thorough understanding of the blastema's role in regeneration (Flowers et. al, 2014, McCusker et al., 2015, Stocum, 2017) Blastema consists of cells with varying lineage with limited differentiation potency (Monaghen and Madden, 2012).

Research has shown that initially, the accumulated blastema initially lacks blood vessels and innervation (Stocum, 2017). However, as nascent blastema cells proliferate, and signals from the peripheral nervous system promote blastemal growth, the blastema undergoes revascularization and reinnervation; this is essential for the continued growth and differentiation of the blastema into the various structures of the regenerated limb (Farkas and Monaghan, 2017).

Cell Differentiation

Gateway to Regeneration

Regeneration in blastema initiates as cells go through a process of differentiation and dedifferentiation. Undifferentiated or less specialized cells become increasingly specialized and acquire specific functions, whereas specialized cells that were already in the body revert from specialized cells to a primitive state to help heal the injury (Stocum., 2019). Undifferentiated stem cells serve as the linchpin in the complex process of regeneration by coordinating the renewal and repair of injured tissues. Their remarkable capability to transform into different cell types is fundamental to the restoration of intricate structures in organisms.

Stem cells are plentiful in embryos and can also be found in adult tissues (Zakrzewski et al., 2019). Stem cells come in various types. Totipotent cells possess the greatest potential for differentiation capable of dividing and transforming into cells for the entire organism, including both embryonic and extra-embryonic structures like the placenta (Zakrzewski et al., 2019). The zygote, which is formed when a sperm fertilizes an egg, is a totipotent cell. In mammals, these cells continue to divide eventually forming the three pluripotent early germ layers- endoderm, mesoderm, and ectoderm, which are responsible for giving rise to the entire organism and its structures. (Zakrzewski et al., 2019). Pluripotent cells have less differentiation potential compared to totipotent cells; as they undergo further differentiation, they transition into cells with reduced potency such as multi, oligo, or unipotent cells. Hematopoietic stem cells are multipotent cells capable of developing into several types of blood cells. However, once these cells undergo

differentiation, they turn oligopotent, meaning that their differentiation is limited to specific cell lineage (Zakrzewski et al., 2019).

Oligopotent stem cells can differentiate into several cell types only within a particular tissue or organ, but not all. They exhibit a more restricted differentiation potential compared to pluripotent and multipotent stem cells. For instance, myeloid cells are oligopotent within the hematopoietic cell system and can differentiate into a few related blood cell types in the body but not all (Zakrzewski et al., 2019). Lastly, unipotent stem cells can only differentiate into one single cell type. For example, satellite cells can differentiate into muscle cells exclusively without giving rise to other cell types (Zakrzewski et al., 2019).

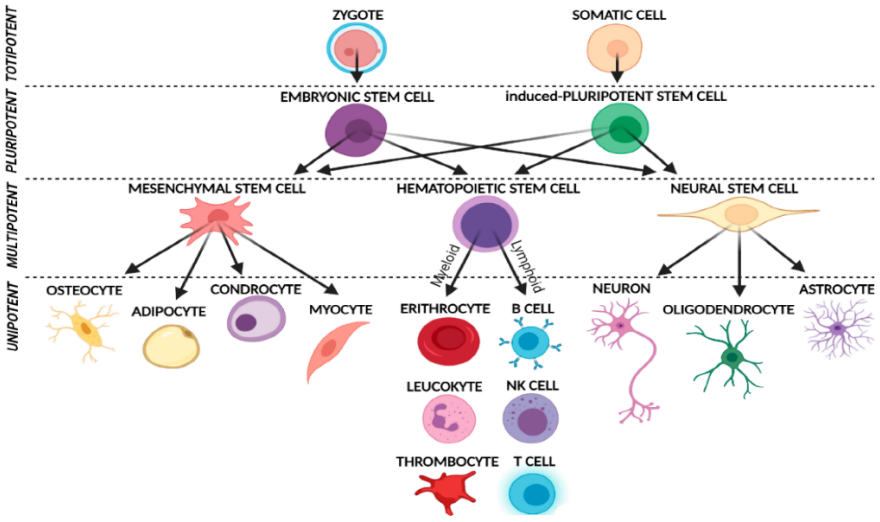

Figure 2: Stem Cell Differentiation

Cellular Specialization Process of Stem Cells (Figure 1, Ossanna R et. al, 2023)

Progenitor cells are partially differentiated cells that have the potential to differentiate into a specific cell type (ClinMedJournals, 2023). They are more committed to a particular cell fate than stem cells and have a more limited ability to self-renew. Progenitor cells are sometimes referred to as intermediate cells since they sit between stem cells and fully differentiated cells (ClinMedJournals, 2023).

Progenitor cells are found in various tissues throughout the body and engage in tissue repair and maintenance. These cells are particularly valuable in the regeneration process as they can replace damaged cells with functional equivalents, aiding tissue repair. By transplanting or stimulating progenitor cells at tissue sites, we can seek to promote tissue regeneration and promote repair.

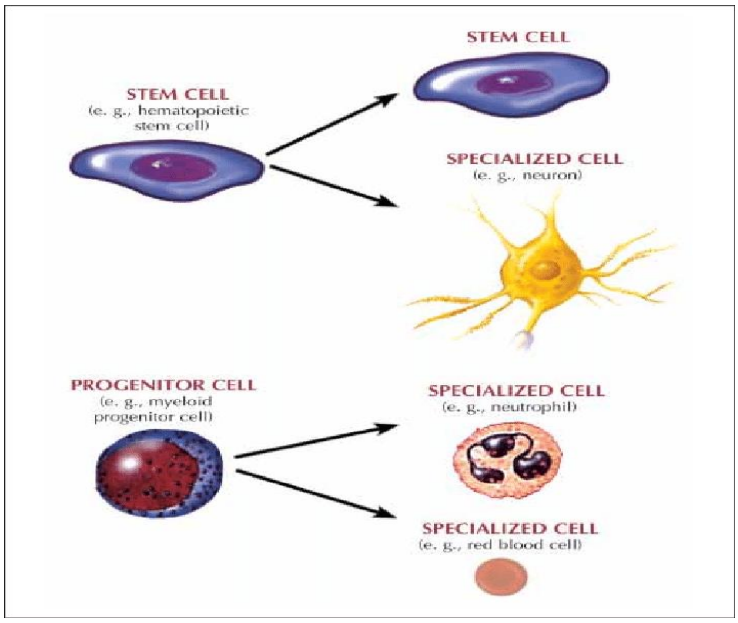

Figure 3: Stem Cell Vs Progenitor Cell

Distinctive trait between Progenitor cells and Stem cells (Figure 3., Zech, 2020)

Upregulated Gene in Blastema – *Kazald2*

The pivotal role of blastema in regeneration has spurred numerous studies aimed at identifying its transcriptome, encompassing all RNA transcripts expressed within blastema cells. One study sequenced the transcriptome of several axolotl tissues including blastema combined with *de novo* transcriptome assembly and computational analysis, without the need for a reference genome (Bryant et al., 2017). They studied the expression analyses of RNA transcripts and genes upregulated in the blastema which resulted in the identification of the gene – *Kazald2* as the most blastema-enriched transcript. *Kazald2* is upregulated >10-fold compared to all the other tissues that were analyzed as shown in Figure 4 below (Bryant et al., 2017).

Further investigation using *in situ hybridization*, also shown in Figure 4 below, throughout regeneration showed that *Kazald2* is upregulated 10 days post-amputation (DPA) but is not detected in an intact limb or a developing limb bud (Bryant et al., 2017). This suggests that *Kazald2* plays a role in axolotl limb regeneration, specifically in blastema.

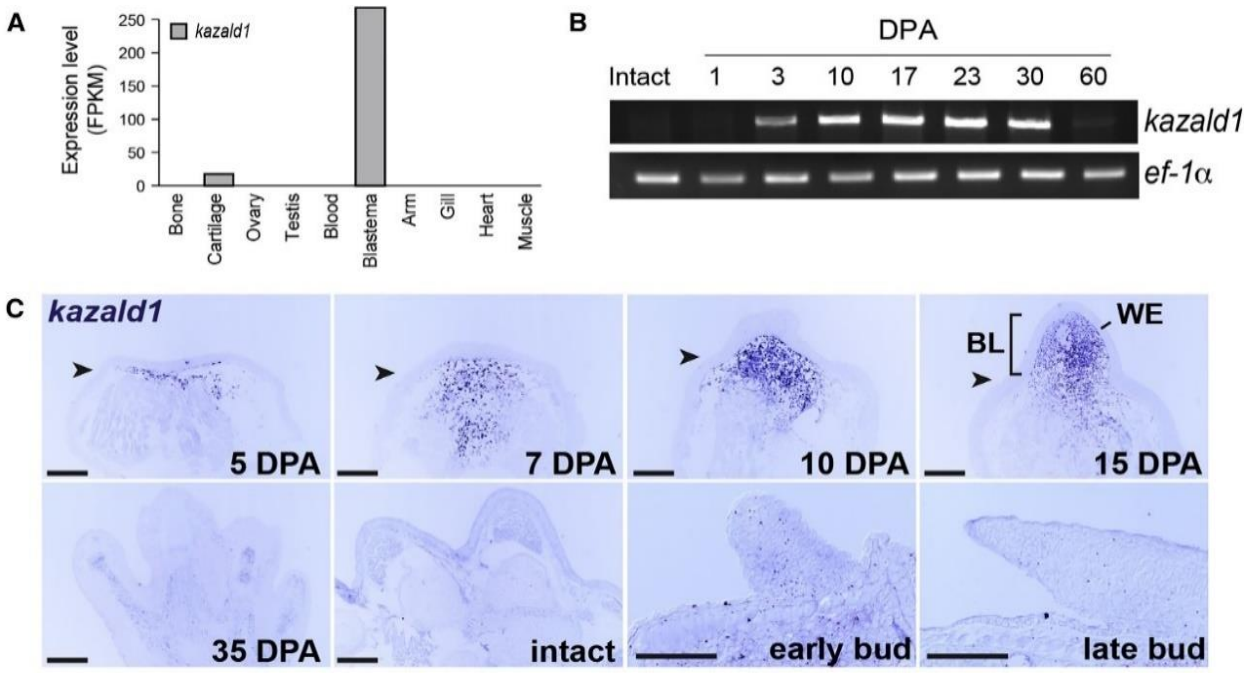

Figure 4: *Kazald2* is necessary for blastema regeneration.

Comparative tissue examination identified Kazald2 as the highly upregulated blastema-enriched transcript in the axolotl limb (Figure 5A, Bryant et al., 2017) The Kazald2 gene referenced in Bryant et al.'s paper has since been determined by additional phylogenetic analyses to be Kazald2, distinct from the Kazald1 ortholog shared with mammals.

They found that *Kazald2* expression increases shortly after amputation, is maintained during the blastema, and dramatically downregulated near the end of regeneration (Bryant et.al., 2017). They used morpholinos to target the *Kazald2* gene; morpholinos are a synthetic molecule used to temporarily block the gene expression by binding to complementary RNA sequences, enabling the study of gene function and developmental process in axolotl. This resulted in delayed limb regeneration and a notably smaller blastema, suggesting the essential role of the *Kazald2* gene in regeneration (Bryant et.al., 2017). Conversely, constitutive expression of *Kazald2* within the blastema led to the regeneration of deformed limbs, suggesting that improper

regulation of *Kazald2* expression has negative consequences for limb regeneration suggesting the importance of the temporal (when) and spatial (where) regulation of *Kazald2* expression during limb as seen in Figure 5 below (Bryant et.al., 2017).

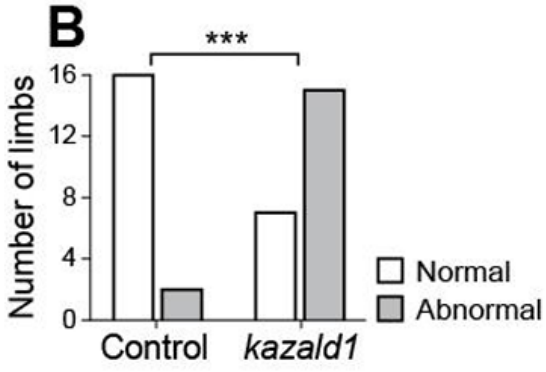

Figure 5: Extended misexpression of *Kazald2* results

Extended misexpression of Kazald2 resulted in significant regenerative defects when compared to control conditions (Bryant et.al., 2017, Supplemental data,S6B). The Kazald2 gene referenced in Bryant et al.'s paper has since been determined by additional phylogenetic analyses to be Kazald2, distinct from the Kazald1 ortholog shared with mammals.

Driven by the discovery of *Kazald2*'s importance in normal regeneration and to complement the morpholino experiment, researchers also targeted *Kazald2* with CRISPR. However, they were dismayed to discover that they did not observe regenerative issues in the gene-edited mosaic animals which they attributed to the low editing efficiency they encountered (Bryant et.al., 2017). This phenotypic discrepancy between morphants created using morpholinos and mutants that were knocked out has been observed in several other comparative studies with other animal models and seems to be the case with

axolotls as well (Peng, 2019). Research suggests that in some cases, employing morpholino knockdown methods can yield more pronounced effects than loss-of-function mutations, (Rossi et al., 2015). This study noted that related genes were upregulated in mutants while morphants did not exhibit this change (Rossi et al., 2015). Considering this finding, through extensive analyses of publicly available data, scientists at the Whited Lab uncovered that Kazald has three closely related genes that might be upregulated by this mechanism (Tajer, B., Unpublished, The Whited Lab).

Kazald Gene Family

Members of the Kazald gene family are characterized by the presence of multiple domains in their proteins –the Kazal-type serine protease inhibitor domain in its central region that acts as an inhibitor of serine protease, an enzyme that cleaves peptide bonds in proteins, Insulin-like-Growth-Factor-Binding- Protein (IGFBP) in its N-terminal, and an immunoglobin-like domain in its C-terminal (NIH, 2023). Domains within proteins serve as structural and functional units, folding to fulfill a diverse range of roles crucial for the proper functioning of cells and organisms (Aziz et. al, 2021). These domains can be useful in elucidating the functions of Kazald family proteins.

*Kazald*1, *Kazald2*, *Kazald3,* and *Kazald4* are paralogs of each other within the Kazald gene family. The axolotl *Kazald1* gene is orthologous to human *Kazald1*. The study of orthologs and paralogs assumes significance as it offers valuable insights into the evolutionary lineage and functional diversity of genes. Paralogs are genes that arise from gene duplication events and have a shared ancestral origin. While they initially possess similar sequences and functions, they have diverged over time due to mutation

and other evolutionary forces. Ortholog genes, on the other hand, are genes that diverged after the speciation event while retaining a similar function to their ancestral gene.

Table 1. Kazald Ortholog Vs Paralog

Species	Kazald1	Kazald2	Kazald3	Kazald4
Human	Orthologs	Not Present	Not Present	Not Present
Axolotl	Orthologs	Paralog	Paralog	Paralog

Human and Axolotl Kazald1 are orthologs while Kazald2, Kazald3 and Kazald4 are paralogs of Axolotl Kazald1 which are not present in humans.

Gene duplication is a fundamental process in molecular evolution, and has significant implications for organisms' genetic makeup, phenotype, and evolutionary trajectory (Ohno, 1967). One of the immediate consequences of gene duplication is the creation of redundant copies of the original gene, safeguarding against deleterious mutations, and ensuring that at least one functional copy of the gene remains in the genome (Wagner,1996). Over time, duplicated genes can accumulate mutations independently, leading to the development of new functions, neofunctionalization, or the splitting of original functions between the copies known as sub-functionalization (Rastogi and Liberles, 2005, Force et.al, 1999).

Duplicated genes may undergo specialization, where each copy becomes optimized for specific functions or conditions contributing to the emergence of novel traits or diversification of species (James and Tawfik, 2003). Additionally, gene duplication can increase gene dosage altering cellular processes and traits, with effects that vary based on the genes' function (Hartwell, 2021). Gene dosage indicates the number of copies of a specific gene housed within an organism's genetic material.

(Hartwell, 2021) Gene dosage effects can have both beneficial and deleterious consequences, depending on the specific gene and its role in the organism. Finally, by providing redundancy in vital pathways, gene duplication enhances genetic robustness, shielding organisms from genetic and environmental stressors.

Gene Robustness

Genetic Robustness refers to an organism's capability to maintain a consistent physical appearance despite genetic variations (El-Brolosy and Stainier, 2017). This trait is measurable and may vary depending on the phyla or organism (Boukhibar and Barkoulas, 2016). Redundant genes, often resulting from gene duplication play a key role in Genetic Robustness (Wagner, 1996; reviewed in Tautz, 1992). Another aspect of robustness stems from precisely regulated cell networks that control metabolism, signaling, and transcription (Davidson and Levin, 2005). Unfortunately, this robustness poses challenges for genetic research studies as it can obscure the expected outcome in mutants, particularly in animal models such as axolotl, which have large genomes with many gaps and repetitive sequences making it challenging to identify the precise function of the original gene as related genes may compensate for the change, referred to hereafter as genetic compensation (El Brolosy et al., 2017).

Genetic Compensation

Genetic compensation is a biological phenomenon where organisms adjust their gene expression to mitigate the effects of genetic mutations (Rossi et. al, 2015). Genetic compensation can enable an organism to maintain cellular or organismal homeostasis despite the presence of genetic mutations. It allows organisms to tolerate genetic

variation and adapt to changes in their genetic makeup. Genetic compensation can be triggered when a gene is knocked out or mutated, leading to the loss of function of a specific protein, often resulting in a mutant phenotype, although it's not always the case (Rossi et. al, 2015) Recent findings suggest that the decay of mutant mRNA is essential for triggering Genetic Compensation, highlighting the intricate interplay between mRNA degradation mechanisms, particularly nonsense-mediated decay, and the cellular response to genetic alterations (El Brolosy et al., 2019).

Nonsense Mediated Decay (NMD) is a mechanism that cells utilize to identify and break down mRNA molecules containing premature stop codons (nonsense mutations), thus preventing the production of truncated and potentially harmful proteins (Tan et. al, 2022). In this research, scientists genetically deactivated key components of the NMD pathway and observed that inhibiting NMD resulted in a reduction in the decay of mutant mRNA (El Brolosy et al., 2019). This disruption in NMD led to the loss of genetic compensation, indicating the active involvement of NMD in cellular adaptation to genetic mutations. Furthermore, the researchers demonstrated that inducing mRNA degradation using uncapped RNAs, susceptible to rapid cellular degradation, could trigger genetic compensation (El Brolosy et al., 2019). This underscores the pivotal role of mRNA degradation pathways, such as NMD, in facilitating cellular adaptation to genetic changes.

This study also suggests that mutant alleles failing to produce mutant mRNA, either due to transcriptional blockade or efficient decay, exhibit more severe phenotypes compared to alleles where mutant mRNA is degraded (El Brolosy et al., 2019). Transcriptome analysis of alleles with degraded mutant mRNA revealed an upregulation

of genes matching the sequence of the mutant mRNA (El Brolosy et al., 2019). These findings prompted us to further investigate the role of Kazald genes in the axolotl, aiming to determine if they provide genetic compensation for *Kazald2*.

Kazald1

In humans, only the *Kazald1* gene exists, hereafter used as *hKazald1,* which is orthologous to axolotl *Kazald1*. By comparing the gene sequences and the expression patterns of these two genes we can try to understand the function of *Kazald1*. A research study investigated the expression pattern of the *hKazald1* and found it to be involved in the developing bones and teeth of mice, and its association with matrix mineralization (James et al., 2004). Research conducted at the Whited Lab has further supported this assumption by revealing similar gene expression patterns of *Kazald1,* particularly upregulated in the cartilage (Payzin et. al, unpublished). We have limited knowledge about the function of any Kazald family member, although little research on humans and mouse Kazald genes suggests its role in bone biology. Since axolotl *Kazald1* is also present in both cartilage and bone, it likely performed similar functions. Additionally, on the molecular level, *Kazald2*, *Kazald3,* and *Kazald4* may operate through a mechanism like the better-understood *Kazald1*.

Hypothesis

Building on the insights gained from the understanding of genetic compensation and paralog functions we have hypothesized that these other Kazald genes – *Kazald1, Kazald3,* and *Kazald4* may be genetically compensating for the loss of function of the *Kazald2* gene, providing a plausible explanation for the observed phenotypic differences

between morpholino Mutant and loss-of-function mutant. To investigate this hypothesis, I sought to test out two experiments; one is to knock out (KO) all Kazald paralogs, either individually or in combination utilizing CRISPR technology; the second experiment involves a Hybridization Chain reaction imagining of RNA in KO mutant.

Research Scope

Scope 1

The Whited Laboratory previously established a KO *Kazald*2 mutant axolotl. Thus, one of the primary objectives of my research was to systematically generate mutants for *Kazald*3, *Kazald*4, and *Kazald*1. This expansion of genetically modified organisms in the laboratory setting aims to enable comprehensive investigations. The goal is to combine these mutants with *Kazald2* mutant to assess if having multiple mutant Kazald paralogs would result in a more severe phenotype. To achieve this, we aimed to disrupt or delete the target genes, *Kazald3*, *Kazald4*, and *Kazald1* utilizing the CRISPR technology.

The resulting KO Kazald mutant can be further utilized to advance the study of genetic compensation. Our hypothesis suggests that mutants with only one Kazald gene KO should exhibit similar phenotypical outcomes as those of normal regeneration. However, we anticipate that all four Kazald KO mutants exhibit abnormal to no regeneration.

Scope 2

In my investigation of Kazald Genetic Compensation, my second research focus involves examining the RNA expression levels of Kazald gene paralogs within the blastema. This investigation employs the Hybridization Chain reaction (HCR) technique, a sophisticated method that provides an unparalleled approach to detecting and visualizing specific RNA sequences with remarkable sensitivity and specificity. HCR enables the direct visualization of target RNA sequences within intact cells and tissues, offering a powerful means to explore the intricate details of genetic expression.

In employing HCR, our objective was to visualize the upregulation of RNA from the other three Kazald genes in mutant animals, aiding in investigating our hypothesis of genetic compensation. Significant upregulation of RNA from the other three Kazald genes in mutant animals compared to wild-type control suggests that the organism may compensate for the loss of function of one Kazald gene, *Kazald2* in this scenario, by increasing the expression of its paralogs. This compensatory mechanism indicates that the organism can maintain normal function despite genetic perturbations, supporting the concept of genetic compensation. Conversely, if there is no significant difference in RNA expression levels, it suggests that there is no compensatory response to loss of function, potentially refuting the hypothesis of genetic compensation.

Chapter II.

Research Methods

This study involved a total of forty (40) WT animals obtained from The Whited Lab. The axolotls used in this study were maintained by the rules of the Harvard Institutional Animal Care and Use Committee (IACUC). We ensured that all animals were treated ethically and in compliance with applicable laws and regulations governing the use of animal models in research. The axolotls utilized in this research study belonged to the white strain, also known as the *leucistic* strain, which we refer to as *Wild Type* (WT) hereafter. However, it is important to acknowledge that in research, WT may denote the most prevalent phenotype or genotype within a population under controlled laboratory conditions, rather than accurately reflecting the phenotype or genotype of the animals in their natural habitat.

No human subjects were involved in this research. The use of human subjects in research involving functional studies such as limb amputation is considered unethical as it could cause unnecessary harm or suffering to the subjects. Axolotls served as the primary animal model for all experiments conducted.

Research Methods in Experiment 1

To mutate the other three KAZALD paralogs- *Kazald1*, *Kazald3,* and *Kazald4,* I employed CRISPR/Cas9 technology for its gene-editing capability. This involved generating embryos with individual gene knockouts and simultaneously targeting all four genes for knock-out. Specifically designed guide RNA matching target gene directed the Cas9 enzyme to cleave DNA, initiating a repair mechanism that may result in insertions

or deletions within the targeted gene. (Lander E., 2016). Injecting axolotl embryos with a combination of guide RNAs and CRISPR-Cas9 enables us to introduce mutations in specific Kazald genes.

Guide RNA and T7 Primer Design

I designed guide RNAs (gRNAs) to target *Kazald1*, *Kazald3*, and *Kazald4* individually using the software tools, CCTop, and Snapgene. Research has shown that efficient gene knockout critically depends on the design of the gRNA, as DNA cleavage efficiency varies among different gRNAs targeting the same locus in axolotl and other species (Fei et al.,2017). CCTop aids in finding the most efficient CRISPR/Cas9 target guide for the locus of interest. CCTop tool identifies and ranks all the potential sgRNA target sites in order of their off-target sites and predicted efficiency. The choice to use CCTop stems from the fact that it is the only gRNA design tool that can look for off-targets. However, its experimental validation for both gene inactivation and non-homologous end-joining repair also aids in decision making (Stemmer. M. et al. 2017). Primer 3 is a widely known tool for designing PCR primers (Koressaar et. al,1937-1938). Using this tool, I was able to find the best forward and reverse T7 primers for each guide. Excel macros were used to ascertain primers' amplification efficiency and to ensure their genomic locations on distinct chromosomes, mitigating the risk of potential off-target effects. The primers and gRNAs that we used for this experiment are:

Table 2. Sequence of gRNA primers.

Gene Name	Direction	Primer sequence
Kazald4	Forward Primer	5’-gccgccagaaaacacctatt- 3’
Kazald4	Reverse Primer	3’- cttcataagactggcgcacC-5’
Kazald3	Reverse Primer	5’- GGACTGAGATATGCGGGTCA-3’
Kazald3	Forward Primer	3’- Agtagtacacggccgaatgt-5’
Kazald1	Forward Primer	5’TGTCCCCACCCTACAACATC-3’
Kazald1	Reverse Primer	3’- acctgacctggagactttgg-5’

Table 3 . Sequence of gRNA.

Gene Name	gRNA sequence
Kazald4	5’-GAAATGTGCGCGTCGTCCCC- 3’
Kazald3	5’- GGGACCAGTTCAACACGTCT -3’
Kazald1	5’- GCACGGACATGCTACTACCT-3’

CRISPR/Cas9

CRISPR, an acronym for Clustered Regularly Interspaced Short Palindromic Repeats, is a genome editing system enabling precise DNA modification in living organisms, widely adopted in. scientific research. CRISPR is a naturally occurring bacterial mechanism used to destroy viral DNA present in bacteria by cutting the DNA at a specific point (Doudna and Charpentier, 2014). This was well-known since the early 2000s but with the advent and knowledge of the Cas9 enzyme, this mechanism has been adapted for use in laboratories to target and mutate DNA since 2013 (Lander E., 2016). Cas9 is an enzyme that can, with the aid of gRNA guiding it to the cut site, cut apart and unzip the double-stranded DNA.

Once CRISPR cleaves the DNA at the cut site, the cell's repair machinery typically engages in the natural DNA repair process. While this repair mechanism generally functions accurately, occasional errors may occur leading to the mutation that can disable the targeted gene, often through non-homologous end joining (NHEJ) (Zaboikin et al., 2017). NHEJ is an error-prone mechanism that can lead to insertions or deletions in the DNA sequence causing frameshift mutations and gene knockouts (Rodger and Mcvey, 2015). By introducing mutations that disable the gene of interest, the CRISPR system can be used to create loss-of-function mutations. Research has demonstrated that using purified Cas9 protein, rather than Cas9 mRNA, leads to the rapid formation of a gRNA-Cas9 ribonucleic protein (RNP) complex (Fei et al., 2017). This significantly enhances CRISPR/Cas9-mediated Knock-out and Knock-In in various model organisms. Therefore, in this research Cas-9 protein was employed for immediate activity post-injection.

CRISPR system in the context of this research consists of three components that are hybridized: endonuclease Cas9 protein, CRISPR RNA (crRNA), and Trans-activating CRISPR RNA (tracrRNA). crRNA is the portion of the gRNA that contains the sequence complementary to the target sequence while tracrRNA serves as a scaffold for Cas9 binding and is necessary for the interaction between cas9 and the crRNA.

I individually co-injected the designed gRNAs targeting the coding sequences in *Kazald1* exon3, *Kazald3* exon 2, and *Kazald4* exon 2 along with together with Cas9 protein, into single-cell stage axolotl eggs. Using a customized CRISPR system with guide RNAs tailored to match specific target genes, I employed CRISPR-Cas9 enzymes to precisely cut all the Kazald genes. It is important to acknowledge that the effectiveness of this method is dependent on several factors, including the targeting efficiency of the gRNAs towards the desired sequences and the injection process itself, which can introduce variability as some embryos may receive better injections than others.

Table 4. Targeted loci.

Kazald1 Exon3.
GACCACAGATCTTGTCCCCACCCTACAACATCTGGAATGTCACGGGGCAAGA
TGTGATATTTGGCTGCGAGGTCTTCGCCTATCCAATGGCATCCATTGAGTGGA
GGAAGGACGGCACGGACATGCTACTACCTGGGGATGATCCTCACATATCTGT
TCAG

Kazald3 Exon 2,
CACCGGAAATTAAGATACCACCCCGGGACCAGTTCAACACGTCTGGACAGGA
TGCCATCTTCTTGTGTGAAGTCGTGGCCTATCCCATGGCACAGGTGGAATGGC
GGAAGAACAGACACAATGTTAGCCTGCCTGGGGATGACCCGCATATCTCAGT
CCAG

Kazald4 Exon 2.
CTCCTGTTATATCTTTGCCGCCGAGAGATGCGCATAATTTTACCGGAAATGAC
ATCATTTTTGGCTGTGAGGTGTCAGCCTATCCCATGCCCCACCTGGAATGGAA
GAAGAAAGGGAACCACGTGTTTCTGCCCGGGGACGACGCGCACATTTCCGTT
CAG

Injections

The axolotl embryo begins as a single cell following fertilization of the egg. The initial round of cell division in an axolotl egg typically takes about six hours. Transgene integration is more probable during the single-cell- stage, as CRISPR is more likely to distribute across all the cells of the axolotl (Fei et al., 2017). As the embryo progresses to multiple cells, it becomes challenging to inject all cells effectively. Hence, we opted to inject the embryos with the Cas9 gRNA/protein complex during the single-cell stage We introduced Cas9 proteins into the single-cell stage axolotl embryos using microinjection to induce mutagenesis of our target genes.

The primary objective of this approach is to attain germline mutations. Not all injected embryos will yield successful mutations, and it is crucial not only to maximize the chances of obtaining viable mutants but also to ensure enough mutants reach adulthood for further analysis, necessitating the injection of a considerable number of embryos. Furthermore, to address the challenge of mosaic mutations in CRISPR F0 mutants, we can employ techniques such as genotyping or sequencing to discern and identify animals that have effectively integrated the desired mutations, which, unfortunately, we were unable to complete.

Each mating session can yield a considerable number of eggs, exceeding 100, providing a substantial pool of embryos for experimentation. Nevertheless, this research was limited by the sporadic timing of axolotl egg-laying. This constraint was compounded by variations in fecundity across mating pairs, with some exhibiting low egg production. Furthermore, the experimental protocol necessitated prompt embryo injection within the initial hours following egg deposition, a condition not consistently

feasible within the constraints of the available research hours. This is not an issue with other animal models such as mice and fish, since it is much easier to time the mating and control the age of the embryos of these animals, which is not the case with axolotl.

Polymerase Chain Reaction

The Polymerase Chain reaction (PCR) is used to amplify a specific segment of DNA rapidly and affordably. Research analyses often require a significant amount of DNA. PCR targets a minute portion of the axolotl's vast genome, amplifying this segment to provide an adequate amount of DNA for downstream analysis. PCR results helped us determine the most effective gRNA primers for targeting the Kazald genes. By analyzing the PCR outcomes of the Kazald genes in both *WT* embryos and lysed embryos, I could ascertain if the gRNA/Cas9 system effectively induced mutations at the intended site.

DNA Purification

I purified DNA from the injected embryos using DNEasy spin columns. DNA purification isolates edited DNA fragments by removing contaminants like cellular debris and proteins. This enhances downstream analyses accuracy and sensitivity, vital for detecting mutations. Once I obtained the purified DNA natant, I proceeded to utilize the PCR method to amplify the target regions using PCR Master Mix as shown in Table 5 below. I used the DNeasy 96 Spin Column Protocol from Qiagen for purification (DNeasy Blood & Tissue 2020).

Table 5 . Master Mix table for PCR Post-DNA Purification

1x (50uL)		12x Master Kazald4	15x Master Kazald3	15x Master Kazald1
31	H20	372	465	465
10	5x Buffer	120	150	150
1	10mM dNTPs	12	15	15
0.5	Phusion Polymerase	6	7.5	7.5
2.5	Forward	30	37.5	37.5
2.5	Reverse	30	37.5	37.5
47.5	Into tube			
2.5	Template	30	37.5	37.5
50	Total volume	570	712.5	712.5

I further purified the PCR product to isolate and remove excess primers, nucleotides, and enzymes before proceeding with T7 digestion using AMPure XP beads, a magnetic bead-based method. This involved binding DNA fragments to the beads, separating them magnetically to remove contaminants, washing them with ethanol for further purification, and eluting the purified DNA. This method yields high-quality DNA suitable for downstream analyses/T7 Digest.

T7 Endonuclease Digestion

I used T7 Endonuclease- an enzyme derived from T7 bacteriophage, to confirm whether mutations have been induced in a target gene using the CRISPR-Cas9 system (New England Biolabs, 2024) . This enzyme specifically recognizes and cleaves heteroduplex DNA structures, formed from mismatches or loops that arose during the repair process of DNA double-strand breaks via NHEJ. These structures produce smaller fragments that are detectable through Gel-electrophoresis. The appearance of smaller fragments indicates successful T7 Endonuclease I cleavage and the presence of mismatches at the target site (New England Biolabs, 2024).

I performed T7 endonuclease I digestion on the DNA sample at 37°C for 15 min using 1 µl of T7 endonuclease I (T7E1; New England BioLabs). Before T7 Digestion, I also conducted T7 PCR to verify the quality of genomic DNA extracted from the embryo to ensure that enough intact DNA is available for the downstream T7 digestion process. We used un-injected embryos, other Kazald genes, and nuclease-free water as a control for T7 digestions. Un-injected embryos and other controls should remain unaltered by the T7 enzyme, exhibiting no multiple bands in the gel.

Research Methods in Experiment 2

Hybridization Chain reaction

Hybridization Chain reaction (HCR) is a highly sensitive and specific method that uses oligonucleotide probes to amplify RNA, revealing expression patterns within cells or tissues (Bi and Zhang, 2017). It is a form of *in-situ* hybridization that generates a fluorescent signal at the site of the target RNA facilitating visualization. The HCR process involves two stages: detection and amplification. It relies on a set of oligonucleotide probes meticulously designed to bind to precise regions of the target RNA. These probes are strategically equipped with an initiator molecule (i1) split between them. In the amplification stage, fluorescent hairpin molecules, H1, and H2, trigger polymer growth when bound to the initiator enabling background suppression. Amplification occurs only when the probe pairs specifically bind to their target RNA sequences, thereby colocalizing the full initiator and triggering amplification. Hairpins that are bound non-specifically are also unable to trigger amplification (Molecular Instruments, 2024).

The availability of heterozygous *Kazald2* mutant (*Kazald2 Het*) at the Whited lab provided an avenue for additional investigation into the Genetic Compensation mechanisms of *Kazald2* in blastema. The overarching objective of this research is to create a progeny with homozygous Kazald KO mutant and observe the phenotypical differences that arise with a focus on the regeneration of clipped limbs. Leveraging the *Kazald*2 Het, HCR techniques we expected to discern potential overexpression of other Kazald genes in the *Kazald2 Het* enabling a comparative analysis of RNA expressions. However, to progress with this investigation, identifying effective HCR probes for the remaining Kazald paralogs proved to be a significant challenge, forming the secondary research aim of my research. It is noteworthy that the Whited Lab already possessed effective HCR probes designed for *Kazald*2 which were used as a control probe along with *PRRX1* probes. We used a maximum of three probes for each HCR experiment.

Table 6. Function of HCR probes.

Gene Name	Function
Kazald4	Target Gene
Kazald3	Target Gene
Kazald1	Target Gene
Kazald2	Control
PRRX1	Control

The results were visualized using fluorescence microscopy and FIJI, an image processing software, which allowed me to visualize fluorescence offering insights into the expression patterns of the Kazald RNA and other control genes used. This process would help detect if there is upregulation of other Kazald RNA present in the blastema of

the regenerating limb of the Kazald mutant. Several HCR experiments were conducted, but the results presented here are from an experiment with the following specifics.

Table 7. Color Channel for the HCR Probes:

Genes	Fluorescence Label	Color Channel
Kazald1	FITC	Green
Kazald3	FITC	Green
Kazald4	FITC	Green
PRRX1	TRITC	Red
Kazald2	Cys5	Far Red
Nuclei	DAPI	Blue

This color-coded scheme allowed for the comparison and visualization of specific gene expressions providing a comprehensive view of the experiment's results in FIJI. The positive controls for *PRRX1* and *Kazald*2 helped validate the specificity and reliability of the HCR experiment. In this instance, these controls played a crucial role in confirming that the probes did not yield the expected results.

As for tissue samples, samples were collected from axolotl limbs at various time points post-amputation (dpa), including 10 dpa, 13dpa, 14 dpa, 15 dpa, and 21 dpa available. However, due to the stump becoming visibly apparent only by 21 dpa, this time point was selected exclusively for the application of the HCR protocol.

Chapter III.

Results

This section presents the results of my study where we explored the CRISPR-Cas9 gene editing system and assessed a newly designed probe for Hybridization Chain Reaction targeting *Kazald2* paralogs.

In-vivo injection results

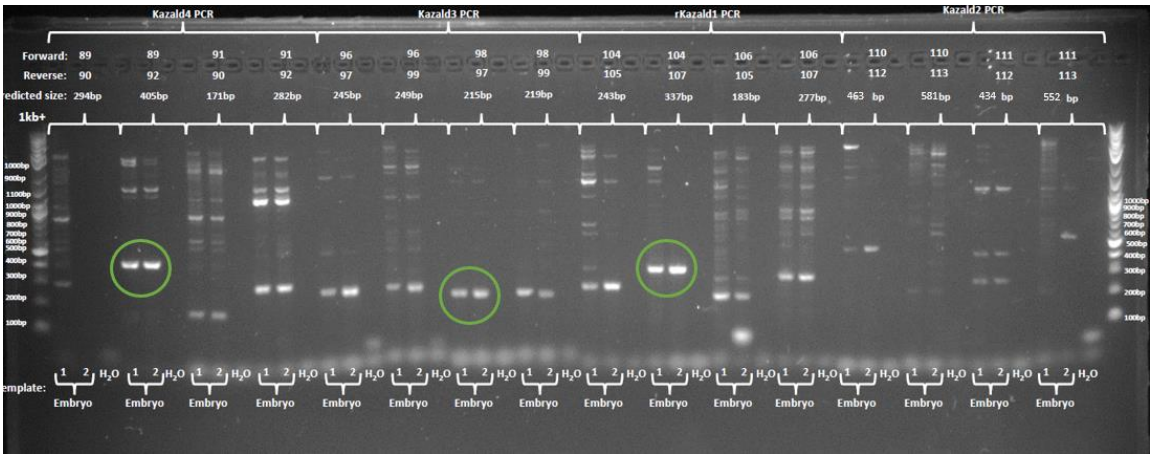

Figure 6. gRNA Primer Test PCR Results

The bands within the green circles were brighter compared to the other primers for each Kazald. They also have fewer bands of the wrong size compared to the other combinations which is why I chose to use those gRNA. The Whited Lab already had Kazald2 primers available which were also tested in this PCR.

Figure 6 above illustrates the assessment of different primers designed for the gRNA, to identify the most effective primer for each gene. We sacrificed a few injected

embryos to determine which primers worked the best for each gene. The bands in Figure 5 represent multiple primer combinations for each Kazald gene that underwent amplification using the designed test primers. Although several options were available at the lab, which we tested as seen in Figure 6, we ultimately used the primer combo for *Kazald2* that the lab had previously deemed effective.

Notably, the bright bands observed correspond to successful primer binding, resulting in bands of the correct size, with a few bands of the wrong size. Conversely, off-target loci contribute to the presence of additional bands, exhibiting varying intensities, and resulting in a fainter appearance. To mitigate the occurrence of such non-specific bands, we increased the PCR temperature to minimize instances of imperfect primer binding. After careful consideration, we decided to use the more prominent bands, corresponding to *Kazald*4 (89,92), *Kazald*3 (98, 97), and *Kazald*1 (104,107).

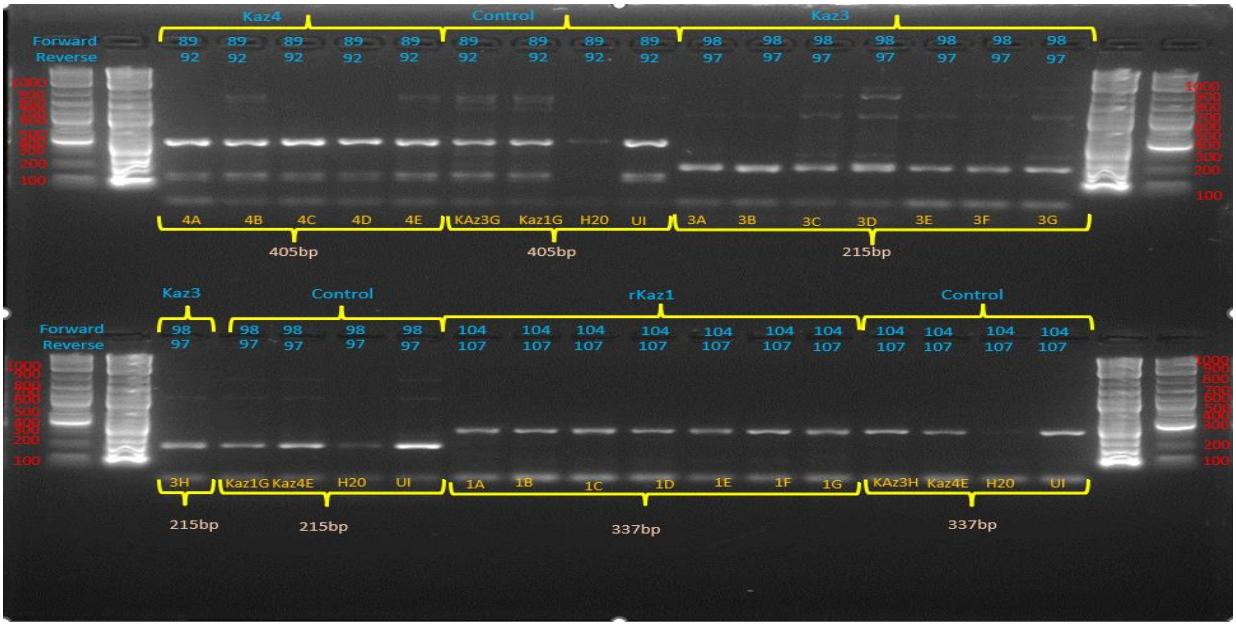

Figure 7. Gel results Before T7 Digestion with Controls

Gel results of the gRNA I chose to use along with controls- Un-injected embryos, water and samples of other injected Kazald genes.

Subsequently, Figure 7 above presents the outcomes testing the effectiveness of gRNA effectiveness, along with controls. The controls utilized for this experiment include embryos from other Kazald genes and un-injected (UI) embryos. UI embryos serve as a good control because they have not been exposed to the gRNA and CRISPR-Cas9 machinery, thus there should be no targeted editing occurring at the genomic level. Without the presence of the CRISPR-gRNA combo, there would be no specific targeting and cleavage of Kazald genes, resulting in the absence of bands in the PCR gel due to the lack of specific DNA fragments to amplify. Samples from other Kazald genes act as a good control because the CRISPR-gRNA combo is highly specific to the target sequence defined by the gRNA. It recognizes and cleaves DNA only at the specific target site

complementary to the gRNA sequence. Therefore, using a DNA sample where *Kazald3* gRNA was injected would act as a good control for checking the efficiency of *Kazald1* or *Kazald4* samples and so forth. However, after repeated experiments, some bands appeared that were not observed in earlier tests, as shown in Figure 6. Despite efforts, we were unable to eliminate these bands, suggesting that the change of PCR cycle of 30x to 35x could have resulted in the appearance of the bands. It is crucial to ensure proper experimental controls and optimization of PCR conditions to minimize non-specific amplification and ensure the reliability of the results.

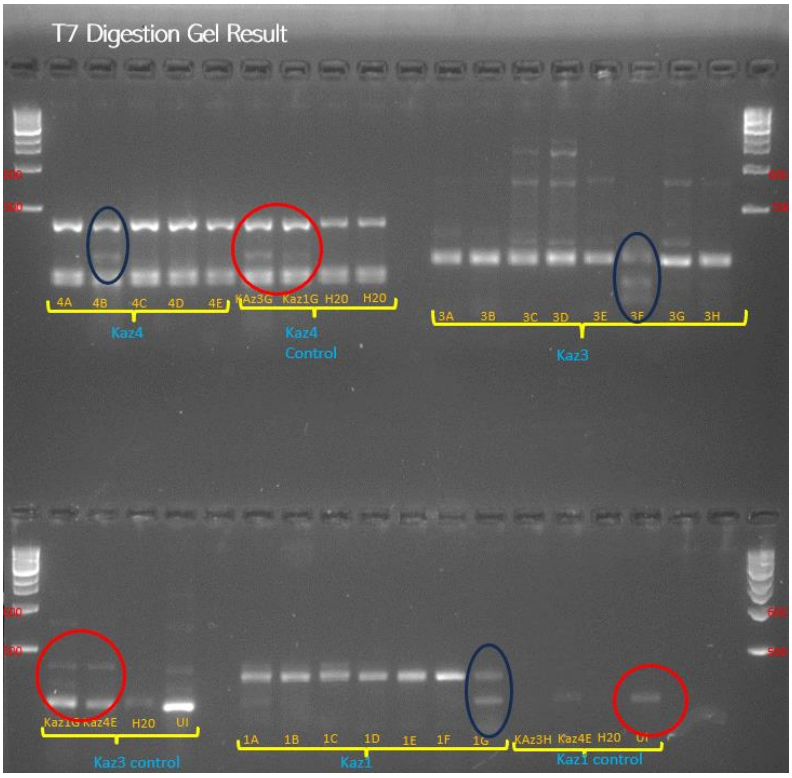

Figure 8: PCR result of T7 Digestion

Gel results in Figure 8 portray T7 digestion results. Kazald3 and Kazald1 were successful while Kazald4 showed some extra bands not expected.

In Figure 8, the PCR results of the sample after T7 Digestion revealed key challenges. Firstly, the *Kazald*1 (Lane 1G and 1A) exhibited two faint bands; but the presence of bands in the control complicated the interpretation of the two bands in the *Kazald1* sample. *Kazald*4 results were beset with issues, necessitating further experiments for validation. Although *Kazald4* T7 Digest gel appeared to work in one lane (Lane 4B), multiple bands were present with this sample, along with extra bands in the *Kazald4* controls (Lane Kaz3G and Kaz1G) Following these observations, attempts were made to obtain a *Kazald*4 PCR with a singular band, a process that extended over several months. While unable to eliminate the multiple bands, we concluded that the T7 digestion itself was successful, as the gel in Figure 9 below appeared as expected, with the T7 digested sample for *Kazald4* having two bands while the non-T7 sample had no cuttings. Conversely, *Kazald*3 (Lane 3E) showed clear success in one lane, with controls exhibiting the expected outcome of no bands, indicating a successful Kazald gene sample with no issues or failures.

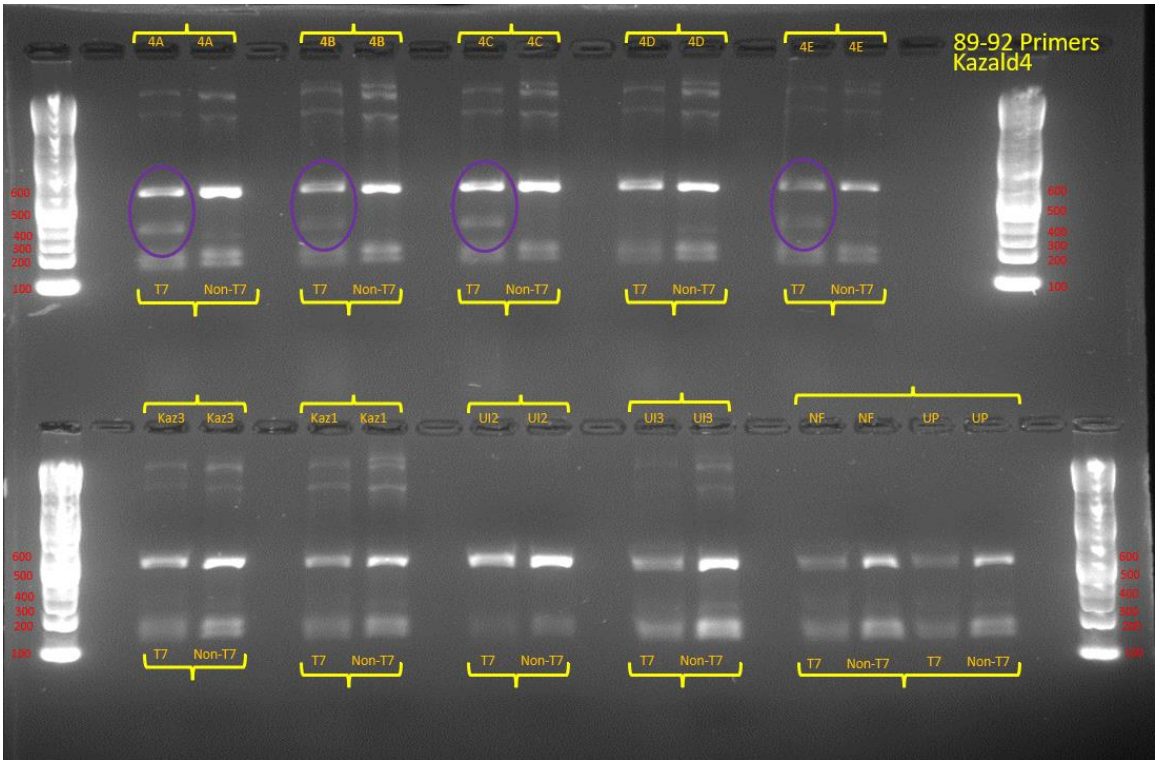

Figure 9: *Kazald*4 T7 Digestion with gRNA

As depicted in Figure 9, the T7 lanes exhibited the presence of additional band on top of the two-band baseline in Kazald4 embryos, while the controls display a singular band without any splitting, aligning with the anticipated outcome.

HCR result

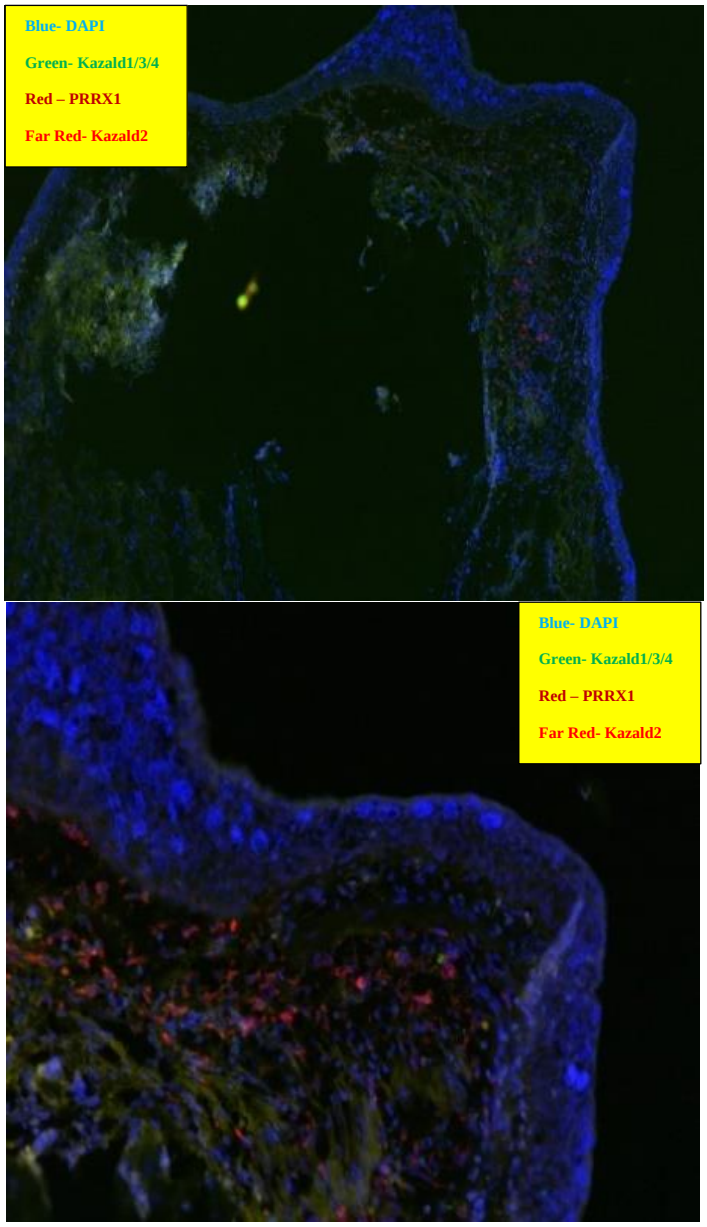

Figure 10*: Kazald1* HCR Composite Imaging (Blastema 21 dpa)

The blue fluorescence depicts DAPI, the green color represents Kazald1, Kazald3 or Kazald4, red color represents PRRX1 and far red represents Kazald2.

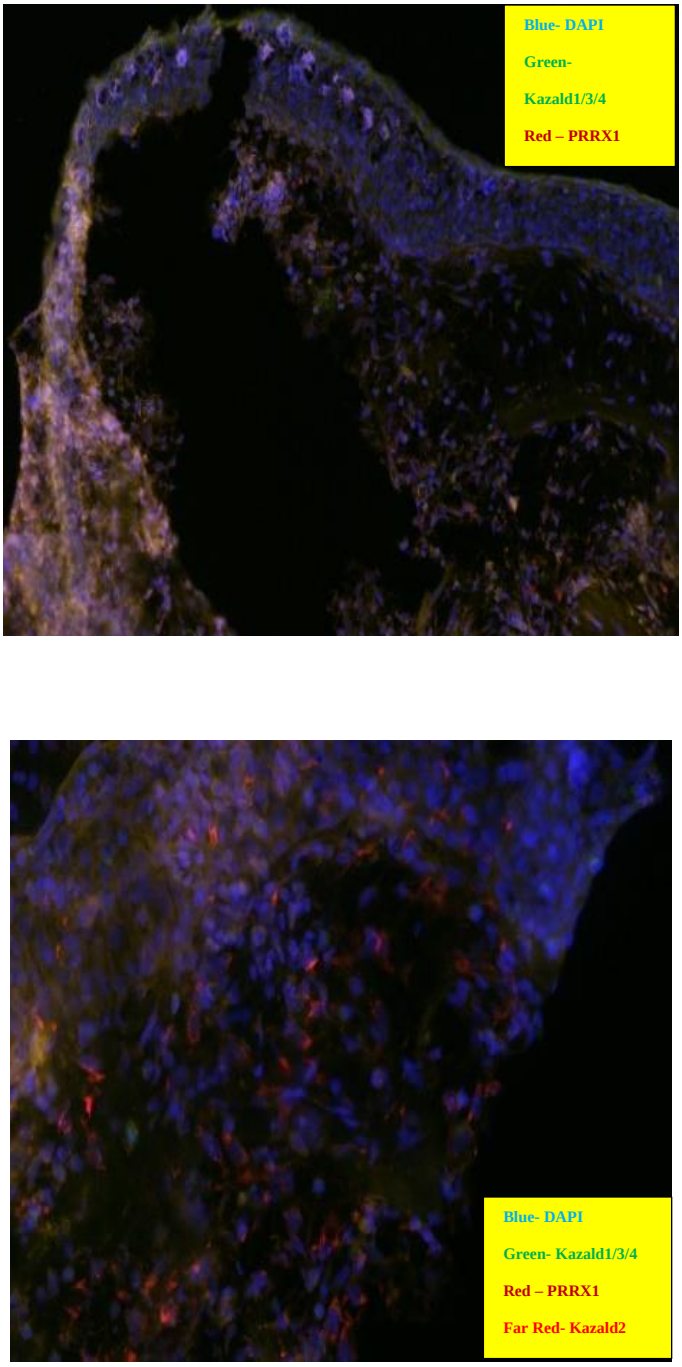

Figure 11: *Kazald*3 HCR Composite Imaging (Blastema 21 dpa)

The blue fluorescence depicts DAPI, the green color represents Kazald1, Kazald3 or Kaald4, red color represents PRRX1 and far red represents Kazald2.

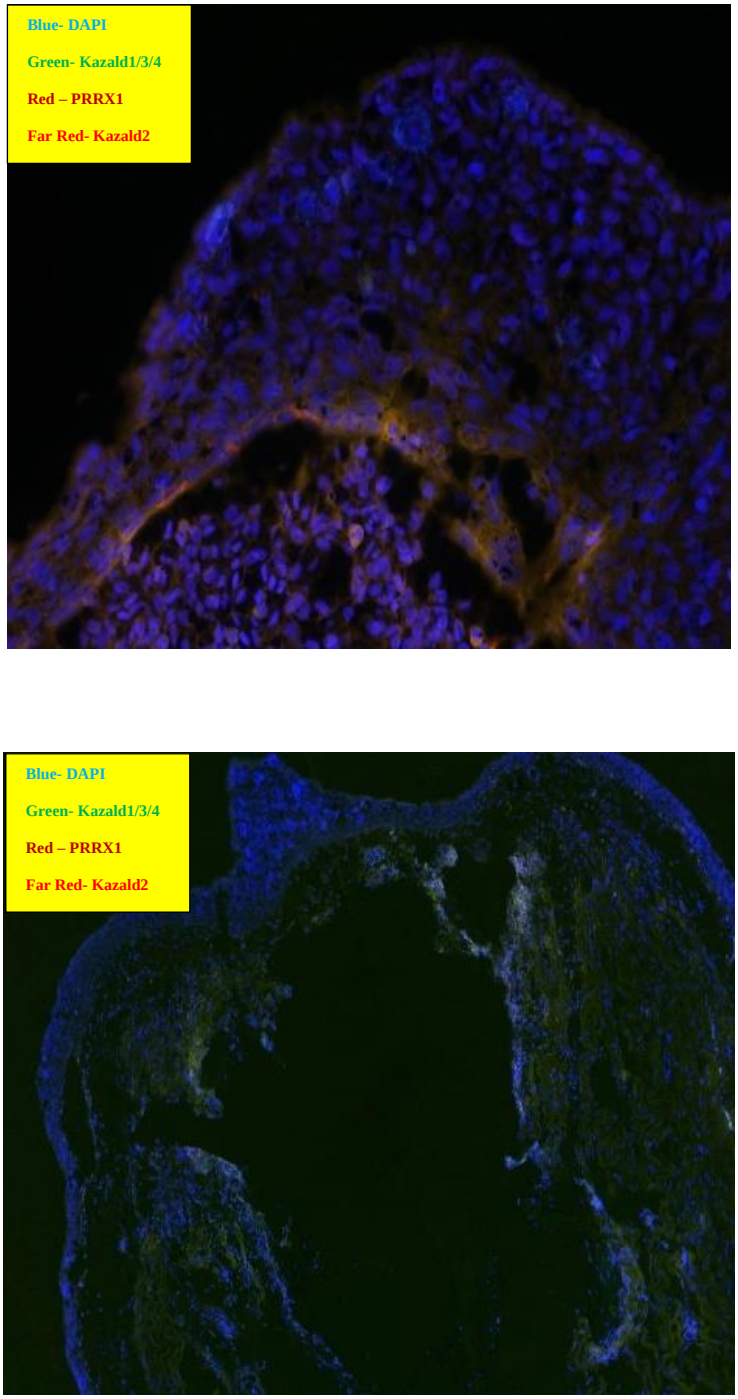

Figure 12: *Kazald*4 HCR Composite Imaging (Blastema 21 dpa)

The blue fluorescence depicts DAPI, the green color represents Kazald1, Kazald3 or Kaald4, red color represents PRRX1 and far red represents Kazald2.

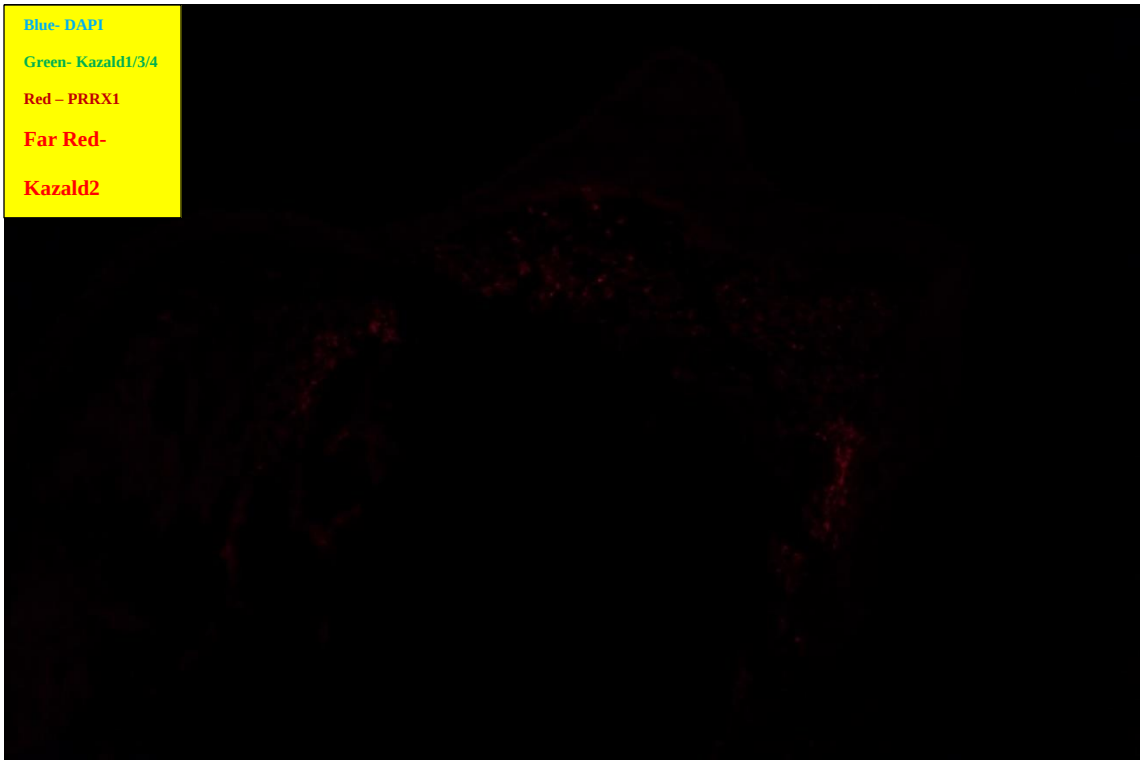

Figure 13: *Kazald2* control

Red dots (far red) in the picture above represent Kazald2 control which states that the protocol itself worked and Kazald2 was a good control. The blue fluorescence depicts DAPI, the green color represents Kazald1, Kazald3 or Kaald4, red color represents PRRX1 and far red represents Kazald2.

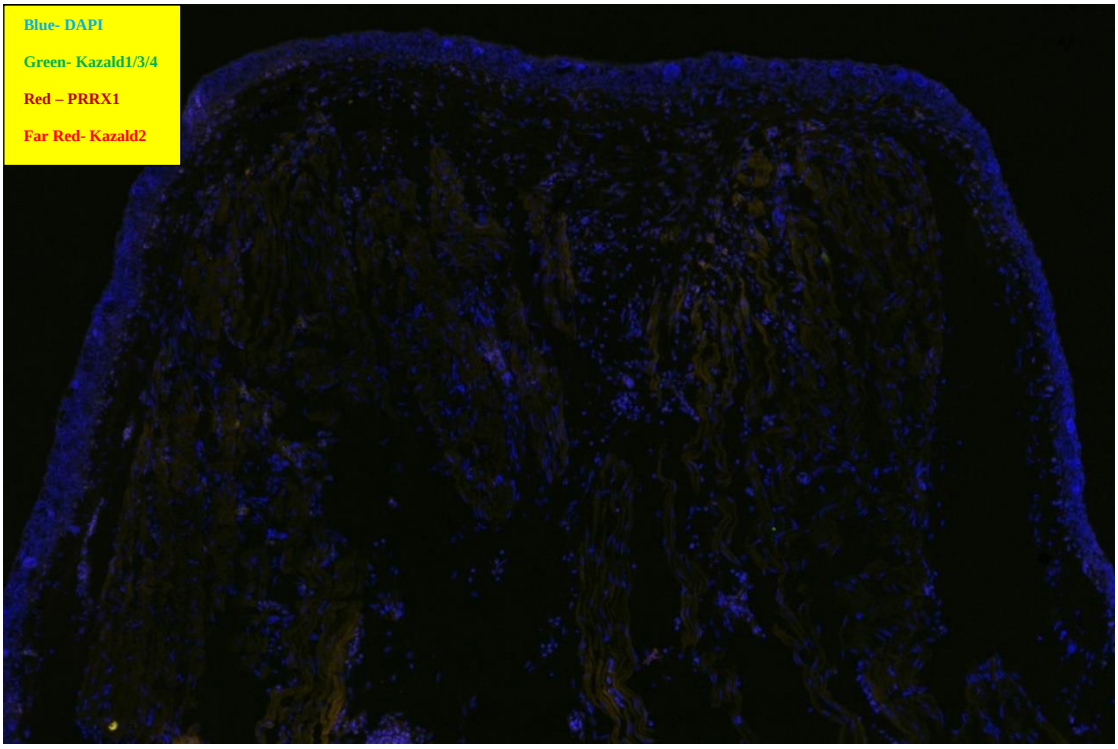

Figure 14: *Kazald1* image with no hairpin

The above picture is an HCR image of Kazald1 without the hairpins which we used as a control. The blue fluorescence depicts DAPI, the green color represents Kazald1, Kazald3 or Kaald4, red color represents PRRX1 and far red represents Kazald2. The results are as expected as there are no dots suggesting that the protocol itself works. There is faint non-specific fluorescence. However, we want to see more fluorescence than this.

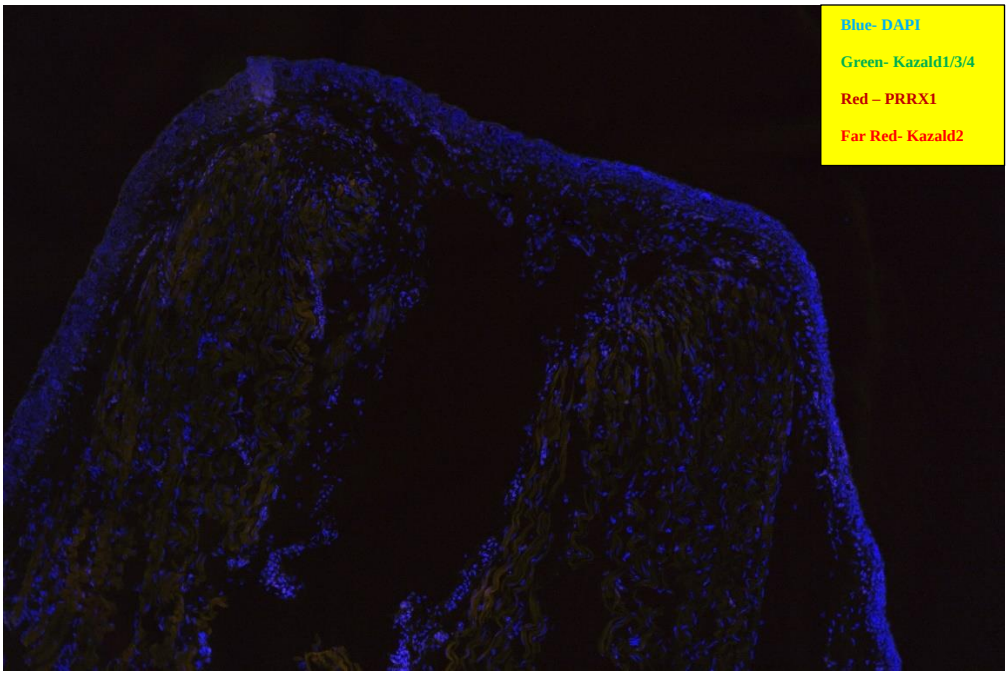

Figure 15: *Kazald3* image with no hairpin

The above picture is an HCR image of Kazald3 without the hairpins which we used as a control. The blue fluorescence depicts DAPI, the green color represents Kazald1, Kazald3 or Kaald4, red color represents PRRX1 and far red represents Kazald2. The results are as expected as there are no dots suggesting that the protocol itself works. There is faint non-specific fluorescence. However, we want to see more fluorescence than this.

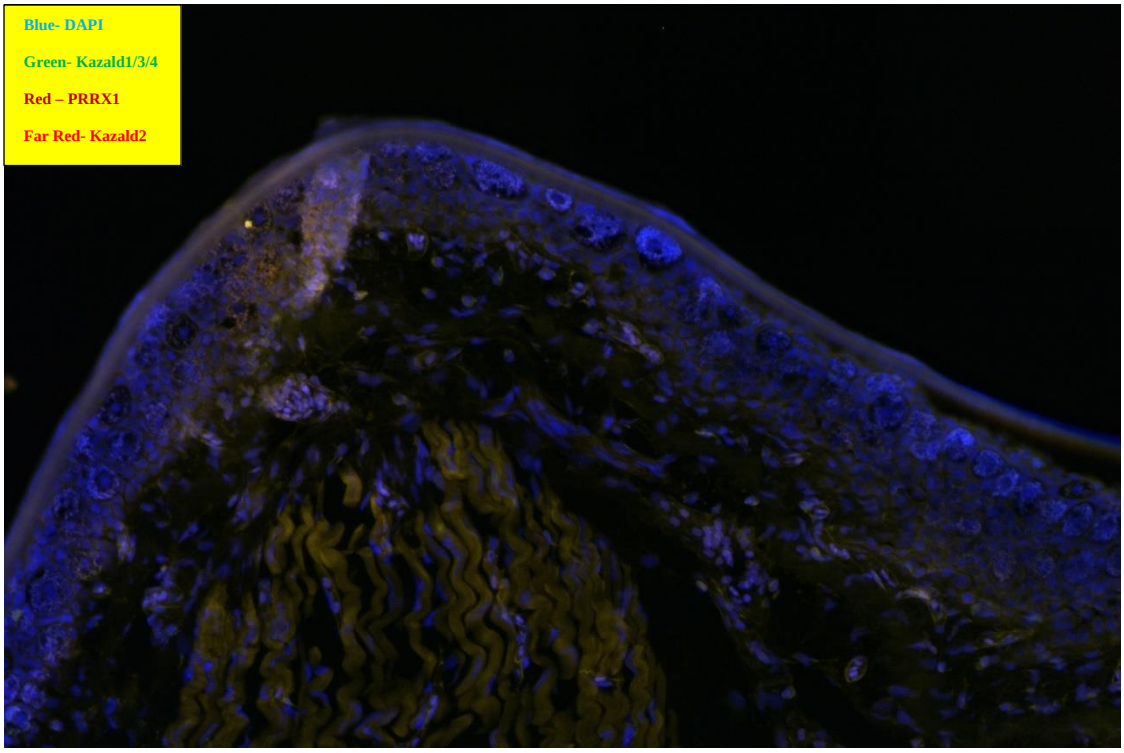

Figure 16: *Kazald4* image with no hairpin

The above picture is an HCR image of Kazald4 without the hairpins which we used as a control. The blue fluorescence depicts DAPI, the green color represents Kazald1, Kazald3 or Kazald4, red color represents PRRX1 and far red represents Kazald2. The results are as expected as there are no dots suggesting that the protocol itself works. There is faint non-specific fluorescence. However, we want to see more fluorescence than this.

While using the HCR protocol to examine the expression patterns of the Kazald genes, we encountered challenges with the HCR probes employed. Our results did not show the anticipated depiction of green dots representing the gene of interest, suggesting potential difficulties with the HCR protocol or challenges specific to the gene under investigation. We expected to observe green, fluorescent signals indicating the presence of the targeted RNA molecules of *Kazald1, Kazald3,* and *Kazald4*. These signals typically appear as bright spots or dots against a dark background, providing insight into the RNA expression patterns of the target gene or RNA molecules within the sample telling us where and when the expression is active. However, we did not see any bright green spots as expected. While there were green dots their fluorescence intensity was not as expected and was attributed to autofluorescence. Utilizing *Kazald2* and *PRRX1* as controls revealed, as seen in Figure 13, that the other Kazald probes did not work consistently in our experiment. Instances of *Kazald2* and *PRRX1* probes not working also suggest the need for refining the precision required to perform the HCR experiment.

Statistics and reproducibility

In both studies, we determined the sample size based on practical considerations and experimental feasibility. We expected dichotomous results from our experiment, i.e., we either see the cuttings from T7 or we do not, and for the HCR experiment, we either see fluorescence or we do not. We calculated the number of animals needed per experiment, ten (10) animals per experiment ensuring that that the sample size was sufficient to achieve meaningful statistical analyses and draw reliable conclusions. In instances where direct comparisons of specific animal tissues were crucial, such as, when positive and negative controls were utilized for control purposes in the HCR experiment,

we ensured that tissues from the same animal were used for both the sample and the control. This approach aimed to maintain consistency and control in the experimental conditions, providing a reliable basis for meaningful comparisons within the study. Each experiment was independently conducted at least twice, unless specified otherwise, to ensure data reliability. It is worth noting that, in the interest of transparency, some experiments encountered occasional errors that were troubleshooted. If the HCR probes had worked, we would have needed more animals to evaluate upregulation in mutants. Despite these challenges, we diligently addressed and learned from any discrepancies, contributing to the overall rigor and integrity of the experimental process.

Chapter IV.

Discussion

In my CRISPR KO study, we emphasized several key findings and challenges encountered during our CRISPR KO experiment. Firstly, we optimized the CRPSIR-cas9 gene editing approach by assessing different primers designed for the gRNA targeting specific genes in axolotl embryos. Through careful experimentation and analysis, we observed the efficient functionality of the primers, evidenced by the presence of bright bands corresponding to the successful amplification of the targeted genes. We observed the presence of the band in the gel, matching the expected sizes of the PCR product for the targeted gene region. We utilized positive controls, such as wild-type DNA samples, and negative controls, such as DNA samples from unedited cells, to ensure that no bands were produced, confirming the absence of contamination or non-specific amplification, and validating the integrity of the PCR assay.

However, we faced a challenge where unexpected bands emerged between different rounds of injections and subsequent PCR analyses. Despite our extensive efforts to optimize the CRISPR-Cas9 gene editing approach, we observed these bands on the gel, distinct from the targeted gene region. They appeared inconsistently across multiple rounds of PCR, posing a considerable obstacle. To mitigate this issue, we employed optimization strategies such as elevating the PCR temperature. This discrepancy was attributed to changes in PCR cycle conditions or potential contamination in the samples used, highlighting the importance of rigorous quality control measures in experimental procedures.

Further analysis of PCR results after T7 digestion revealed additional challenges, particularly in the interpretation of band patterns. For example, the *Kazald1* sample exhibited multiple bands, albeit faint, complicated data interpretation. The *Kazald3* samples exhibited heavy off-target bands. Despite these challenges, we observed notable successes, especially in one lane of the *Kazald3* sample. However, issues with the *Kazald4* results necessitated further experimentation for validation. Overcoming these challenges demanded persistent efforts over several months to obtain PCR results with a singular band for *Kazald4*, underscoring the complexity and intricacies involved in gene editing experiments. Further investigations, including sequencing analyses and additional experimental validation, are warranted to elucidate the precise outcomes of the knockout attempts.

While successfully demonstrating the effectiveness of CRISPR-Cas9 gene editing in axolotl embryos targeting specific genes, we encountered challenges such as non-specific amplification, unexpected results, and complexities in data interpretation. These challenges underscore the importance of careful optimization and implementation of quality control measures in experimental design and execution. The guides we designed effectively target all Kazald genes, laying the groundwork for future studies involving the generation of Kazald mutants and investigations into genome compensation, among other potential applications.

In my HCR experiment, targeting the Kazald genes- *Kazald*1, *Kazald*3, and *Kazald*4 with HCR, presented certain challenges, primarily related to the performance of the HCR probes employed. Despite expecting to observe green dots representing the gene of interest, our results did not reflect the expected expression patterns. This outcome

suggests that *Kazald1*, *Kazald3*, and *Kazald4*, may not be expressed sufficiently in the limb to be visualized with HCR.

One approach to validate the effectiveness of the involves utilizing *Kazald2* and *PRRX1* as controls. The functionality of *PRRX1* controls, which initially exhibited efficacy, ceased with the introduction of a new batch. This discrepancy may potentially arise from improper handling and storage of the *PRRX1* as a shared resource between multiple labs. However, consecutive verification using these controls revealed that the probes for the other Kazald genes failed to produce the expected results. While we endeavored to create probes with high specificity for the target genes, the expression of the gene was likely insufficient in the examined tissues. This further emphasizes the limitations encountered in the HCR experiment and suggests potential shortcomings in probe design or experimental executions.

The observed instances of Kazald2 and PRRX1 probes not functioning as expected also highlight mistakes made while performing the HCR experiment. Achieving reliable and reproducible results with HCR assays necessitates meticulously optimizing experimental conditions, including probe design, hybridization conditions, and imaging parameters. It is essential to also acknowledge that imaging techniques, especially in complex biological contexts, are easily influenced by various parameters such as autofluorescence, environmental factors, etc.

In conclusion, while the HCR protocol holds promise as a valuable tool for visualizing gene expression patterns, this study revealed challenges that warrant further investigation and improvement. Designing optimal probes for comprehensive targeting of the entire transcript poses challenges. Future investigation may benefit from sequencing

analyses to determine whether there is upregulation of these genes in *Kazald2* mutants. Furthermore, considering alternatives to HCR, such as RNAscope, which we have yet to explore, or traditional *in-situ* hybridization, could offer valuable insights and complement our research efforts.

Study Limitations

CRISPR experiment

During the experiment, I encountered multiple limitations that may have impacted its outcomes. Firstly, while it was ideal to inject cells at the single-cell stage, the timing of egg laying did not always align with optimal injection hours. Hence, sometimes the eggs were too old to inject rendering those eggs unusable for the experiment. Additionally, the axolotl's lengthy period to reach sexual maturity, spanning approximately a year, posed a challenge in conducting timely mating. Furthermore, not all eggs were viable, limiting the number of successful injections. These limitations highlight the inherent challenges of working with axolotls, emphasizing the necessity for careful planning in future studies to overcome these constraints. Given the absence of viable alternatives to injection, enhancing our injection techniques holds promise for improving survival rates and effectively addressing these challenges.

HCR experiment

The experiment encountered various limitations that might have impacted the reliability and interpretation of the results. Initially, there were concerns about whether the probe design could accurately detect RNA expression patterns, hinting at potential

gaps or inaccuracies in the data regarding the gene's expression. However, the main issue seems to be its lack of sensitivity which refers to the inability of the probe design to detect low levels of RNA expression accurately. Moreover, there were challenges in preparing tissue samples for analysis, potentially introducing inconsistencies in the experimental results.

Furthermore, the presence of autofluorescence posed a challenge in accurately detecting fluorescence signals, complicating the interpretation of the results. Lastly, the lack of comprehensive knowledge about the function of the gene of interest limited the interpretation of experimental findings. These limitations highlight the need for prioritizing the refinement of the HCR protocol and probe design to enhance sensitivity and specificity. By improving these aspects, a more precise characterization of gene expression in axolotl tissue samples can be achieved in future studies.

Future research directions

The outcomes of our CRISPR-cas9 study will be helpful to subsequent research endeavors in laboratories focusing on axolotls, thereby facilitating a profound comprehension of Kazald *in vivo* functionalities. These findings will push research forward in understanding how changes in gene expression affect blastema formation and lead to differences in appearance when Kazald genes do not work correctly. It is crucial to understand how axolotls can fully regenerate.

As for my HCR results, given that HCR targets the entire transcript, the optimal design of new probes remains ambiguous. It is plausible that the observed limitation stems from insufficient gene expression levels for detection via HCR. Future avenues may involve sequencing analyses to ascertain whether these genes experience

upregulation in *Kazald2* mutants. Additionally, evaluating the efficacy of previously designed probes in alternate tissues exhibiting potentially heightened expression levels could offer valuable insights into probe design optimization.

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