

**Understanding Positional Information During
Zebrafish Fin Regeneration**

by

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University Program in
Genetics and Genomics
Duke University

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program in
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ABSTRACT

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Abstract

Regeneration is a remarkable feat that varies across the animal kingdom. For example, certain salamanders and fish completely regenerate lost appendages while the mammalian capacity to regenerate either limbs or digits is limited. Successful regeneration requires not only generation of new tissues but also patterning the newly formed tissues into suitable replacement structures. The information responsible for this patterning is called positional memory. Despite years of study, many of the factors responsible for positional memory during vertebrate appendage regeneration remain to be discovered. To help bridge this knowledge gap, here zebrafish pectoral fin regeneration was utilized to identify positional memory factors in a genetically tractable vertebrate. The identification of candidate positional memory factors was accomplished through morphological measurements, gene expression analysis, the generation of transgenic zebrafish, and chemical treatments. Using these methods, two striking examples of positional information were found. The first is a region-specific defect in male pectoral fin regeneration, which is governed by androgen's influence on GSK3 activity. The second is the role of *hand2* in the posterior region of the pectoral fin, where *hand2* limits bone growth in part by regulating vitamin D signaling. These findings reveal regulatory mechanisms of positional memory and provide a basis for future work exploring positional memory during zebrafish fin regeneration.

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1. Introduction

1. Introduction

Man's fascination with regeneration likely predates recorded history. A broad definition of regeneration would include all cycles of life, death, and renewal in the natural world. Thus the existence of deities responsible for these cycles such as the harvest gods Renenutet and Hou Ji in ancient Egyptian and Chinese culture, respectively, provide evidence that the importance of regeneration was acknowledged in early human societies. However, the formalized study of life as an academic discipline probably did not begin in earnest until the ancient Greeks courtesy of Aristotle and his *History of Animals* (Aristotle et al., 1862). Even at this early date the idea that animals might regenerate after an acute injury was considered spectacular. So spectacular, in fact, that it was confined to mythical tales. The second of Hercules' Twelve Labors was to kill the Hydra of Lerna (Ruck and Staples, 1994), and this creature possessed a remarkable regenerative capacity: specifically removing one head resulted in the regrowth of two heads. Although Hercules and the Hydra of Lerna would remain in the public conscience, nearly two millennia would pass before regeneration would move from the realm of mythology to experimental science.

1.1 Defining regeneration

Regeneration is the process by which a structure is renewed and reformed. In the context of biology, regeneration happens on many levels ranging from cellular

components to ecosystems. Interestingly, the regenerative capacity of biological systems is not uniform. Variations occur between organisms, organs, and stimuli (Bely and Nyberg, 2010; Brockes and Kumar, 2008; Sanchez Alvarado and Tsonis, 2006). For example, humans have significant regenerative capacity in the hematopoietic system but lack the capacity to replace a lost limb. While certain species of salamanders can completely regenerate amputated limbs. Identifying the factors responsible for regeneration became an area of interest shortly after animal regeneration began to be seriously studied. One modern hope is that uncovering these factors will define potential strategies for human regenerative medicine.

1.1.1 The beginning of experimental study of animal regeneration

Regeneration was first described experimentally by René-Antoine Ferchault de Réaumur (Dinsmore and American Society of Zoologists., 1991). In 1712, he reported that crayfish could regenerate missing limbs in, "Sur les diverses reproductions qui se font dans les Ecrevisse, les Omars, les Crabes, etc. et entr'autres sur celles de leurs Jambes et de leurs Ecailles."

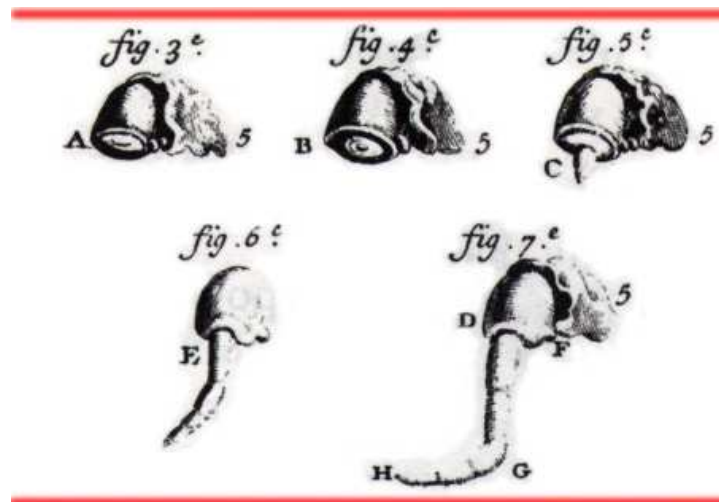


Figure 1: The first known experimental report of regeneration from René-Antoine Ferchault de Réaumur 1712. Crayfish are capable of regenerating amputated legs after molting.

As is often the case with the first report of any phenomenon, Réaumur's work failed to inspire a great deal of interest in regeneration. It was the work of Abraham Trembley on the amazing regenerative capacity of freshwater polyps that really first captured the attention of the science community (Trembley, 1744). The anatomy and robust regenerative responses of these polyps reminded him of the mythical Hydra of Lerna, so he began to refer to these polyps as Hydra. Since many people were familiar with the Greek myth, this name evoked an excitement for these creatures and regeneration. While enthusiasm for Hydra regeneration may wax or wane the name Trembley assigned these polyps is still used (Galliot, 2012).

Réaumur's and Trembley's reports of animal regeneration inspired others to search for the phenomenon in a variety of organisms. However, it is worth noting that

much of this work was not entirely motivated by an interest in regeneration per se, but rather it was driven by an interest in embryonic development. In the 18th century, there were two competing theories that provided general models of development. The first was the preformation theory, which stated that the organism arose from a preformed preexisting germ. This theory can be epitomized by the homunculus, a tiny preformed man present inside either the human sperm or egg. The second theory was epigenesis, which stated that the development occurred from initially unorganized material. It was hoped that regeneration might provide some insight as to which one of these two competing ideas was most accurate. Interestingly in this environment, noteworthy work on regeneration was performed by the discoverer of parthenogenesis, Charles Bonnet, and an ordained Jesuit, Lazzaro Spallanzani, who challenged the spontaneous generation of life theory. Bonnet described the regeneration of rainwater worms, snails, and tritons. Spallanzani studied the regeneration of earthworms, snails, aquatic salamanders, and frogs (Dinsmore and American Society of Zoologists., 1991). Although these studies may have failed to achieve some of their initial goals such as providing a definitive answer regarding the origin of development, they established a great deal of information about the regenerative capacity of various organisms.

The controversy about the nature of development continued, and it motivated experiments in urchin by Hans Adolf Eduard Driesch and in frog by Wilhem Roux.

Roux found that destroying one cell in a two-cell stage frog embryo lead to the formation of a half embryo. However, Driesch described that in urchins separating two-cell stage blastomeres could produce two complete urchins. These conflicting results indicated the technical challenge of manipulating blastomeres and the problems of drawing conclusions based on the results of these manipulations.

It was through this developmental lens that Morgan first viewed regeneration. He hoped that studying the growth of tissue in a more experimentally tractable system than the embryo might produce more interpretable results (Sunderland, 2010). Morgan emulated Spallanzani's approach of examining regeneration in many different contexts in various animals. Morgan gave lectures on his regeneration studies, and he compiled the results into a manuscript which he published in 1901 simply entitled, *Regeneration*. There Morgan described his work on the regeneration of animals such as the hydra, crabs, urchins, earthworms, snails, planarians, frogs, and fish. He also critiqued the works of others and attempted to establish definitions for regenerative events (Dinsmore and American Society of Zoologists., 1991; Sunderland, 2010). Many of his findings are the foundation for questions currently being asked by those studying regeneration today. Perhaps hindered by the diverse nature of the animals he studied, Morgan was unable to generate a universal theory of regeneration. He subsequently turned his attention to other areas such as the genetics of flies. After Morgan the study of

regeneration lost a considerable amount of momentum.

1.1.2 Stem cell research and the reawakening of interest in regeneration

For the better part of the 20th century, regeneration was studied without much fanfare, and little progress toward identifying either cellular or molecular mechanisms of regeneration was made. The reawakening of interest in regeneration was once again linked to research on developmental biology. In the early 1960s the clonal nature of cell colonies derived from mouse bone marrow was first observed (Becker et al., 1963). This discovery of cells capable of self-renewal and differentiation provided the basis for the modern definition of stem cells. Subsequent work has identified stem cells in many tissues including the skin, central nervous system, and intestine. Research into the mechanisms by which stem cells self-renew and differentiate has greatly increased our understanding of development. Additionally, the successful use of stem cells to generate genetically-modified organisms has spurred interest in potential clinical applications of stem cells such as replacing damaged or diseased human organs. However, in many cases the technical and ethical challenges to employing such an approach remain daunting. While there may not be any simple solutions to ethical dilemmas, one possible route to overcome the technical hurdles is through the study of organisms with robust natural regenerative capacity. Specifically, defining the cellular and molecular mechanisms underlying regeneration in other organisms may provide insight useful

during the development of regenerative medicine-based therapies in humans.

While still in early days with much of the promise of regenerative medicine yet to be fulfilled, a great deal has been accomplished in terms of developing a deeper understanding of natural regeneration. One area where this growth is readily apparent is in the field of appendage regeneration. There classical questions are finally beginning to be answered. Some questions being addressed include: what initiates the formation of the proliferative cell mass known as the blastema, what signals drive regenerate outgrowth, and what are the blastemal contributions to the regenerate structures?

Initiation of vertebrate appendage regeneration requires the induction of mitogenic signaling molecules near the injury site (Poss, 2010). This elevated expression of known mitogenic molecules can happen rapidly. For example, in the zebrafish fin the process begins within a first few hours following amputation. In fins, limbs, and tails the events leading to the increased proliferative signaling is being investigated with numerous candidates being explored. One group of potential mediators are the bioelectric signals resulting from changes in ion channel expression and activity soon after injury (Adams et al., 2007). Intriguingly, altered currents and loss of a voltage-gated sodium channel correspond to a non-regenerative or refractory stage during xenopus tadpole tail development (Tseng et al., 2010). Another possibility is that the change in mechanical stimuli after injury could initiate a program leading to a

regenerative response in certain organisms. This might also be related to the bioelectric hypothesis as certain ion channels are mechanically-gated. Additional candidates are signals from immune cells. This is an attractive hypothesis because these cells have been shown to arrive at the injury site in the first half hour after amputation (Niethammer et al., 2009). Although it remains uncertain if the first regeneration-specific event has been identified, work on this question of how regeneration is initiated has defined key early events in regeneration. In the future, understanding the regulation of these early events should help answer the question of why some appendages regenerate while others do not.

Appendage regeneration requires many of the same pathways that are necessary for limb bud formation and outgrowth during embryonic development. These pathways include Fgf, Wnt, Hedgehog, and Bmp signaling (Poss, 2010). While the same pathways are involved in both development and regeneration, whether or not regeneration is simply a recapitulation of the embryonic program remains uncertain. Further dissection of the functions of these pathways during regeneration will need to be done prior to concluding just how similar regeneration is to embryonic development. At least in the case of Shh signaling, there appears to be a distinction between fin development and regeneration (Hadzhiev et al., 2007; Laforest et al., 1998; Lee et al., 2009). One reason these pathways lack defined roles or interactions is that traditionally

genetic manipulations in regenerate organisms have lagged far behind the technological developments in developmental models such as mice. However, the prospects for new approaches in vertebrate regenerate model organisms appear promising. If this potential is realized, it will allow for more thorough examinations during regeneration of pathways with established roles in embryonic development.

For many years the doctrine, based on cells' morphological similarities, was that blastemal cells were stem-like cells, endowed with the potential to contribute to multiple lineages (Kragl et al., 2009). This idea may have appeared wondrous and unique to regeneration, but the stunning de-differentiation and reprogramming displayed in the generation of induced pluripotent stem cells (iPSCs) made this stem-like mechanism for regeneration seem more plausible (Takahashi and Yamanaka, 2006). However while the induction of stem-like cells may be possible in cell culture, lineage-tracing experiments during axolotl limb regeneration demonstrated the model of blastemal cell de-differentiation to a multipotent state to be inaccurate in vivo (Kragl et al., 2009). Currently, all the evidence indicates that multiple cell types contribute to form the regenerate through mechanisms that allow the cells undergo only a limited de-differentiation and thereby retain some lineage identity or restriction.

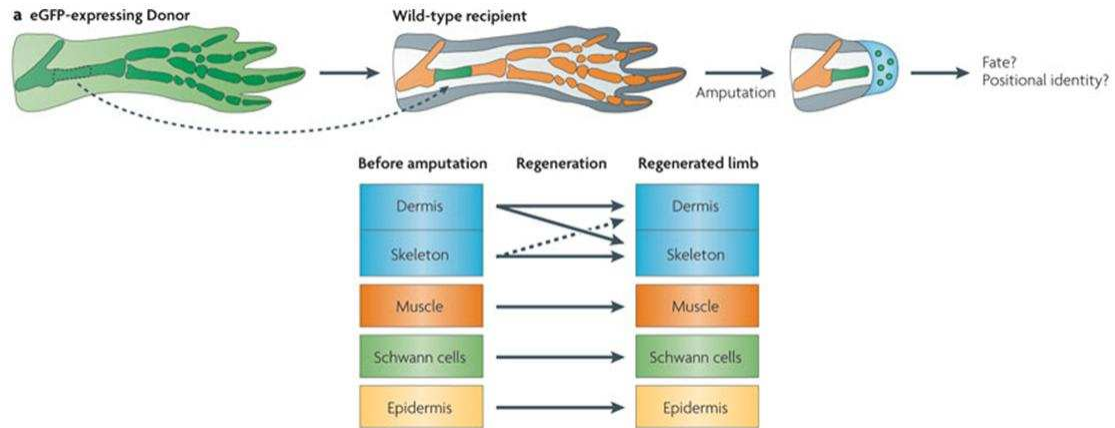


Figure 2: Summary of axolotl work detailing lineage restriction during appendage regeneration. Adapted from Poss 2010. Lineage restriction during axolotl limb regeneration was discovered with grafting experiments. Fragments of a defined tissue in the limb were transferred from an axolotl expressing GFP under the control of a ubiquitous promoter to a wild-type axolotl. The recipient axolotl limb was then amputated and allowed to regenerate. GFP positive regenerate tissues were found only in the same lineage as the original graft.

With some answers to these basic questions and the potential for more discoveries on the horizon, the promise of inducing appendage regeneration in either non- or poorly regenerative conditions may not just be a pipe dream.

1.2 Positional memory, deciding what to regenerate

Although our knowledge regarding the inner workings of regeneration has increased dramatically over the last 30 years, some facets of regeneration still remain to be illuminated. One of these dark regions is understanding how the regenerate tissue is shaped into a near perfect replacement for the damaged or lost tissue. Unlike the

mythical Hydra of Lerna, the faithful recovery of only the lost structures is observed during regeneration in multiple contexts in both vertebrates and invertebrates. Initially, the question of proper recovery was thought of in terms of preformationism versus epigenesis. Now ideas can be framed in terms of how cells and their genomes contain or process information about cells' location (Wolpert, 1971). This positional information is thought to be central to the regeneration of pattern structures, a phenomenon generally termed positional memory. The desire to identify the mechanisms of positional memory has been long standing, but until recently technical limitations prevented much beyond descriptions of the phenomenon. However, improved tools have permitted the development of positional memory models and the description of a few molecules integral to the phenomenon.

1.2.1 Planarian regeneration displays stunning positional memory

Positional memory was extensively studied by Morgan. He described the regeneration of various fish fin shapes after amputation injuries (Morgan, 1901c). While this work is interesting, Morgan's most famous work on regeneration and positional memory was done using small freshwater flatworms called planarians. These worms have a relatively simple anatomy with several defining landmarks. The most noteworthy are the photoreceptors or eyespots which are located in the anterior region of the worm and the pharynx which is located on the ventral side of the worm. Morgan

found that these creatures could regenerate entire properly patterned worms from small amputated fragments. In fact, Morgan so thoroughly explored the regenerative potential of planarians that he concluded a piece of planarian $1/279^{\text{th}}$ the size of the original was capable of regenerating an entire worm (Morgan, 1901a, b).

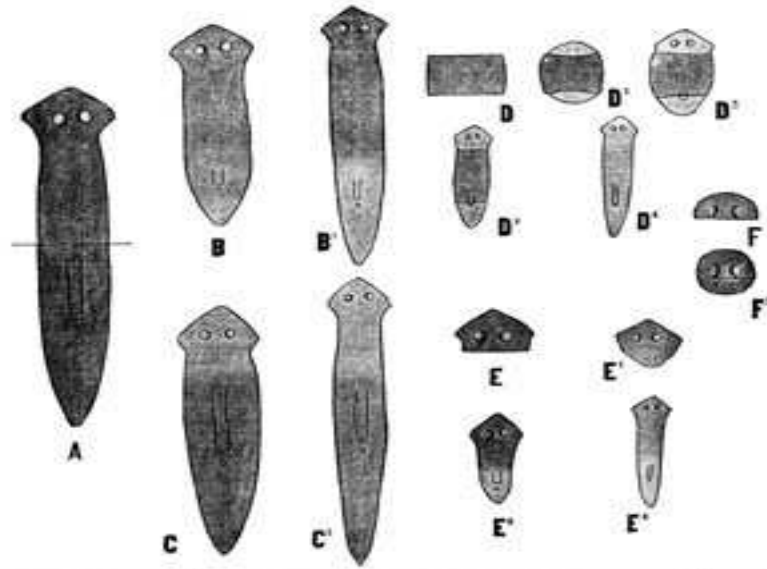


Figure 3: Planarian regeneration displays remarkable recovery of pattern. Adapted from Morgan 1901. Figure from Morgan's work detailing the regeneration of planarian heads, tails, or both heads and tails simultaneously.

This implies that even such a minuscule amount of tissue contains sufficient information to enable the reformation of all the worm's structures. For many years after Morgan's reports what endows the planarian with this remarkable ability remained unknown.

1.2.1.1 Revival of interest in planarian positional memory

For nearly a century, planarians and their stunning regenerative capacity were

largely forgotten. However, in the late 20th century the reawakening of interest in regeneration and the adaptation of a molecular tool led to the revival of planarians as a model organism. The discovery that similar to nematodes, planarians were susceptible to gene knockdown through ingestion of dsRNA has allowed for the importance of various genes during regeneration and homeostasis to be tested in these worms (Sanchez Alvarado and Newmark, 1999). Planarian regeneration and homeostasis is regulated through the actions of a stem cell population called neoblasts (Aboobaker, 2011). Many genes that alter regeneration do so via influencing neoblast proliferation and differentiation. Strikingly, genes and pathways that govern the pattern of the planarian without having significant effects on neoblast proliferation and differentiation have also been identified. Perhaps the most dramatic example of this is the role of Wnt/Bcatenin signaling in the maintenance of the planarian head versus tail identity during regeneration and homeostasis (Gurley et al., 2008; Petersen and Reddien, 2008).

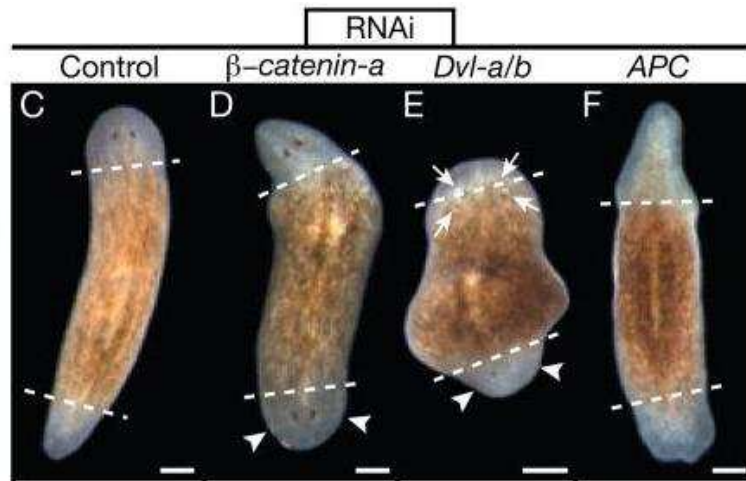


Figure 4: β -catenin defines head versus tail positional memory in planarians. Adapted from Gurley et al., 2008. (C) Planarians appropriately regenerate either a head or a tail. (D) When β -catenin is reduced posterior regenerates produce a head instead of a tail. (E) When negative regulators of β -catenin are reduced anterior regenerates produce a tail instead of a head.

Additionally, the BMP pathway has been implicated in the regulation positional information along the dorsal/ventral axis of the worm (Reddien et al., 2007). The striking finding that altering these pathways changes the pattern uninjured worms demonstrates just how plastic these worms are and just how important positional information is for their homeostasis. Interestingly, both of these pathways involved secreted signals which possibly indicates that in the adult worm gradients of secreted molecules specify positional information similar to how many embryonic tissues are patterned. Whether this mechanism for regulating positional information is also found in the adults of other organisms including vertebrates or is restricted to relatively simple invertebrates such as planarians remains to be determined.

1.2.2 Intercalary regeneration in insects and proximodistal (PD) positional memory

Another significant demonstration of the importance of positional information during regeneration was found when the regeneration of insect legs was examined. These studies found that cockroaches regenerated lost or injured leg segments after molting. The regenerated leg possessed a length and pattern equivalent to the original regardless of where the injury occurred along the PD axis of the leg. But the most provocative results in terms of indicating the presence of positional information came from grafting experiments.

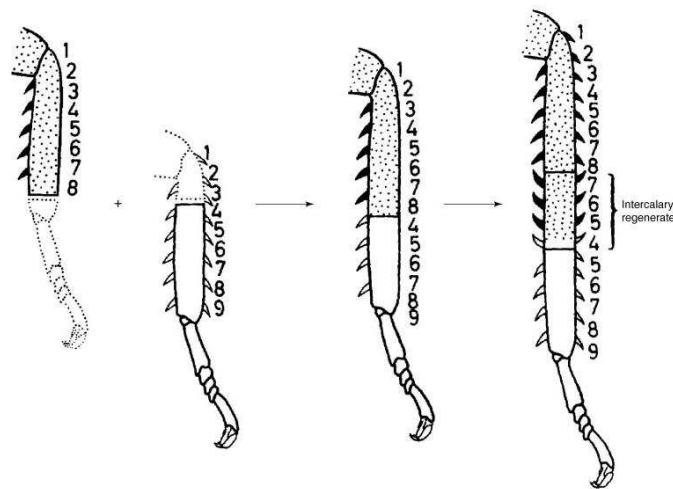


Figure 5: Intercalary regeneration in the cockroach leg. Adapted from Meinhardt 1982. Grafting the leg fragment from a proximal amputation onto a distal stump results in an elongated leg. Interestingly after molting instead of regenerating a leg of the same length and pattern as either the original or the grafted and elongate leg a new leg that is even longer is produced. This growth is caused by the addition of tissue known as the intercalary regenerate between the recipient leg and graft.

When a fragment from a distal amputation was grafted onto a proximal amputation stump thus forming a shortened leg, after molting the regenerated leg was not shortened but rather the appropriate length. However, when a proximal fragment was grafted onto a distal stump forming a longer leg the resulting regenerated leg was longer than either the grafted or the original uninjured leg. This was interpreted to mean that the leg segments contained positional information and that grafts resulted in gaps of positional information. These information gaps were then filled in as efficiently as possible, referred to as the shortest intercalation rule. This interpretation was supported by the orientation and number of exoskeletal spikes in elongated regenerated legs (Bohn, 1970). The molecular mechanisms responsible for intercalary regeneration are now being interrogated (Bando et al., 2009).

1.2.3 Positional memory during salamander limb regeneration

Other than in planarians, positional memory has probably been studied in the greatest detail in urodele amphibians. There are three well-known findings associated with positional information in the amphibian limb. The first is that depending upon the PD location of the amputation plane the rate of regenerate growth varies, with more proximal amputations resulting in regenerates with higher growth rates (Spallanzani et al., 1769). This position-dependent growth is robust enough that amputations at either the shoulder or wrist-level will complete regeneration in nearly the same amount of

time. A full explanation of this result requires further research. The second is the production of supernumerary structures when regenerating limb blastemas are removed, rotated, and regrafted (Maden, 2002). While this demonstrates some change or juxtaposition of positional information results in severely altered regenerates a detailed understanding of this phenomenon has remained elusive. The third and most well-defined characteristic is the proximalizing effect of retinoids, which is that transient treatment of wrist-level blastemas with retinoic acid (RA) produces shoulder-level regenerate limbs (Maden, 1982).

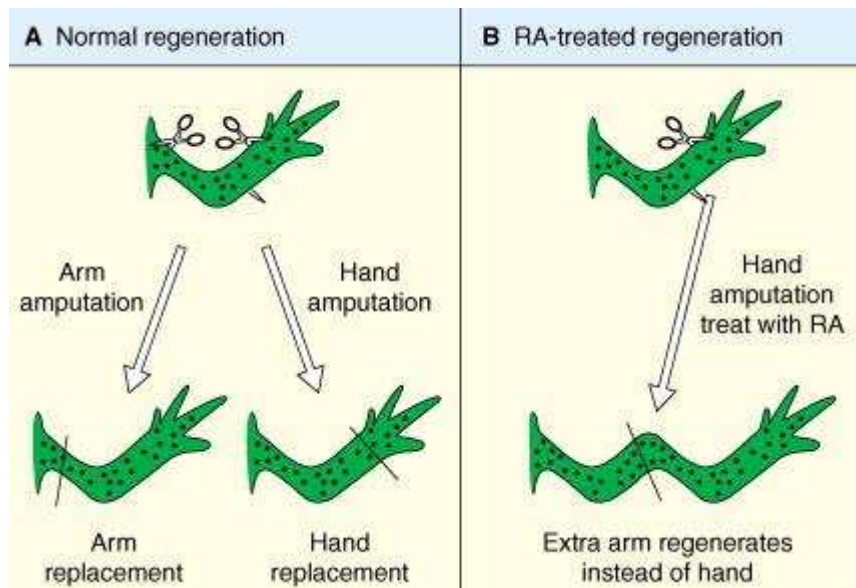


Figure 6: Summary of the effects of RA on salamander limb regeneration. Adapted from Maden, 2002. In salamanders treatment of distal amputations with RA can result in proximal-type regenerates and thus duplications of arm segments.

It is possible that these characteristic positional responses will allow for the

identification of molecules important for positional memory during limb regeneration.

1.2.1.1 Identifying factors that govern positional memory in salamander limbs

However, while the RA finding is spectacular, the role of endogenous RA in positional memory is unclear, since there is no discernible PD gradient of RA in the intact limb. Another candidate factor is Prod1, a proposed receptor for the recently discovered newt blastema mitogen, Anterior gradient (da Silva et al., 2002; Kumar et al., 2007b). Prod1 is induced by exogenous RA and expressed at slightly higher levels (~1.8-fold) in proximal intact limb regions as compared to distal regions (Kumar et al., 2007a). While the *in vivo* function remains uncertain, inhibiting Prod1 in cultured blastemas blocks characteristic proximal behaviors, and blastema cells electroporated with excess Prod1 distribute more proximally than control cells (da Silva et al., 2002; Echeverri and Tanaka, 2005). A potential link between RA and Prod1 could be provided by Meis proteins, which are important for the proximalizing effects of RA on regeneration and may regulate Prod1 (Mercader et al., 2005; Shaikh et al., 2011). In amphibian limb regeneration these three gene products and one signaling molecule are the sum total of positional memory factors identified to date.

1.3 Studying appendage regeneration in fish

Besides the salamander limb another established model of vertebrate appendage regeneration is the fish fin (Broussonet, 1786). While the regenerative capacity of fins

can vary between fish species, the majority of teleost fish are capable of rapid and full fin regeneration (Morgan, 1901c). When comparing teleosts as potential model systems for studying fin regeneration zebrafish, *Danio rerio*, offer several important advantages.

Zebrafish have a relatively short generation time, produce large numbers of offspring, have a sequenced genome, are amenable to forward genetic approaches, and are also amenable to various methods of transgenesis. Consequently although fin regeneration has been observed in many fish species, zebrafish have become the predominant model.

1.3.1 Stages and timeline

The zebrafish has five distinct fin types, and all fins are capable of regenerating (Poss et al., 2003). While their shapes and developmental origins may vary all zebrafish fins share a common anatomy. Zebrafish fins can be viewed relatively simply as being composed of bone rays, which provide the shape and structure of the fin, and the tissue surrounding the bone rays. Bone rays are formed by two sets individual hemirays that are segmented into individual bones called lepidotrichia. Fins grow and regenerate through the addition of new ray segments. The fin ray bones are believed to be formed via direct ossification rather than through the mineralization of a cartilage precursor. Surrounding the lepidotrichia are multiple tissue types including fibroblasts, blood vessels, nerves, and pigment cells in area generally referred to as the fin mesenchyme. A multi-layered epidermis encases the fin mesenchyme. All the cell types and tissues of

the fin robustly regenerated and repartitioned after an amputation injury.

Of all zebrafish fins, the caudal fin is the most commonly used in regeneration experiments for a few reasons. The caudal fin is the largest and most easily accessible fin, and its amputation does not significantly alter swimming ability of the zebrafish. Thus the fish tolerates its amputation quite well, and the caudal fin quickly and reliably regenerates. Surprisingly, amputation of the caudal fin with either a razor blade or scissors results in little blood loss. Within the first few hours after injury the epidermal cells of the fin migrate and form a single cell layer covering the wound. Over the course of the following day the epidermis thickens and become multiple cell layers. This new epidermal structure over the wound has been referred to as the apical epidermal cap (AEC) because of its potential similarity to the apical ectodermal ridge (AER) observed during limb development.

After the formation of this epidermal structure which is generally completed at 1 to 2 days post amputation (dpa) the underlying fin mesenchyme undergoes a massive entry into the cell cycle forming the proliferative cell mass known as the blastema. Blastemal proliferation continues at a high rate until 4 or 5 dpa at which point cell division slows and differentiation into mature cell types begins in earnest. This can be observed as the formation of regenerate bone becomes evident at 4 dpa. The fin then continues to regenerate and grow out until original length and shape of the fin is

restored which is generally around 14 dpa (Poss et al., 2003).

The speed of fin regeneration coupled with the available genetic tools in zebrafish has allowed for some important questions to begin to be addressed. These questions include: What are the contributions of the various cell types in the fin to the regenerate process? And what are the molecular signals that control blastema formation and regenerative outgrowth?

1.3.2 Cellular sources

Similar to the salamander limb, the sources of regenerate tissues during fin regeneration has been a traditional and important area of research. However, only in the past several years with the adaptation of recombinase-based lineage tracing techniques to zebrafish have the definitive experiments been feasible. To date the majority of work on cell lineages and regeneration in the fin has focused primarily on the osteoblast lineage. This is a logical choice because these cells are responsible for the most striking aspect of fin regeneration, the formation of new bone structures. The current model is that mature osteoblasts will de-differentiate and enter the blastema. In the blastema they proliferate but will subsequently re-differentiate and produce the regenerate bone rays (Knopf et al., 2011; Sousa et al., 2011). This indicates the fin blastema composed of a heterogenous group of cells that have undergone a limited or transient de-differentiate similar to what has been seen during salamander limb regeneration. However, there

also exists the potential for some lineage plasticity in the fin. Ablation of the vast majority osteoblast population immediately prior to amputation does not grossly alter fin regenerate length or the ability of the fin to form new bone rays (Singh et al., 2012). This suggests that there are likely some additional sources of osteoblasts such as the fin fibroblasts that can be activated during regeneration. Such sources might be uncovered in the future by combining osteoblast ablation with clonal analysis. Additionally, recombinase-based lineage analysis of other fin cell types will need to be done to determine if the phenomenon of lineage-restriction observed in salamander limb regeneration is also present in zebrafish fin regeneration.

1.3.3 Regulation of fin regeneration

A second area where the zebrafish fin has been informative is in defining the molecules that initiate and control regeneration with many potential regulators already identified. However, beyond the identification of molecules still remains the more important and daunting task of understanding how these molecules are regulated and integrated to form a network capable of initiating and controlling appendage regeneration. This task appears so daunting because of the lack of readily available tools and techniques for altering gene expression or pathway function in either a spatiotemporal or a cell type specific manner in the adult fin. Traditional methods such as morpholino injections, drug treatments, or ubiquitous expression dominant-negatives

alone offer little information about the regulation or function of any gene product or pathway other than to demonstrate its importance for some facet of regeneration. Additionally, without an understanding of how cell lineages behave during fin regeneration the only phenotypes easily assessed are cell proliferation and regenerate length. Thus although many of the critical players have likely been identified, our knowledge of how fin regeneration is actually regulated is still relatively limited. Perhaps the most informative way to classify the molecules and pathways that are believed to have important roles in fin regeneration is to divide them into two classes (1) those that are produced near the injury site and act locally or (2) molecules that are systemic and influence regeneration.

1.3.3.1 Local signaling pathways

The development and outgrowth of mammalian limbs is coordinated through the actions and interactions of the Fgf, Shh, Bmp, and Wnt signaling pathways. All of these pathways have also been implicated in the regulation of fin regeneration. Through pharmacological and genetic methods, proper levels of Fgf signaling have been shown to be essential for blastema formation (Poss et al., 2000; Lee et al., 2005). Hedgehog and Bmp signaling are probably important for both blastemal cell proliferation and regenerate bone formation (Quint et al., 2002). Wnts are thought to be upstream of Fgf signaling in blastema formation. Wnts may also regulate proliferation and the rate of

regenerate outgrowth directly. Canonical and non-canonical Wnt signaling likely play specific and distinct roles to accomplish these feats (Stoick-Cooper et al., 2007).

RA plays an important role during the earliest stages of limb development and as previously described has been shown to have remarkable effects on amphibian limb regeneration. RA is also important during zebrafish fin regeneration as RA serves both pro-survival and pro-proliferative functions (Blum and Begemann, 2012). Key roles in the formation of the regenerative epidermis have been suggested for Activin-betaA and IGF signaling (Chablais and Jazwinska, 2010; Jazwinska et al., 2007). Finally, recent work has implicated Notch signaling in the control of blastemal proliferation and differentiation (Munch et al., 2013). The involvement these many pathways argues that the control of fin regeneration is a complex process. Perhaps the best way to develop a deeper understanding of how these molecules work to control the spatial and temporal aspects of fin regeneration will be through the application of live-imaging techniques where the resolution will be superior to end point analysis.

1.3.3.2 Systemic signaling pathways

In addition to the many signals that originate locally in the fin, there are at least a few systemic molecules that can affect regeneration. The concept that circulating factors may be critical regenerative regulators in fish is not a particularly new idea. In fact there was a substantial amount of work done last century attempting to determine if the

pituitary gland, a master component of the endocrine system, plays pivotal role in fin regeneration (Liversage, 1973). Intellectually these experiments were based on the finding in frogs that thyroid hormone controlled metamorphosis (Gudernatsch, 1912). Unfortunately, the fish studies are challenging to interpret as often the overall health of the fish was severely compromised by the surgical procedure employed to either damage or removal the gland. However, recent work in zebrafish has implicated a direct role in fin regeneration for thyroid hormone (Bouzaffour et al., 2010). Male sex hormone, androgen, has been shown control both fin morphology and the regeneration of caudal fin swords and specialized anal fin rays in certain platyfish (Grobstein, 1947, 1948; Offen et al., 2009). It is likely that other hormones contribute in some manner to the regenerative process although their influence might be relatively minor.

1.3.4 Fish fins as a model of positional memory

Fish fins have characteristic shapes and patterns. Examples include either the oblong-lobed shape of the caudal fin of *Fundulus heteroclitus* or the bi-lobed shape of the zebrafish caudal fin. Fish faithfully regenerate these stereotyped fin morphologies suggesting there must be positional information governing this recovery. The regeneration of fin pattern was well documented by Morgan. He described the regeneration of many fin shapes in response to various types and combinations of amputation injuries, and he postulated some form of positional information was

conveyed by or utilized in response to the injury (Morgan, 1901c). However, for many years these initial descriptions were the sum of our knowledge regarding positional memory in fish fins.

1.3.4.1 Fgf signaling and the PD axis during caudal fin regeneration

The discovery that RA proximalized limb regenerates reinvigorated interest in positional information during regeneration. However, treatment of regenerating zebrafish caudal fins with RA did not result in PD duplications or patterning alterations but rather more subtle changes to bone morphology including ray fusions (White et al., 1994). While the proximodistal effect of RA may not be conserved in zebrafish, another characteristic of positional information in limb regeneration is conserved between salamanders and zebrafish. Zebrafish fins also display position-dependent regeneration rates (Lee et al., 2005). Amputations more proximal, or closer to the body of fish, result in regenerates that proliferate and grow out at higher rates than distal amputations.

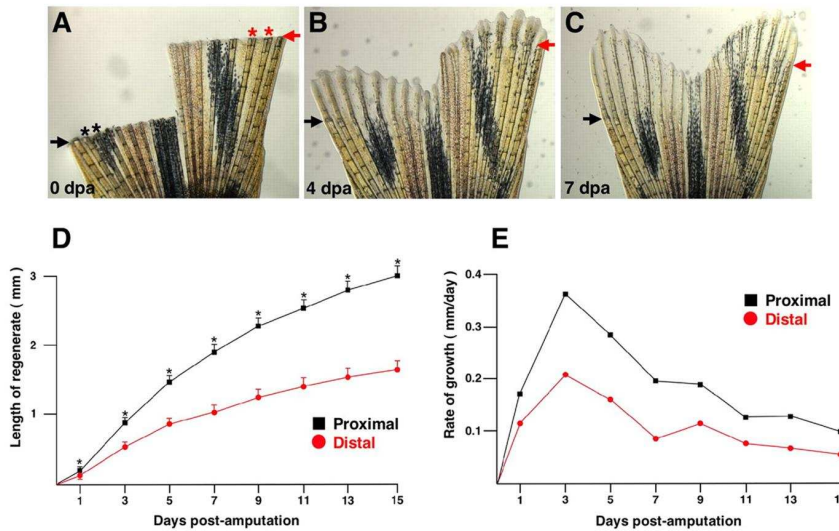


Figure 7: Position-dependent growth rate during zebrafish caudal fin regeneration. Adapted from Lee et al., 2005. (A-D) Amputation of the zebrafish caudal fin at either a proximal (black arrow) or distal (red arrow) level will result in a regenerative response that will complete in the same timeframe. (E) This result is explained by position-dependent growth of the regenerate with proximal regenerates growing more rapidly than distal regenerates.

This position-dependent rate of regeneration is mediated at least in part via the levels of Fgf signaling. In the majority of fins, proximal regenerates have higher levels of Fgf signaling than distal regenerates (Lee et al., 2005). The PD positional information responsible for location-specific amount of Fgf signaling remains to be determined.

1.4 Developing the zebrafish fin as a system to study positional information during appendage regeneration

This dissertation focused on developing a second model of positional information in zebrafish fins as a potential complement to the PD axis of the caudal fin. To accomplish this goal, in multiple fin types both uninjured and regenerate pattern

were examined with the hope of identifying a fin axis with a distinctive pattern that is faithfully regenerated. Upon finding a suitable model, the anteroposterior (AP) axis of the pectoral fin, gene expression analysis was undertaken and transgenic reporters and function reagents were generated to identify and test candidate positional memory genes during zebrafish pectoral fin regeneration.

1.4.1 Positional memory is likely present across multiple fin axes

Previous work on regenerate patterning or positional memory has focused almost exclusively on the PD axis. One reason to focus on the PD axis is that it appears to directly control amount of regenerate tissue formed and thus has the greatest potential benefit in terms of understanding regenerative capacity. Although this logic is appealing, studying the PD axis can be problematic. The majority of successful regeneration studies have been built on or incorporated knowledge that has been learned by studying the developing embryo. When compared to the AP or dorsoventral (DV) axis, the PD axis is probably least well understood. The AP axis of the developing limb has been extensively studied with secreted signaling molecules such as Shh and transcriptional regulators like Hand2, Gli3, and the 5'HoxD genes all having defined roles in AP patterning (Gilbert, 2010). This knowledge base should provide a strong foundation for studying AP positional information during appendage regeneration. Additionally, crosstalk between the AP and PD has been shown to regulate the

outgrowth of the developing limb (Zeller et al., 2009). This crosstalk suggests that positional information regardless of its axis may influence regenerative capacity. Consequently, this work explored the possibility of studying positional memory using fin axes other than the PD axis.

1.4.2 Identifying positional memory factors

Once a promising fin axis was found the focus of work shifted to identifying genes that control the regeneration of pattern across that axis. Regenerate pattern is influenced by both regenerative capacity and positional memory. Positional memory genes should have at least two defining attributes. First, they should maintain differential expression in the uninjured fin as they must provide positional information for both the uninjured and regenerating fin. This is maintenance of expression in the uninjured fin is critical, because aside from regeneration the fin grows and retains pattern throughout life. Second, altering the expression of a positional memory gene should change the regenerate pattern. Finding genes that fulfill the first requirement was accomplished using unbiased expression profiling, quantitative real-time PCR (qPCR), and the generation of transgenic reporter lines. The potential tools for altering the expression domains in the adult zebrafish fin are limited. The gold standard for drawing conclusions about gene function remains a loss-of-function technique. However, when this project was initiated genome engineering in zebrafish was not

technically feasible. So the tools for loss-of-function in the adult zebrafish fin were and for all intents and purposes are still limited to dominant-negatives or morpholinos. Limitations with these approaches include that morpholinos can only be efficiently injected into the unpaired medial fins of the zebrafish and that depending upon the gene being investigated a dominant-negative is not always possible. Moreover, in terms of patterning it is also unrealistic to expect to achieve sufficient knockdown in a large enough percentage of cells via morpholino injection in the adult fin to create reproducible phenotypes. To circumvent these limitations, the strategy that was employed was the creation of heat-shock inducible transgenic lines to misexpress promising putative positional memory factors during regeneration. This misexpression strategy was analogous to previous approaches used to successfully identify important factors before conditional knockout mice could be made with relative ease (Charite et al., 2000; Maccabe et al., 1973; Riddle et al., 1993).

1.4.3 Determining positional memory mechanisms

Initial functional screening for positional memory factors was accomplished using the heat-shock transgene strains. Pectoral fins were amputated, and the fish subjected to daily heat shocks throughout the course of regeneration. Fin regenerates from transgenics and wild-type clutchmate controls were then measured to determine if there were any changes in either regenerate length or ray patter. After finding a factor

whose misexpression produced consistent changes in fin ray length and width, focused shifted to determining how this factor regulated regenerate pattern. First a candidate approach was undertaken based on the known roles of the factor during limb development. However, upon finding this mechanism unlikely during regeneration a more unbiased approach was deemed necessary. Using the AP expression data and by varying the timing and duration of heat shock transgene induction another candidate mechanism was identified. Drug treatments altering this pathway were performed to verify the importance of this mechanism in regulating the regeneration of fin ray pattern. This success provides a general framework for combining gene expression data, the transgenic reagents, and pharmacology to define plausible mechanisms and downstream mediators of positional memory factors.

1.4.4 Evaluating the zebrafish fin as a positional memory model

A final goal of this dissertation is to produce a general assessment zebrafish fins as a positional memory model system. Prior to any experimentation the advantages of the zebrafish on paper are compelling. These advantages include the ease of transgenesis, the speed of regeneration, the availability of genome information, and the relatively short generation time. Some potential obstacles included the difficulty of finding a suitable fin axis, a lack of tools for determining downstream effectors of positional memory, and that even transformations of pattern in the fin would appear

uninspiring given the simple repetitive architecture of the fin. However, based on the results and experiences detailed in this dissertation it is now possible to make a definitive statement about the future prospects and directions of positional memory research in the zebrafish fin.

2. Sexually dimorphic regeneration in zebrafish

The experimental work was performed by Greg Nachtrab, and the microarray analysis was done by Michael Czerwinski.

This work has been published:

Sexually dimorphic fin regeneration in zebrafish controlled by androgen/GSK3 signaling.

Nachtrab G, Czerwinski M, Poss KD.

Curr Biol. 2011 Nov 22;21(22):1912-7.

2. Sexually dimorphic regeneration in zebrafish

Previous work has shown that the regenerative capacity of fins can vary depending upon the fin and the fish species. It is often presumed that zebrafish regenerate all fins with the same efficiency, and the caudal fin is the used in most experiments as a manner of convenience. Given that the caudal fin may not be the best fin for studying positional memory, the regenerative capacity and pattern of all the fins of the zebrafish was more closely examined.

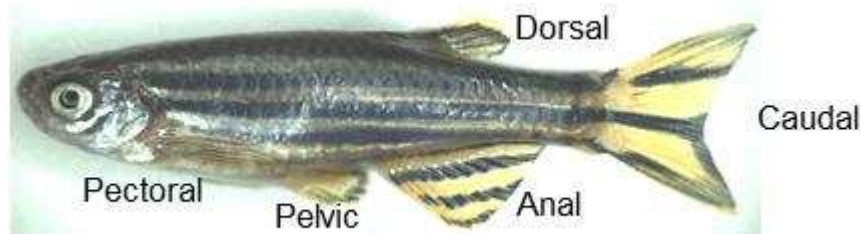


Figure 8: Fins of an adult female zebrafish. Zebrafish have five distinct fin types. Three unpaired fins: dorsal, anal, and caudal. Two sets of paired fins: pectoral and pelvic.

This analysis uncovered some surprising differences in the regenerative capacity between zebrafish fins, strains, and sexes.

2.1 Regeneration of male zebrafish pectoral fins is defective

To identify possible modifiers of positional memory during appendage regeneration, fin regeneration was assessed in 6 month-old adult zebrafish of several commonly used strains. Zebrafish have 2 sets of paired fins, the pectoral and pelvic fins,

and 3 unpaired fins, the anal, caudal, and dorsal fins (Figure 8). Fish of all strains displayed reliable regeneration of amputated anal, caudal, dorsal, and pelvic fins at 5 dpa (Figure 9).

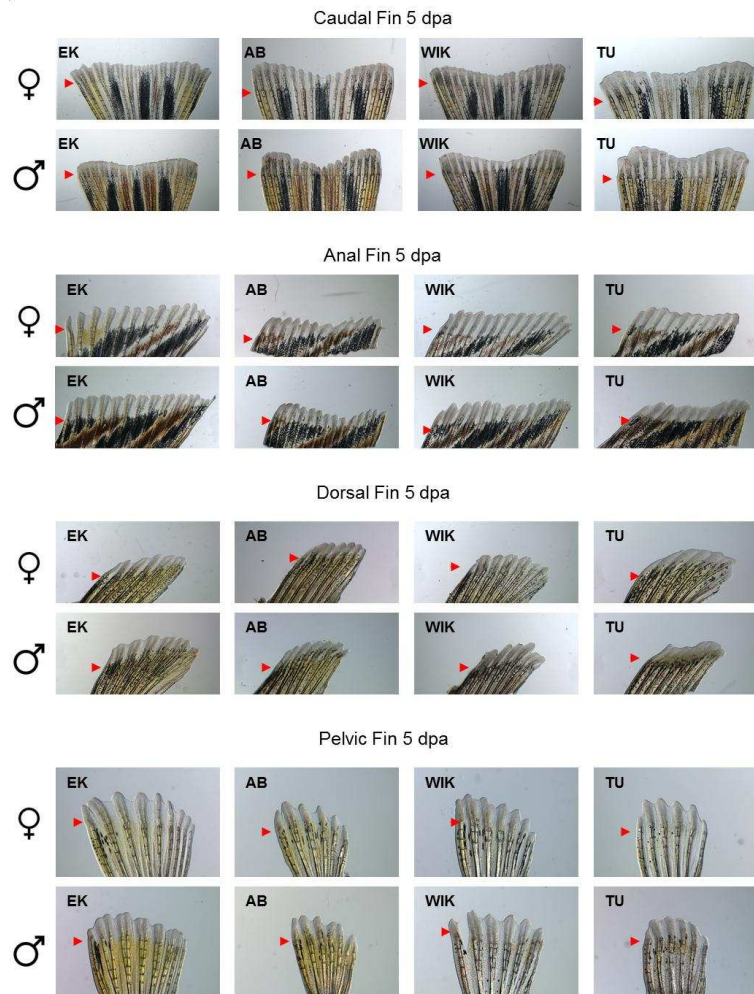


Figure 9: Equal regenerative capacity between strains and sexes for all fins other than the pectoral fin. Both sexes in all four strains tested efficiently regenerated their caudal, anal, dorsal, and pelvic fins.

However, male fish from all strains displayed defects in regeneration of their

pectoral fins (Figure 10). The defects were most prominent and consistent in the EK and AB strains. The WIK and TU strains had a substantial percentage of males with relatively normal regeneration. Searches through published literature did not reveal any previous reports of sex or strain differences in fin regenerative capacity in zebrafish.

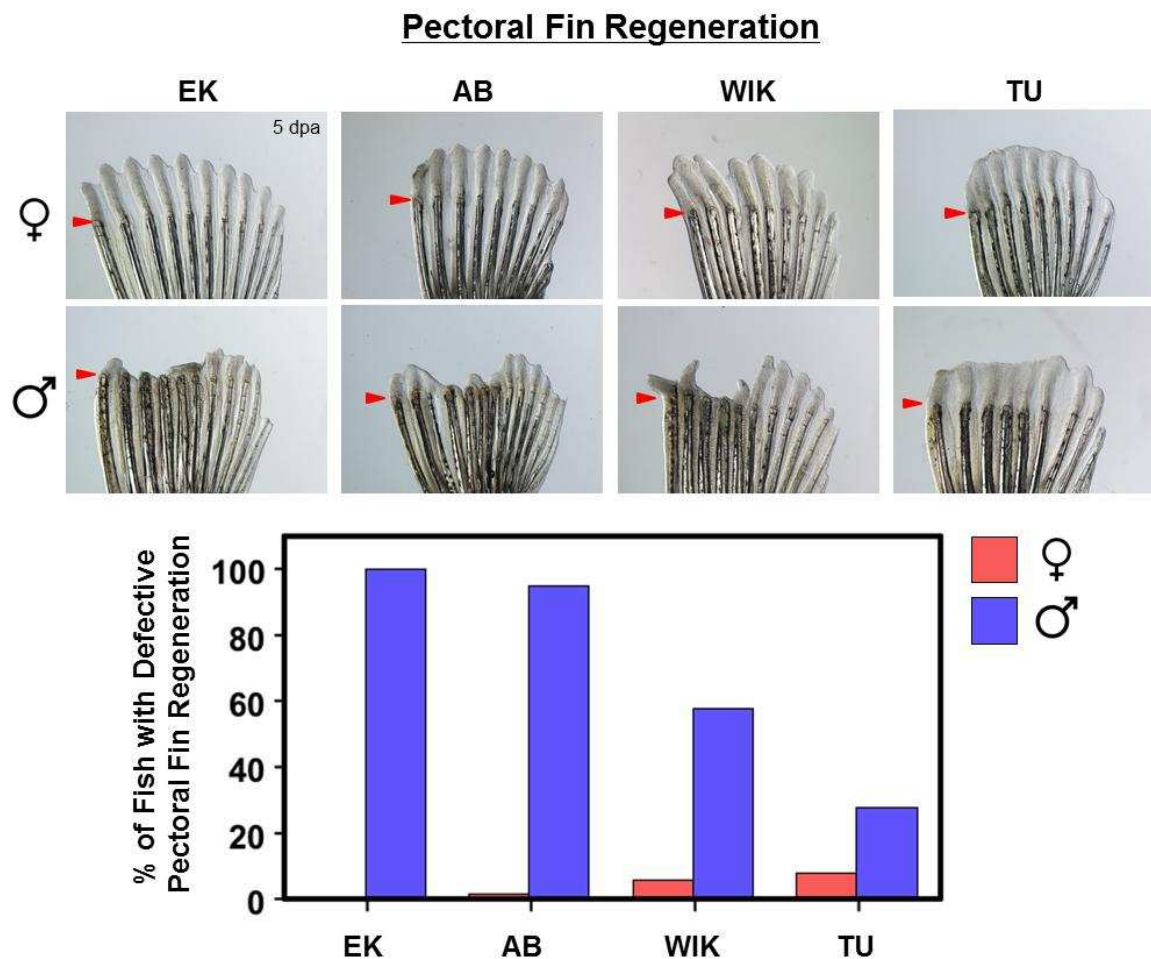


Figure 10: Defective regeneration in male zebrafish. (Top) EK, AB, and WIK males show defects in regeneration of the anteromedial region of the pectoral fin. (Bottom) Quantification of the percentage of fish by strain and sex that had regenerative defects at 5 dpa. A significant percentage of males in all strains display defects.

Regenerative defects in males of the EK strain, on which subsequent experiments were conducted, were mainly present in anterior and anteromedial rays. While ranging somewhat in severity, male regenerates were on average 79% shorter at 5 days post-amputation (dpa) than the corresponding female regenerates. By 10 dpa, when all female zebrafish had nearly completed regeneration, less than 15% of males had regenerated a fanned pattern with multiple bone segments in each fin ray (Figure 11).

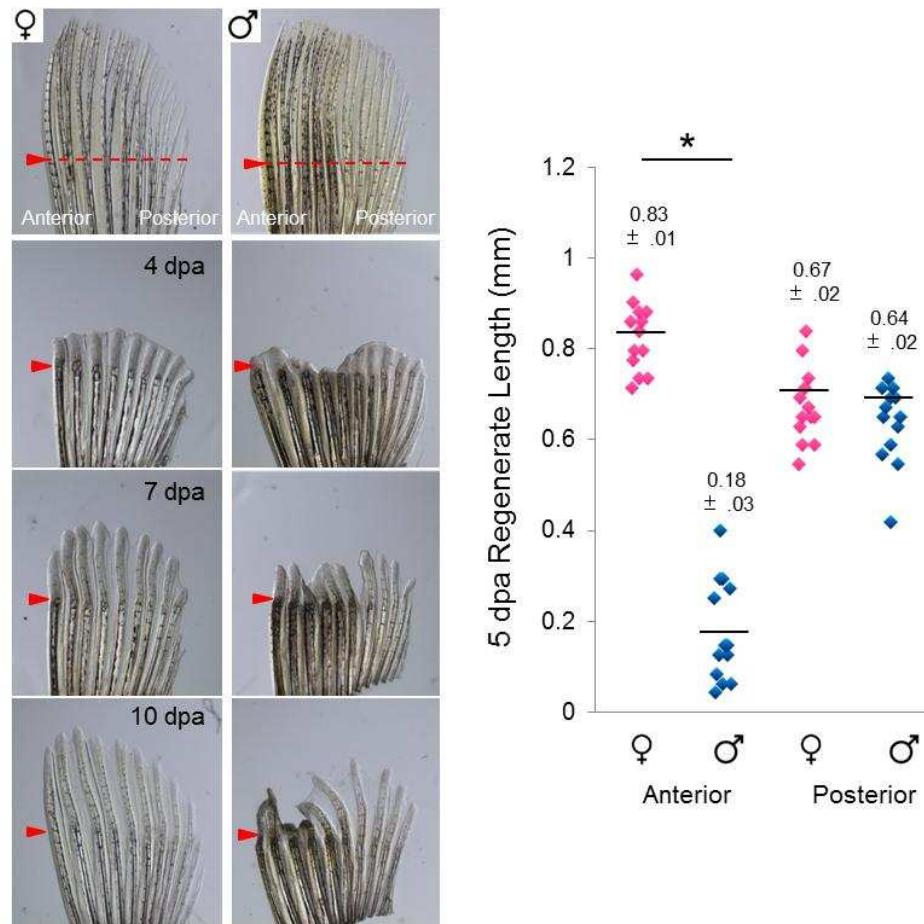


Figure 11: Time course of male regenerative defects. (Left) Comparison of female and male pectoral fin regeneration. Male regenerative defects were clear at 4 dpa and remained through 7 and 10 dpa. Arrowheads indicate amputation plane. (Right) Male regenerative failures were focused to anterior rays. $n = 13$, mean \pm SEM, Student's t-test, $*P < 0.001$.

These results indicated that the regenerative capacity of zebrafish pectoral fins is sexually dimorphic and results in a significant decrease in the ability of male pectoral fins to recover from amputation.

2.2 Male-specific regenerative defect results from decreased blastemal proliferation

Appendage regeneration is initiated by formation of a specialized epidermis after amputation. This regeneration epidermis stimulates the creation and maintenance of a blastema, a mass of proliferative tissue that is comprised at least in part by cell-type-restricted progenitor cells. To determine the cellular basis of sexually dimorphic regeneration, blastemal cell proliferation was assessed in regenerating anterior pectoral fin rays of male and female animals. Anterior blastemas of regenerating male pectoral fins had 66% fewer cells positive for phosphorylated Histone 3-positive (H3P), a marker of mitosis, than the corresponding female regions at 4 dpa (Figure 12).

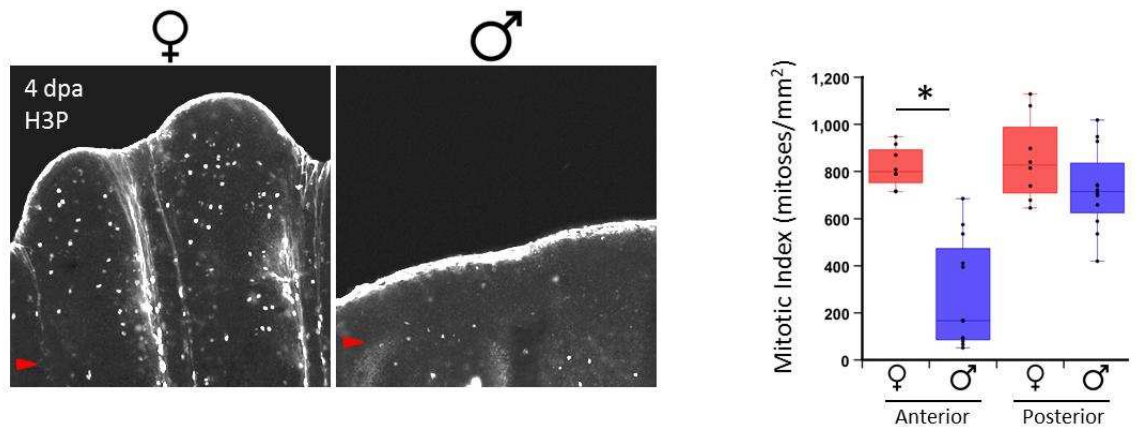


Figure 12: Decreased blastemal proliferation in anterior region of male pectoral fins. (Left) Blastemal proliferation in anterior portions of male and female 4 dpa pectoral fin regenerates, assessed by phosphorylated histone-3 (H3P) staining. (Right) Quantification in females and males confirms defect is specific to the anterior region of the male fin. $n = 12$, Student's t -test, $*P < 0.001$.

2.3 Androgen signaling reduces male regenerative capacity

To test whether regenerative capacity is normal prior to acquisition of male sexual characteristics, the pectoral fins of juvenile animals that displayed only subtle sexual features were amputated and regenerate was examined at 5 dpa.

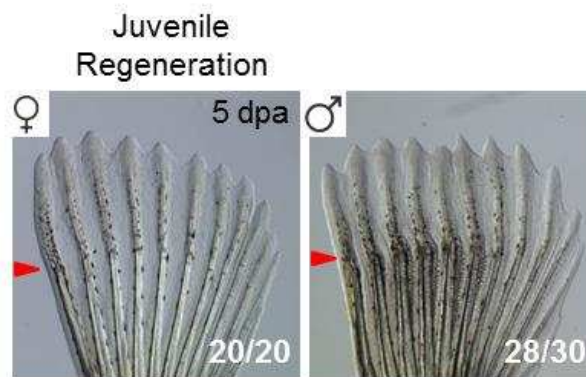


Figure 13: Efficient regeneration in juveniles of both sexes. Juvenile fish (2 months old) of either sex proficiently regenerated pectoral fins.

All 8-week old females and 93% of 8-week old males regenerated fins of normal length and pattern (Figure 13; $P = 0.25$, Fisher-Irwin exact test), indicating that regenerative capacity is present in pectoral fins of young male zebrafish, but then diminishes during maturation.

As in other vertebrates, androgen levels rise as male teleosts grow and sexually mature (Borg, 1994). This suggests that androgen production was responsible for the stage-specific regenerative defects observed in male pectoral fins. To test this idea, adult female zebrafish were treated by bath incubation with the androgen norethindrone

acetate (NA; 1 $\mu\text{g}/\text{mL}$) for 4 days after pectoral fin amputation (Lemus et al., 1997).

Anterior pectoral fin regenerates of NA-treated animals were 62% shorter than those of vehicle-treated fish, while posterior regenerates were 43% shorter (Figure 14). These experiments indicated the inhibitory effects of androgen on pectoral fin regeneration.

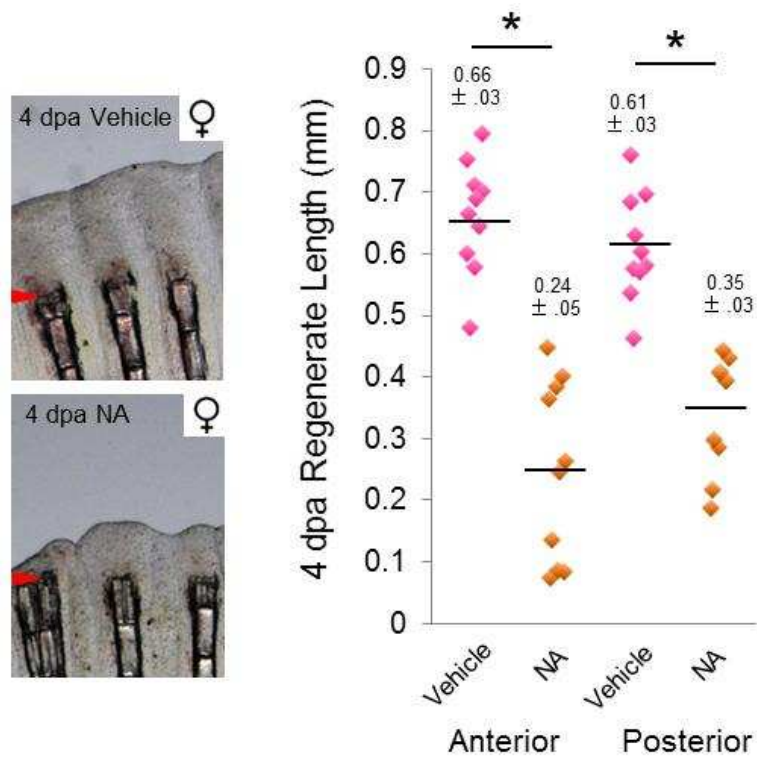


Figure 14: Androgen treatment inhibits female pectoral fin regeneration. (Left) Four days of androgen treatment (1 $\mu\text{g}/\text{mL}$ NA) after amputation inhibited pectoral fin regeneration in females. (Right) Quantification of effects of NA on female fin regeneration (n = 10, Student's t-test, *P << 0.001).

To experimentally decrease androgen levels, the majority of testes tissue was surgically removed from male fish and both pectoral fins were amputated 2 days later. In these experiments, 38% of animals showed morphologically normal regeneration in

both pectoral fins at 5 dpa (Figure 15; $P < 0.05$, Fisher-Irwin exact test).

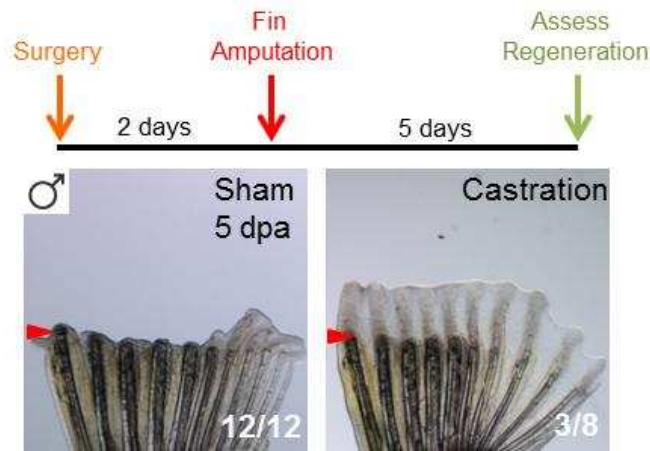


Figure 15: Surgical removal of testis can rescue male pectoral fin regeneration. Castration rescued regeneration of both pectoral fins in 38% of male animals, assessed at 5 dpa.

This result suggested that any structural features of male pectoral fins acquired during maturation do not preclude regeneration, and implicated circulating androgens in control of regeneration.

To specifically inhibit androgen receptors during fin regeneration, male animals were treated with fenitrothion (FEN), a competitive androgen receptor inhibitor. Bath treatment with FEN (1 $\mu\text{g}/\text{mL}$) increased the length of regenerating male anterior rays by 52% at 4 dpa compared to vehicle-treated animals (Figure 16) (Sebire et al., 2009; Tamura et al., 2001).

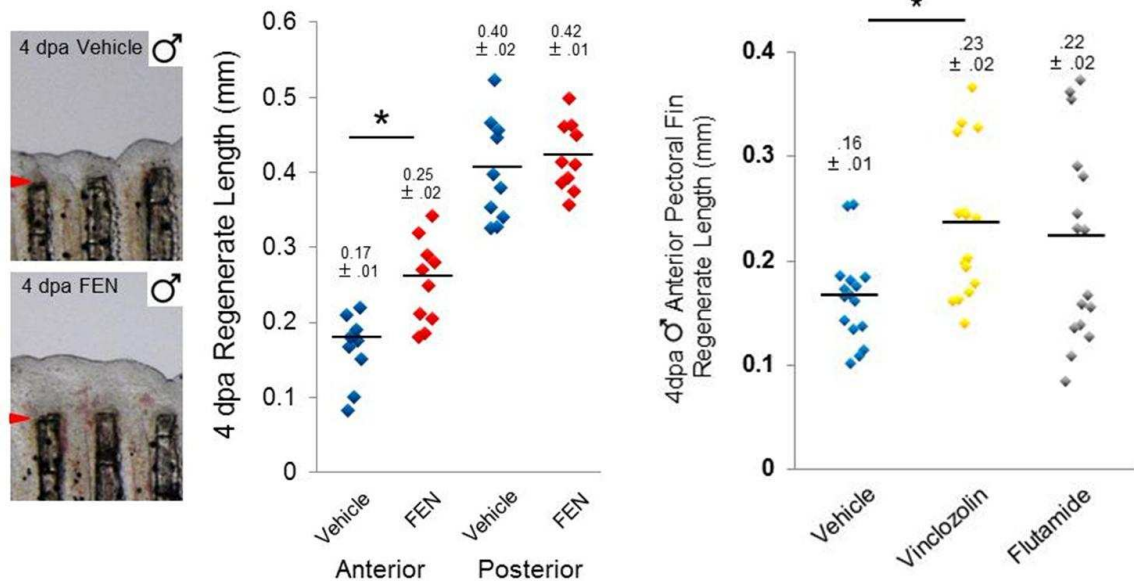


Figure 16: Androgen receptor antagonism improves male pectoral fin regeneration. (Left) Androgen receptor antagonist treatment (1 $\mu\text{g}/\text{mL}$ FEN) for 4 days after amputation improved male fin regeneration. (Center) Quantification of effects of FEN on male regeneration ($n = 10$, Student's t-test, $*P < 0.005$). (Right) Treatment with other androgen receptor inhibitors vinclozolin and flutamide improves male regeneration. $n = 16$, Student's t-test $*P < 0.05$.

Bath treatment with other androgen receptor inhibitors, vinclozolin (250 ng/mL) and flutamide (250 ng/mL), increased male regenerative length by 44% ($P < 0.05$) and 38% ($P = 0.063$), respectively (Figure 16) (Martinovic-Weigelt et al., 2011). Androgen receptor antagonism did not significantly affect the lengths of male posterior regenerates. These findings indicated that androgen presence inhibits regeneration in male animals, and that regenerative potential can be recovered by impeding androgen signaling.

2.3.1 Androgen signaling regulates blastemal proliferation

NA treatment reduced the number of H3P⁺ cells by 53% and 40% in anterior and posterior blastemas, respectively, of female fin regenerates (Figure 17). Conversely, androgen receptor blockade increased the number of H3P⁺ cells in the anterior blastemas of regenerating male pectoral fins by 112%, with no significant effects on posterior blastemal proliferation (Figure 17). These results indicate that androgen signaling inhibits cell proliferation in the appendage blastema.

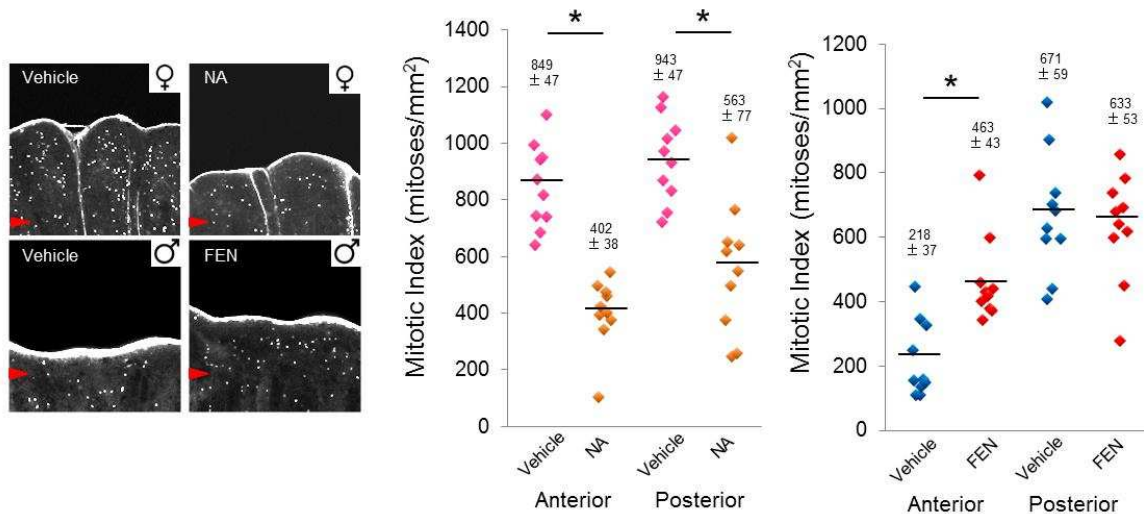


Figure 17: Androgen signaling regulates blastemal proliferation. (Left upper) Blastemal proliferation in females treated with vehicle or NA. (Left lower) Blastemal proliferation in males treated with vehicle or FEN. (Center) Quantification of effects of NA on female blastemal proliferation. n = 10, Student's t-test, *P < 0.001. (Right) Quantification of effects of FEN on male blastemal proliferation. n = 10, Student's t-test, *P < 0.001.

2.4 Androgen signaling regulates Wnt and Igf signaling inhibitors

To define molecular differences between male and female regenerative

responses, gene expression was examined by performing microarrays with adult EK female or male pectoral fins. Analysis of uninjured fins as well as 4 dpa regenerates permitted identification of genes that are differentially regulated by sex and/or regeneration. There were 700 genes with significant, sex-specific expression differences in the absence of injury. A total of 4653 genes displayed differential expression between uninjured and 4 dpa samples, including 400 of the 700 sexually dimorphic genes (data available on NCBI GEO website, entry GSE31871). This subgroup of sexually dimorphic, regeneration-responsive genes represented diverse cellular and molecular functions. In particular, genes that participate in DNA replication were induced at greater levels upon injury and regeneration in female fins compared with male fins, consistent with the sex-specific differences in blastemal proliferation that we observed. Thus, uninjured and regenerating male and female fins exhibit distinct gene expression profiles.

As mentioned earlier, previous studies of zebrafish caudal fin regeneration identified many locally secreted factors that influence blastemal proliferation, including Fgfs, Wnts, retinoic acid, Bmps, Activin- β A, Shh, and Igf2 (Blum and Begemann, 2012; Chablais and Jazwinska, 2010; Jazwinska et al., 2007; Kawakami et al., 2006; Laforest et al., 1998; Lee et al., 2005; Stoick-Cooper et al., 2007; Whitehead et al., 2005). Examination of the microarray dataset to detect sex-specific differences in regulation of these

upstream factors identified male-specific expression of *dkk1b* and *igfbp2a*, encoding secreted inhibitors of Wnt and Igf signaling, respectively. In previous studies, ectopic expression of *dkk1* decreased blastemal proliferation and blocked fin or limb regeneration, as did pharmacological inhibition of Igf signaling (Chablais and Jazwinska, 2010; Kawakami et al., 2006; Stoick-Cooper et al., 2007). Quantitative PCR using uninjured pectoral fin anterior tissue revealed that male *dkk1b* and *igfbp2a* expression levels were 48- and 4.6-fold higher than those of females. Expression of these inhibitors decreased in males after amputation, but remained 8.2-fold and 7.2-fold, respectively, higher than those of regenerating female fins (Figure 18).

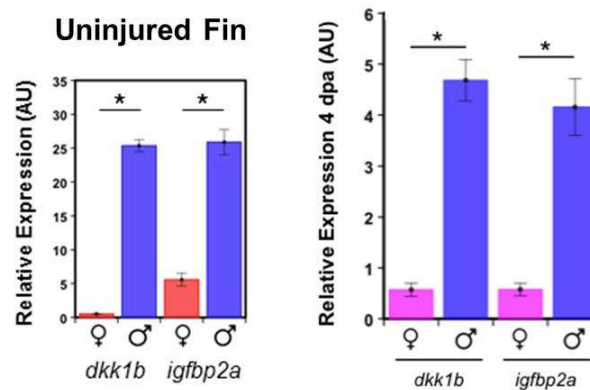


Figure 18: Sexually dimorphic expression of *dkk1b* and *igfbp2a*. (Left) Expression in uninjured pectoral fins. *dkk1b* and *igfbp2a* are expressed in male fins at 48-fold and 4.6-fold the levels of females. $n = 3$, mean \pm SEM, Student's t-test, $*P < 0.001$, normalized to β -actin1, AU = arbitrary units. (Right) Increased expression of *dkk1b* and *igfbp2a* in the anterior regions of regenerating male pectoral fins compared with females. $n = 3$, mean \pm s.e.m., Student's t-test, $*P < 0.005$, normalized to β -actin1, AU = arbitrary units.

These inhibitors were present at low or undetectable levels in the posterior rays of male pectoral fins, and were similarly diminished in male caudal fins. Additionally, levels of *dkk1b* and *igfbp2a* were also lowered in pectoral fin anterior regions of the better regenerating TU strain when compared to EK males (Figure 19).

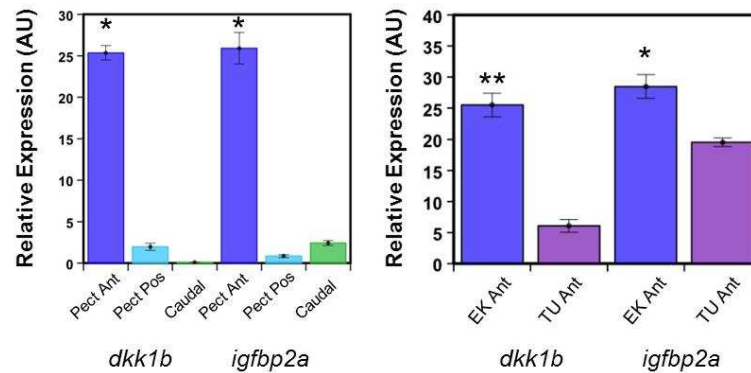


Figure 19: Expression of *dkk1b* and *igfbp2a* is highest in the anterior region of EK pectoral fins. (Left) Biased expression in poorly regenerative fin structures. *dkk1b* and *igfbp2a* are highly expressed in the anterior region of male pectoral fins, but not in fin tissues that regenerate efficiently like the posterior region of pectoral fins or the caudal fin. n = 3, mean \pm s.e.m., Student's t-test, *P < 0.001, normalized to β -actin1, AU = arbitrary units. (Right) Expression of *dkk1b* and *igfbp2a* is lower in the males of the TU strain which had less severe regenerative defects. n = 3, mean \pm s.e.m., Student's t-test, *P < 0.05, *P < 0.005 normalized to β -actin1, AU = arbitrary units.

To assess whether androgen signaling influences *dkk1b* and *igfbp2a* during regeneration, females were treated with NA and males were treated with FEN for 4 days after fin amputation. Anterior fin regenerates from NA-treated females had *dkk1b* and *igfbp2a* levels that were 2.5- and 21-fold, respectively, higher than those from vehicle-treated animals (Figure 20). The particularly high expression of these inhibitory factors

after NA treatment might explain its effects on regeneration across the AP axis of female pectoral fins. NA treatment did not significantly induce *dkk1b* and *igfbp2a* expression in female caudal fins. FEN treatment of males reduced *dkk1b* and *igfbp2a* expression in anterior pectoral fin regenerates by 47% and 57%, respectively (Figure 20).

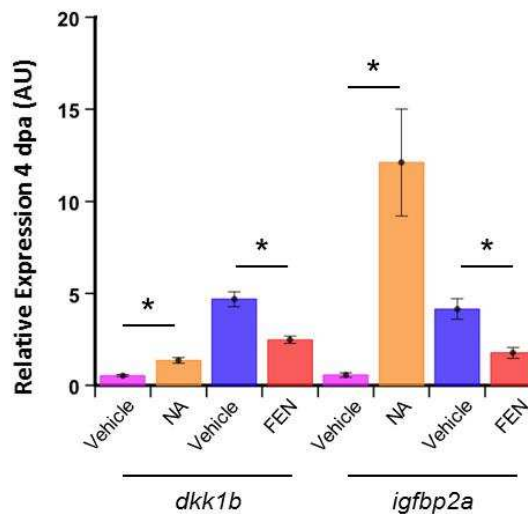


Figure 20: Regulation of *dkk1b* and *igfbp2a* by androgen. NA treatment of females increased *dkk1b* and *igfbp2a* levels during fin regeneration, while FEN treatment of males reduced their expression. n = 3, Student's t-test, *P < 0.05, normalized to β -actin1, AU = arbitrary units.

Thus, secreted inhibitors of key pathways required for blastemal proliferation are positively regulated in male pectoral fins by androgen signaling.

2.4.1 GSK3 activity is a regulatory target of androgen signaling

A common mode of Wnt and Igf signaling activity is inhibition of GSK3 β , a multifunctional kinase that, among other regulatory roles, targets β -catenin, cyclin D, and other protein substrates for degradation (Cohen and Frame, 2001; Kim et al., 2009;

Kimelman and Xu, 2006; Logan and Nusse, 2004; MacDonald et al., 2009). A recent study indicated that Wnts inhibit GSK3 β through its sequestration in endosomes, while Igfs have been shown to inactivate GSK3 β through phosphorylation of serine 9 (Cross et al., 1995; Desbois-Mouthon et al., 2001; Taelman et al., 2010). At 4 dpa, the amounts of inactive P-GSK3 β were present at 2.7-fold higher levels in female anterior pectoral fin regenerates than in males (Figure 21). Treatment of males with FEN was able to increase by 1.7-fold the amount of P-GSK3 β in the regenerate (Figure 21).

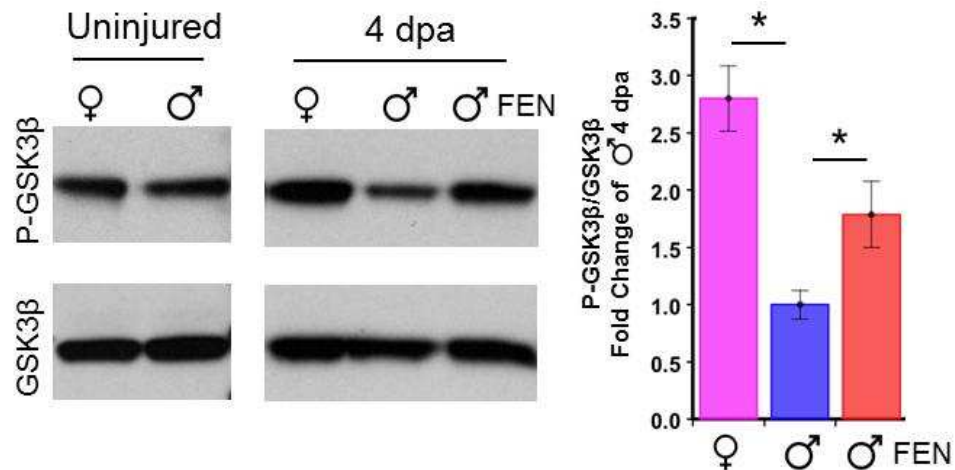


Figure 21: Androgen signaling regulates GSK3 β activity. (Left) Uninjured male and female zebrafish pectoral fins have similar levels of inactive P-GSK3 β . (Center) Upon injury and regeneration, females increase P-GSK3 β levels. Male P-GSK3 β levels appear stable during regeneration but can be enhanced by androgen receptor antagonism. (Right) Quantification of androgen regulation of P-GSK-3 β levels during pectoral fin regeneration. n = 4, Student's t-test, *P < 0.05.

These experiments indicated that GSK3 β activity is a regulatory target of androgen signaling during fin regeneration, likely via control of *dkk1b* and *igfbp2a*

expression.

2.5 Altering GSK3 activity rescues male pectoral fin regeneration

To determine the significance of GSK3 β activity on sexually dimorphic regenerative capacity, male zebrafish were exposed to the GSK3 inhibitor, (2'Z, 3'E)-6-Bromoindirubin-3'-oxime (BIO), a manipulation expected to be epistatic to influences of Dkk1b or Igfbp2a (Meijer et al., 2003). Animals were treated with 100 nM BIO by bath incubation following amputation and assessed for blastemal proliferation at 4 dpa. This treatment increased blastemal mitoses by 56% in male regenerates (Figure 22). BIO treatment had no significant effect on female blastemal proliferation, suggesting that its proliferative effect in males is specific to normal functions of GSK3 β signaling during regeneration (Figure 22).

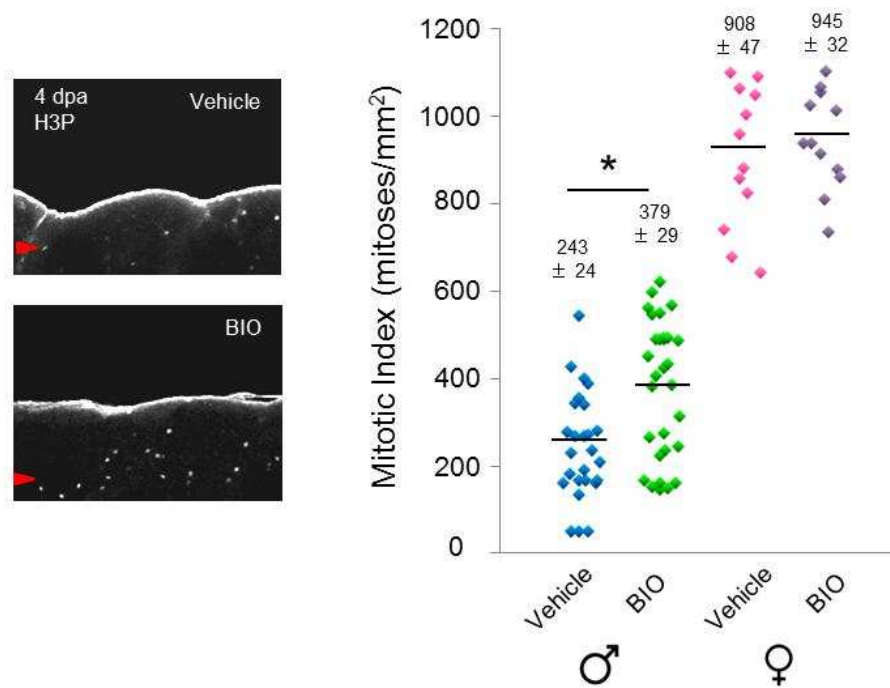


Figure 22: Inhibiting GSK3 increases male blastemal proliferation. Treatment with 100 nM BIO for 4 days after amputation increased male anterior blastemal proliferation by 56%. $n = 26-30$, Student's t-test, $*P < 0.001$.

To detect a possible morphological improvement resulting from the increased blastemal proliferation the regenerates from male animals that had undergone 4 days of BIO treatment after amputation, plus an additional 3 days in the absence of BIO, were examined. GSK3 inhibitor treatment markedly improved regeneration, frequently restoring normal or near-normal fin pattern and increasing its regenerate length by 47% compared to vehicle-treated animals (Figure 23). This extent of regenerative recovery was similar to the effects of the same treatment regimen with FEN instead of BIO (Figure 23).

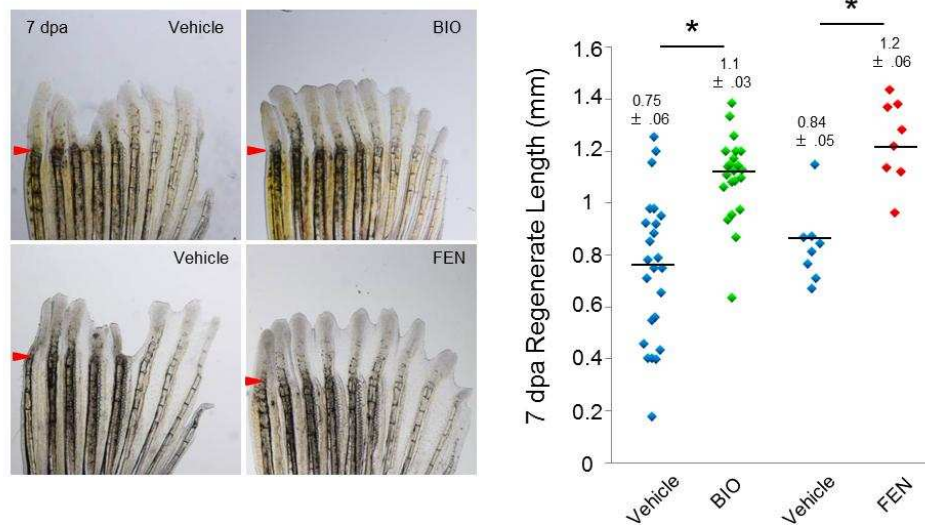


Figure 23: Inhibiting GSK3 activity rescues male pectoral fin regeneration. Four days of BIO or FEN treatment, followed by 3 days without treatment, increased male anterior regenerate lengths by 47% and 48%, respectively, over vehicle alone. n = 22-24 (BIO), n = 8 (FEN), Student's t-test, *P < 0.001.

Thus, transient pharmacological inhibition of GSK3 signaling in zebrafish was sufficient to de-repress the regenerative responses of male pectoral fins.

2.6 Discussion

Together, these findings support a model in which the sex- and age-specific systemic factor, androgen, influences the regenerative potential of appendage tissue through modulation of its GSK3 β activity. Locally, amputation and wound healing trigger synthesis of Igf2, Wnts, and possibly other ligands that contribute to inactivating the GSK3 β pool and enabling blastemal proliferation. Androgen counters these effects in male pectoral fins through the maintenance of ligand antagonists, repressing GSK3 β

inactivation mechanisms and blunting regenerative capacity. Notably, androgen signaling has been implicated in multiple contexts of tissue homeostasis and regeneration. These include positive effects on neuron survival and bone density, and negative effects on wound healing and hair follicle maintenance; androgens impact antler regeneration in red deer in both positive and negative fashions (Ashcroft and Mills, 2002; Frenkel et al., 2010; Goss, 1968; Inui and Itami, 2011; Kierdorf and Kierdorf, 2011; Rosario et al., 2004). Thus, it will be important to determine the range of functions performed by androgen/GSK3 interactions in fins and other tissues, and what may be the physiological consequences of sexually dimorphic regeneration. Interestingly, atrophied pectoral fins are much more common in aging male zebrafish than females (Figure 24). Homeostatic maintenance of zebrafish fin structures has been shown to rely on factors important for amputation-induced regeneration; therefore, this sex-biased aging phenotype might be caused by reduced regenerative capacity (Wills et al., 2008).

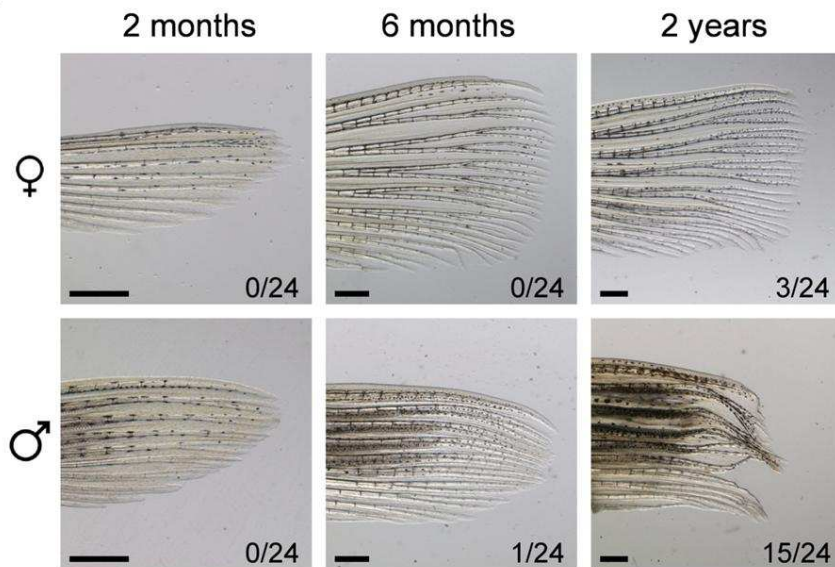


Figure 24: Atrophied pectoral fins in aging male zebrafish. A high proportion of males display bilateral pectoral fin defects at 2 years, including atrophy, ray folding, and ray fusion, while these conditions are rare in females. $n = 24$, Fisher-irwin exact test, $*P < 0.05$.

Most interesting in terms of positional information was that the androgen-regulated gene expression and diminished regenerative capacity were mainly localized to anterior pectoral fin structures of male zebrafish. While androgen receptor expression was slightly higher in anterior pectoral fin regions than posterior regions, it was expressed at similar levels in all fin types (Figure 25).

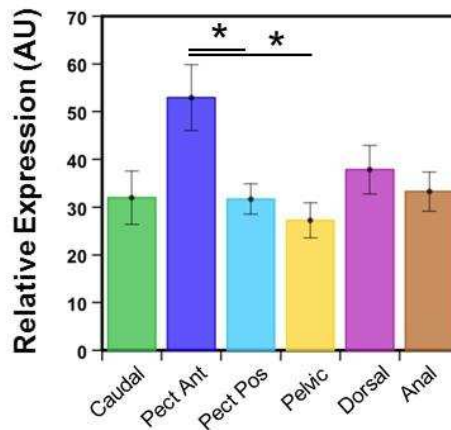


Figure 25: Androgen receptor expression in various zebrafish fins. The androgen receptor is expressed in all zebrafish fins. While the expression of *dkk1b* and *igfbp2a* is restricted to the anterior region of the pectoral fin the androgen receptor is expressed in all fins. n = 3, mean ± SEM, Student's t-test, *P < 0.05, normalized to β -actin1, AU = arbitrary units.

One possible explanation for the regional specificity is the differential expression and/or activity of androgen receptor cofactors. Such differential expression or activity is likely a manifestation of positional memory, and future work to further define the regulation of *dkk1b* or *igfbp2a* may lead to advances in understanding positional memory.

In addition to appendages, stage- or age-dependent losses in regenerative potential have been described for mammalian tissues like the heart, blood, and pancreas (Janzen et al., 2006; Krishnamurthy et al., 2006; Porrello et al., 2011). Murine skeletal muscle regeneration, which is also less effective in old animals versus young, can be modulated by an unidentified circulating factor(s) whose presence changes with age (Conboy et al., 2005). An implicated target of this factor is Wnt signaling, which displays an age-

dependent increase in myogenic cells that is associated with conversion to a fibrogenic lineage and inhibition of regeneration (Brack et al., 2007). In a similar vein to this previous work, pharmacological blockade of activities of either circulating androgen or a target within appendage tissue, GSK3, considerably increased the regenerative capacity of amputated male zebrafish pectoral fins. Approaches to retain or increase the regenerative capacity of injured human tissues remain challenging, but these studies suggest that elucidating and modulating interactions between systemic factors and local regenerative programs maybe important.

2.7 Methods

2.7.1 Animals, surgeries, and drug treatments

Zebrafish between 2 and 9 months of age of the outbred Ekkwill (EK), AB, Tubingen, and WIK strains were used for fin regeneration studies. Fins were amputated at ~50% of their original length, using a razor blade for unpaired fins and iridectomy scissors for paired fins. Lengths of tissue from the amputation plane to the distal tips of the third and fourth rays from the anterior were measured using Openlab software and averaged for the anterior measurement; the seventh and eighth rays were used for the posterior measurement. For castration surgery, we anaesthetized males and made an incision in the ventral body wall caudal to the pericardial area and rostral to the pelvic fins. Each of the two testes was extracted from the animal using forceps, removing ~80-

90% of testis tissue. Vetbond was used to seal the incision, and fish were returned to water and revived. Fins were amputated 2 days after the procedure.

NA (Sigma N6127) and FEN (Sigma 45487) were dissolved in ethanol and methanol, respectively, at a concentration of 10 mg/mL. Vinclozolin (Sigma PS1049) and flutamide (Sigma F9397) were dissolved in DMSO to a concentration of 10mg/mL. After fin amputation, fish were placed in a 1.5 L solution of fish water containing 1 µg/mL of NA, 1 µg/mL FEN, or 150 µl of vehicle. (2'Z, 3'E)-6-BIO (Calboichem 361550) was dissolved in ethanol to a stock concentration of 4 mM. Aliquots of the stock were diluted in 1 L of fish water for a 100 nM working solution. Working solutions were changed every 24 hours. Analyses of H3P⁺ cells were performed as previously described (Lee et al., 2005). The anterior mitotic index was averaged from the third and fourth rays, and the posterior index from rays seven and eight. Quantification of regenerate lengths at 4 dpa was performed with the same fins that were used for the proliferation analysis.

2.7.2 RNA isolation and quantitative PCR

RNA was isolated from the distal half of anterior or posterior regions of uninjured or 4 dpa fins using Tri-Reagent (Sigma). cDNA was synthesized from 1 µg of total RNA using the Roche First Strand Synthesis Kit. Quantitative PCR was performed using the Roche LightCycler 480 and the Roche LightCycler 480 SYBR Green I Master.

All samples were analyzed in biological triplicates and technical duplicates, and all reactions were performed with an annealing temperature of 60°C. The analysis was performed using the $\Delta\Delta C_T$ as previously described (Yin et al., 2008). Primers sequences are listed in Table 1.

Table 1: Primer sequences used to study sexually dimorphic regeneration

<i>b-actin1 F</i>	5' - tggactttgagcaggagatgggaa - 3'
<i>b-actin1 R</i>	5' - aagggtgtctcatggataccgcaa - 3'
<i>dkk1b F</i>	5' - acatgattcagcagcttggcatgg - 3'
<i>dkk1b R</i>	5' - acagttctctccctccagacctt - 3'
<i>igfbp2a F</i>	5' - gcagagcctcggaagtca - 3'
<i>igfbp2a R</i>	5' - tcttagacttcagctgttggcgg - 3'
<i>ar F</i>	5' - acacgactgatggactcactgcaa - 3'
<i>ar R</i>	5' - atcttggcacctggaccgagatt - 3'

2.7.3 Gene expression microarray and analysis

RNA was isolated by Tri-Reagent extraction from the distal portions of the both pectoral fins from uninjured female, uninjured male, 4 dpa female and 4 dpa male fins in biological triplicates. Double stranded cDNA was synthesized following the manufacturer's protocol (Nimblegen). Nimblegen (100718 Zv7 EXPR XH12) chips were labeled and hybridized by MOgene (St. Louis, MO), using 2 μ g cDNA for samples (total of 12 chips). Analysis of microarray data was conducted with Bioconductor. MOgene

provided .XYS files for each chip, which were read and annotated using the Oligo package in Bioconductor. Probe intensities were normalized using robust multiarray average (RMA), and differentially expressed genes were identified using a moderated t-statistic with a p-value cutoff of 0.01 and a minimum log fold-change of one. Significantly differentially expressed genes were identified between sexes for both regenerating and uninjured samples, as well as within each sex and between injury states. Probe sets were annotated by aligning their sequences with the zebrafish genome and assigning an Entrez ID based on sequence alignment. Gene ontology (GO) functional annotation analysis was done using DAVID software (<http://david.abcc.ncifcrf.gov/>). Array files are available on NCBI GEO website as series entry GSE31871 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31871>).

2.7.4 Protein extraction and western blots

Fins were collected, snap frozen in liquid nitrogen, and mechanically homogenized in a modified RIPA buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, and 0.1% SDS) with Roche Phosphostop and Complete Mini Protease inhibitor supplements. After a brief spin to pellet debris, protein extracts were quantified by BCA assay (Pierce). Gel electrophoresis was performed using NuPage Bis/Tris gels (Invitrogen), and proteins were transferred to Immobilon-FL membranes (Millipore). Anti-GSK3 β (1:1000, BD Biosciences #610201)

and anti-P-GSK3 β (1:500, Cell Signaling Technology #9336) were use as primary antibodies. Blots were developed using ECL detection (Pierce).

3. Pectoral fin AP positional memory

The experimental work was performed by Greg Nachtrab. The *hand2*:EGFP fish was generated by Kazu Kikuchi.

This work has been submitted to Development:

Hand2 regulates anteroposterior patterning during zebrafish pectoral fin regeneration.

Nachtrab G, Kikuchi K, Poss KD.

3. Pectoral fin AP memory

Finding a position-dependent effect along the AP axis of the pectoral fin on the regenerative capacity was encouraging. However, the underlying question regarding how fin pattern is restored in female pectoral fins remained unaddressed.

3.1 AP pattern in pectoral fins

The skeletal components of fins, a set of cylindrical segmented rays, are composed of dermal bone that is formed by the deposition and subsequent mineralization of collagen and other proteins. Fin rays are connected by intraray tissue and covered by epidermis, and encase fibroblasts, nerves, blood vessels, and pigment cells. Clear differences in fin ray lengths and widths are observed along the AP axis of pectoral fins.

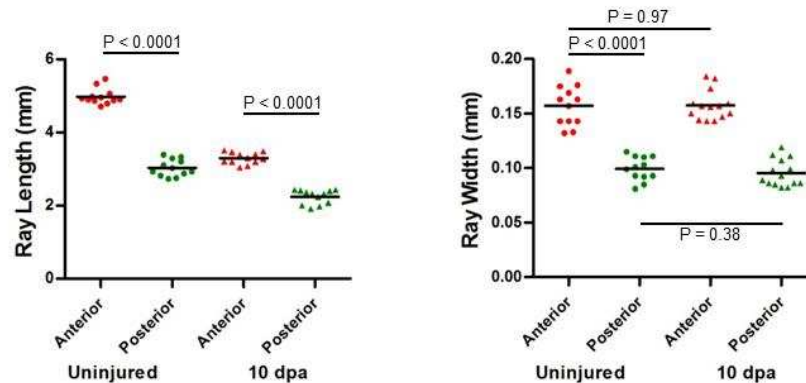


Figure 26: Pectoral fin displays AP pattern in bone ray morphology. (Left) Anterior rays are longer than posterior rays in both uninjured and regenerating fins. $n = 12$, Student's t-test. (Right) Anterior rays are wider than posterior rays in both uninjured and regenerating fins. $n = 12$, Student's t-test.

For instance, the third (anterior) rays were on average 47% longer and 65% wider than the eighth (posterior) rays (Figure 26).

3.1.1 Regeneration of pectoral fin AP pattern

Fin regeneration in zebrafish is initiated by the formation of an individual blastema per ray by 2-4 days post-amputation (dpa). Fin ray regeneration proceeds by distal maintenance of proliferative blastemal tissue; followed by proximal osteoblast patterning and bone formation (Akimenko et al., 2003; Knopf et al., 2011; Poss et al., 2003). To quantify the pattern and fidelity of bone regeneration in pectoral fins, regenerate bone morphology and osteoblast differentiation events were analyzed in *Tg(osterix:EGFP-CAAX)^{pd51}* regenerates. The *osterix* promoter fragment marks both early and mature osteoblasts (Singh et al., 2012).

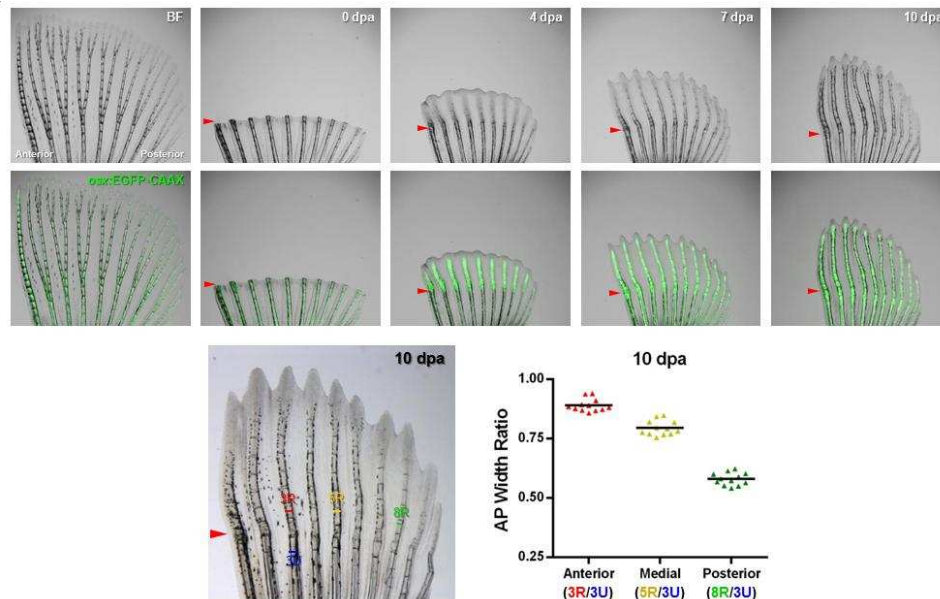


Figure 27: Regeneration of pectoral fin AP pattern. (Top) Zebrafish pectoral fins possess and regenerate an AP skeletal pattern. Early osteoblasts marked by *osx:EGFP-CAAX* (green) are present in the regenerate prior to the onset of AP differences, which arise between 5 and 7 dpa. (Bottom) Zebrafish robustly regenerate AP bone pattern. Quantification of the relative ratios between the width of the uninjured portion of anterior ray 3 (blue) and widths of regenerating rays across the AP axis. n = 12.

By 4 dpa, bone forming osteoblasts were detectable in early regenerates. AP differences in aligned osteoblasts began to manifest from 5 to 7 dpa, and by 10 dpa, the AP ray pattern was restored (Figure 27).

3.2 Differential gene expression across pectoral fin AP axis

A regulator of regenerative positional memory should possess two main characters: 1) presence in a gradient or restricted pattern within the intact and regenerating adult appendage; and 2) its misexpression or blockade impacts

regenerative pattern. To identify genes in the fin that fulfilled the first requirement of positional memory, RNAs collected from the most anterior (AP1) and most posterior (AP5) regions of adult zebrafish pectoral fins were sequenced (Figure 28).

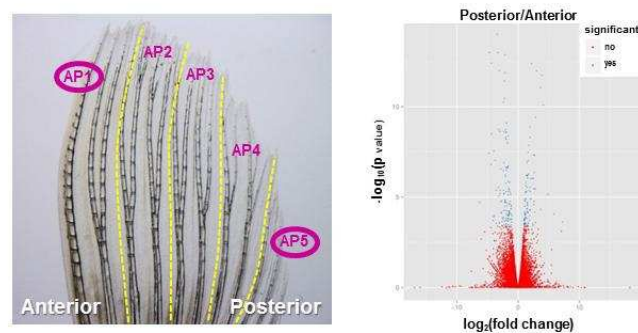


Figure 28: RNA sequencing across AP axis for differential gene expression.

(Left) Diagram of a pectoral fin defining the 2 regions used for RNA-Seq (circled AP1 and AP5) and the 5 regions (AP1-AP5) used for subsequent qPCR validations. (Right) Volcano plot of RNA-Seq data illustrates the relatively small percentage of genes with significant differences in AP expression. Student's t-test.

235 potential genes, or approximately 0.8% of the transcriptome, was found to have significantly different expression across the AP axis of pectoral fins (Figure 27).

The 235 potential genes comprised 195 annotated genes and 40 putative novel genes.

Of the 195 AP genes, 105 were elevated in the anterior and 90 were elevated in the posterior, which may suggest the presence of positional information in both the fin anterior and posterior.

3.2.1 Region-specific transcriptional codes in pectoral fin

To assess if certain category or class of genes was enriched in our AP dataset, a

simple gene ontology search was performed. Using the GO category biological process, the differentially expressed AP genes were found to be enriched for general terms such as developmental process and biological regulation. However, there was also enrichment for more specific terms such as fin development, nervous system development, and regulation of transcription, DNA-dependent (Figure 29).

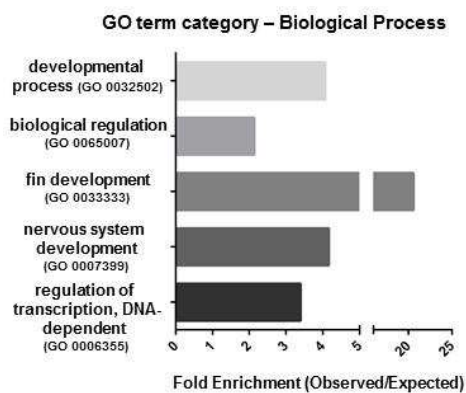


Figure 29: Gene ontology analysis differentially expressed genes. Gene ontology analysis of the differentially expressed AP genes, revealing an enrichment of genes with roles in fin development, as well as transcriptional regulators.

Encouraged by the gene ontology result, the decision was made to focus more detailed analysis and study on the differentially expressed transcription factors for several reasons. First, transcription factors are often central regulators of developmental pathways; suggesting, their regionalized expression stands to impact multiple downstream genes. Second, a rich field of embryonic limb development has detailed the relationship between transcription factors and AP patterning (Charite et al., 2000;

Fernandez-Teran et al., 2000; Galli et al., 2010; Qu et al., 1997; Sheth et al., 2012; Tzchori et al., 2009; Zakany et al., 2004). Third, the AP dataset revealed many examples of developmental transcription factors with significant expression differences along the AP axis. Strikingly, the transcription factor *hand2*, critical for pectoral fin development and localized to the posterior region of developing fin buds (Charite et al., 2000; Fernandez-Teran et al., 2000; Yelon et al., 2000), showed the most polarized expression of all genes enriched in the posterior. Additionally, the embryonic anterior patterning factors *alx4a* and *lhx9* were fifth and eighth on the list of polarized anterior genes (Figure 30).

Gene	Anterior (FPKM)	Posterior (FPKM)	log ₂ (fold_change)	p_value
<i>hand2</i>	0.0968681	18.9788	7.61415	0
<i>scpp7</i>	0.286602	50.5264	7.46185	0
<i>ucp1</i>	0.105493	16.5504	7.29358	0.00023498
<i>si:dkey-22i16.3</i>	0.520251	35.5538	6.09465	0
<i>and2</i>	0.409826	12.3869	4.91766	0
<i>tbx4</i>	0.0261007	0.780067	4.90144	6.93E-06
<i>ASIP</i>	2.14036	36.8699	4.10652	8.04E-11
<i>and3</i>	0.571774	8.42031	3.88036	1.85E-12
<i>si:dkey-189n19.4</i>	5.10311	66.1357	3.69598	4.76E-05
<i>hoxd12a</i>	0.983108	12.6017	3.68012	8.73E-12
<i>and1</i>	4.98078	60.0581	3.59192	0
<i>hoxd11a</i>	1.12486	12.7787	3.50593	0
<i>frem2a</i>	0.143168	1.57487	3.45944	0
<i>hoxd13a</i>	0.674127	5.49144	3.02609	1.05E-12
<i>cyp24a1</i>	6.77993	52.1819	2.94421	0

Gene	Anterior (FPKM)	Posterior (FPKM)	log ₂ (fold_change)	p_value
<i>rspo4</i>	1.4779	0	-1.79769e+3i	0.00012407
<i>zgc:165423</i>	1.35336	0.0176704	-6.25907	1.89E-05
<i>mpz</i>	20.0033	0.757093	-4.72363	3.77E-08
<i>rspo1</i>	2.93687	0.112627	-4.70465	1.03E-13
<i>alx4a</i>	5.25698	0.220614	-4.57464	0
<i>CR925813.1</i>	3.39955	0.16331	-4.37966	1.53E-07
<i>BX927244.2</i>	40.4926	1.99251	-4.345	3.92E-13
<i>lhx9</i>	8.47527	0.424337	-4.31998	3.16E-09
<i>chodl</i>	0.939737	0.0593396	-3.98519	0.00012273
<i>FCGBP</i>	1.19301	0.0773798	-3.9465	2.77E-08
<i>si:ch211-36i19.1</i>	1.29692	0.0868631	-3.90021	0.00010676
<i>tyrp1b</i>	23.0805	1.59837	-3.852	0
<i>SLC7A8 (2 of 2)</i>	2.97957	0.221598	-3.74909	9.25E-08
<i>plcx3</i>	1.0103	0.0849633	-3.57179	4.05E-06
<i>scinta</i>	55.6951	4.68688	-3.57085	0

Figure 30: Most significantly differentially expressed genes across fin AP axis. (Left) List of the top 15 genes enriched in the fin posterior. *hand2* displays the greatest differential expression. Student's t-test. (Right) List of the top 15 genes enriched in the fin anterior. Student's t-test.

To define the transcription factor expression signatures across the AP axis, quantitative RT-PCR (qPCR) was performed for several transcription factor genes. This approach confirmed restriction of *hand2* to posterior fin rays, and also verified region-

specific expression for 8 other transcription factors: *alx4a*, *lhx9*, *id4*, *pax9*, *tbx2a*, *hoxc8a*, *hoxd11a*, and *hoxd13a*. 6 of these 8 transcription factors have roles in AP patterning during limb or fin development (Harrelson et al., 2004; McGlinn et al., 2005; Qu et al., 1997; Tzchori et al., 2009; Zakany et al., 2004), whereas *id4* has been implicated in bone homeostasis (Tokuzawa et al., 2010). The anterior expression *hoxc* genes has been described pectoral fin development (Molven et al., 1990), and *HOXC8* specifically has been shown to be expressed in the anterior region of developing chick wings (Nelson et al., 1996). Gauged by expression of these 9 factors, each set of two rays each across the AP axis displayed a unique gene signature (Figure 30). Notably, *hand2*, *hoxd13a*, *alx4a*, and *lhx9* displayed regional expression in adult fins similar reported expression in embryonic limbs or fin buds (Ahn and Ho, 2008; Charite et al., 2000; Qu et al., 1997; Wang et al., 2011). If they are important for patterning regenerating structures, these transcription factors would be expected to retain their restricted character during regeneration. At 4 dpa, all nine factors had a profile similar to the uninjured fin (Figure 31).

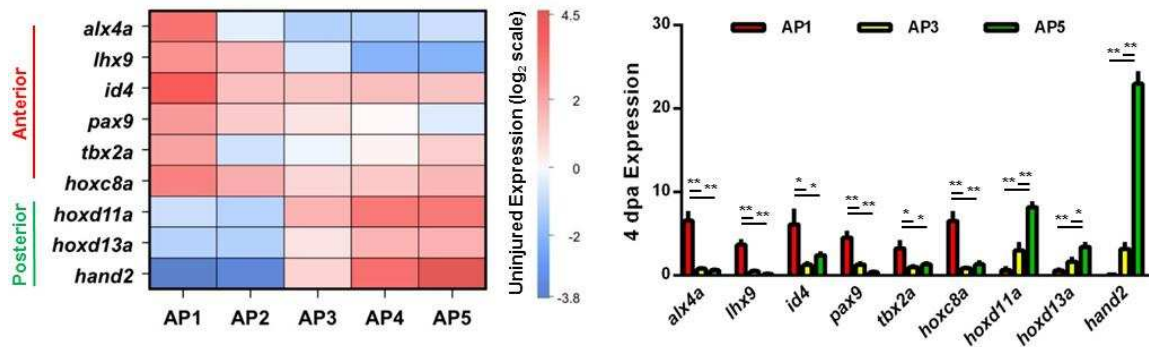


Figure 31: Adult fish maintain region-specific transcription factor expression in uninjured and regenerating pectoral fins. (Left) Each AP region of uninjured zebrafish pectoral fins expresses a unique AP code of patterning transcription factor genes. Results shown reflect qPCR confirmation of RNA-Seq data, normalized to β -actin1 levels, log₂ scale. n = 3. (Right) AP-regionalized expression of transcription factor genes is maintained during regeneration. qPCR expression profiles at 4 dpa, normalized to β -actin1 levels. n = 3, *p < 0.05, **p < 0.005, Student's t-test, mean \pm SEM.

These expression studies indicate that adult zebrafish pectoral fins retain region-specific signatures of transcription factor genes important for AP patterning of embryonic limbs/fins. This is consistent with a potential role in positional memory for these genes.

3.3 Visualizing expression of two most differentially expressed regional transcription factors, *alx4a* and *hand2*

To visualize differential expression of anterior and posterior gene expression, a BAC transgenic reporter line (*Tg(alx4a:DsRed2)^{pd52}*) was generated, and the fin expression of the *hand2:EGFP* BAC transgenic line was assessed (Kikuchi et al., 2011).

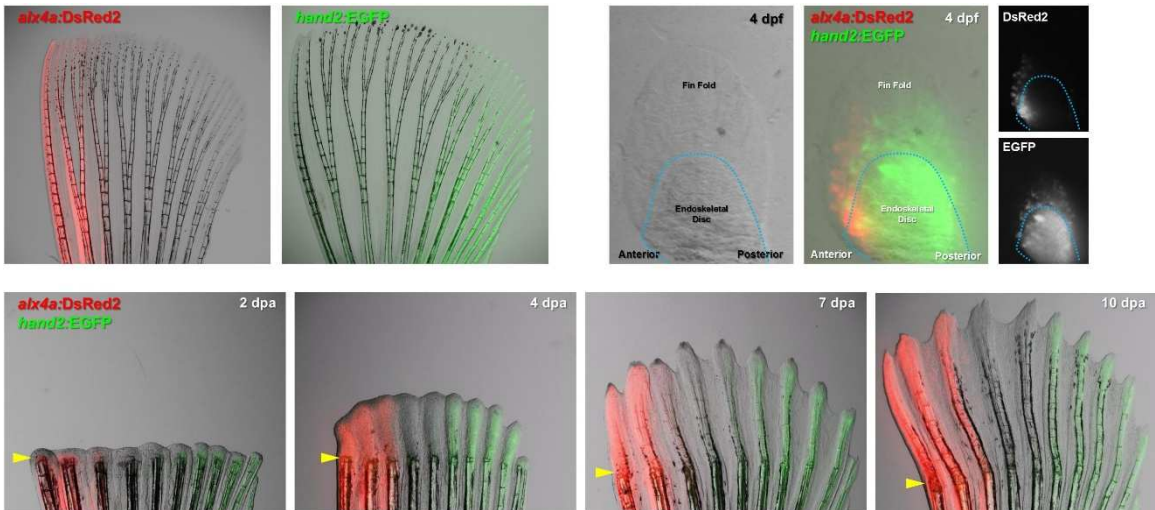


Figure 32: BAC transgenic reporters visualize *alx4a* and *hand2* expression. (Upper left) Expression of fluorescent transgenic reporters in adult pectoral fins. The *alx4a*:DsRed2 domain ranges from the most anterior to the third ray. The *hand2*:EGFP domain extends from the posterior edge to the sixth ray of the fin. (Upper right) Expression of fluorescent reporters in embryonic pectoral fins. At 4 days post-fertilization (dpf), both reporters display region-specific expression. The expression is similar to the adult, but the double-negative medial region is not yet defined. (Bottom) The AP expression characteristics of *alx4a*:DsRed2 and *hand2*:EGFP are maintained throughout regeneration.

The *alx4a* region extended from the most anterior to the third ray of the pectoral fin. Conversely, the *hand2* domain extended from the sixth ray to the posterior edge of the fin (Figure 32). These adult expression domains were reminiscent of developmental expression (Figure 32). In uninjured fins both reporters extended along the entire PD axis of the fin and during regeneration, both reporters maintained regional expression that extended to nearly the distal tip of the fin (Figure 32 and Figure 33).

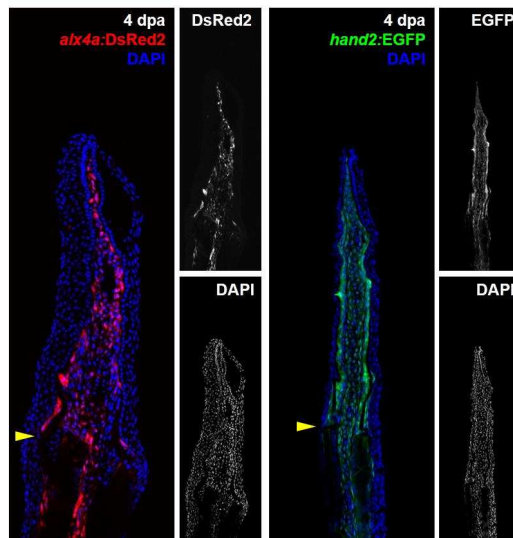


Figure 33: Sections at 4 dpa showing that *alx4a* and *hand2* are expressed in pectoral fin regenerate. Longitudinal sections at 4 dpa confirm reporter expression to nearly the distal tip of the fin.

3.3.1 Expression in fin mesenchyme and osteoblasts

To determine which pectoral fin cells expressed *hand2* and *alx4a*, the reporter strains were examined in transverse section and co-stained with cell type markers.

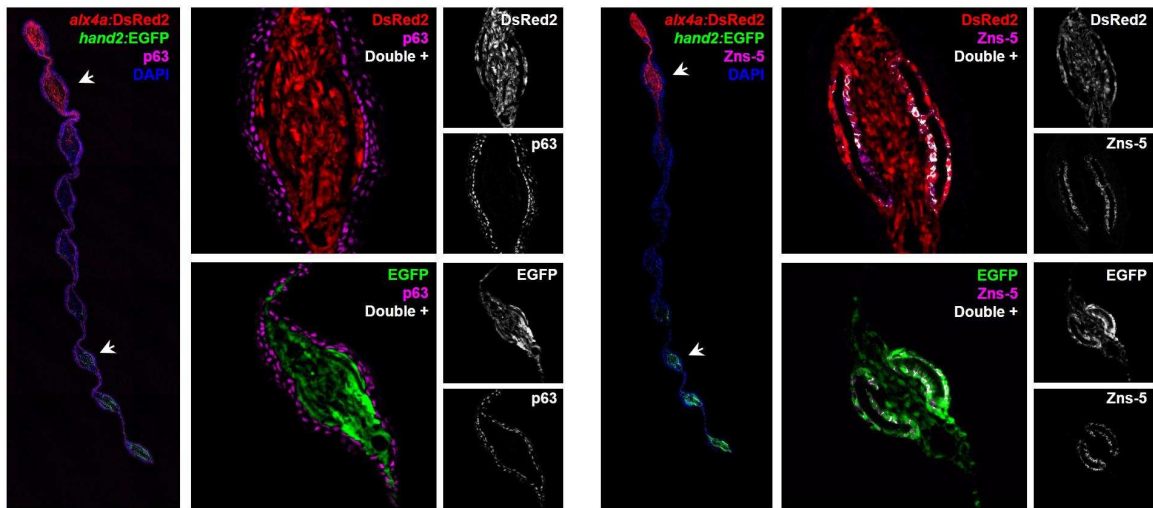


Figure 34: Expression of *alx4a* and *hand2* in fin fibroblasts and osteoblasts. (Left) Transverse sections indicate that expression of either reporter is restricted to the fin mesenchyme. The antibody against p63 marks fin epidermis adjacent to the mesenchymal compartment. (Right) Transverse sections indicating that both *alx4a*:DsRed2 and *hand2*:EGFP are expressed in a population of fin osteoblasts, identifiable by Zns-5 immunoreactivity.

These analyses revealed that the expression of *hand2* and *alx4a* was limited to the mesenchymal compartment of the fin (Figure 34). While the majority of the positive cells in the mesenchyme are fibroblasts, there were many fin osteoblasts lining the bone rays that were distinctly positive for either of these transcription factors (Figure 34). Thus, AP transcription factors are maintained in the critical bone-forming cells of regenerating fins.

3.3.2 Expression in other fins

alx4a- and *hand2*-driven transgenic reporter expression was assessed in other to

determine if the region-specific was restricted to the pectoral fin or a general feature of all adult zebrafish fins (Figure 35).

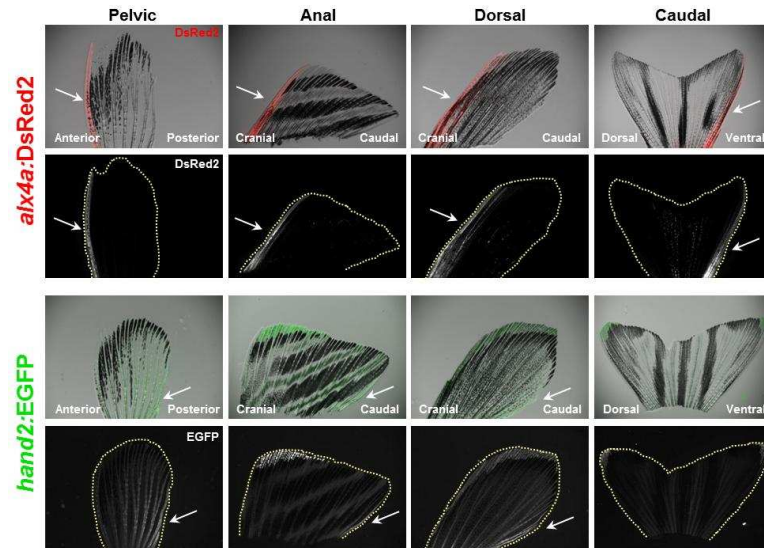


Figure 35: Expression of *alx4a* and *hand2* in all other adult fins. Expression of *alx4a*:DsRed2 and *hand2*:EGFP in pelvic, anal, dorsal, and caudal fins of adult fish. Fins show distinct expression domains (arrows) of *alx4a* and *hand2*. Other fluorescence in *hand2*:EGFP pelvic, anal, and dorsal fins is background.

This analysis revealed a similar pattern of *alx4a* and *hand2* expression in pelvic fins as in pectoral fins. In the anal and dorsal fins, *alx4a* was expressed in the most anterior marginal ray, while *hand2* was expressed weakly in the most caudal ray (expression marked by arrows). In the caudal fin, *alx4a* was expressed only in the most ventral ray, and *hand2* expression was not detectable. These observations revealed differential expression domains for *alx4a* and *hand2* in multiple patterned fins.

3.4 Identifying hand2 as a positional memory factor

To test the idea that regionally restricted transcription factors fulfill the second criterion, regulating regenerative pattern, and thus are positional memory components, transgenic zebrafish permitting heat-inducible expression of the anterior genes, *alx4a*, *lhx9*, or *id4*, and the posterior gene, *hand2* were generated. Since the pectoral fin faithfully regenerates an AP bone pattern, the expectation was that misexpression of a positional memory factor would change fin ray length or width during regeneration.

3.4.1 Expression of anterior transcription factors has little effect on regenerative pattern or capacity

Inducible transgenic lines for the anterior genes, *alx4a*, *id4*, and *lhx9*, (*Tg(hsp70l:alx4a)^{pd53}*, *Tg(hsp70l:id4)^{pd54}*, *Tg(hsp70l:lhx9)^{pd55}*), enabled ~13, 10, and 13-fold increases, respectively, in expression of these genes in posterior rays after a single heat-shock (Figure 36A).

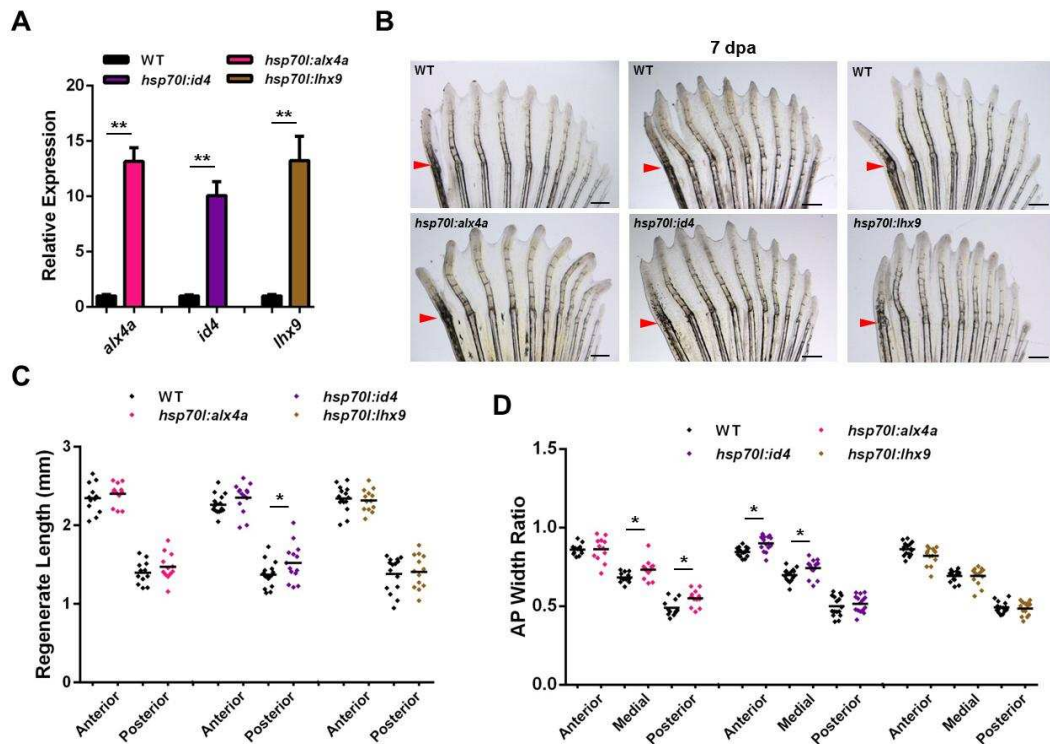


Figure 36: Anterior transcription factor misexpression has minimal effects on regenerate pattern. (A) Anterior transcription factor expression is induced in posterior regions of pectoral fins 4 hours after a single heat shock. Values are normalized to β -actin1 levels and relative to wild-type controls, $n = 3$, $**p < 0.005$, Student's t-test, mean \pm SEM. (B) Appearance of transgenic and wild-type clutchmate fins at 7 dpa, after a series of daily heat shocks. Transgenic fin rays appear have a pattern grossly to wild-type. (C) Quantification of regenerate lengths indicates a minor change in some rays. $n = 12, 12, 16, 14, 15$, and 13 , $*p < 0.05$, Student's t-test. (D) Quantification of width ratios indicates a minor change in some rays. $n = 12, 12, 16, 14, 15$, and 13 , $*p < 0.05$, Student's t-test.

To test whether this misexpression could alter regenerative pattern pectoral fins in these lines were amputated in the afternoon after an initial heat-shock and then daily heat-shocks were continued throughout the course of regeneration. At 7 dpa, there was no gross change in the appearance of the fins (Figure 36B). Quantification of the

regenerate length and AP pattern was performed to ascertain if there were any subtle alterations. Heightened *id4* levels during regeneration produced an 11% increase in posterior fin length (Figure 36C). Elevated *alx4a* increased relative medial and posterior ray width by 4 and 6%, and there was a 6% increase in the anterior and medial regions with *id4* misexpression (Figure 36D). Such modest changes indicate that these anterior factors are not on their own sufficient control positional memory in pectoral fins.

3.4.2 Expression of *hand2* causes change in regenerate pattern

An inducible transgenic line for *hand2* misexpression (*(Tg(hsp70l:hand2)^{pd56}*), increased *hand2* expression by 58-fold in anterior fin regions after a single heat-shock (Figure 37A). This marked change more reflects the negligible endogenous levels of *hand2* mRNA in anterior rays, opposed to the sheer magnitude of *hand2* induction (Figure 31) By 7 dpa with daily induction of *hand2* moderate to severe defects in the patterns of regenerating became evident (Figure 37B). Overall, there was a 42% decrease in the lengths of regenerated anterior rays at 7 dpa, and a 56% decrease in lengths of posterior rays (Figure 37C). There were also significant changes in relative rays' widths indicative of less bone regeneration and further evidence for altered AP fin patterning resulting from *hand2* misexpression. The width ratio reductions across the fin ranged from 14 to 22% (Figure 37D).

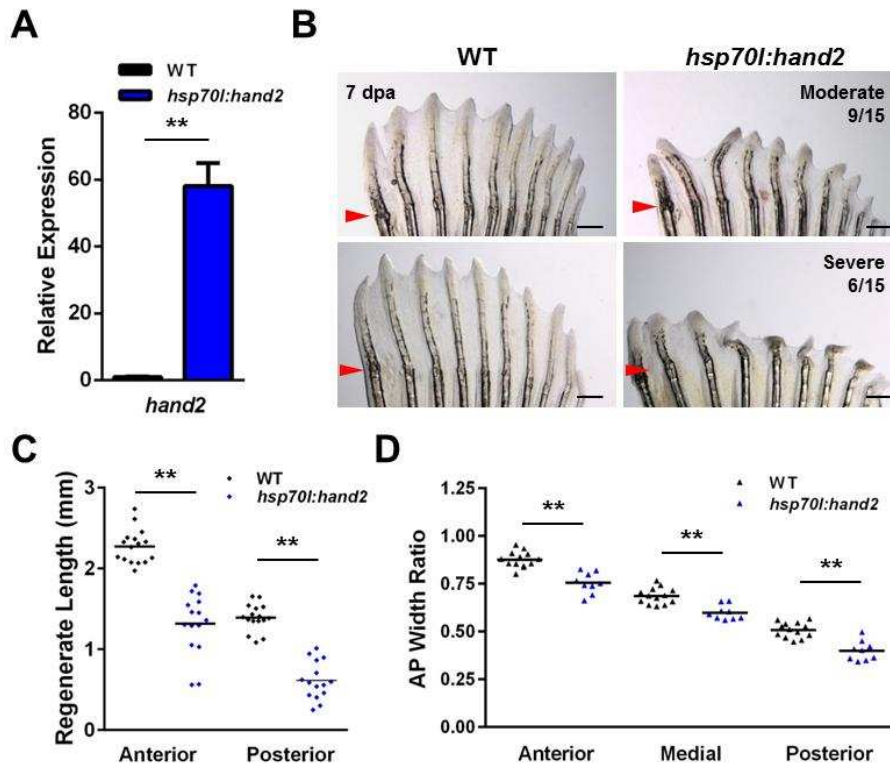


Figure 37: *hand2* misexpression alters regenerate pattern. (A) *hand2* expression is induced 58-fold in anterior regions of *hsp70:hand2* pectoral fins 4 hours after a single heat shock. Values are normalized to β -actin1 levels and relative to wild-type controls, $n = 3$, $**p < 0.005$, Student's t-test, mean \pm SEM. (B) Appearance of *hsp70:hand2* and wild-type clutchmate fins at 7 dpa, after a series of daily heat shocks. *hand2* misexpression generates shorter rays with a reduced number of bone segments. Phenotypes range from moderate (upper right) to severe (lower right). Representative regenerative growth of wild-type rays 3 and 8 are denoted by dashed lines. (C) Misexpression of *hand2* reduces lengths of regenerating rays across the AP axis of pectoral fins. $n = 16$ (wild-type) and $n = 15$ (*hsp70:hand2*), $**p < 0.005$, Student's t-test. (D) Misexpression of *hand2* during regeneration reduces the widths of regenerating fin rays across the AP axis. Transgenic fish that displayed a moderate phenotype were quantified. $n = 13$ (wild-type) and $n = 6$ (*hsp70:hand2*), $*p < 0.05$, Student's t-test.

3.4.2.1 Continuous expression of *hand2* results in sustained alterations of pattern

To assess if the *hand2* misexpression phenotype was the result of a transient delay in regeneration the daily heat-shock regime was extended to 30 dpa. This protocol produced shortened regenerates with smaller rays in the *hsp70l:hand2* transgenics at 30 dpa similar to the morphological phenotypes at 7 dpa (Figure 38A-C).

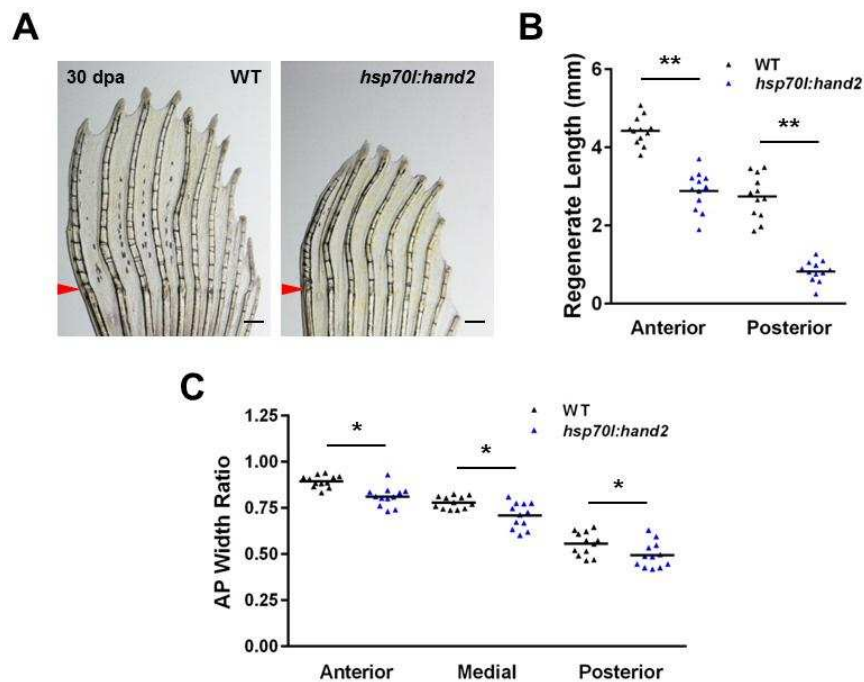


Figure 37: Sustained *hand2* misexpression alters regenerate pattern. (A) Appearance of *hsp70l:hand2* and wild-type clutchmate fins at 30 dpa, after daily heat shocks. *hsp70l:hand2* regenerates remain stunted with shorter and thinner fin rays. Representative regenerative growth of wild-type rays 3 and 8 are denoted by dashed lines. (B) Quantification of *hsp70l:hand2* ray lengths at 30 dpa. $n = 12$, $**p < 0.005$, Student's t-test. (C) Quantification of *hsp70l:hand2* ray widths at 30 dpa. $n = 12$, $*p < 0.05$, Student's t-test.

3.4.3 Hand2 has its most significant effects during later stages of regeneration

hand2 regulates the localization of *sonic hedgehog* in the posterior region of developing embryonic forelimbs, wings, or pectoral fins (Charite et al., 2000; Fernandez-Teran et al., 2000; Wang et al., 2011; Yelon et al., 2000). However, *shha* is expressed in the distal tips of every uninjured and regenerating adult fin ray regardless of where or not *hand2* is expressed in the region (Lee et al., 2009; Quint et al., 2002). Since this embryonic role is likely the not the mechanism through which *hand2* influences adult positional memory it was important to define the critical window for *hand2* action during pectoral fin regeneration.

3.4.3.1 Hand2 does not inhibit blastemal proliferation

hand2 misexpression for 3 days after amputation, which is prior to the onset of substantial bone regeneration, resulted in no significant change in regenerate length (Figure 39A, B). Consistent with this there was no difference in blastemal proliferation at 3 dpa between *hand2* transgenics and clutch-mate controls (Figure 39C, D).

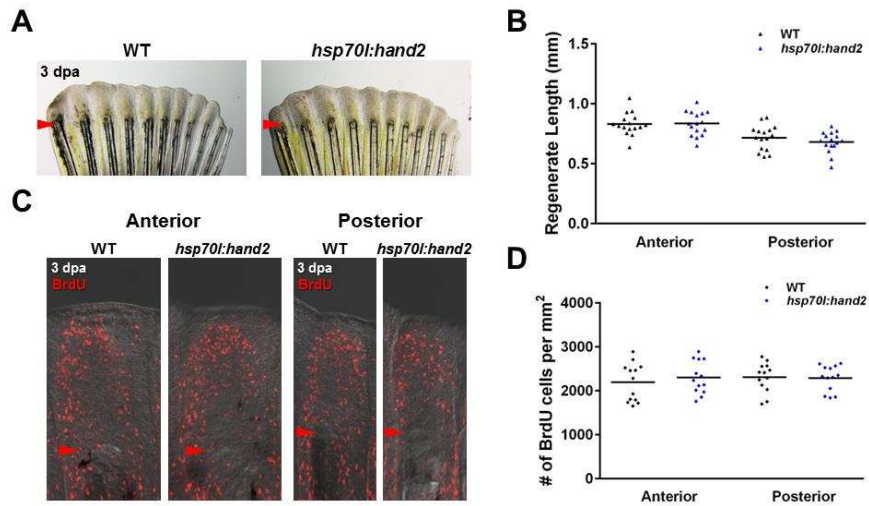


Figure 39: Hand2 does not inhibit blastemal proliferation. (A, B) No difference in the appearance of *hsp70l:hand2* and wild-type clutchmate fins at 3 dpa, after a series of daily heat shocks. $n = 16$, Student's t-test. (C, D) Blastemal proliferation as measured by BrdU incorporation is also unaffected by *hand2* misexpression. $n = 13$, Student's t-test.

3.4.3.2 Hand2 reduces fin regenerate outgrowth

However, daily *hand2* induction starting at 3 dpa resulted in a 20% decrease in regenerate length and an 8% decrease in bone formation by 7 dpa (Figure 40A-C). Thus, elevating levels of the normally posterior-restricted *hand2* throughout the fin did not disrupt early regenerative growth, but in later stages altered regeneration by reducing ray lengths and widths.

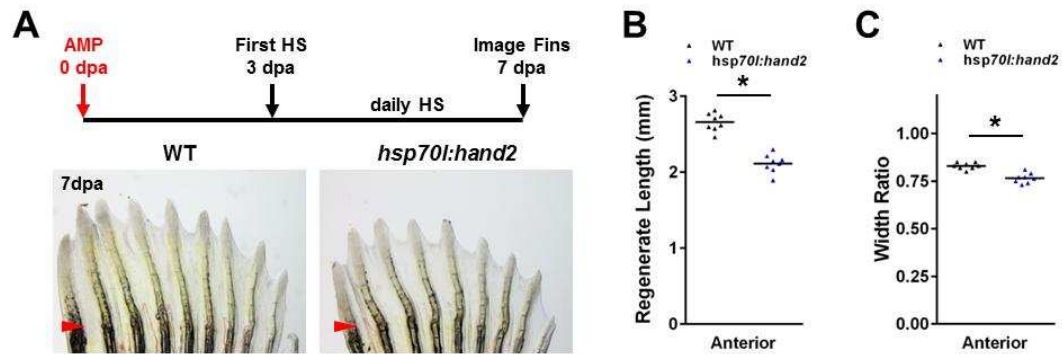


Figure 40: Hand2 reduces regenerate bone growth. (A) Stunted regeneration in a *hsp70:hand2* pectoral fin compared to a wild-type clutchmate fin at 7 dpa, after a series of daily heat shocks beginning at 3 dpa. Representative regenerative growth of wild-type ray 3 is denoted by a dashed line. (B, C) Significant reductions in anterior ray lengths and widths manifest when *hand2* is misexpressed during later stages of regeneration. $n = 8$, $*p < 0.05$, Student's t-test.

3.5 *hand2* regulates vitamin D signaling in the pectoral fin

To identify possible mechanisms for the actions of *hand2* the RNA-Seq AP dataset was reexamination. Given the late stage regenerate phenotype, the primary area of focus was on genes known to be involved pathways regulating bone formation or homeostasis. A significant AP asymmetry was observed in the expression of the vitamin D inactivating enzyme, *cyp24a1* (Knutson and DeLuca, 1974). Vitamin D is a known regulator of calcium and bone homeostasis in mammals and has also been shown to influence bone formation in zebrafish larvae (Baldock et al., 2006; Fleming et al., 2005; Gardiner et al., 2000). To confirm that vitamin D signaling might be able influence bone formation and mineralization during fin regeneration, fish were treated by bath

application with 1ng/ml calcitriol from 0 to 5 dpa. The formation of mineralized bone was then assessed with alizarin red staining. There were small but significant increases in the amount of bone that had formed in the regenerate (Figure 41). This indicates a potential role for vitamin D signaling in bone formation during zebrafish fin regeneration.

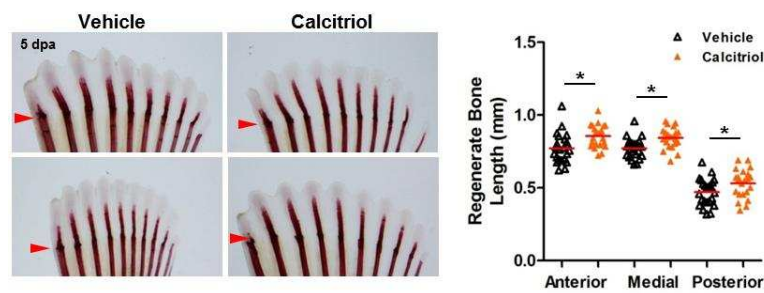


Figure 41: Vitamin D can increase bone formation during zebrafish fin regeneration. (Left) Alizarin red staining of fins treated with calcitriol (1 ng/ml) from 2 dpa to 5 dpa. (Right) Calcitriol treatment increases the lengths of regenerated bone. $n = 23$ (vehicle) and $n = 22$ (calcitriol), $*p < 0.05$, Student's t-test.

Like *hand2*, the vitamin D degrading enzyme, *cyp24a1*, was restricted to the posterior regions of the pectoral fin. Although overall expression was decreased, the AP pattern of *cyp24a1* was maintained at 4 dpa (Figure 42).

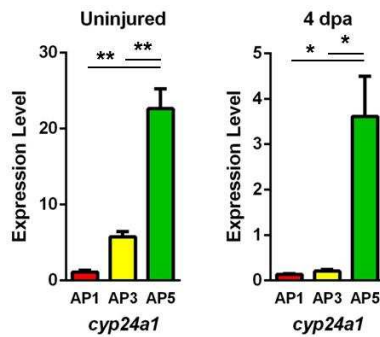


Figure 42: Elevated posterior expression of *cyp24a1*. (Left) *cyp24a1*, a vitamin D-inactivating enzyme, is more highly expressed in posterior regions of uninjured pectoral fins. Expression is normalized to β -actin1, n = 3, **p < 0.005, Student's t-test, mean \pm SEM. (Right) Posterior enrichment of *cyp24a1* is maintained during regeneration. Expression is normalized to β -actin1, n = 3, *p < 0.05, Student's t-test, mean \pm SEM.

This distribution suggested the presence of differential vitamin D signaling across the fin's AP axis. Using mammalian literature as a guide *calb2a*, *bglap*, and *sparc* were identified to be vitamin D-responsive in zebrafish by induction of these genes in the fin after intraperitoneal (IP) injection of vitamin D (Figure 43) (McDonnell et al., 1989; Wasserman and Fullmer, 1989; zur Nieden et al., 2003). The expression of these genes was significantly higher in the *cyp24a1*-negative anterior region of the pectoral fin (Figure 43).

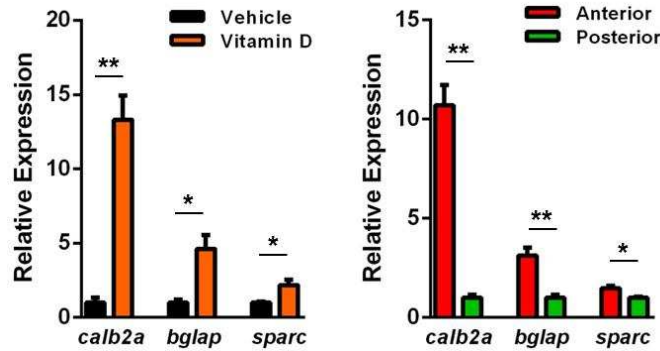


Figure 43: Differential vitamin D signaling across the pectoral fin AP axis.

(Right) Induction of *calb2a*, *bglap*, and *sparc* in the posterior regions of zebrafish pectoral fins 8 hours after a single intraperitoneal injection of vitamin D. Expression is normalized to β -actin1, relative to vehicle-injected clutchmates, n = 3, **p < 0.005, *p < 0.05, Student's t-test, mean \pm SEM. (Left) Expression of vitamin D-regulated genes across the pectoral fin AP axis is inversely correlated with *cyp24a1* expression. Expression is normalized to β -actin1, relative to posterior expression, n = 3, **p < 0.005, *p < 0.05, Student's t-test, mean \pm SEM.

3.5.1 Expression of *hand2* alters *cyp24a1* levels and downstream targets of vitamin D signaling

To test whether *hand2* regulates vitamin D signaling during fin regeneration, the relationship between transgenic *hand2* elevation and *cyp24a1* levels was explored.

Induction of *hand2* raised *cyp24a1* expression 7.5 fold, indicative that *hand2* lies upstream of *cyp24a1* during fin regeneration (Figure 44).

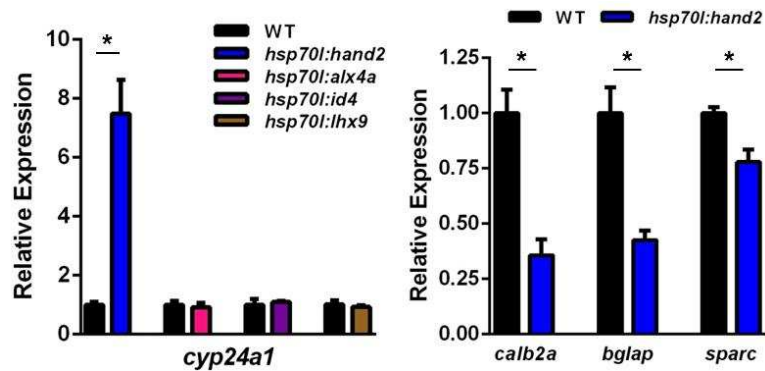


Figure 44: *hand2* influences *cyp24a1* expression and vitamin D signaling. (Left) *hand2* induction elevates *cyp24a1* expression approximately 7.5 fold, normalized to β -*actin1*, relative heat-shock wild-type clutchmate controls, n = 3, *p < 0.05, Student's t-test, mean \pm SEM. (Left) *hand2* induction reduces expression of genes stimulated by vitamin D, normalized to β -*actin1*, relative heat-shock wild-type clutchmate controls, n = 3, *p < 0.05, Student's t-test, mean \pm SEM.

hand2 misexpression reduced *calb2a*, *bglap*, and *sparc* levels 64%, 57%, and 22%, respectively, a finding that also associates reductions in vitamin D signaling with bone defects during regeneration (Figure 44). These data suggest a vitamin D signaling gradient in the adult pectoral fin regulated by *hand2*.

3.5.2 Expression of *hand2* does not alter expression of the posteriorly enriched actinodin genes

To determine if *hand2* might be upstream of multiple posterior and thus more of “master regulator” or “determinant” of positional memory its effects on second class of posterior genes that could reflect altered bone formation and fin ray pattern were examined. The actinodin genes are structural components of actinotrichia, which are unmineralized fibrils found in early pectoral development (Zhang et al., 2010).

Surprisingly the entire family, *and1-4*, was detected by RNA-Seq to be significantly elevated in the posterior region of the adult pectoral fin, and this expression pattern was validated by qPCR (Figure 29, Figure 45). But unlike *cyp24a1*, induction of *hand2* failed to significantly increase the level of the actinodin gene family in the anterior region of the pectoral fin (Figure 44).

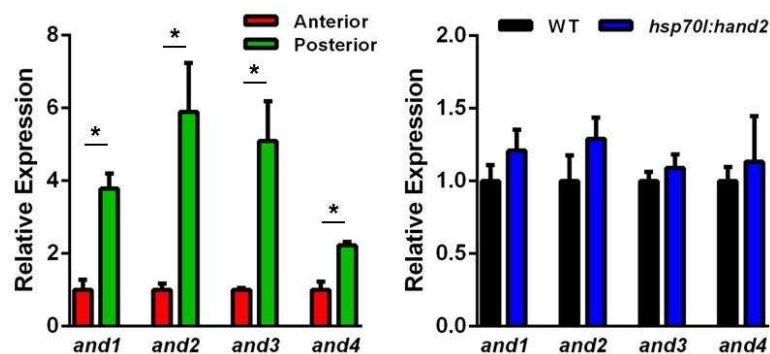


Figure 45: *hand2* does not regulate posterior actinodin genes. (Left) qPCR verification of posterior enrichment of *actinodin* gene expression. Expression is normalized to β -*actin1*, relative to anterior expression, $n = 3$, $*p < 0.05$, Student's t-test, mean \pm SEM. (Right) *hand2* misexpression not does alter expression of *actinodin* genes. Expression is normalized to β -*actin1*, relative to heat-shocked wild-type clutchmate controls, $n = 3$, $*p < 0.05$, Student's t-test, mean \pm SEM.

This demonstrates that *hand2* controls only a subset of posterior genes and thus is almost certainly not the sole factor in the pectoral fin controlling AP positional memory. This is also likely an important reason for partial nature of the transformation observed when *hand2* is misexpressed.

3.6 hand2 acts as a positional memory factor via its actions on vitamin D signaling

To define the significance of vitamin D signaling as a target of *hand2*, zebrafish were supplemented with vitamin D throughout the course of pectoral fin regeneration. Because the heat-shock protocol requires immersion in the circulating water system daily IP injection was used instead of bath incubation. Daily injection of fish with vitamin D had no effects on pectoral fin regeneration in wild-type fish. By contrast, although delivered systemically, this regimen partially rescued the *hsp70l:hand2* patterning phenotype (Figure 46A-C). Compared vehicle injection transgenics, vitamin D dosing led to 24% and 20% increases regenerate length of anterior and posterior rays respectively (Figure 46B). Accompanying the length increase was an ~11% increase in relative widths of the regenerate rays across the AP axis (Figure 46C).

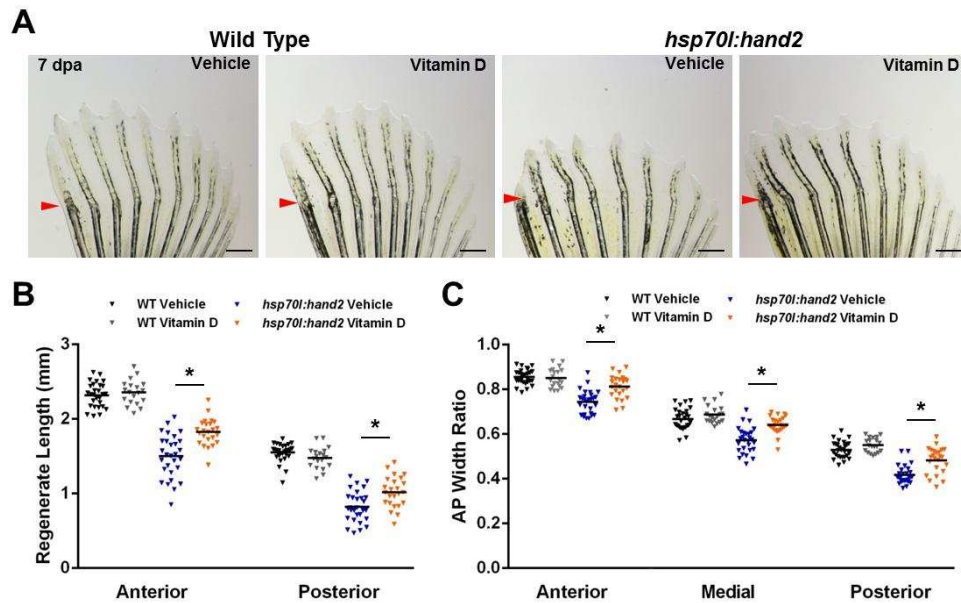


Figure 46: Increasing vitamin D levels partially rescues *hand2* phenotype. (A) Appearance of 7 dpa fins from *hsp70l:hand2* and wild-type clutchmates, given a daily heat-shock and either daily vehicle or vitamin D injections. Vitamin D injection had little effect on wild-type regeneration, but partially suppresses the effects on bone patterning caused by *hand2* misexpression. Representative regenerative growth of *hsp70l:hand2* vehicle-injected rays 3 and 8 are denoted by dashed lines. (B) The effects of daily vitamin D injection on ray lengths, indicating improvement in *hsp70l:hand2* animals. $n = 20-28$ $**p < 0.05$, Student's t-test. (C) Vitamin D injection increases ray widths in regenerating *hsp70l:hand2* fins. $n = 20-28$ $**p < 0.05$, Student's t-test.

Together, these gene expression and rescue experiments reveal that *hand2* controls patterning in regenerating pectoral fins at least in part by modulating vitamin D metabolism and signaling (Figure 47).

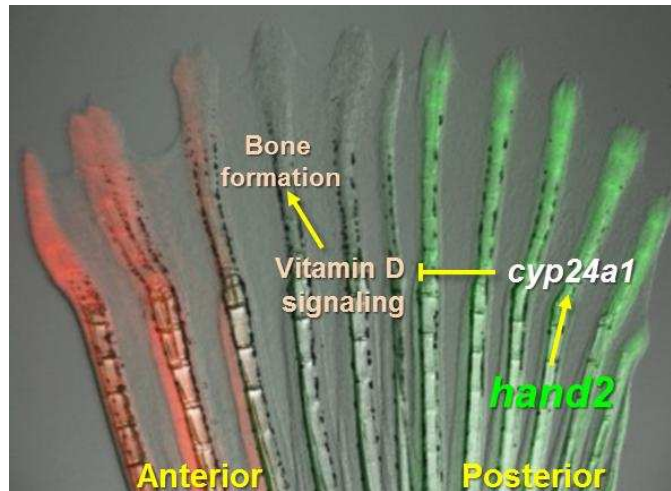


Figure 47: *hand2* acts as a positional memory via regulation of vitamin D signaling. Model for Hand2 influences on positional memory. Adult zebrafish pectoral fins maintain region-specific expression of transcription factors. Levels of the posterior transcription factor gene *hand2* can help control bone patterning during pectoral fin regeneration. Hand2 levels regulate bone formation during regeneration by direct or indirect regulation of the vitamin D-inactivating enzyme, *cyp24a1*, restricting bone formation in the posterior rays of pectoral fins.

3.7 Discussion

A defining feature of appendage regeneration is the maintenance and recall of positional information. Here a transcription factor gene expression signature and a downstream signaling pathway that helps explain AP positional memory in zebrafish pectoral fins has been discovered. Many of these genes displayed region-specific expression across the AP axis of uninjured adult fins, of which the impact of 4 on patterning during regeneration was examined via inducible transgenic misexpression. One of these transcription factor genes, *hand2*, displayed key aspects of a regulator of positional memory. First, *hand2* expression is maintained in an AP region-specific

manner in bone-forming cells of uninjured and regenerating fins. Second, *hand2* misexpression changes the AP patterning of bone during fin regeneration. Furthermore, the data indicates that *hand2* regulates posterior bone formation in part through local control of the activity of a systemic signal, vitamin D.

These findings reveal that zebrafish pectoral fins maintain a basal level of transcription factors in preferential domains through all life stages, as opposed to turning off expression after patterning and differentiation. This is likely to be a central mechanism of positional memory in zebrafish fins. The expression of embryonic signaling factors in uninjured adult structures has been observed in other examples of appendage regeneration (Nicolas et al., 2003; Poss, 2010; Schnapp et al., 2005; Wills et al., 2008), and mammalian fibroblasts are known to express region-specific Hox gene codes (Chang et al., 2002; Rinn et al., 2006). Although positional memory factors are likely to recapitulate aspect of the embryonic developmental program, they are also likely to regulate pattern by mechanisms that are distinct from embryogenesis. Relevant to this second idea, *hand2* likely does not influence pattern via Shh signaling in adult pectoral fins as it does in embryonic pectoral appendages. Rather, *hand2* controls of vitamin D signaling, an interaction that has not been previously reported.

A great deal of research has demonstrated the complex role of vitamin D in mammalian bone homeostasis (Lieben and Carmeliet, 2012). While implanting vitamin

D soaked beads was shown to have somewhat minor effects on axolotl limb regeneration (Washabaugh and Tsonis, 1995), there has been little or no evidence that vitamin D signaling helps define AP patterning in developing or regenerating limbs. This lack of evidence can be explained in part by the fact that only when signaling was compromised by *hand2* misexpression was a phenotype observed (Figure 46). Aside from appendage regeneration, vitamin D has been shown to influence liver, axon, and skeletal muscle regeneration (Chabas et al., 2008; Ethier et al., 1990; Stratos et al., 2012). It will be interesting to examine if the regulation of vitamin D signaling is important in additional regenerative contexts.

By what mechanism(s) do adult cells maintain regionalized expression of patterning transcription factors? There are at least two important gene regulatory components of regeneration, which might on the surface appear contradictory. First, cells must be capable of rapid gene expression changes, to enact major changes like de-differentiation in response to injury. Second, as shown in this chapter, cells also “lock-in” expression of key developmental regulators in a region-specific manner throughout life. Novel epigenetic regulation, possibly at the chromatin level, is likely to underlie this versatility.

While differential AP expression of numerous transcription factors was found, functional manipulation of just one of these produced clear patterning effects during

regeneration. This might reflect the limitations of the misexpression technologies currently available for use in adult zebrafish fins. Alternatively, it is possible that multiple transcription factors act in concert as a code to appropriately specify positional information. Consistent with this notion, there were posterior-enriched genes that were not impacted by the misexpression of *hand2* in anterior rays (Figure 45). Moreover, complete reprogramming of positional memory is likely to require simultaneous increases and decreases in gene expression. Emerging genetic toolsets for zebrafish and other highly regenerative vertebrates should enable the discovery of core modules of positional memory (Bedell et al., 2012; Dahlem et al., 2012; Meng et al., 2008)(102-104), bringing the community closer to an understanding of how and why regeneration occurs.

3.8 Methods

3.8.1 Zebrafish, surgeries, measurements, and drug treatments

Males of several zebrafish strains show defects in pectoral fin regeneration (Chapter 2), making it necessary to use females for these experiments. All animals were between 4 and 12 months of age and in an outbred Ekkwill (EK) strain background. Pectoral fins were amputated proximal to the first bifurcation point at approximately one-third of their original length using iridectomy scissors. Fin lengths and widths were measured from images using Leica Application Suite V3.6 software. Widths of the seg-

ment proximal to the first bifurcation of the ray were measured in uninjured fins, and the second ray segment distal to the amputation plane was measure in fin regenerates. Heat-shock experiments were performed by giving transgenic and clutchmate controls a daily 38°C heat-shock as previously described (Wills et al., 2008). Calcitriol (Sigma 17936) was performed making a 5µg/ml stock solution in ethanol then diluting in 2.5L of fish water to the 1ng/ml final concentration with daily water changes. 1α,25-Dihydroxyvitamin D₃ (Sigma D1530) was dissolved in ethanol to make a 10 µM stock solution and stored at -20°C. For intraperitoneal injections, this stock solution was diluted 1:10 with water and 10 µl was injected per fish.

3.8.2 Construction of transgenic animals

osx:EGFP-CAAX was generated by subcloning an EGFP-CAAX cassette that had been amplified from Tol2kit plasmid #384 (Kwan et al., 2007) downstream of published promoter sequences of medaka *osterix* (Renn and Winkler, 2009). The full name of this transgenic line is *Tg(osterix:EGFP-CAAX)^{pd51}*. For *alx4a:DsRed2*, the first exon of *alx4a* in the BAC clone CH211-107P11 was replaced with a DsRed2 cassette at the translational initiation site by Red/ET recombineering (GeneBridges). The full name of this transgenic line is *Tg(alx4a:DsRed2)^{pd52}*. For *hsp70l:alx4a*, *hsp70l:id4*, *hsp70l:lhx9*, and *hsp70l:hand2*, full-length cDNAs were cloned from adult pectoral fins and then subcloned downstream of the inducible *hsp70l* promoter (Halloran et al., 2000). The full names of these

transgenic lines are *Tg(hsp70l:alx4a)^{pd53}*, *Tg(hsp70l:id4)^{pd54}*, *Tg(hsp70l:lhx9)^{pd55}*, and *Tg(hsp70l:hand2)^{pd56}*. An *α-crystallin:EGFP* cassette was inserted in reverse orientation to make lens fluorescence an identifier of transgenic animals (Waxman et al., 2008). Purified plasmid or BAC DNA was coinjected with I-SceI into single-cell embryos.

3.8.3 Alizarin red skeletal staining

Fins were removed from the fish and fixed in 4% paraformaldehyde (PFA) at overnight at 4°C. Fixative was removed and fins were dehydrated in 50% ethanol for 30 minutes at room temperature. Fins were then stained in a solution containing 0.5% potassium hydroxide and 0.01% alizarin red S (Sigma A5533) overnight with gentle agitation at room temperature. After staining fins were bleached in a 1.5% hydrogen peroxide and 1% potassium hydroxide solution for 20 minutes at room temperature. After bleaching the fins were then cleared with a 50% glycerol and 0.25% potassium hydroxide solution.

3.8.4 RNA isolation and quantitative PCR

For gene expression analysis, fin regions from 3 fish were dissected and pooled for each sample. RNA was isolated using Tri-Reagent (Sigma). cDNA was synthesized from 1 µg of total RNA using the Roche First Strand Synthesis Kit. Quantitative PCR was performed using the Roche LightCycler 480 and the Roche LightCycler 480 SYBR Green I Master. All samples were analyzed in biological triplicate and technical dupli-

cate, and all reactions were performed with an annealing temperature of 60°C. The analysis was performed using the $\Delta\Delta C_T$ as previously described (Yin et al., 2008). Primers sequences are listed in Table 2.

Table 2: Primers sequences used in studying AP positional memory

Primer	Sequence
zebrafish qPCR	
<i>alx4a</i> F	AGAGAGCGTTTCGGACAGATGCAA
<i>alx4a</i> R	TGTGGATGAGGTGGCATAACAGGAA
<i>lhx9</i> F	GACCAAGCGCATGCGTACATCATT
<i>lhx9</i> R	ACGTTTCTTCTGAATTTGGCCCGTG
<i>hoxc8a</i> F	ACCTTAGCGCAATACCCAGACTGT
<i>hoxc8a</i> R	ATCGGCTGTATGTCTGCCTCCAT
<i>id4</i> F	TGCCCACTATTCCGAGGATAAGA
<i>id4</i> R	TATTGACACCCGTTGTCTCTGCT
<i>hand2</i> F	CAGACGCCAAGAAGAAAGGCGAA
<i>hand2</i> R	CATTGCTTCAGTCCAATGCCAA
<i>hoxd13a</i> F	TGGGACTGGTCAAACAATTGCAGC
<i>hoxd13a</i> R	TTTCTCCCAGCTGACAGAATGAA
<i>hoxd11a</i> F	TGCATGTAACCCGTCTACAGATAGCG
<i>hoxd11a</i> R	ACGGGCATCTCTTTCTGGACT
<i>pax9</i> F	TAAAGTACCAACTCCGCCTGGCAT
<i>pax9</i> R	TGGATGCTGGGAGCTGTGACATAA
<i>tbx2a</i> F	TGACCAGCAACAAGTCCACTGAA
<i>tbx2a</i> R	AACGTGGGCTGAATGGTGATTGTG
<i>actb1</i> F	TGGACTTTGAGCAGGAGATGGGAA
<i>actb1</i> R	AAGGTGGTCTCATGATACCGCAA
<i>cyp24a1</i> F	TGTCGGACATCTCCATAACGGCA
<i>cyp24a1</i> R	TGGATCTCTTCAGGAGTTTGCCT
<i>calb2a</i> F	GCTGAGCAGTTCAACGCCATCTTT
<i>calb2a</i> R	CAGGGCCATGATGCTTTGCTTGTA
<i>bglap</i> F	CGTCTGGAATCATCGCCGCCTATAAA
<i>bglap</i> R	TCAGCATCTTCACTGCTGGTGT
<i>sparc</i> F	GTGGAGACCGGAGAGTTTGATGA
<i>sparc</i> R	CTGGTTGCTGTCATCTACCTACA
<i>and1</i> F	CGACTGTGACCCAGAGTACGAC
<i>and1</i> R	TAAGCGTCATATTGCTGCTGGT
<i>and2</i> F	AGCAAGTCACATATCCGCAAAA
<i>and2</i> R	TGGCACCCCTTCTACTCTGTG
<i>and3</i> F	CAAAAATCCCAGCTTTCAGTCC
<i>and3</i> R	CATAGGTGAGCTCCAGATGACG
<i>and4</i> F	ATGTCTGCGGTGTATGCTGT
<i>and4</i> R	CTGAGGATCTCCATCAGGGATT
Cloning	
<i>alx4a</i> cDNA F	atatagatccaccATGAACGCCGAGACGTGCGTTTCATAC
<i>alx4a</i> cDNA R	tatacctcgagTCATGTAGCCCAAGAGATGGCTGCACTGTG
<i>id4</i> cDNA F	atatagatccaccATGAAGGCCAGCGTCCCGTTCCGCC
<i>id4</i> cDNA R	tatacctcgagTCAGCGACACAAAAAAGTGAGTCTTCTTATTG
<i>lhx9</i> cDNA F	atatagatccaccATGGAAGTTGTGGGTGCAAGGCCAAAG
<i>lhx9</i> cDNA R	tatacctcgagTTAGAAAAGTTTGTCAAGGTAGTTTG
<i>hand2</i> cDNA F	atatagatccaccATGAGTTTAGTTGGAGGTTTCCCC
<i>hand2</i> cDNA R	tatacctcgagTCATTGCTTCAGCTCCAATGCCCAAAC

3.8.5 RNA-Seq

The two most anterior and posterior rays (AP1 and AP5) were collected and pooled from pectoral fins of 20 6-8 month old zebrafish in duplicate. RNA was isolated using Tri-Reagent (Sigma). Samples were then submitted to the Duke Genome Sequencing and Analysis Core for library preparation and run on an Illumina HiSeq2000. The data were analyzed using Tophat, Bowtie, and Cufflinks according to described protocols (Trapnell et al., 2012). Ensembl's Zv9.70 was used for genome annotation. Gene ontology analysis was performed using the Princeton University Lewis-Sigler Institute for Integrative Genomics website (<http://go.princeton.edu/>)

3.8.6 Immunofluorescence and BrdU incorporation

Fins were removed and fixed in 4% PFA at room temperature for an hour. Staining of fin cryosections was performed as previously described (Johnson and Weston, 1995; Wills et al., 2008) using a p63 antibody (mouse 4A4; Santa Cruz Biotechnology) at 1:200 or Zns-5 (ZIRC) at 1:200. Imaging and co-localization analysis was done using a Zeiss LSM 700 confocal microscope. Quantification of pectoral fin BrdU incorporation was performed as previously described in Chapter 2.

4. Future Directions and Discussion

The ChIP experiments were a collaboration between Greg Nachtrab and Joseph Goldman. The *osx:INTACT* and *osx:EGFP-TVA* fish were generated by Greg Nachtrab. The *dkk1b:EGFP* fish was generated by Junsu Kang.

4. Future Directions and Discussion

Positional memory is one of the least understood aspects of regeneration. To address this lack of knowledge, a goal of this dissertation was to identify additional examples of positional information in zebrafish fins. The ultimate purpose of this being that once an intriguing example of positional information was identified, the technical advantages of zebrafish would facilitate the discovery of the underlying positional memory factors. These goals were accomplished through the examination of the patterning and regenerative capacity of all fins of the zebrafish. In terms of the patterning of the fin rays, the AP axis of pectoral fins displayed a pronounced asymmetry indicating the likely presence of positional information. Unexpectedly, only female fish were found to be capable of faithfully regenerating this AP fin pattern. Adult male pectoral fin regenerates showed region-specific regenerative defects. The complete recovery of AP pattern in female fish and the region-specific male defects are two examples of the importance of positional information during fin regeneration. The mechanisms responsible for both of these occurrences were identified. These results indicate the promising potential of zebrafish pectoral fins as a model to study positional memory, and the identified mechanisms provide starting points for future work.

Much of the current interest in regeneration is predicated on the hope that one day human regenerative responses might be either enhanced or stimulated. It may be

tempting to speculate about how any findings in fish could be applied to humans, but given the obvious structural differences between a fish fin and human limb it is probably wise to remain cautious. However, basic questions about the initiation and the control of regeneration are more easily addressed in zebrafish fins than would be possible in either a mammalian or amphibian limb. Although the simple structure of a fin compared to a limb could necessitate that when studying fins a full mechanistic explanation of any finding will be required for the general importance to become apparent. In terms of control of regeneration or positional memory in fins, this evaluation mandates both the generation of novel reagents and the identification of interesting biological phenomena. This dissertation has defined two examples of positional information that can be utilized as starting points for further interrogation of positional memory in the zebrafish pectoral fin. The potential implications of these findings are discussed below. Additionally, some ideas and initial work on developing the next generation of tools for studying positional memory are described below. Hopefully, these results and ideas will prove useful during future investigations of positional memory.

4.1 New examples of positional memory in zebrafish fins

Positional memory is most often studied in the context of the PD axis. This axis is extremely challenging for a few reasons. The first is that many highly regenerative

organisms such as fish or salamanders continue to grow throughout life. To accommodate this growth the PD axis must display some plasticity, be capable of continuous reorganization, or specify new proximodistal values throughout life. Zebrafish fins grow by the addition of new ray segments (Goldsmith et al., 2003). While the mechanisms regulating these additions remain uncertain, the studies of growth in other repetitive structures have yielded some surprising insights. During somitogenesis a clock-wavefront mechanism occurs with oscillations in Fgf, Wnt, Notch signaling play pivotal roles in somite boundary formation (Saga, 2012). Similarly, during Arabidopsis root growth, oscillating gene expression along the length of the root marks the locations from which lateral root branches emerge (Moreno-Risueno et al., 2010). If similar mechanisms are present along the PD axis in vertebrate appendages, this would make finding PD positional information during regeneration even more daunting.

Although extensively studied for many years, the control of PD growth during vertebrate limb development still appears complex. The current model involves the proper integration Shh, Fgf, Wnt, and Bmp signaling, but there still remains a great deal to be learned how these crosstalk functions to produce a properly proportioned limb (Gilbert, 2010; Zeller et al., 2009). Many regeneration experiments use a candidate approach based on knowledge from developmental studies. Thus this developmental complexity makes picking the proper candidates for PD positional memory quite

challenging.

While there may be significant complications associated with the PD axis, the AP and dorsoventral (DV) axes offer promising alternatives. Although zebrafish fins continue to grow throughout life, the positional values along these axes likely do not require drastic resetting to accommodate for adult growth or regeneration. Additionally, during development many factors and signaling pathways have well defined roles in establishing and specifying these axes. Such attributes make both the AP and DV axes of fins intriguing avenues for investigating positional memory.

This dissertation has not previously discussed the DV axis of fins. Fin rays are composed two adjacent crescent hemi-rays that form a cylindrical shape. This stereotypical shape and orientation of the hemirays indicates the likely presence of positional information. Given the proximity of the hemirays a dissection and profiling approach, similar to what was done for the AP axis, is not feasible for the DV axis. However, an alternative approach would to focus on candidate molecules based on knowledge of DV patterning during embryonic limb development. For instance, using either in-situ hybridizations or transgenic reporters the fin expression of the zebrafish orthologues of embryonic DV patterning genes, such as *Wnt7a*, *Lmx1*, and *En* could be examined (Logan et al., 1997; Riddle et al., 1995). If one of these genes displayed a restricted DV expression, it would provide another intriguing jumping off point.

Overexpression or loss-of-function of a DV restricted gene might alter the orientation or crescent shape of the hemirays. Such a phenotype would be quite striking. Moreover, the DV axis might be critical for more than just the curved nature of the hemirays. During limb development, manipulation of DV axis factors has been shown to influence AP and PD patterning (Parr and McMahon, 1995; Yang and Niswander, 1995).

The AP axis of the developing vertebrate limb has been intensely examined and re-examined. The importance of many factors ranging from secreted molecules for example Shh and Bmps to transcription factors such as Hand2 and Hoxd13 has been established. Although the importance of Hand2 and Hoxd13 during limb development is clear, a precise understanding of how their functions are achieved remains elusive. One potential reason for this lack of mechanistic clarity is that similar to the problem that Thomas Hunt Morgan faced over a century ago, manipulating development to make concrete interpretations is still extremely challenging. Additionally, in the case of the developing limb it appears that the same signaling pathway may help specify multiple axes, which can make interpreting phenotypes problematic. Like Morgan did, one might hope that studying this patterning information by examining the maintenance and recovery of adult pattern during regeneration could yield some unique insights that are not readily apparent when examining the embryo. The work described in this dissertation uncovered two examples of AP positional information and its

manifestations in zebrafish pectoral fins. Although these discoveries by themselves do not fully explain all facets of AP information in a fin or a limb, they provide some general insights and a framework that can be expanded upon in the future.

4.1.1 Anteromedial nature of male-specific defects

The most surprising finding of this dissertation is that zebrafish display sexually dimorphic regeneration. Interestingly, male-specific defects are confined to the anteromedial region of pectoral fins. This is paradoxical as the cause of the male defects is the increased presence of the male sex hormone androgen. It is not obvious how a factor produced primarily by a distant tissue, the testis, and is delivered systemically could lead to such a dramatic region-specific effect.

There is almost certainly an interaction between androgen signaling and AP positional information in pectoral fins. There does not appear to be sufficient positional information at the level of androgen receptor expression to be the sole reason for the region-specific phenotype. In pectoral fins the *ar* expression was only 1.7-fold higher in the anterior region when compared to the posterior region (Figure 25). This was in contrast to the striking asymmetry in *dkk1b* expression as the *dkk1b* level was ~13-fold higher in the anterior region (Figure 18). An alternate hypothesis is that the AP differences in expression of *dkk1b* and *igfbp2a* could also be regulated by the topography of the genome. Meaning that only in the anteromedial region of pectoral fins are the

androgen responsive regulatory elements in the *dkk1b* and *igfbp2a* promoter accessible. A simple initial method to examine the genome topography is to check for post-translational modifications of Histone 3 at lysine 27. Methylation and acetylation of this residue have been demonstrated to be tightly correlated with gene accessibility and transcriptional activity. However, chromatin immunoprecipitation (ChIP-qPCR) experiments comparing the anterior and posterior regions of pectoral fins failed to detect any difference in either H3K27 tri-methylation (H3K27me3) which is associated with gene silencing or acetylation which is associated with gene activation around either the *dkk1b* or *igfbp2a* gene locus. This result suggests that a simple gene accessibility based mechanism is not likely driving the region-specific expression of these genes in male pectoral fins.

Perhaps the region-specific phenotype and *dkk1b* expression result from the differential distribution of an androgen receptor cofactor. Although the androgen receptor could interact with many transcriptional regulators, one group of intriguing candidates given the positional component are the Hox factors. Recent work has described how Hox proteins can bind and redirect the androgen receptor. Normally the androgen receptor targets regions containing androgen response elements (ARE), but in the presence of a Hox protein the androgen receptor was redirected to promoters or enhancers containing the Hox responsive element (Norris et al., 2009). Additionally, the

human orthologue of *hoxc8a*, an anterior-specific Hox gene in pectoral fins, has been shown to interact with the androgen receptor (Axlund et al., 2010).

Focusing specifically on *dkk1b*, an additional striking fact is that when a *dkk1b*:EGFP reporter was generated by a postdoctoral researcher, Junsu Kang, the male-specific expression was found to be restricted to the epidermis on one axis of the DV axis (Figure 48).

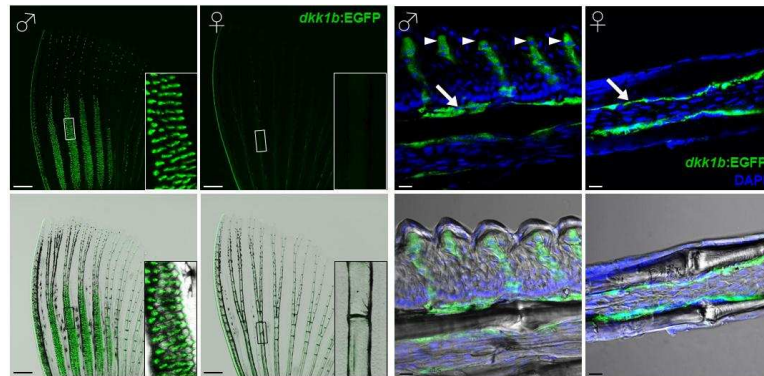


Figure 48: Expression of *dkk1b* in epidermal male secondary sexual structures. Courtesy of Junsu Kang. A BAC *dkk1b*:EGFP reporter indicates both sexes express *dkk1b* in cells adjacent to fin rays (right). However, a significant epidermal expression is found in male fins and restricted to sex-specific projections.

This suggests both positional information in the epidermis as well as some form of dual specification or interaction between the AP and DV axis in the adult fin. During development, DV factors such as *Wnt7a* and *En* are expressed in the ectoderm as well as the transcription factor *Hoxc8*. This makes further examination of a potential Ar-Hox mechanism via *Hoxc8a* even more intriguing. One possible first experiment would be to

inject *hoxc8a* mRNA with or without *ar* mRNA into *dkk1b*:EGFP embryos. If *dkk1b* can be regulated by Hoxc8a there should be some change in EGFP fluorescence after *hoxc8a* mRNA injection. If the combination of Hoxc8a and Ar can interact, the prediction would be that treating the injected embryos with androgen would yield an even greater increase in EGFP fluorescence. Whatever the cause, the combined DV and AP nature of the *dkk1b* expression pattern make the regulators of this region-specific expression in male fin pectoral fins both more interesting and significant.

Finally, a generally provocative aspect of the male defects are that *dkk1b* is expressed in and correlated with the development of the male-specific denticles or papillar structures on pectoral fins (Figure 48). These structures are likely critical for the mating behavior of zebrafish (personal communication, Junsu Kang). That signaling presumably involved in the formation or maintenance of this secondary sexual characteristic can compromise regenerative capacity is fascinating especially when one considers the potential evolutionary implications. The relationship between regenerative capacity and evolution is an area of research with a dearth of data and an excess of speculation. In zebrafish pectoral fins, what appears to have evolved is a situation where a structure more beneficial for reproduction compromises the regenerative capacity of the fin. While a molecular understanding of this phenomenon is still uncertain, one hypothesis is that Dkk1b from the epidermis blocks some of the

Wnt signaling necessary for fin regeneration. Currently, this idea remains a hypothesis based on gene regulation, expression patterns, and misexpression reagents. Hopefully in the future, rigorous testing of this hypothesis will be possible with either inducible tissue-specific knockouts or cell ablation models.

4.1.2 Hand2 and pectoral fins

While the difference in female pectoral fin AP pattern in terms of ray width was modest, it was faithfully regenerated. Thus providing an opportunity to identify the positional information responsible for this recovery. Position-dependent gene expression was observed for a number of genes, including transcription factors with known roles in AP patterning during limb development. Inducible misexpression transgenic zebrafish lines were generated to test if the regional expression of these transcription factors is critical for proper regenerate patterning. Appropriate expression of one factor, *hand2*, was found to be essential for the regeneration of the correct AP pattern in pectoral fins.

Hand2 misexpression or knockout alters vertebrate limb development. It is thought that the actions of Hand2 during development result primarily from its regulation of Shh expression levels. However, based on expression domains it appears unlikely that Hand2 is regulating *shha* expression in adult zebrafish fins. This means that there must be alternative roles for Hand2 in adult pectoral fins. Through the use of

expression analysis, misexpression transgenics, and drug studies, vitamin D metabolism and signaling were found to be regulated by Hand2. This was the first report of any relationship between this transcription factor and signaling pathway.

In pectoral fins the expression of *hand2* remains confined to the posterior region of the fin through the life of the zebrafish. This is in contrast to its expression in the developing mouse limb. By E12.5 Hand2 is expressed more broadly across the AP axis of the developing mouse limb (Charite et al., 2000). It is during this time period in the mouse that Hand2 is thought to have important functions in regulating both osteoblast and chondrocyte differentiation (Abe et al., 2010; Funato et al., 2009). The difference in Hand2 domains between the fish and mouse as well as the proposed murine mechanisms cast doubt upon the possibility that the regulation of vitamin D signaling by Hand2 is conserved across species. However, none of these data exclude the idea that some of Hand2's actions on differentiation of skeletal cells in mouse could be mediated through the regulation of vitamin D signaling. Thus it remains possible that small differences in Hand2 levels and thus vitamin D signaling along the AP axis of the mammalian limb contribute to the digit pattern observed in mice and humans. These subtle differences in the levels of *Hand2* are likely not detectable by whole mount in-situ hybridization, which is the traditional standard for analyzing gene expression in the limb development. Further work will need to be done to determine how relevant the

relationship uncovered here between *hand2* and vitamin D is across all species.

However, it is worth noting that there is at least some conservation between fish species.

Adult females from, *Oryzias latipes* commonly known as medaka, a species that diverged from zebrafish over 100 million years ago also maintain elevated expression of *hand2* and *cyp24a1* in the posterior regions of pectoral fins (Figure 49).

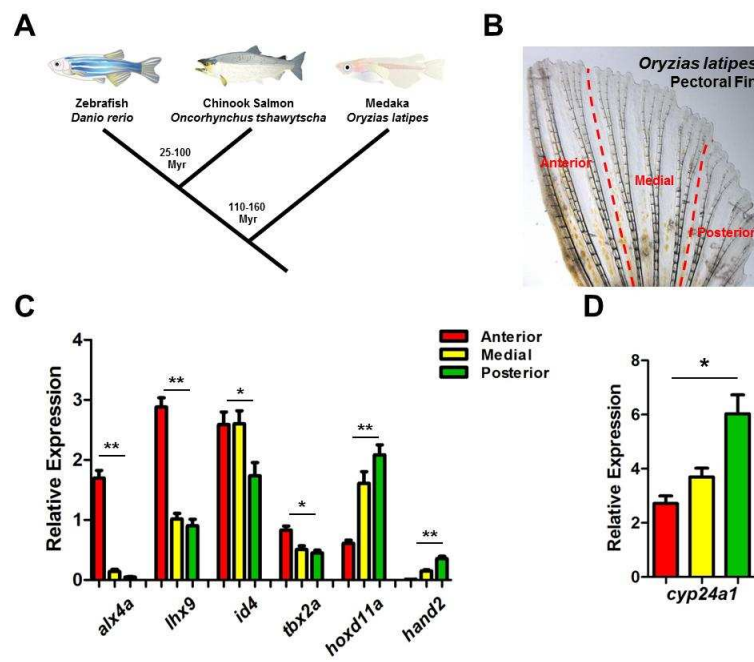


Figure 49: Elevated expression of *hand2* and *cyp24a1* is also maintained in the posterior region of the medaka pectoral fin. (A) Phylogenetic tree showing the distant relationship between medaka and zebrafish. (B) Medaka pectoral fins have an AP ray pattern that is similar to zebrafish. (C) An AP transcription factor code is also conserved in medaka. Expression is normalized to β -actin1, n = 3, *p < 0.05, **p < 0.005, Student's t-test, mean \pm SEM. (D) Expression of *cyp24a1* is also higher in the posterior region of the medaka pectoral fin. Expression is normalized to β -actin1, n = 3, *p < 0.05, Student's t-test, mean \pm SEM.

One more important point about the initial study of pectoral fin AP patterning is

that many transcription factors other than *hand2* such as *alx4a* and *lhx9* maintain region-specific expression in the adult fin. Although singular misexpression of three of these factors was insufficient to drastically alter regenerate pattern, the probable cumulative significance of their continued expression should not be overlooked. Even if many of these factors have only a minor function in the adult, their continued expression likely indicates at least a partial maintenance of an embryonic-like state in the adult fin. Questions such as if this embryonic-like state is necessary or sufficient to enable fin regeneration are intriguing. To tackle questions such as this will require a deeper understanding of how these genes are regulated in adult fins. While new reagents or techniques will have to be developed and adapted to this system, the regulation of the regional expression of embryonic AP patterning factors in adult pectoral fins should be a fruitful area for future research.

4.2 A general modus operandii for positional memory

This dissertation described two examples of positional effects on the regeneration of the pectoral fin. On the surface there may not appear to be a common theme shared between sexually dimorphic regeneration and the posterior actions of Hand2. However, a common theme is that both are cases where there is region-specific effect of a systemic signal. It may be that local sensitivity to systemic signals is a general modus operandii for maintaining form and structure in the adult.

In order for this hypothesis to be valid, systemic signals must play pivotal roles in fin regeneration and homeostasis. As described early in the introduction, the idea that systemic signals impact regeneration is not a recent one, but there are still relatively few document examples its occurrence. Studying systemic signaling in regeneration is quite a technical challenge. Approaches such as the surgical removal of putative source cells or the generation genetic mutants will likely significantly affect not only the regenerate but also many other organs and tissues throughout the animal. In terms of systemic signals and bone biology in mice, theses complexities are being tackled using Cre/lox based strategies. Using such techniques, intriguing roles for molecules such as serotonin and vitamin D have been uncovered. In the future it appears that manipulations similar to what has been done in mice will also be possible in zebrafish. As these technical developments progress it will be important to consider examining or re-examining the roles of systemic signals in fin regeneration.

Are there any systemic signaling molecules that already appear to be interesting candidates? One obvious answer given the effects of androgen is to evaluate the importance of estrogen signaling. Estrogen has well documented roles in bone development and homeostasis. Although bath treatment of fish with estrogen did not produce any gross regenerate defects (data not shown), this is likely the result the inefficient drug delivery using that approach. Hopefully, with a targeted genetic

method more informative results can be attained. Another intriguing candidate is growth hormone. A fair amount of work has been done trying to determine if growth hormone can be sufficient to drive limb regeneration in hypophysectomized newts. However, those experiments have the obvious surgical caveat and provide little information regarding the role of endogenous growth hormone. Much of growth hormone's actions in the bone are thought to be mediated through crosstalk with IGF signaling. Given the demonstrated importance of IGF signaling in fin regeneration, perhaps there already is an established mechanism for how growth hormone might influence regeneration. Additionally, the initial IGF work focused on concept that Igf2b was produced and acted locally, but the majority of the experimental manipulations targeted the IGF receptor which responds to both systemic Igf1 as well as local Igf2. Thus it remains possible that some portion of the IGF signaling requirement in the fin is normally fulfilled by systemic Igf1. There are many other potential candidates including serotonin, leptin, and the full gamut of putative neuroendocrine factors. Perhaps the best method for selecting targets is to evaluate the expression of various receptors during fin regeneration in conjunction with knowledge that can be gleaned from both classical regeneration literature and more recent developmental biology publications.

An appealing aspect of the possibility that location regulation of systemic signals contributes to positional memory is that this mechanism allows for potent development

pathways to continue to control cell proliferation and fate decisions. In fin regeneration these roles for developmental signaling pathways such as Fgf or Shh have been well established. It is intriguing to picture the fin as a system where the number, proportions, and possibly even alignments of various cell types are control by classical developmental pathways and then the overall functional output of these cells such as how much bone is produced is governed by systemic pathways. This is a potentially elegant solution for integrating local and organism-wide events and signaling to control adult homeostasis and regeneration.

It is also striking that the regional control of these pathways at least in zebrafish appears to be built on the foundations of the differential gene expression that is critical for proper embryonic development. This implies that at least in a subset of genes expression is maintained throughout development and then possibly re-tooled for an adult-specific function. The evolution of this phenomenon is fascinating. One could envision a few possible scenarios. The first is that this represents a general feature of positional information found throughout the animal kingdom, and supporting this interpretation is that region-specific transcription factor expression has been found in adult mammals. Another is that this interaction between developmental factors and adult systemic pathways evolved in lower vertebrates and is a common feature of organisms with robust regenerative capacity. Overall, the work presented here suggests

that the regulation of local responses to systemic signaling is a central component of positional memory. Future work in zebrafish and other organisms such as mice and salamanders will help determine if this is the general *modus operandi* for positional information or just one particular type of positional information.

4.3 Regulation of positional memory

To date the most convincing mechanistic explanations of positional memory have been described in invertebrates and more specifically in planarians. In planarians the current model is that positional memory is coordinated by spatial gradients of secreted molecules that specify either head or tail and dorsal versus ventral. This model invokes gradients of secreted molecules and is similar to often how positional information is thought to be specified in developing tissues in both invertebrates and vertebrates alike. However, the many tissues in adult vertebrates such as a limb are complex with numerous physical boundaries. This suggests that simple gradients of secreted molecules alone are unlikely to be sufficient or effective for specifying positional information in an adult vertebrate appendage. Bearing this prediction out are the RNA-Seq results described in this dissertation. Across the pectoral fin AP axis, many genes with differential expression were identified, but the analysis did not reveal any vastly polarized signaling molecules or morphogens classically associated with patterning the developing fin or limb. Thus, positional memory in vertebrate appendages is likely

controlled by more subtle mechanisms such as a combinatorial code of a few transcription factors. The likelihood that vertebrate positional memory is more complex than simple gradients of secreted molecules makes the identification of positional memory factors in adult vertebrates intellectually and technically challenging.

4.3.1 Developing reagents to identify DNA regulatory regions crucial for positional memory in zebrafish fins.

The techniques to identify and study the roles of DNA regulatory regions or epigenetic regulators in any biological phenomenon traditionally have been somewhat limited. In terms of identify DNA sequences critical for the gene regulation, the generation of reporter constructs containing fragments of putative regulatory sequence i.e. promoter bashing has been and remains the standard assay. Such an approach is laborious and to examine expression in the adult requires the generation of stable transgenic lines. Interpretation of promoter bashing results in the adult is made more complicated by the potential confounding effects that the transgene integration site itself might have. For epigenetic regulators the most direct experiments are candidate based using inhibitory drugs or knocking-out key components of a histone-modifying complexes such as Ezh2 in the Polycomb Repressive Complex 2. Besides the issue of targeting the correct complex, these manipulations are broad strokes. As such they would not only likely influence positional memory but also would likely directly impact regenerative capacity.

Many of the potential problems discussed above result from a lack of information regarding which genome regions or epigenetic regulators are focusing on with more detailed and elegant experimental approaches. One way to overcome this obstacle in the study of positional memory is through region-specific profiling of histone modifications (ChIP-Seq or ChIP-qPCR). Depending on the nature of the histone modifications chosen the profiling results could implicate either DNA regions as potential regulatory loci or the profiling results could indicate the importance of a certain epigenetic complex. With this information follow up experiments would be much more directed. Potential regulatory regions could be verified through the generation of reporter constructs taking care to establish multiple transgenic lines. Additionally, the increased power of genome engineering may soon make it feasible to delete or alter regulatory regions in the zebrafish genome. If a particular histone modifying complex appeared interesting in the near future it should be possible to develop reagents to alter the complexes activity with cell type and temporal specificity. Ideally this approach will be compatible with positional memory reporter transgenic strains such as *hand2*:EGFP. In summary, region-specific histone modification profiles should greatly enhance the chances that there would be a significant return on investments into the study of position-specific gene expression in zebrafish fins.

One technical requirement for successfully profiling histone modifications is that

the cell population used must be fairly homogenous. In the case of cell culture experiments this is rarely a problem, but it is a serious complications when considering ChIP experiments from tissue. Recently, a technique to efficiently purify nuclei in a cell type specific manner was developed in Arabidopsis, and this technique has been subsequently adapted to worms and flies (Deal and Henikoff, 2010; Steiner et al., 2012). This technique is called INTACT for Isolation of Nuclei TAGged in specific Cell Types, and it utilizes in vivo biotin-tagging of the nuclear envelope. Realizing the potential power of this approach in overcoming the purity issues inherent in tissue ChIP experiments an attempted was made to adapt this technique to zebrafish fins. Generating cell type specific profiles of histone modifications would likely have important applications for both the study of positional memory and the study of the general dynamics of regeneration (Figure 50).

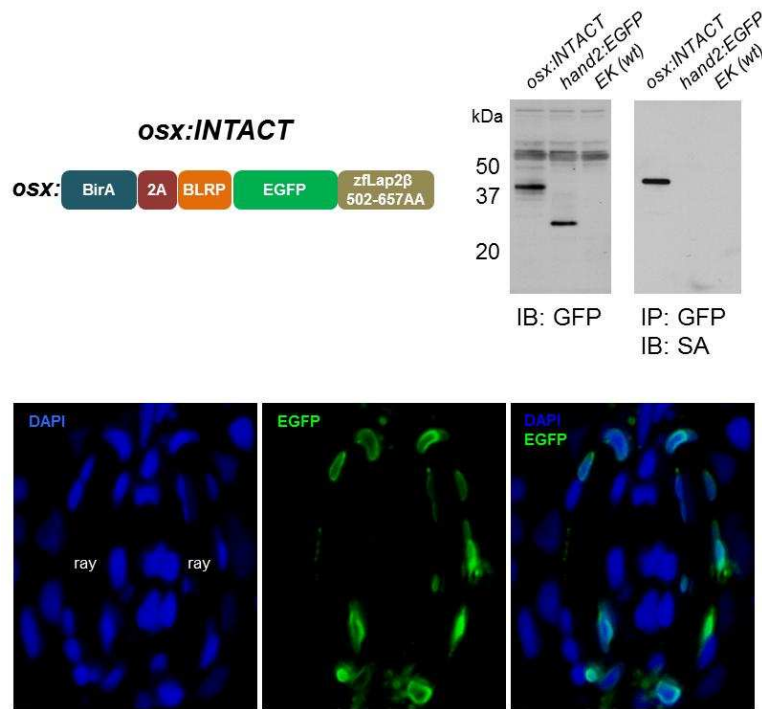


Figure 50: Initial design and testing of biotin-mediated osteoblast nuclei purification zebrafish. (Upper left) Schematic of initial construct design. (Upper right) Immunoprecipitation and blot showing the presence of biotinylated GFP in only the *osx:INTACT* fish. (Bottom) Localization of EGFP to the nuclear envelope in cells adjacent to fin rays in *osx:INTACT* founder fin.

The initial design focused on purifying osteoblasts for several reasons. First is that these cells are responsible for bone formation in the fin. Second at least some of these cells contain positional memory. Finally these cells can be labelled by a promoter fragment rather than requiring BAC recombineering and transgenesis. A single transgene approach was used with a 2A linker separating the *E. coli* biotin ligase and the nuclear envelope fusion protein. The biotin ligase recognition peptide (BLRP) sequence was designed based on previous reports (Howarth and Ting, 2008). Two nuclear

envelope fusion proteins (POM121 and LAP2 β) were tested by injection into embryos, but only the LAP2 β fragment resulted in viable positive embryos. Several lines were generated with the primary testing focused on the line most highly expressed in the adult as gauged by EGFP fluorescence. Western blots and IPs confirmed *in vivo* biotinylation of the fusion protein, and inspection of fin sections stained with DAPI indicated nuclear membrane localization of the fusion protein (Figure 50).

However in spite of these successes, repeated attempts to purify osteoblast nuclei with this reagent have failed to achieve sufficient purify for successful ChIP experiments. This is made apparent when the biotin-mediate purification is performed on EK control fish and there is still a significant amount DNA present; when there should be little to no DNA recovered as the nuclear envelopes of wild-type cells is not reported to be biotinylated. Additionally, although the H3K27me3 antibody concentration was titrated to be specific for only repressed genes (Figure 51), there was detectable enrichment for this repressive mark in genes presumed to be expressed in osteoblasts in the samples from the *osx:INTACT* fish. Furthermore, in the *osx:INTACT* samples there was also detectable enrichment for the active mark H3K4me3 in genes that are not expressed in osteoblasts (Figure 52).

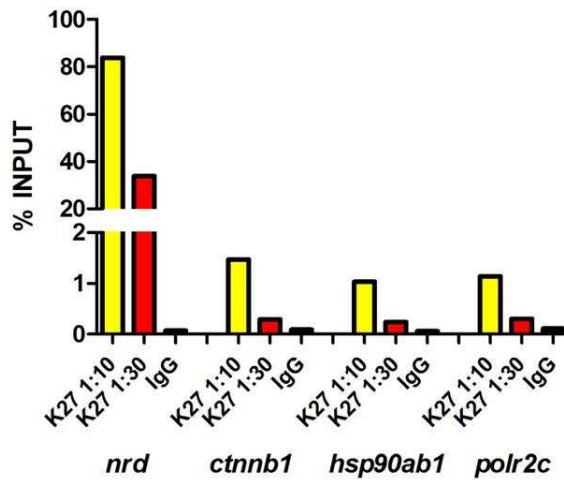


Figure 51: Titration of H3K27me3 antibody concentration examining active (*cttnb1*, *hsp90ab1*, and *polr2c*) and inactive (*nrd*) genes. By using the proper ratio of chromatin to antibody specific ChIP signal can be achieved. In the case of the fin, a ratio of 1 μ g of antibody to 10 μ g of chromatin resulted in nonspecific enrichment, but a ratio of 1 μ g of antibody to 30 μ g of chromatin detected only the repressed gene, *nrd*.

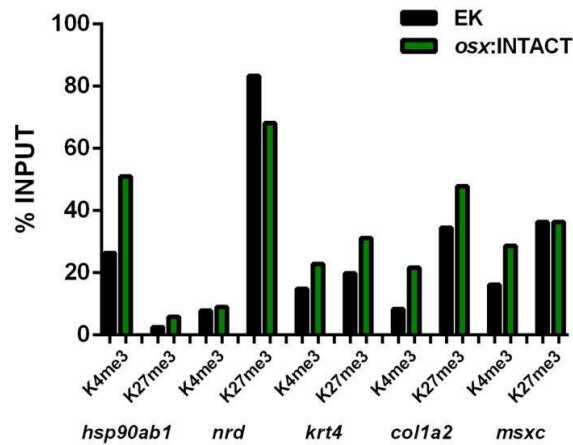


Figure 52: Insufficient purification of osteoblast nuclei from the *osx:INTACT* fish. Positive signal was found in both the wild-type and transgenic fish. Active and repressed marks were observed in both expressed and non-expressed genes.

Based on the papers describing the successful adaptation of INTACT to other model organisms there are likely at least three factors contributing to this failure. The first is the accessibility of the BLRP tag. Although the fragment used was reported to have some outer nuclear membrane localization the majority probably remains on the inner side and thus is inaccessible for nuclei purification (Lang and Krohne, 2003). The initial paper using *Arabidopsis* took advantage of a unique nuclear envelope targeting peptide sequence (Deal and Henikoff, 2010). Subsequent work in other systems reports having to empirically test different fusion proteins to find an effective one, although RanGAP may be a safe general bet (Steiner et al., 2012). Unfortunately, using the medaka *osx* promoter only a small number of cells express the transgene during early embryonic development which makes rapid testing with transgene injected embryos impossible. Along a similar line the percentage of osteoblasts in the adult fin is low making successful nuclei purification challenging. Additionally, the treatments required to isolate intact nuclei from one tissue or another may vary dramatically depending upon the composition of the tissues. While numerous iterations were tried, the possibility that the optimal conditions for isolating osteoblast nuclei from zebrafish fins were never achieved remains. Overall, the initial attempt provided some valuable information such as *in vivo* biotinylation is feasible. This finding has been the basis for

other reagents developed in the lab (personal communication, Joseph Goldman). The most important lesson learned is that future attempts should use a promoter fragment that enables embryonic testing of the fusion protein and nuclei purification prior to the generation of adult transgenic lines.

Although a fairly homogeneous cell population is important for ChIP experiments examining histone modifications associated with gene repression, the requirement is less stringent when focusing on marks linked to gene activation. The reason for this being that when looking at repressive marks any cell not expressing the gene of interest will result in positive signal. However, when examining active marks only the cells expressing the gene of interest will produce enrichment, and this enrichment will occur regardless of the presence of non-expressing cells. This can result in a tolerable signal to noise ratio from a heterogeneous sample such as whole tissue. To test if this simple approach might be useful in the pectoral fin the enrichment H3K27ac (a mark associated with active genes and enhancers) and H3K27me3 were examined near the transcription start sites for the AP transcription factors (Figure 53).

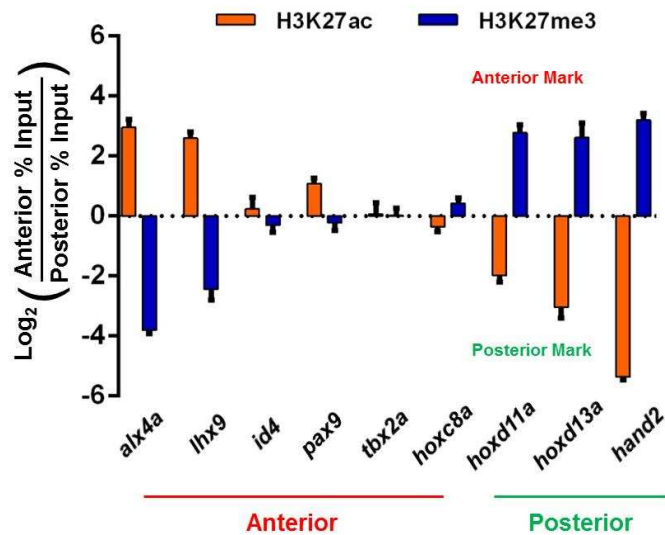


Figure 53: The AP distribution of active versus repressive marks on H3K27 correlates with differential AP gene expression. For *alx4a*, *lhx9*, *hoxd11a*, *hoxd13a*, and *hand2* the localization of the H3K27 acetylation and methylation is reflective of the gene expression pattern.

These data indicate in the case of genes that display a significant AP polarity in expression such as *alx4a* and *hand2* ChIP experiments from isolated regions of whole fin tissue have the potential to be informative. This statement is based on the fact that the gene expression pattern correlated with the presence of either the active or repressive histone mark. Encouraged by these results, the regions around the *hand2* gene locus were examined further. Previous ChIP experiments on whole 24 hour post fertilization embryos identified some candidate regulatory regions (Aday et al., 2011). Using this as a guide ChIP-qPCR was performed to test H3K27ac deposition at these putative enhancers in both the anterior and posterior region of adult pectoral fins (Figure 54).

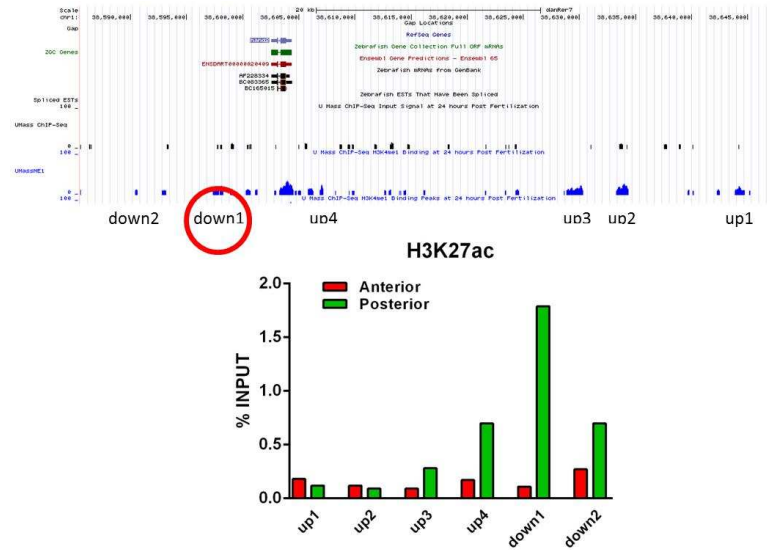


Figure 54: Identifying possible positional enhancers of *hand2* with H3K27ac. (Upper) UCSC genome browser shows areas of H3K4me1 around the zebrafish *hand2* locus. (Bottom) Differential H3K27ac deposition between the pectoral fin anterior and posterior in areas that correspond to potential *hand2* enhancers.

One region approximately 5 kilobases downstream of the *hand2* termination site displayed significant H3K27ac enrichment in posterior of the fin. This enrichment was absent in the anterior region of the pectoral fin. Attempts to determine if this region actually represents a functional enhancer or merely is a by-product of gene transcription through injection of reporter constructs into embryos have been unsuccessful. This negative is uninterpretable as it may result from an inefficient minimal promoter (used a version of mouse *c-fos* minimal promoter), insufficient regulatory information from this region alone, or this region also could have no regulatory function whatsoever. In the future more work will need to be done to develop a robust and controlled system for

examining candidate regulatory regions. In summary, some initial experiments directed at examining positional memory at the level of regulatory sequences and histone modifications have not been wildly successful. However these experiments have provided evidence that there is likely information at this level as well as being the foundation for the development of more useful reagents.

4.3.1.1 ChIP protocol

This protocol for native ChIP from fin extractions was developed in collaboration with Joseph Goldman. Harvest fins into 1.7mL tube with 1mL HBS. Wash 2X with 1mL HBS. Dilute DH Liberase stock 1:11 in HBS. Add 1mL to tube and vortex 15-20 min. Remove supernatant and put over 150micron filter with 125uL serum at the bottom to stop the DH reaction. Add 1mL fresh DH solution and repeat 2-3 times until fins are mostly gone. Spin down 100g for 30min in swinging bucket rotor. Bring up in 500uL-1mL Fin Mnase buffer and pipette up and down 2-3X (all buffers listed in Table 3). Add appropriate amount of Mnase. Usually 40-50U/mL for 30-40min but needs to be re-titrated periodically. Want mostly mononucleosomes but should see up to 4N-6N. Stop by adding .5M EGTA for a final [5mM]. Spin down 15min at 10,000g. Remove supernatant and incubate o/n with antibody of interest. Remember to keep ~5% as input to analyze Mnase digestion. Add washed ProtA beads to IP rxn for 3-4 hrs. Remove

unbound material with ~8min on the magnet. Wash 3 X with 1mL N-ChIP wash buffer with a 15-30min incubation on the nutator in between. Only need 5 min on the magnet here. Elute with 200uL N-ChIP elution buffer for 15min at RT. Equal volume phenol followed by phaselock (spin 2.5min at 12,000g). Repeat with chloroform. Isolate on Zymo-column-5 exactly as directed in the manual and elute in 6-12uL.

Table 3: ChIP buffers.

Mnase Buffer (working)

960uL stock
 2uL spermidine (500X)
 2uL spermine (500X)
 1uL DTT
 33.3 uL albumin (30% stock)

N-ChIP wash buffer

50mM TrisHCl pH 7.5	(2.5mL 1M stock)
200mM NaCl	(2mL 5M stock)
5mM EDTA pH 8.0	(500uL 500mM stock)
45mL H ₂ O	

N-ChIP elution buffer

50mM TrisHCl pH 7.5	(2.5mL 1M stock)
200mM NaCl	(2mL 5M stock)
5mM EDTA pH 8.0	(500uL 500mM stock)
1% SDS	(5mL 10% stock)
40mL H ₂ O	

4.4 Remaining challenges in zebrafish pectoral fin

Although this dissertation was successful in identifying two examples of positional information in zebrafish pectoral fins, there are still some inherent challenges to overcome when considering future use of zebrafish fins to investigate positional memory. Current approaches in zebrafish have some technical limitations, but possible

solutions to these limitations are being rapidly developed. Also all vertebrate model organisms capable of robust appendage regeneration face similar significant technical problems that must be addressed before positional memory can be effectively studied. However, there are a few issues specific to fins one should be mindful of when designing future attempts to explore positional memory in zebrafish fins.

4.4.1 Shallow nature of gradients

One of the most basic challenges in studying positional memory is the nature of the phenotypes that are expected from alterations of positional information. Ideal phenotypes for positional memory alterations are regional transformations of structures, but not necessarily skewed cell lineage decisions or complete losses or gains of structures. The requirement for this category of phenotype means that a positional memory model is only as powerful as the range of identifiable morphological transformations it can produce. This maybe a problem in regards to zebrafish fins as positional memory models, because many fins have a high degree of symmetry across multiple axes.

While this symmetry does not necessarily preclude the presence of positional information, symmetry does limit the morphological changes one would expect to be able to identify after altering positional information. When focusing on bone or fin ray pattern, the pectoral fin AP axis has the most polarized gradient. Unfortunately, this gradient still reflects only an approximately sixty percent change between the anterior

and posterior regions of pectoral fins. That is a relatively narrow window in which to achieve compelling morphological transformations.

4.4.2 Lack of defining landmarks

In a similar vein to shallow gradients, zebrafish fins lack of defining landmarks or striking position-dependent changes in anatomy. A limb has both multiple joints such as the elbow and wrist and defined bones such as the metacarpals and phalanges. Analogous distinct anatomical features are not present in zebrafish fins. Without readily apparent reference points determining the extent pattern recovery or transformation is challenging. Analysis of fin ray pattern in this dissertation attempted to circumvent the lack of landmarks by assessing morphology in terms of ratios between rays. This method produced consistent and interpretable results. Overall the absence of obvious landmark structures or large gradients might be a blessing in disguise. Their absence should force future work to be focused on mechanisms of positional gene regulation rather than seeking striking changes in fin specific anatomy.

4.4.2.1 Movement of bifurcation points during regeneration

One potential landmark feature in a zebrafish fin are the ray branching or bifurcation points. However, the position and pattern of ray bifurcation points is not recovered during fin regeneration. In general, regardless of the PD location of the amputation ray bifurcation appears to be inhibited for the first few days of fin

regeneration. Thus the branch points assume more distal locations in the regenerate fin compared to the original. This repositioning has led some to suggest that the newly formed regenerate initiating assumes a proximal identity (Azevedo et al., 2012). However, the molecular evidence for this hypothesis is scant as the factors regulating ray bifurcation remain to be determined.

4.4.3 Indeterminate growth of the fin

The indeterminate growth capacity of the zebrafish possess another potential confound to the use of zebrafish fins as positional memory models. In the adult fin growth occurs through the addition of new ray segments. This addition is preceded by a burst of cell proliferate at the distal tip of the fin. One has to wonder if this growth requires a readjustment of positional information across the fin, and while this growth may be grossly imperceptible it has the potential to produce significant changes in gene expression between fish.

A more hopeful interpretation of this phenomenon of indeterminate growth is that it might be able to be exploited to identify regulators of positional information. There are mutant zebrafish that develop longer than average fins, hence the general name for the group, long fin (Goldsmith et al., 2003). These fish may offer a tantalizing glimpse at PD positional information as it is very likely altered in these fish. If PD information is changed in these fish, it will be critical to assess if this alternation is a

cause or an effect of the phenotype. However, to date no one has capitalized on this opportunity and the genes disrupted in the several of the long fin fish have yet to be either identified or published.

4.4.4 Lack of endoskeletal elements

The idea of a fin-to-limb transition is a popular one in evolutionary biology (Shubin et al., 2006; Zhang et al., 2010). However, in the case of zebrafish one has to be careful not to apply this concept too literally as molecularly there are a few important distinctions between their fins and mammalian limbs. The most obvious difference is in the developmental process through which many of the bones formed. A vertebrate tetrapod limb contains many long endochondral bone elements like the femur or humerus. This type of bone is formed through the ossification of a cartilage precursor. The formation of cartilage also plays pivotal roles in endochondral bone response to injuries such as a fracture. But the zebrafish fin rays are composed of dermal elements, this type of bone is formed through direct ossification without the cartilage component. A few bones connected to the fin but located inside the body of the fish may form via an endochondral process. Interestingly whether or not these bones regenerate well is still uncertain as they are technically challenging to amputate or remove. This difference in the type of bone leads to the inevitable question: how relevant to mammals is the fin as a model of appendage regeneration given the lack of endochondral bone? While this is a

difficult question, regardless of the specifics of bone formation zebrafish fins should remain useful for big picture questions such as what initiates, controls, and terminates the regenerate process.

4.4.4.1 Scleroblasts, a hybrid cell lineage?

Further complicating matters regarding the importance of the fin is the nature of the bone-forming lineage in zebrafish. In mammals there are at least two defined cell lineages responsible for forming the skeletal. The first is the osteoblast lineage which is responsible for mineralized bone and the second is the chondrocyte lineage which produces cartilage. However, in the zebrafish fin the same cells appear to express genes and produce proteins associated with both lineages (Avaron et al., 2006). If zebrafish bone-forming really do have this dual nature it makes defining the precise steps by which rays regenerate and how that might be applicable to the regeneration of mammalian bone convoluted. Perhaps the zebrafish community should return to more cautiously calling the bone-forming cells in the fin scleroblasts rather than osteoblasts.

4.5 New reagent development for future fin studies

As described above zebrafish fins may have some less than desirable characteristics. However, the biggest obstacle facing zebrafish fin research is the lack of techniques and genetic tools for identifying and defining specific mechanisms in vivo. Current approaches such as overexpression transgenics and pharmacology can be

criticized for their inability to adequately assess endogenous gene function. Fortunately recent advances in genome engineering and microscopy may offer significant promise in terms of aiding future fin research. There are at least three areas where the development of new tools for the fin have the potential to quickly produce profound findings

4.5.1 Live or intensive imaging of fin regeneration

One of the biggest advantages of the zebrafish fin when compared to the salamander limb is the prospect for live-imaging regeneration. Within the last year, two papers were published where the authors successfully used live-imaging to examine fin regeneration in zebrafish embryos (Li et al., 2012; Mateus et al., 2012). With some adjustments live-imaging protocols likely could be applied to the adult caudal fin. The adult caudal fin regenerate is thin and relatively translucent. The regenerate process is quite rapid, and for several days fins can regenerate robustly without the need to feed the fish. It should be possible to immobilize the fish via surgical, physical, or pharmacological means, and image regeneration over the course of a few days. Imaging of regeneration also would allow for one of the strengths of zebrafish, the relative ease of transgenesis, to be exploited. Live-imaging could be combined with the generation of reporters and fusion protein transgenics to get a more detailed picture of what is occurring during fin regeneration. Ideally, the integration of live-imaging with reporters would be used to create data for descriptive and predictive modeling. Such as approach

would be similar to what has been done to study shoot growth in *Arabidopsis* (Roeder et al., 2011). Perhaps the best course of action would be focus on the early regenerative events so the imaging window could be small, and the potential impact of the differences between the fin and limb would be minimized.

4.5.2 Loss-of-function reagents

Along with adapting existing technologies to fin research, new genetic techniques will also have to be developed. One of utmost importance is the generation of a reliable strategy for making genetic inducible loss-of-function reagents. The lack of a reliable way to reduce gene expression almost surely hindered the attempts to define AP positional memory. During this project one thought was that fish missing one copy of *hand2* might show reduced expression and a morphological phenotype in the adult. However, adult female fish with a deficiency for the region containing the *hand2* gene locus (*Df(Chr01:hand2)s6/+*) had neither a morphological phenotype nor any detectable reduction in *hand2* mRNA levels (Figure 55). This result further emphasizes the importance of developing a technique for inducible adult loss-of-function as heterozygous adult fish may not prove useful.

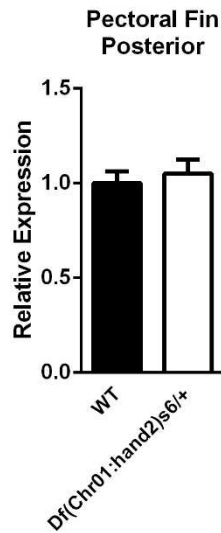


Figure 55: Pectoral fin *hand2* expression in *hand2* heterozygous fish. Similar level of *hand2* expression in the pectoral fin between wild-type and heterozygous fish. Expression is normalized to β -*actin1*, relative to wild-type level, n = 4, Student's t-test, mean \pm SEM.

There are at least three possible approaches to reduce gene expression in the adult. The most traditional strategy would be the use of some type of RNAi mediated method. That strategy requires both proper processing and high target specificity. Many of the algorithms used to design RNAi sequences are inefficient, so the most reliable method is in vivo screening (Fellmann et al., 2011). This likely would require a cell culture system that would also need to be established. Furthermore, the packing of the RNAi likely would also need to be optimized from the reported mir-30 based backbone (Dong et al., 2009). The two other methods would rely on genome engineering, and currently the methods showing the most promise are the TALE-based

techniques. It is possible that by exchanging the nuclease domain for a repressive domain such as SID or an engrailed repressive domain one might be able to use a single custom TALE-repressor to knockdown expression of an endogenous gene (Cong et al., 2012). While certainly exciting, this approach awaits additional verification. Another strategy would be to use TALENs and homologous recombination to insert loxp sites or possibly larger fragments. If the preliminary reports are accurate this method is possible, but would be time-consuming to produce adult knockouts as it would require several generations (Bedell et al., 2012). However, using this technique one could envision generating floxed alleles that could be used with Cre recombinase to generate knockouts just as in mice. Another genome engineering using the bacterial-derived CRISPR mechanism has recently been described (Hwang et al., 2013), and this system may offer additional avenues for manipulating the zebrafish genome. The future for generating zebrafish adult inducible loss-of-function reagents appears much more promising than it did a few years ago when the projects described in this dissertation were initiated.

4.5.3 Higher throughput testing of candidate genes

While tools for profiling gene expression are progressing rapidly, methods to quickly functionally assay any candidate molecules identified lag behind. This limitation was evident in the previously described AP transcription factor project. It is

also likely the lack of a rapid functional assay will become an even larger issue as RNA-Seq costs continue to drop, and thus the feasibility of using RNA-Seq to find potential candidate genes and mechanisms increases. RNA-Seq experiments to identify interesting candidates also capitalize on one of the advantages zebrafish has over salamanders, which is the sequenced genome. However, without a secondary assay to assess the biological significance of any gene, prioritizing potential hits generated from RNA-Seq experiments is problematic.

One possible solution to this problem is to develop cell or tissue culture based assays, but these approaches would likely have significant limitations. An alternative would be to create an in vivo system where either candidate gene products or RNAs could be readily introduced. One way to accomplish this in vivo introduction would be through the use of a virus. A major potential drawback to any viral approach is that achieving sufficient levels of infection to cause morphological phenotypes can be difficult. An additional drawback is the possible lack of any cell type specificity. A possible way around these issues would be to render certain cells in the zebrafish highly susceptible to viral infection. This could be accomplished by creating transgenic strains expressing a receptor for the virus in a cell type specific fashion. This approach has been successful used in mouse models where the receptor (TVA) for the subgroup A avian leukosis viruses (ALV-A) was transgenically expressed astrocytes. These mice were

subsequently injected with virus and the expression of alkaline phosphatase or bFGF was introduced specifically in astrocytes (Holland and Varmus, 1998). This approach is currently being tested with lines expressing the TVA receptor in osteoblasts (*osx:EGFP-TVA*) and an ALV-A psuedotyped lentivirus. If it is successful it could significantly increase the speed of functional experiments in the adult zebrafish.

4.6 Evaluation of zebrafish fin as a positional memory model

This dissertation has shown that the zebrafish pectoral fin can be used as a positional memory model system. Two examples of positional information during regeneration were discovered. Zebrafish fins still pose some inherent challenges, including the shallow nature of morphological gradients and questions about the mammalian relevance of the model. However, in the foreseeable future zebrafish fins will likely remain superior to amphibian limbs in terms of the potential for dissecting the mechanisms of positional information. Tools for editing the zebrafish genome are rapidly improving, and transgenesis allows other technologies such as cell type specific nuclei purification or viral gene transfer to be adapted to zebrafish. The combination of new techniques and the findings described here makes the potential future for using zebrafish fins to explore positional memory a bright one.

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Biography

Gregory Thomas Nachtrab was born on April 18, 1983 in West Chester, PA. He attended the Massachusetts Institute of Technology from September 2001 to June 2005 and was awarded an SB in Biology. After college he worked for two years as research technician in the lab of Henry Kronenberg at Massachusetts General Hospital Endocrine Unit. This led to a 2008 publication in *Molecular and Cellular Biology* entitled, A-raf and B-raf are dispensable for normal endochondral bone development, and parathyroid hormone-related peptide suppresses extracellular signal-regulated kinase activation in hypertrophic chondrocytes. He entered graduate school at Duke University in the Developmental Biology Training Program in the fall of 2007. In spring of 2008 he joined Ken Poss's lab to study zebrafish fin regeneration. In 2011 he published his first author paper in *Current Biology*, Sexually dimorphic fin regeneration in zebrafish controlled by androgen/GSK3 signaling. He was also a second author on a 2010 publication in *Disease Models and Mechanisms*, Ras controls melanocyte expansion during zebrafish fin stripe regeneration. He has also published a 2009 preview in *Developmental Cell*, Genetic DISC-section of regeneration in *Drosophila*, and a 2012 meeting review in *Development*, Toward a blueprint for regeneration. He currently has a first author manuscript in the submission process, Hand2 is a component of positional memory during zebrafish fin regeneration, and a second author manuscript also in the

submission, Signaling interference from a sexually selected feature compromises tissue regenerative capacity. Once his dissertation is accepted he will be moving onto a postdoctoral position dissecting neural circuits and behavior.