The roles of FGF and Wnt signaling during maxillary barbel regeneration in the Zebrafish (Danio rerio).

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Recommended Citation
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The Roles of FGF and Wnt Signaling During Maxillary Barbel Regeneration in the Zebrafish (*Danio rerio*)

A Thesis

Presented in

Partial Fulfillment of the

Requirements for the Degree of

Master of Science

November, 2011

BY

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Acknowledgements

First and foremost I thank my thesis adviser Dr. Elizabeth LeClair for allowing me to join her lab and take on this project. It has been an honor for me to work with Dr. LeClair and I am grateful for her endless enthusiasm and support. I could not have hoped for a better mentor. I also thank my thesis committee members, Dr. Dorothy Kozlowski and Dr. Tim Sparkes, for their contributions to this project and guidance along the way. I extend my profound gratitude to my major collaborators, Dr. Jacek Topczewski, Dr. Jolanta Topczewska and members of their lab. They were kind enough to let me use their materials, equipment and facilities. This thesis would not have been possible without their help. I am grateful for the assistance of my lab members Paulina Pawluczuk, Alex Moore, Natali Chavez, Tiffany Mark and Ann Hogan. Lastly, I would like to extend a great deal of love and appreciation to my family for their continual support and encouragement.

Financial support for this project was provided by the National Institutes of Health (R15HD064169 to E. LeClair, R01DE016678 to J. Topczewski), the DePaul University Research Council, the Department of Biological Sciences, and the DePaul Graduate Research Fund. Resources from the Zebrafish International Resource Center were supported by grant P40-RR012546 from the National Institutes of Health’s National Center for Research Resources (NIH-NCRR).
# Table of Contents

List of Tables and Figures................................................................. vi

Abstract .................................................................................................. vii

Introduction ........................................................................................... 8

Literature Review ..................................................................................... 9

Regeneration varies among vertebrate species.......................................... 9
Zebralsh as a model for regeneration research .......................................... 9
The Zebralsh Maxillary Barbel, (ZMB), a novel model for appendage regeneration ................................................................. 18
Anatomy of the ZMB ........................................................................... 19
The ZMB Regenerates ......................................................................... 19
Morphological processes in zebralsh fin regeneration................................. 20
Molecular events in fin regeneration ....................................................... 22
FGF and Wnt Signaling pathways regulate fin regeneration ....................... 16
Experimental Rationale ....................................................................... 17

Methods ................................................................................................. 16
Fish Husbandry .................................................................................... 16
Surgical Procedures ............................................................................. 17
Nucleic Acid Extraction ....................................................................... 18
Polymerase Chain Reaction (PCR).......................................................... 19
Reverse-Transcription Polymerase Chain Reaction (RT-PCR)...................... 19
Fish Lines and Genotyping .................................................................. 20
Barbel Regeneration Experiments .......................................................... 22

Results .................................................................................................. 25
FGF and Wnt signaling molecules are expressed during barbel regeneration ................................................................. 25
The FGF pathway regulates maxillary barbel regeneration through the receptor fgfr1 ................................................................. 26
The ligand fgf20a is essential for tail, but not barbel regeneration ................... 30
Canonical Wnt signaling regulates ZMB regeneration .................................. 33
Notum1a is potentially a novel regulator of zebrafish regeneration .................. 35
Discussion.........................................................................................................................................................38

The regulatory mechanisms of early stages in barbel regeneration are unknown ........................................38
The initiation of a barbel blastema does not depend on FGF or Wnt signaling...............................................39
Notum may be a critical regulator of many signaling pathways during blastema formation..................41
FGF and Wnt signaling are highly conserved regulators of cell proliferation during regeneration ....42
Fgf20a is a fin specific regulator of regeneration .........................................................................................44
The importance of comparing regeneration within zebrafish.......................................................................45
Regeneration among different species ........................................................................................................47
Conclusion .........................................................................................................................................................48

References..........................................................................................................................................................49

Tables and Figures ...........................................................................................................................................53
List of Tables and Figures

Table 1. Reverse transcription polymerase chain reaction (RT-PCR) primers ............................................. 54

Figure 1. An adult zebrafish (Danio rerio) ........................................................................................................ 55
Figure 2. Anatomy of the ZMB on a wild type adult zebrafish ........................................................................ 56
Figure 3. Proximal amputation of the ZMB induces regeneration .................................................................... 57
Figure 4. Major morphological stages and molecular signaling events during fin regeneration .................. 58
Figure 5. The fibroblast growth factor (FGF) signaling pathway ................................................................. 59
Figure 6. The canonical and noncanonical Wnt signaling pathways ............................................................ 60
Figure 7. Thermal cycle parameters for polymerase chain reactions (PCR) ............................................... 61
Figure 8. The heat-shock inducible transgenic dnfgfr1 experimental design .................................................. 62
Figure 9. The temperature sensitive fgf20a^zp3 mutant experimental design .................................................. 63
Figure 10. The small-molecule canonical Wnt inhibitor IWR-1 experimental design .................................... 64
Figure 11. Schematic of appendage measurements used to quantify ZMB regeneration ............................... 65
Figure 12. FGF and Wnt signaling members are expressed during barbel regeneration ............................... 66
Figure 13. FGFR1 inhibition reduces ZMB regeneration .............................................................................. 67
Figure 14. Heat-shock treatments do not inhibit barbel regeneration in wild types ...................................... 68
Figure 15. The mutation fgf20a^zp3 has no effect on ZMB regeneration .......................................................... 69
Figure 16. The canonical Wnt inhibitor IWR-1 causes decreased ZMB regeneration .................................... 70
Figure 17. Overexpression of nom1a inhibits tail and barbel regeneration .................................................... 71
Abstract

The zebrafish (Danio rerio) maxillary barbel is an integumentary sense organ that contains skin, glands, pigment cells, taste buds, nerve fibers, blood vessels and a putative lymphatic. Like other zebrafish organs, the maxillary barbel can regenerate most of its tissues after amputation. However, little is known about the molecular mechanisms that control this regeneration. Fibroblast growth factor (FGF) and wingless (Wnt) signaling are required for the control of zebrafish caudal fin regeneration; due to the similarities between the fin and the maxillary barbel, these regulatory networks may play a role in barbel regeneration as well. In this study, I explored the roles of FGF and Wnt signaling during barbel regeneration by detecting the expression of crucial genes in each pathway. These experiments revealed that many FGF and Wnt pathway members are expressed during early barbel regeneration. To test the necessity of these signaling pathways, I used a variety of in vivo molecular techniques to inhibit candidate genes in barbel regenerates. Results indicated that both pathways play important roles in barbel regrowth; however, some differences between barbel and fin regeneration were observed. Activating a dominant negative fibroblast growth factor receptor (dnfgfr1) reduced the length of barbel regenerates by 60% but completely prevented fin regeneration in the same fish. Interestingly, zebrafish homozygous recessive for a point mutation in fgf20a did not show any inhibition of barbel regeneration, although this ligand was essential for fin regeneration. When the canonical Wnt inhibitor drug IWR-1 was applied to wild type fish, barbel regenerate length decreased by nearly 50%, but was not completely prevented as in fin regenerates of the same fish. Finally, experiments transgenically overexpressing the Wnt antagonist notum1a (nom1a) dramatically inhibited regeneration in both barbels and tails, highlighting this molecule as a potential novel regulator of Wnt-dependent regeneration in zebrafish. These are the first results that describe the molecular mechanisms of zebrafish barbel regeneration. Understanding the similarities and differences between regenerating barbels and other organs could help uncover any molecular themes or, in contrast, any variations that control regeneration. Altogether, this project has established the zebrafish maxillary barbel as yet another accessible and productive model for vertebrate regeneration studies.
Introduction

As humans, we are limited in our ability to regenerate certain adult tissue types. In contrast, non-mammalian vertebrates including newts, salamanders, and zebrafish can regenerate a wide variety of structures. Most of what is known about regeneration comes from studying these animal models. Recently, the molecular basis of this ability has received a great deal of scientific attention due to the potential therapeutic applications for human disease and injury. It is uncertain, however, what genes and molecular pathways are needed to regulate regeneration and how common these mechanisms are. Developing effective therapies could be made easier if similarities exist among the mechanisms controlling regenerative processes. For this reason, researchers are asking whether there are specific signaling pathways controlling regeneration that are conserved both among species and among tissues within the same species.

The zebrafish, Danio rerio, is at the forefront of these research efforts because it is capable of regrowing retinal, brain, heart, and liver tissue, as well as entire appendages such as fins. Zebrafish fins regenerate in a morphologically stepwise manner using conserved gene regulatory networks like the fibroblast growth factor (FGF) and wingless (Wnt) signaling pathways. These networks regulate regeneration by choreographing when and where certain downstream genes will be expressed during each step. Studies inhibiting FGF and/or Wnt signaling in zebrafish have shown that multiple genes in these pathways are required for fin regeneration. FGFs and Wnts are also required for regulating a variety of other regenerative processes within the zebrafish; therefore, these pathways may be part of a conserved network of regulators controlling regeneration.

The maxillary barbel, a sensory skin appendage of the zebrafish, has also been shown to regenerate. Barbels are excellent models for regeneration because they are external, transparent, and regenerate quickly. They contain several tissue types, including skin, glands, pigment cells, taste buds, nerve fibers, blood vessels and a putative lymphatic. However, our knowledge of barbel regeneration is
very limited. It is uncertain what genes are expressed during barbel regeneration, what regulatory networks control gene expression, and what similarities there are between regeneration in the barbel as compared to the fin. Using methods for detecting and inhibiting FGF and Wnt signaling, we can determine if these genes play conserved roles in zebrafish appendage regeneration. Elucidating these roles will aid in our understanding of mechanisms controlling regeneration and how these mechanisms may have evolved.

**Literature Review**

*Regeneration varies among vertebrate species.* Many organisms have the ability to rapidly replace lost or damaged tissue. “Lower” vertebrates such as newts, salamanders and some fish can regenerate many tissues including heart muscle (Ausoni and Sartore, 2009; Lepilina et al., 2006), optic nerve (Becker and Becker, 2007), spinal cord (Becker and Becker, 2008; Chernoff et al., 2003), liver (Hata et al., 2007), limbs (Beck et al., 2006; Stocum and Cameron, 2011) and fins (Tal et al., 2009). Mammals cannot regenerate these structures entirely, but can in some cases replace limited cell types (Gurtner et al., 2008). Investigating similarities among species that can regenerate may reveal certain molecular regulators that are simple and widely used. These “primitive” mechanisms may have evolved in earlier vertebrates but are either not conserved or not expressed in mammals.

*Zebrafish as a model for regeneration research.* Among “lower” vertebrates, the zebrafish, *Danio rerio* (Order Cypriniformes, Family Cyprinidae), has emerged as popular model system for studying the morphological and molecular aspects of regeneration (Figure 1; Ward and Lieschke, 2002). Zebrafish are capable of regenerating retina (Becker and Becker, 2007), optic nerve (Becker and Becker, 2007), heart (Poss, 2007), kidney (Diep et al., 2011), the lateral line sensory system (Dufourcq et al., 2006) and spinal cord (Becker and Becker, 2008). More remarkably, zebrafish can regenerate entire appendages such as the caudal fin (reviewed by Tal et al., 2009). Because so many zebrafish tissue types can regenerate, it is possible that these tissues share common molecular mechanisms of wound healing,
regenerative cell proliferation, and redifferentiation. Understanding the similarities and differences among regulatory mechanisms within the zebrafish would help determine if the molecular basis for regeneration evolved once or multiple times in this species, and if these mechanisms are conserved among organs, or tissue specific.

**The Zebrafish Maxillary Barbel (ZMB), a novel model of appendage regeneration.** Our laboratory has recently established the zebrafish maxillary barbel (ZMB) as another zebrafish appendage that can regenerate (LeClair and Topczewski, 2009, 2010). The ZMB is a skin sensory appendage that extends as a “whisker-like” protrusion from the posterior ventral corner of the zebrafish maxilla (Figure 2A). Similar structures can be found in other fish species, as well as amphibians and reptiles. Barbels carry many taste buds, as well as other types of sensory cells, which together are used for taste and/or mechanoreception (Fox, 1999). Although barbels are primarily associated with locating food, some hypothesize that they could play a role in courtship and mating (Fox, 1999).

Although the ZMB has not received much research attention, many aspects of this structure make it an ideal model for studying regeneration. The ZMB is transparent throughout the lifespan of the zebrafish, making it possible to observe all of the internal anatomy using light microscopy. The appendage is external, and large enough to manipulate easily for surgeries and tissue processing. At standard zebrafish rearing temperatures (28°C), maxillary barbels regenerate in 2-3 weeks, allowing for efficient data collection. Although there is no human structure similar to the ZMB, the cell and tissue types found within the barbel are highly conserved among vertebrates. This makes the ZMB a novel model for regenerative studies of the skin, glands, pigment cells, nerves, blood vessels and lymphatics. Finally, the molecular procedures already established for zebrafish studies, particularly those of fin regeneration, can be applied to the ZMB.
Anatomy of the ZMB. The ZMB is the larger of two sets of barbels located on the zebrafish head. In adults, the ZMB usually reaches 2-4 millimeters in length and tapers from ~250 microns at the base to 50 microns at the tip (LeClair and Topczewski, 2010). The ZMB develops 30-40 days post fertilization from both mesodermal and ectodermal origins and contains multiple cell types (Figure 2B; LeClair and Topczewski, 2010). In the center of the ZMB is a dense rod of connective tissue. Just ventral to the central rod are two small blood vessels, between which weave large bundles of nerve fibers. Dorsal to the central rod is a narrow vessel tentatively identified as a lymphatic. These mesodermal tissues of the ZMB are wrapped in a thick glandular epithelium covered with taste buds. Each taste bud contains three types of sensory cells, all connected to the aforementioned nerve bundles. It is this network of taste buds and nerve fibers that allows the ZMB to function as a taste organ (Hansen et al., 2002; LeClair and Topczewski, 2010). Although the ZMB is transparent, it does contain a few pigment cells (black melanophores and yellow-orange xanthophores) along its length.

The ZMB regenerates. Recently, our lab discovered that the ZMB and most of its cell types can regenerate after surgical amputation (Figure 3; LeClair and Topczewski, 2010). Barbel regeneration appears to follow the classic morphological stages of regeneration, including wound healing, blastema formation, elongation and termination. By 7 days post amputation, the regenerated ZMB is a smaller version of the original, with a complete capillary loop, regularly spaced melanophores, differentiated taste buds and connecting sensory axons. The regenerated barbel continues to grow 14-28 days post amputation, but maintains a similar appearance (LeClair and Topczewski, 2010).

The dramatic morphological regeneration of the ZMB was the focus of our lab’s previous studies. To date, however, there is no published information on the molecular regulation of this appendage. In order to understand the molecular mechanisms that might be controlling ZMB regeneration, I used the well-studied process of zebrafish fin regeneration as a starting point for comparison.
Morphological processes in zebrafish fin regeneration. The zebrafish fin is a popular model in regeneration research, leading the way in the broader field of vertebrate limb regeneration. A quick PubMed search of “zebrafish fin regeneration” provides over 120 articles that date back almost 20 years. The zebrafish tail, or caudal fin, is conveniently accessed, is thin and transparent, and has a relatively rapid regeneration time at standard rearing temperatures (3 days for larval fish and 2 weeks for adults). The fin is composed of 16-18 bony fin rays that protect a core of mesenchymal cells, vascular tissue, nerves, melanocytes and fibroblasts. An epithelial layer covers the entire complex. Conveniently for this study, fin regeneration can be compared to barbel regeneration within the context of the same species – zebrafish – and its completely mapped genome. These resources help us to study if both appendages use similar (or different) mechanisms to replace appendage-specific cell types and morphology.

Regeneration researchers recognize five different stages of regeneration: wound healing, epithelium thickening, blastema formation, regenerative outgrowth and termination. These steps have been extensively characterized in zebrafish fin regeneration (Tal et al., 2009; Figure 4). Immediately after surgical amputation, an apical epidermal cap (AEC) forms from migrating epithelial cells (Akimenko et al., 2003) and covers the wound. Next, the epithelial tissue of the cap starts to thicken and mesenchymal cells proximal to the AEC disorganize. Growth factors from the AEC signal the mesenchymal tissue to proliferate and differentiate, as well as migrate proximally (Nechiporuk and Keating, 2002). In the third stage a blastema forms, which is a mass of progenitor cells that can differentiate into the cell types required to replace the missing fin (Akimenko et al., 1995). The fourth stage of fin regeneration, outgrowth, includes the division, differentiation and migration of blastema cells into the appropriate locations for each tissue type (Lee et al., 2009). The final step is termination, when growth ends. This regeneration sequence produces a complete caudal fin in less than 2 weeks, and can be repeated at any time in the life of the organism (Azevedo et al., 2011).
Although the fin is different from the ZMB, our preliminary studies show that both appendages regenerate through similar morphological stages (Figure 3). As with all biological processes, there is a framework of molecular events that orchestrates these broader morphological changes, and regeneration is no exception. I therefore hypothesized that similar molecular pathways may control the response of the fin and ZMB when these organs are injured or amputated. In the next section, I will review what gene regulatory networks are, how they function, and how zebrafish researchers can control these networks in order to study the molecular mechanisms of fin regeneration.

Molecular events in fin regeneration. Molecular regulatory networks are a series of cellular signals that determine when and where genes are transcribed and translated. Conservation of regulatory networks is common, meaning that the same genes are active in different species for similar physiological functions (Adams, 2005). A major goal of modern molecular biology is to discover what signaling pathways are active in particular biological contexts and to manipulate those pathways to affect cellular and physiological outcomes. Researchers working on zebrafish fins have developed many molecular techniques that allow for the precise control of conditions during regeneration. These include injecting DNA, RNA or protein into regenerating tissue (Prykhozhij and Neumann, 2008), heat-shocking transgenic zebrafish to increase or decrease gene expression (Poss et al., 2000a), screening mutants for defective regeneration (Whitehead et al., 2005), chemically inhibiting signaling pathways (Bouzaffour et al., 2009; Poss et al., 2000a; Poss et al., 2000b; Prykhozhij and Neumann, 2008), and electroporation of antisense molecules that inhibit translation (Thummel et al., 2006). As a result, we now know a great deal about how caudal fins regenerate and the molecular pathways involved (reviewed by Iovine, 2007; Tal et al., 2009; Figure 4). Two highly conserved gene pathways – the fibroblast growth factor (FGF) and wingless (Wnt) pathways – play critical roles during multiple stages of regeneration (Lee et al., 2005; Poss et al., 2000a; Stoick-Cooper et al., 2007; Whitehead et al., 2005). Other signaling pathways that interact with FGF/Wnt signaling to affect fin regeneration are retinoic acid signaling, hedgehog signaling
and microRNA regulation (Géraudie et al., 1995; Laforest et al., 1998; Yin and Poss, 2008; Yin et al., 2008), which are beyond the scope of this project.

**FGF and Wnt signaling pathways regulate fin regeneration.** FGFs are a family of secreted proteins or “growth factors” that bind and activate specific membrane-bound receptor tyrosine kinases (Figure 5). FGF signaling occurs during many physiological processes in developing and adult mammals, including wound healing, angiogenesis, and tumor growth (reviewed by Barrientos et al., 2008; Turner and Grose, 2010). Within zebrafish, FGF signaling regulates the development and regeneration of many tissue types (Poss et al., 2003), including the caudal fin previously described (Figure 4). Experiments inhibiting the FGF receptor 1 protein (fgfr1) show that FGF signaling is required for blastema formation and regenerative outgrowth (Poss et al., 2000a). FGF inhibition also decreases the expression of downstream genes such as muscle segment homeobox (msx) genes, which are associated with blastema formation (Poss et al., 2000a; Thummel et al., 2006). Furthermore, a zebrafish mutagenesis screen for impaired fin regeneration revealed that the FGF ligand fgf20a is specifically required for blastema formation during fin regeneration (Whitehead et al., 2005). Heat-shock transgenic studies inhibiting FGFR1 later in regeneration shows that FGF signaling is not only involved in blastema formation but also in controlling cell distribution and regenerative growth rates (Lee et al., 2005). The presence of FGF signaling as a key regulator in many aspects of fin regeneration allows us to predict that FGF signaling may be a conserved mechanism of regeneration among many zebrafish tissue types, including the maxillary barbel.

A second critical pathway regulating fin regeneration is the Wnt signaling pathway (Figure 6). Wnts are the vertebrate homologs of the fruit fly (Drosophila melanogaster) Wingless gene. Wnts are secreted glycoproteins that are important during the development of epidermal-dermal appendages, the maintenance of skin stem cells and the response of cells to injury (reviewed by Huelsken and Behrens, 2002; Kawakami et al., 2006; Stoick-Cooper et al., 2007). Once secreted, extracellular Wnts bind to
members of the Frizzled (FZD) family of receptors, and this interaction is facilitated by Glypican co-
receptors. Ligand binding can activate a “canonical” and/or a “non-canonical” pathway. The canonical
Wnt pathway involves transcriptional activation by nuclear localization of the cytoplasmic protein β-
catenin (Chen et al., 2009; Gao and Chen, 2010; MacDonald et al., 2009). After nuclear transport, β-
catenin is able to interact with transcription factors in the nucleus, promoting specific gene expression.
Wnt signaling can also activate “non-canonical” signaling pathways that act independently of β-catenin
(Chien et al., 2009; Gao and Chen, 2010; Sugimura and Li, 2010); these pathways include the planar cell
polarity (PCP) pathway and Ca^{2+} pathway. Paradoxically, these non-canonical pathways ultimately
regulate other nuclear transcription factors that may oppose canonical Wnt activity (Huelsken and
Behrens, 2002; Stoick-Cooper et al., 2007).

During zebrafish fin regeneration, Wnt target genes such as lymphoid-enhancer-binding factor
(lef1) are newly expressed in the wound epithelium distal to the amputation plane, suggesting the
importance of Wnt signaling in blastema formation. Conversely, if an inhibitor of “canonical” Wnt
signaling, the protein Dickkopf (Dkk1), is expressed, blastema formation and associated marker genes are
blocked, halting regeneration (Kawakami et al., 2006; Stoick-Cooper et al., 2007). At a slightly later
stage, once a blastema has formed, inhibiting Wnts using Dkk1 reduces epithelial and mesenchymal
proliferation, indicating that Wnts also control blastema maintenance. Interestingly, molecular
communication between the FGF and Wnt signaling pathways occurs. For example, expression of the
fgf20a ligand is regulated through Wnt signaling. In turn, fgf20a regulates transcription of Wnt signaling
targets, such as lef1 (Poss et al., 2000b).

Experimental Rationale. The biological requirement for FGF/Wnt signaling in zebrafish fin
regeneration, as well as in other regenerating tissues, led to the hypothesis that these two networks are
potential molecular regulators of ZMB regeneration. To test this hypothesis, I:

1) detected the expression of potential FGF/Wnt pathway members in regenerating barbel tissue,
2) activated a transgenic dominant negative copy of the FGF receptor 1 (fgfr1),
3) utilized a fish line that contained a mutation in the FGF ligand, fgf20a,
4) applied a small-molecule drug to block canonical Wnt signaling, and
5) overexpressed a canonical Wnt signaling antagonist, the enzyme notum 3 (nom3)

Taken together, these experiments provide the first evidence as to whether or not these molecular pathways are active in ZMB regeneration, and document their phenotypic effects.

Uncovering similarities and differences between known regenerative gene networks and those of the ZMB could help to solve several unanswered questions surrounding regeneration. Firstly, zebrafish have the ability to regenerate numerous organs; comparing the mechanisms controlling regeneration of each organ may help to explain how species like this can regenerate so broadly. These comparisons are important in determining if regulatory mechanisms are conserved or tissue specific. Secondly, comparing the genetics of barbel regeneration may also help us understand how regeneration has evolved. Similarities among regenerative mechanisms may indicate that these mechanisms evolved only once while differences may indicate mechanisms evolved independently both among tissue and among species. Finally, determining the molecular basis for regeneration may help to uncover why some species have the ability to regenerate and others do not, and how we may be able to design therapies that compensate for missing regulatory mechanisms. Overall, this project helps to establish the ZMB as yet another accessible and productive model in vertebrate regeneration research.

Methods

Fish Husbandry:

All animal protocols were approved by the Institutional Animal Care and Use Committees (IACUCs) of DePaul University and/or Children’s Memorial Research Center (CMRC; Chicago, IL). Adult fish were kept either in a heated, recirculating rack system (Aquaneering, Inc.) or small aquaria equipped with heaters, aerators and sponge filters. Adult fish density was kept below 1 fish/200 mL and
all fish were fed live brine shrimp and/or commercial fish flakes once or twice daily until used for experiments. Fish were crossed by placing one male and one female into a 500 mL crossing tank and letting them sit overnight. Clutches were collected the following morning by straining the crossing tank water and removing unfertilized and unhealthy embryos from the clutch. Healthy eggs were placed in a Petri dish of egg water (40 g Instant Ocean/L dI water) at 28°C. After 5-6 days, embryos were placed in the recirculating rack or aquaria.

**Surgical Procedures:**

*Caudal Fin Clips:* Fish were anesthetized using 0.015% µg/mL buffered Tricaine (Sigma-Aldrich: E102521) in system water. The distal half of the caudal fin was then removed with a razor blade. Fish were allowed to recover in a crossing tank of system water with a drop of methylene blue for at least 20 minutes before being returned to the recirculating system or aquaria.

*Barbectomies:* Fish were anesthetized as previously stated (Caudal Fin Clips). Barbels were then surgically removed near the distal end of the maxilla with a pair of small spring-scissors. For RNA collection and expression experiments, both the left and right barbels were removed. For regeneration experiments, only the left barbel was amputated. Fish were allowed to recover as previously described (Caudal Fin Clips).

*Euthanasia and Tissue Collection:* For terminal tissue collection, fish were first anesthetized (Caudal Fin Clips) and then placed in ice water until gill ventilation stopped. Fish were then decapitated or, to keep the head and tail attached, the spinal cord was severed immediately behind the head using a sharp razor blade. For tissue preservation, fish were fixed in 4% paraformaldehyde/1x phosphate-buffered saline (PF-PBS, pH 7.4) at 4°C overnight, then extensively rinsed in 1x PBS and stored in 70% ethanol.
Nucleic Acid Extraction:

**DNA Extraction:** DNA isolation was performed in order to genotype individuals. Fish were anesthetized as previously described (Caudal Fin Clips) and pieces of the caudal, dorsal and/or anal fins were removed with a razor blade and placed in a PCR tube with 50 μL DNA extraction buffer (10 mM Tris pH 8.2, 10 mM EDTA, 200 mM NaCl, 0.5% SDS and 200μg/mL proteinase K). Tissue was digested at 55°C for 18 hours, heat-inactivated at 98°C for 10 minutes and stored at 4°C. 100 μL of 100% ethanol was added to each tube and the samples were placed on ice for 30 minutes. Tubes were centrifuged at 16,000g for 10 minutes and the supernatant was decanted. 200 μL of 70% ethanol was added and tubes were spun again at 16,000g for 2 minutes. The supernatant was again decanted, and pellets were allowed to air dry for 10 minutes before being resuspended in 20-50 μL Tris-EDTA and stored at -20°C.

**RNA Extraction:** Total RNA isolation was performed for analysis of mRNA expression during regeneration. Fin clips and barbectomies were performed on wild types as previously described (Surgical Procedures) and fish were allowed to regenerate for 3 – 4 days. Fish were euthanized as previously described (Euthanasia) and transferred to cold PBS. The regenerating caudal fin and/or ZMB tissue were removed just proximal to the amputation plane and placed in a tube of 250 μL RNA isolation reagent (RNAzol, Molecular Research Center). The tube was immediately snap frozen in liquid nitrogen. Tubes were then thawed and the tissue was homogenized with a motorized pestle. Samples of the same tissue type were combined into 1 mL aliquots with an additional 0.4 mL of RNase free water. Tubes were vortexed for 15 seconds and allowed to incubate for 15 minutes at room temperature. After incubation, samples were centrifuged at 16,000g for 15 minutes at 4°C. 1 mL of the supernatant was removed and 0.4 mL 75% ethanol was added before the tube was stored at -20°C for 30 minutes. Tubes were centrifuged again at 16,000g for 20 minutes, and the pellet was then washed twice with 0.4% EtOH, spinning at 6,500g for 3 minutes each time. The ethanol supernatant was removed and RNA was resuspended in 25 μL RNase free water at room temperature for 5 minutes. Aliquots were stored at -80°C.
**Nucleic Acid Quantification and Detection:** Nucleic acid concentrations were determined by measuring the absorbance at 260nm of 2 μL of each sample on a NanoDrop 1000c spectrophotometer. To visualize nucleic acids, 3 μL of each sample were run on a 1% lithium borate gel with ethidium bromide for 20 – 30 minutes at ~200 mV. Gels were imaged on an Alpha Innotech FluorChem HD2 camera with AlphaView 3.0 software.

**Polymerase Chain Reaction (PCR):**

PCR was used to amplify specific segments of DNA for genotyping. Reactions were performed using the JumpStart REDTaq PCR Reaction kit (Sigma-Aldrich: P0982). Each PCR reaction was performed in a total of volume of 20 μL containing 10 μL RedTaq ReadyMix, 1 μL forward primer (0.5 μM), 1 μL reverse primer (0.5 μM), 1 μL DNA template (50 – 200 ng) and 7 μL nuclease-free distilled water. Samples were then placed in an Applied Biosystems 2720 Thermal Cycler and cycle parameters for denaturing, annealing and extension were set depending on the primer combinations (*Figure 7*). Samples were then stored at 4°C. Using 10 μL of this PCR product, amplified DNA was evaluated by gel electrophoresis and imaging as previously described (Nucleic Acid Quantification and Detection).

**Reverse-Transcription Polymerase Chain Reaction (RT-PCR):**

RT-PCR was performed in order to observe the mRNA expression of select genes. RNA was collected from 3 dps regenerating caudal fins and 4 dps regenerating ZMB as previously described (RNA Extraction). The SuperScript III One-Step RT-PCR System with Platinum Taq Polymerase kit was used to amplify mRNA transcripts. For each gene, a reaction of 1.4 μL RNA (~70 ng), 5 μL SuperScript reaction mix, 0.4 μL SuperScript III RT/Platinum Taq Mix and 2.8 μL RNase free distilled water were mixed in separate PCR tubes. Then 0.2 L (10 M) of both a forward and reverse primer for select gene transcripts (*Table 1*) was added to each tube. The thermal cycler was set to perform a “touchdown PCR” to accommodate for the range of primer annealing temperatures (*Figure 7A*). Gel electrophoresis and imaging were performed as previously described (Nucleic Acid Quantification and Detection).
**Fish Lines and Genotyping:**

*Wild type zebrafish:* Zebrafish of the wild type strain “AB” were obtained from stocks at CMRC. This strain was used for outcrossing, gene expression studies, heat-shock controls and pharmacological inhibition experiments.

*Dominant negative fibroblast growth factor receptor 1 (dnfgfr1) zebrafish:* The heat-shock transgenic dominant negative fgfr1 line (Tg(hsp70l:dnfgfr1-EGFP)dl) (Lee et al., 2005) was ordered from the Zebrafish International Resource Center (ZIRC; Catalog ID #: ZL1476). These fish contain a transgenic insert with a heat-sensitive promoter (hsp70l) that is activated when a cell reaches temperatures of 37-38°C (Shoji and Sato-Maeda, 2008). This promoter drives the transcription of a dominant negative copy of dnfgfr1 that has the kinase domain replaced by an enhanced green fluorescent protein (EGFP) sequence (Lee et al., 2005), inactivating downstream signaling. Adults were genotyped using PCR as previously described (Polymerase Chain Reaction) with the following primers for EGFP and the cycling parameters recommended by ZIRC (Figure 7A):

Forward – EGFP6 = 5’ TTC TTC AAG TCC GCC ATG CCC G 3’
Reverse – EGFP8 = 5’ GCA CGC TGC CGT CCT CGA TGT T 3’

Surprisingly, 100% of the fish tested by PCR had the transgenic insertion (data not shown), so a heat-shock test was needed to identify those fish whose insertions were functional. ZIRC males of unknown genotype were outcrossed with wild type (AB) females and fertilized eggs were collected as described (Fish Husbandry). After 3 days, embryos were heated to 38°C for 30 minutes in a microcentrifuge tube of egg water placed in a water bath. Embryos were then incubated for 3 hours at 28°C in a Petri dish to allow for protein synthesis. Embryos were screened for EGFP using a Nikon Eclipse 50i fluorescent microscope. Dnfgfr1 fathers either produced 100% GFP positive offspring, 50% GFP positive offspring or no GFP positive offspring. Dnfgfr1 males that produced 50:50 offspring were
separated for future crosses and clutches of 50:50::transgenic:wild type were saved for regeneration experiments.

Fibroblast growth factor 20a (fgf20a) mutant zebrafish: The fgf20a<sup>p3</sup> temperature-sensitive mutant fish line (Whitehead et al., 2005) was obtained as a single clutch of ~100 embryos from ZIRC (Catalog ID #: ZL1047). This clutch of embryos contained fish heterozygous, homozygous dominant and homozygous recessive for the mutation. 3-4 months later, selected adult fish were genotyped by performing caudal fin clips and observing tail regeneration. The fgf20a<sup>p3</sup> allele is recessive and, in order for tail regeneration to be inhibited, an individual must be homozygous for the mutation. Homozygous recessive individuals had clear tail inhibition at 28°C, while heterozygous and homozygous dominant individuals were able to fully regenerate.

Genotypes were confirmed by PCR using a line-specific protocol supplied by ZIRC and primers specific for the fgf20a<sup>p3</sup> mutation:

**Forward – FGT_01d = 5’ TTT GAG GAG AAT TGG **<u>AAC AAC ACT T</u> 3’

**Reverse – FGT_02 = 5’ TTT TTG GGG TGG TTT TGA GTT T** 3’

PCR was performed as previously described (Polymerase Chain Reaction) using ZIRC recommended thermal cycles (**Figure 7B**). The FGT_01d forward primer contains a base pair mismatch (bold, underlined), which is introduced into each PCR product. This mismatch is adjacent to the fgf20a<sup>p3</sup> mutation and together they form a restriction site that does not exist in wild type PCR products. This novel restriction site is recognized by the enzyme Alo1, which cleaves the mutant PCR product, creating 207 and 39 nucleotide length fragments. Wild type PCR products are uncleaved and are 246 nucleotides long. To produce these fragments and determine genotypes, 8 μL of each PCR product was added to a restriction enzyme reaction mixture containing 2 μL Alo1 enzyme, 2 μL Buffer R and 8 μL distilled water. The digestion reaction was run at 30°C overnight. Products were analyzed by running 10 μL of digestion product on a 50:50 3% agarose:3% MetaPhor gel (Cambrex, Inc.) with ethidium bromide in
TBE buffer for ~3 hours at 90 mV. Visualization was performed as previously described (Nucleic Acid Quantification and Detection).

*Notum1a (nom1a) transgenic zebrafish:* The heat-shock transgenic line Tg(hsp70l:nom1a) was kindly donated by our collaborators at CMRC. These fish contain a transgenic insert with a heat-sensitive promoter (hsp70l) that drives the overexpression of the zebrafish Notum homolog, notum1a (nom1a) at 37-38°C. Nom1a is an enzyme that acts as an antagonist of canonical Wnt-signaling by cleaving glypicans co-receptors from the cell surface (Flowers et al., 2010 J. Topczewski, pers. comm.). This transgenic construct also contains a sequence that constitutively expresses EGFP in cardiac tissue even in the absence of heat-shock promoter activity (J. Topczewski, pers. comm.). This allows transgenic fish to be genotyped under a fluorescent microscope without exposing them to heat shocks. It should be noted that at the time of this study, transgenic insertions in this line were not mapped and multiple copies could have existed within the genome of transgenic carriers.

**Barbel Regeneration Experiments:**

*Heat-shock inhibition of fgfr1:* To test the necessity of fgfr1 during ZMB regeneration, I designed an experiment to inhibit this receptor during barbel and caudal fin regeneration (Figure 8). Dnfgfr1 males were outcrossed (Figure 8A) and offspring were genotyped (Figure 8B). Clutches of 50:50::wild type:transgenic fish (Fish Genotyping and Outcrosses) were allowed to grow to 3-6 months old. 58 fish from multiple, pooled 50:50 clutches had barbectomies and fin clips performed as described (Caudal Fin Clips and Barbectomies; Figure 8C). Fish were haphazardly split into four tanks: two with heat-shock treatment and two without (Figure 8D). In order to induce the Tg(hsp70l:dnfgfr1-EGFP)pd1 transgenic, fish in the heat-shock treatments received a semi-automated heat shock of ~ 37-38°C once per day starting at 24 hours post surgery (Duszynski et al., 2011). Fish not receiving heat shocks remained at 28°C. After 14 days, fish were collected as described (Euthanasia and Tissue Collection; Figure 8E). To ensure an experimenter’s bias would not affect the results of this study, each fixed fish was kept in an
individual tube and assigned a random number (www.random.org). The number, treatment group and genotype for each individual were recorded, and then all of the tubes were reordered sequentially. While imaging and measuring fins and barbels I was kept blind to the treatment and genotype of each fish and only identified each individual by its number code. Not until after all data were collected and ready to be analyzed was the genotype and treatment of each individual revealed.

**Inhibition of fgf20a:** To test the necessity of fgf20a during ZMB regeneration, I designed an experiment to block this ligand during barbel and caudal fin regeneration (Figure 9). The temperature sensitive mutant fish line fgf20a<sup>z3</sup> has a known defect in tail regeneration. Males identified as homozygous for the fgf20a<sup>z3</sup> allele (Figure 9A) were outcrossed to wild type females (Figure 9B). Heterozygote female offspring were raised to 3-4 months old and backcrossed to homozygous fathers to produce 50:50 clutches of homozygous dominant:heterozygote fish (Figure 9C). This means that half of this clutch will show inhibited caudal fin regeneration, while the other half is wild type. 50:50 clutches were allowed to grow to 3-6 months old. Barbectomies and fin clips were performed on 66 fish from multiple 50:50 clutches as described (Caudal Fin Clips and Barbectomies; Figure 9D). After 24 hours to allow for wound healing, fish were haphazardly split into two tanks, one heated to 33°C and one control tank at 28°C. After 14 days, fish were collected as described (Euthanasia and Tissue Collection). To prevent any bias during data collecting, I was kept blind to the treatment and genotype of each fish as before (Heat-Shock Inhibition of fgfr1).

**Pharmaceutical inhibition of Wnt signaling:** Canonical Wnt signaling was inhibited by the application of the small molecule IWR-1 (Sigma-Aldrich: I0161; Figure 10). IWR-1 inhibits the β-catenin-dependent Wnt signaling pathway by stabilizing the destruction complex that leads to the degradation of β-catenin (Chen et al., 2009). Barbectomies and caudal fin clips on adult wild type fish were performed as previously described. Fish were kept at 28°C in an aerated glass beaker with 500 mL of fish water (60 mg Instant Ocean/L of distilled water) for 24 hours. After 24 hours, fish were either
moved to a new beaker of 10 mM IWR-1 compound in 0.15% dimethyl sulfoxide (DMSO) or to a control beaker of 0.15% DMSO in fish water. Solutions were changed once daily for up to 10 days, the maximum interval approved by the IACUC. To make the 10 mM IWR-1 solution, the IWR-1 stock of 25 mg was diluted in 3.06 mL DMSO to make a 20 mM solution and then separated into 250 μL aliquots. Each time a new solution was made, 500 mL fish water was “primed” with 500 μL DMSO and the pH was brought to 7.6-7.7 with sodium bicarbonate. The 250 μL aliquot of IWR-1 was poured in and stirred until completely dissolved into solution. Fish were fed commercial fish flakes once per day and the fish water was oxygenated with a bubbler/aerator. After 10 days, fish were collected as previously described.

Transgenic overexpression of nom1a: Transgenic inhibition of canonical Wnt signaling was performed using the heat-shock transgenic notum1a (nom1a) line. Barbectomies and tail fin clips were performed as previously described on 22 fish from a 50:50::transgenic:wild type clutch. The experimental conditions were identical to those previously described (Heat-Shock Inhibition of fgfr1; Appendix A). Qualitative observations were made on 10 heat-shock and 12 non-heat shock treated fish; however, no measurements were taken.

Digital photography, image processing, and measurement: To measure the regrowth of barbel regenerates, matched pairs of maxillary barbels (regenerates and controls) were dissected at the base of the barbel still attached to their maxillae. The matched barbels were photographed in a Petri dish of PBS on a Leica MZ6 dissecting microscope with a Leica DFC295 3-megapixel digital camera and AS Suite Software. The AS Suite Software calibrated the scale for each level of magnification and digitally attached scale bars to each image. This calibration was confirmed by measuring a picture of a ruler. Photographs were cropped and adjusted for overall contrast and brightness in Adobe Photoshop.

Barbel lengths were measured using the segmented line tool in ImageJ (http://rsbweb.nih.gov/ij/) to plot points along the midline of each barbel (Figure 11A-B). ImageJ line lengths were calibrated by
tracing the attached scale bar for each picture and entering this length in millimeters. To determine the extent of regeneration relative to original length, three measurements needed to be collected. First, the total length (TL) of the unamputated control barbel was measured from the proximal end of the central rod to the tip of the barbel. Next, regenerating barbel length was divided into two measurements: Stump Length (SL), or the length of the barbel that remained after amputation, and Regenerate Length (RL), or the length of the barbel that was caused by regeneration. SL was measured from the proximal end of the central rod to the amputation plane (end of central rod). RL was measured from the amputation plane to the tip to the barbel. Our previous data have shown that the left and right barbels of an adult zebrafish are typically the same length (on average, 3.07 ± 0.48 mm; N_{left} = 15; N_{right} = 15) and only differ by an average of 0.076 ± 0.074 mm (2.1%). Therefore, we can use the right, unoperated control as a proxy for the original length of the left, regenerating barbel. Finally, the percent regeneration relative to control was calculated as (% regenerated = (Regeneration Length / (Control Total Length - Stump Length))) (Figure 11C).

Statistical analysis: Percentage values were exported to GraphPad Prism for graphing and statistical analysis. Most data distributions had a small sample size (n < 20) and were skewed; therefore, the non-parametric Mann-Whitney U test was used. This test was performed on barbel regenerate percentages to determine if there was a significant difference between median values of two groups. Significant differences were examined at \( \alpha = 0.05 \).

Results

FGF and Wnt signaling molecules are expressed during barbel regeneration

Many FGF and Wnt ligands, receptors, and downstream targets are expressed during caudal fin regeneration. As previously reviewed, the expression of these pathway members is critical for various cellular and morphological regenerative events. To determine if the same genes are also expressed during barbel regeneration, I designed a set of polymerase chain reaction (PCR) primers targeting components of
FGF and Wnt signaling. These primers were first tested using reverse-transcription PCR (RT-PCR) on wild type regenerating fin RNA (Figure 12A-B). After confirming that each primer set worked, I examined mRNA transcript expression of each gene in wild type regenerating barbels. All tested FGF pathway genes were expressed in regenerating barbels at 4 days post surgery (Figure 12A). This included expression of the FGF ligands fgf20a and fgf24, receptors fgfr1-4, and downstream targets interleukin 17 receptor D (il17rdTsang et al., 2002) and ETS-domain transcription factor pea3 (pea3; Brown et al., 1998). The canonical Wnt-signaling transcription factor lymphocyte enhancer binding factor 1 (lef1Porfiri et al., 1997) was also expressed in 4 days post surgery barbels (Figure 12B). In addition, our lab has previously used the same technique to observe expression of non-canonical Wnt signaling pathway members in regenerating barbels at 3 days post surgery (Figure 12C). These include the non-canonical Wnt ligands wnt5a, wnt5b, wnt11 and wnt11R, and the non-canonical Wnt co-receptor glypican4. In each experiment, primers for the housekeeping gene β-actin served as positive controls for RNA integrity, and all samples produced a band of expected size. These results indicate that FGF and Wnt signaling members are expressed during barbel regeneration at the appropriate time and place to act as regulatory molecules.

The FGF pathway regulates maxillary barbel regeneration through the receptor fgfr1

The FGF signaling pathway has been previously identified as a critical controller of zebrafish caudal fin regeneration (Lee et al., 2005; Poss et al., 2000a; Thummel et al., 2006) and is thus an excellent candidate for ZMB regeneration studies. Typically, research on FGF signaling in fin regeneration has focused on the FGF receptor 1 (fgfr1) because it is the most highly expressed FGF receptor during this process (Poss et al., 2000a). Fgfr1 is initially expressed in cells forming the blastema at 18 hours post surgery. Expression continues within both the proximal and distal blastema at 48 hours, and during regenerate outgrowth (Poss et al., 2000a). Consistent with the spatial and temporal expression of fgfr1, studies inhibiting this molecule using heat-shock transgenics, small-molecule drugs and antisense-mediated knockdown have indicated that it plays a major role in fin regeneration (Poss et al.,
I was therefore interested in manipulating \textit{fgfr1} expression to determine the potential roles of FGF signaling in ZMB regeneration.

To test whether FGF signaling was involved in ZMB regeneration, I chose to inhibit the FGFR1 receptor \textit{in vivo} by using a heat-shock inducible transgenic fish line (\textbf{Figure 8}). The \textit{dnfgfr1} transgenic line is the most widely used, well-validated and efficient way to inhibit FGFs during a variety of zebrafish developmental stages and processes. For these experiments, I produced clutches of 50:50::transgenic: wild type fish. When the fish reached 3-6 months of age, I performed fin clips and unilateral barbectomies. Fish were then haphazardly split into heat-shock (HS) and non-heat shock (NHS) tanks. After 24 hours, fish in the heat-shock tank received automated heat shocks of \textgreater{}37°C for 1 hour/day. Non-heat shock controls (wild type + \textit{dnfgfr1}) remained at 28°C throughout the experiment and, in the absence of functional genotyping, were analyzed as one group. After 14 days, fish were collected and regeneration was observed and measured. All regenerate lengths were expressed as a percent of the contralateral control barbel (for details, see Methods).

I previously hypothesized that the regulatory mechanisms controlling ZMB regeneration would be similar to those controlling fin regeneration. Therefore, I expected similar results when inhibiting \textit{fgfr1} in both appendages. Under heat-shock conditions, I expected that fin regeneration would be completely inhibited in \textit{dnfgfr1} fish, as previously described. If \textit{fgfr1} is involved in ZMB regeneration, blastema formation and/or elongation would also be inhibited under the same conditions. If \textit{fgfr1} is not involved in ZMB regeneration, I would still see inhibition of tail regeneration, but complete re-growth in the ZMB. Finally, I predicted that wild type fish receiving heat shocks and all non-heat shock controls (wild type + \textit{dnfgfr1}) would both regenerate their fins and barbels normally.

As predicted, inhibiting \textit{fgfr1} caused complete inhibition of fin regeneration (\textbf{Figure 13A}). There was a complete blockage of blastema formation and outgrowth in the amputated fins of all \textit{dnfgfr1}
individuals that received heat shocks (14/14 = 100%; Figure 13A). Only a thin layer of cells covered the amputation plane, presumably an epithelium produced by wound healing in the first 24 hours. These individuals also showed global EGFP expression, an indicator of successful transgene expression (Figure 13B, B’). In contrast, fin regeneration was normal in wild type fish receiving heat shocks, and in all non-heat shocked fish (data not shown). These fins grew back to original lengths and completely restored their bony fin rays, pigment cells and other anatomy. Thus, I was able to successfully replicate the conditions necessary to induce dnfgfr1 expression, inhibit FGF signaling and prevent fin regeneration.

ZMB regeneration in dnfgfr1 fish was also strongly inhibited. After 14 days of heat shocks, the median percentage of barbel regrowth among dnfgfr1 fish was 19.6 ± 13.8 (n = 14; Figure 13C). This was two and a half times shorter than heat-shocked wild type controls (median ± SD = 48.6 ± 11.6, n = 14; Figure 13C), a statistically significantly difference (Mann-Whitney U test; U = 14; p = 0.0001):

<table>
<thead>
<tr>
<th>treatment</th>
<th>genotype</th>
<th>N</th>
<th>% regeneration (median)</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>HS</td>
<td>dnfgfr1</td>
<td>14</td>
<td>19.61</td>
<td>3.07-50.7</td>
</tr>
<tr>
<td>HS</td>
<td>wild type</td>
<td>14</td>
<td>48.64</td>
<td>26.7-68.1</td>
</tr>
<tr>
<td>NHS</td>
<td>dnfgfr1 + wild type</td>
<td>30</td>
<td>56.7</td>
<td>35.3-69.0</td>
</tr>
</tbody>
</table>

The median percentage of barbel regrowth among heat-shocked dnfgfr1 barbels was almost three times shorter than that of the non-heat shock group (median ± SD = 56.7 ± 7.06; n = 30; Figure 13C). These results indicate that inhibiting fgfr1 causes a decrease in ZMB regeneration length.

Although heat shock completely repressed fin regeneration in dnfgfr1 individuals, all the barbels of these fish had some regenerative outgrowth. These regenerates varied not only in length (3.07 – 50.73 % regeneration) but in gross morphology. Subjectively, I grouped regenerating dnfgfr1 barbels according to differences in these features. The first group of barbels (4/14 fish = 29%; 42.3 – 50.7% regeneration) regrew comparable to wild types. These barbels clearly contained extensive connective tissue, a connected blood vessel loop, distally located taste buds and scattered melanophores. Most dnfgfr1 barbels did not regenerate this completely, however, forming the second group of intermediate
regeneration lengths (7/14 fish = 50%; 16.3 – 25.5 % regeneration). These barbels all had taste buds and melanophores well past the amputation plane. However, unlike barbels of the first group, it was unclear to what extent mesodermal tissue regenerated in these samples. Finally, a third group of heat-shocked 
dngfgr1 barbels produced minimal outgrowths of less than 8.0% (3/14 fish = 21%). All of these barbels had a distinct epithelial layer covering the amputation plane, indicating wound healing. Two of these barbels contained a small mass of cells beneath the epithelium but past the amputation plane, while the third contained only a thin layer of these underlying cells. These small masses appeared disorganized, with no indication of differentiated mesodermal tissue. Lastly, taste buds and melanophores did appear immediately distal to the amputation plain on the epithelium of the two larger cell masses. However, it is unknown if these were the products of regeneration or epithelial migration. These results indicate that inhibiting 
fgfr1 produces a range of barbel lengths and morphologies, which is not seen in fin regeneration.

Finally, I had to compare ZMB regeneration between heat-shock and non-heat-shock wild types to ensure that the heat-shock treatments themselves were not the cause of inhibited barbel regeneration. This control experiment was important because zebrafish are ectotherms and their body temperatures rely on ambient temperature. Studies have indicated that changes in environmental temperature can affect the rate of development, metabolism and transcription (Parichy et al., 2009; Vergauwen et al., 2010). Furthermore, zebrafish express endogenous heat-shock proteins, which are upregulated with increased temperatures (Basu et al., 2002; Krone et al., 1997). Some endogenous heat-shock proteins are even required for blastema formation and maintenance during fin regeneration (Makino et al., 2005). Although previous research indicates that transient heat shocks (1 hour/day) do not affect fin regeneration in wild types (Lee et al., 2005), it possible that simply increasing water temperatures may have affected barbel regeneration, which has not previously been studied in this context.
To test the effects of heat shocks on barbel regeneration, wild type adult fish were fin clipped and barbectomized. After 24 hours, 20 fish received automated heat shocks of >37°C for 1 hour/day, while 18 fish remained at 28°C. Heat-shocked fish (n = 10) were collected and compared to non-heat shock fish (n = 9) at 14 days or 21 days. After 14 days, there was no significant difference in median barbel regeneration comparing non-heat shocked and heat-shocked wild types (44.5% vs. 40.0% respectively; Mann-Whitney U test; p = 0.50; U = 36; Figure 14). Longer heat-shock treatments (up to 21 days) also did not affect barbel regeneration (NHS vs. HS; 62.6% vs. 66% regeneration; Mann-Whitney U test; U = 43; p = 0.90; Figure 14). However, this longer heat-shock period caused the range of regenerate lengths to expand, which was not observed at 14 days post surgery (Figure 14). Therefore, the decrease in the regenerative length of heat-shock treated dnfgfr1 barbels was caused by inhibited fgfr1 signaling and cannot be attributed to the heat-shock treatments themselves:

<table>
<thead>
<tr>
<th>days post surgery</th>
<th>treatment</th>
<th>N</th>
<th>% regeneration (median)</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>NHS</td>
<td>9</td>
<td>44.5</td>
<td>28.2-62.0</td>
</tr>
<tr>
<td>14</td>
<td>HS</td>
<td>10</td>
<td>40.0</td>
<td>23.3-59.3</td>
</tr>
<tr>
<td>21</td>
<td>NHS</td>
<td>9</td>
<td>62.6</td>
<td>53.8-74.2</td>
</tr>
<tr>
<td>21</td>
<td>HS</td>
<td>10</td>
<td>66.0</td>
<td>33.8-78.4</td>
</tr>
</tbody>
</table>

The ligand fgf20a is essential for tail, but not barbel regeneration

The dnfgfr1 experiments clearly indicated that ZMB regeneration is controlled by FGF signaling, but which FGFs are involved? In a 2005 issue of the journal Science, Whitehead et al. demonstrated that the ligand fgf20a is essential to initiate fin regeneration. In their experiment, an ENU-mutagenesis screen in zebrafish produced a temperature-sensitive null mutation in fgf20a. Fish homozygous for this mutation formed an abnormal regenerative epithelium, did not produce a blastema and failed to express typical blastemal markers. Furthermore, this mutation did not seem to have any clear developmental significance and did not hinder embryonic survival. Therefore, the authors argued that fgf20a was specific for the initiation of regeneration.
The *fgf20a* mutant line contains a temperature-sensitive null mutation, which causes a change in the binding site of the *fgf20a* ligand, preventing downstream signaling (Whitehead *et al*., 2005). This mutation inhibits fin regeneration most effectively at 33°C, but can be active at lower temperatures. To test the necessity for *fgf20a* in regeneration, I compared fin and barbel regeneration between wild types and *fgf20a* mutants at both 28°C and 33°C (Figure 9). I had to first produce a 50:50 clutch of *fgf20a* homozygous mutants:heterozygous wild types. Then, at 3-6 months old, fin clips and unilateral barbectomies were performed. After 24 hours, these fish were haphazardly split into two tanks, one held at 33°C and the other held at 28°C. Finally, fish were collected after 14 days and regeneration was observed and measured.

I predicted that *fgf20a* mutant barbels would show regeneration defects similar to caudal fins. Specifically, I expected that fin regeneration in *fgf20a* fish would be completely inhibited at 33°C. If *fgf20a* were essential for barbel regeneration, blastema formation and elongation of barbels would also be inhibited under the same conditions. If *fgf20a* were not essential for barbel regeneration, fin regeneration would still be prevented, but barbels would regenerate normally, even within the same fish. I also expected that fin regeneration could be inhibited in controls (28°C). Whitehead *et al*. (2005) indicated that 72% of mutants had regeneration defects at this temperature, which is standard for most zebrafish facilities. If this were the case, I would predict similar amounts of inhibition in both the fin and the barbel at 28°C. Any fish with fin regeneration defects at 28°C would have shown comparable defects in its barbel regeneration as well.

As predicted, all wild types at 33°C regenerated fins normally (n = 18; Figure 15A), but *fgf20a* mutants at the same temperature had substantial fin regeneration defects (n = 13; Figure 15B). Six out of 13 mutant fins (46%) were only able to cover the wound with an epithelium, and did not produce a blastema or any outgrowth. The other seven *fgf20a* mutants (54%) produced minimal amounts of fin regeneration. In these fish, only one or two fin ray segments were able to regenerate (Figure 15B).
These regenerated segments were typically very thin, short, and never reached lengths comparable to wild type regeneration. In addition, all *fgf20a* mutants at control temperatures (28°C) also regenerated fins abnormally (data not shown). Most *fgf20a* mutant fish at 28°C (10/14 = 71%) regenerated fins to only approximately half of the fin’s original size. These partial regenerates were usually opaque, disorganized and resembled nothing like a normal wild type regenerating fin. The other four fish at 28°C (29%) showed complete inhibition of regeneration. Thus, despite minimal regeneration in a few fish, I felt confident that I effectively used temperature to inhibit *fgf20a* signaling, reproducing known defects in fin regeneration.

Interestingly, I found that *fgf20a* mutant fish regenerate the ZMB normally. After 14 days at 33°C, *fgf20a* barbels regenerated a range of 20.3 – 56.3%, with a median of 41.3% (n = 13; Figure 15C). Surprisingly, this was nearly the same as wild type controls at the same temperature, and not significantly different (Mann-Whitney U test; U = 100; p = 0.51):

<table>
<thead>
<tr>
<th>treatment</th>
<th>genotype</th>
<th>N</th>
<th>% regeneration (median)</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>33°C</td>
<td>wild type</td>
<td>18</td>
<td>42.9</td>
<td>18.2-62.7</td>
</tr>
<tr>
<td>33°C</td>
<td><em>fgf20a</em></td>
<td>13</td>
<td>41.3</td>
<td>21.6-57.3</td>
</tr>
</tbody>
</table>

Morphologically, regenerating barbels of *fgf20a* mutants and wild types at 33°C were indistinguishable (Figure 15D-E). Both groups appeared to completely regenerate most tissue types. These included numerous melanophores, a distally located taste bud cluster, a complete blood capillary loop and extensive connective tissue (Figure 15F). Wild type and *fgf20a* barbels also shared similar shape, thickness and color. These findings indicate that inhibiting *fgf20a* at 33°C affected fin regeneration but not ZMB regeneration.

Similar results were observed in *fgf20a* mutants regenerating at 28°C. As described above, *fgf20a* mutant fin regeneration was also inhibited at this temperature, indicating lack of *fgf20a* signaling. I therefore predicted that I would see comparable amounts of inhibition in the ZMB. However, this was
not the case. Median barbel regeneration in \textit{fgf}20\textit{a} mutants at 28°C was comparable to wild types at the same temperature (40.8\% vs. 44.2\%, respectively) and not significantly different (Mann-Whitney U test; \( U = 133; p = 0.82; \textbf{Figure 15C})): 

<table>
<thead>
<tr>
<th>treatment</th>
<th>genotype</th>
<th>N</th>
<th>% regeneration (median)</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>28°C</td>
<td>wild type</td>
<td>18</td>
<td>42.9</td>
<td>18.2-62.7</td>
</tr>
<tr>
<td>28°C</td>
<td>\textit{fgf}20\textit{a}</td>
<td>13</td>
<td>41.3</td>
<td>21.6-57.3</td>
</tr>
</tbody>
</table>

In this experiment, I was able to completely block tail regeneration, replicating the landmark results reported by Whitehead \textit{et al.} (2005) in \textit{Science}. However, barbel regeneration was normal within the very same individuals. These results indicate very clearly that barbels can regenerate normally in the absence of \textit{fgf}20\textit{a} signaling. Therefore, \textit{fgf}20\textit{a} is not essential to barbel regeneration.

\textbf{Canonical Wnt signaling regulates ZMB regeneration}

A second regulatory pathway that is essential for fin regeneration, and thus another excellent candidate for controlling ZMB regeneration, is the Wnt signaling pathway. Wnts are expressed in the wound epithelium of fins and are essential for blastema formation and maintenance (Kawakami \textit{et al.}, 2006; Stoick-Cooper \textit{et al.}, 2007). Interestingly, Wnt signaling also plays an important role regulating other gene networks during fin regeneration, including FGF signaling (Stoick-Cooper \textit{et al.}, 2007). As I previously described, the regenerating barbel expresses many canonical and non-canonical Wnt signaling pathway members (\textbf{Figure 12}). I was therefore interested in inhibiting Wnt signaling and observing the effects this had on ZMB regeneration.

Typically, researchers block Wnt signaling during fin regeneration with heat-shock transgenic lines expressing either the Wnt inhibitor \textit{Dkk}1 or a defective form of the transcription factor \textit{tcf}. Unfortunately, these lines were unavailable for my project. Instead, I used the small-molecule drug IWR-1, which effectively inhibits canonical Wnt signaling and blocks fin regeneration (Chen \textit{et al.}, 2009). This compound stabilizes the destruction complex that degrades the signaling molecule \(\beta\)-catenin (\textbf{Figure}
6). This prevents the accumulation of β-catenin in the nucleus, inhibiting canonical Wnt signaling; however, non-canonical Wnt signaling is left unaffected.

To test if canonical Wnt signaling is necessary for ZMB regeneration, I applied the small-molecule inhibitor IWR-1 to wild type fish undergoing fin and barbel regeneration (Figure 10). For this experiment, fin clips and barbectomies were performed on 18 wild type adults 3-6 months old. Fish were initially placed in clean fish water to allow for wound healing. After 24 hours, fish were moved to new tanks of water containing either 10 mM IWR-1 + 0.15% DMSO as a carrier (n = 9), or 0.15% DMSO as a control (n = 9). After 10 days in IWR-1 solution, I expected to see that fin regeneration would be completely inhibited. I also predicted that Wnt signaling would be essential for ZMB regeneration, and that IWR-1 would inhibit blastema formation and/or outgrowth in these appendages as well. If Wnts were not essential for ZMB regeneration, fin regeneration would still be inhibited, but barbels would grow back normally.

As predicted, administering IWR-1 for 10 days caused a drastic inhibition of fin regeneration compared to controls. All DMSO control fins (10/10 = 100%) had full and robust regeneration at every fin ray segment (Figure 16A). After only ten days, fins have not had enough time to regrow to their original size; however, these appendages regenerated every fin ray and display a typical forked pattern. In contrast, most of the wild type fish treated with IWR-1 (5/9 = 56%) did not show any signs of fin regeneration (Figure 16C). The remaining IWR-1 fish (4/9 = 44%) regenerated minimal amounts of tissue (data not shown). These fins resembled the phenotype of some fgf20a mutants at 33°C. This tissue was usually only 2-3 fin rays wide, very short, disorganized and colorless. These results indicate that the IWR-1 treatment successfully blocked Wnt signaling in vivo and inhibited fin regeneration.

IWR-1 treatment also caused a decrease in ZMB regeneration. Median barbel regeneration of the IWR-1 treated fish (n = 9; median ± SD = 6.4% ± 3.0%; Figure 16D, E) was nearly half that of DMSO
controls (n = 9; median ± SD = 12.1% ± 3.9%; Figure 16B, E). This was a statistically significant difference (Mann-Whitney U test; U = 14; p = 0.02; Figure 16E). Interestingly, DMSO controls had a larger range (3.0-14.7% regeneration) than IWR-1 treated fish (1.6-10.5); however, DMSO controls were more negatively skewed towards greater regenerate lengths (Figure 16E). These results clearly indicate that I was able to block canonical Wnt signaling and cause a decrease in regenerating barbel outgrowth:

<table>
<thead>
<tr>
<th>treatment</th>
<th>genotype</th>
<th>N</th>
<th>% regeneration (median)</th>
<th>range</th>
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<tbody>
<tr>
<td>IWR-1 + DMSO</td>
<td>wild type</td>
<td>9</td>
<td>6.4</td>
<td>1.6-10.5</td>
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<tr>
<td>DMSO</td>
<td>wild type</td>
<td>9</td>
<td>12.1</td>
<td>3.0-14.7</td>
</tr>
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</table>

In previous experiments, I examined regenerating barbels at 14 days post surgery. These barbels are “older” and have had enough time for extensive cell proliferation and differentiation. This is why normal regenerating barbels at this time point are long and contain distinct tissue types. However, the limited interval approved for this experiment (10 days) produced “younger” regenerating barbels that are typically shorter and less developed. To the naked eye, clearly differentiated cells and distinguishable tissue types may not be visible at 10 days post surgery. This explains why, other than a significant difference in length, there was no clear distinction between the overall structure and anatomy of control and IWR-1-treated barbels (Figure 16B, D). Both DMSO and IWR-1 regenerating barbels produced a thin epithelial cover over the wound. Except for the two longest DMSO regenerating barbels, epithelia did not contain melanophores past the amputation plane. No clearly visible taste buds were observed in either group. Finally, regenerated mesodermal tissue appeared disorganized and undifferentiated in barbels of both groups. These results are consistent with the experiment’s duration; however, it is still evident that blocking canonical Wnt signaling caused a significant decrease in regenerating barbel length.

Notum1a is potentially a novel regulator of zebrafish regeneration

In the previous section, I demonstrated that canonical Wnt signaling affects barbel regeneration, but how are extracellular Wnt ligands regulated during this process? One known mechanism is through the cell surface protein called Glypican (Figure 6). Glypicans are widely conserved proteoglycans that
act as co-receptors trafficking many extracellular ligands including Wnts (Filmus et al., 2008). Interestingly, I have previously identified one zebrafish glypican homolog (glypican4) that is expressed during barbel regeneration (Figure 12). These co-receptors are capable of both positively and negatively regulating Wnt signaling, as well as other pathways (Filmus et al., 2008; Kreuger et al., 2004). Adding to their complexity, individual glypican molecules can be either membrane-attached or shed from the surface, and each type may have distinct regulatory capabilities (Filmus et al., 2008). The secreted enzyme called Notum is capable of cleaving glypicans and other proteoglycans from the cell surface (Filmus et al., 2008; Kreuger et al., 2004; Traister et al., 2007). Furthermore, Notum homologs seem to play a role in Wnt signaling during zebrafish development. Specifically, the zebrafish notum homolog nom1a acts as an antagonist to canonical Wnt signaling but does not affect non-cannonical Wnt signaling (Flowers et al., 2010; J. Topczewski pers. com.). For these reasons, I hypothesized that overexpressing nom1a would inhibit the regeneration of both appendages, most likely by inhibiting canonical Wnt signaling.

To test the regulatory capabilities of notum and, indirectly, glypicans during zebrafish caudal fin and barbel regeneration, I transgenically overexpressed nom1a using the fish line Tg(hsp70l:nom1a). This is a heat-shock transgenic line that is activated at 37°C-38°C; therefore, I used the same experimental heat-shock design for this study that I used for the dnfgr1 experiment. I performed caudal fin clips and barbectomies on a clutch of 22 adult fish presumed to be genotypically 50:50::transgenic:wildtype. After 24 hours, 10 fish received heat shocks and 12 fish remained at 28°C. Finally, all fish were collected at 14 days. As I previously presented, canonical Wnt signaling is important for both fin and barbel regeneration. Therefore, if nom1a is a canonical Wnt signaling antagonist in adult zebrafish, I would expect to see that overexpressing nom1a in heat-shocked transgenic fish would inhibit both fin and barbel regrowth. If nom1a was not an antagonist of Wnt signaling during regeneration, fins and barbels would grow back normally in heat-shocked transgenic fish.
Using fixed fish, I attempted to genotype *nom1a* fish by cardiac EGFP expression as described (*Notum1a Transgenic Zebrafish*). While genotyping, however, I was not able to successfully sort all wild type and transgenic individuals by observing cardiac EGFP. In the absence of clearly defined treatment and control groups, no significant claims could be made; therefore, I did not measure regenerative length but only observed the presence or absence of regenerating tissue. I then predicted that all non-heat shock fish would regenerate both fins and barbels normally. Assuming that the population of fish receiving heat shocks was genotypically 50:50::transgenic:wildtype, I predicted that I would see both normal and inhibited caudal fin and barbel regeneration in a similar phenotypic ratio.

Within this context, I compared the amount of fin and barbel regeneration in heat-shocked and non-heat shocked groups. The heat-shocked fish group contained individuals that regenerated fins normally (4/10 = 40%) and individuals with inhibited fin regeneration (6/10 = 60%). Inhibition of fin regeneration ranged from complete absence of regenerated tissue (Figure 17A) to partial regrowth of multiple fin rays (data not shown). On the other hand, nearly all non-heat shock treated fish regenerated their fins normally (11/12 = 92%; Figure 17B). Interestingly, the patterns of barbel regeneration were almost identical to those of fin regeneration. The regeneration of many heat-shock barbels was blocked (Figure 17D), while all non-heat shocked barbels regenerated normally (Figure 17D). Strikingly, the amount of inhibition was nearly identical between fins and barbels within the same fish (compare Figures 17B and C). Because regeneration was only prevented in individuals of the heat-shocked fish population, I assumed that these phenotypes were caused by activating the heat-shock transgenic promoter. Inhibited regeneration can then be attributed to the overexpression of *nom1a*. This result suggests that *nom1a*, by regulating glypicans, may be a novel regulator of regeneration in zebrafish. Although the effects of *nom1a* have been investigated in zebrafish embryonic development, this experiment is the first to test the effect of *nom1a* in adult fish physiology.
Discussion

Regeneration is a complex process, making it relatively difficult to understand its molecular basis. Therefore, researchers hope to discover regulatory mechanisms that are simple and widely conserved. Just a few gene networks control many regenerative processes; however, the specific ways these networks regulate regeneration may be different among species, structures, organs and tissues. The zebrafish maxillary barbel provides a novel system for studying these similarities and differences. In this study, I observed that FGF and Wnt signaling pathways regulate both barbel and caudal fin regeneration. However, these pathways regulate each appendage differently. My results are the first to highlight the molecular mechanisms of regeneration in zebrafish barbels and how these mechanisms relate to other known regenerative processes. In this section, I will discuss how FGF and Wnt signaling may regulate the different stages of barbel regeneration, how this compares to fin regeneration, and what ecological and evolutionary implications these conclusions may have.

The regulatory mechanisms of early stages in barbel regeneration are unknown

My goal for this study was to uncover any roles that FGF and Wnt signaling may have during barbel regeneration. Specifically, I wanted to determine if these signaling pathways were essential for regulating certain regenerative stages. In order to examine the function of each pathway, I measured the effects of inhibiting FGF or Wnt signaling in regenerating barbels. This was done by inducing the expression of a transgenic dominant negative FGF receptor (dnfgfr1) or by administering the canonical Wnt inhibitor drug IWR-1, respectively. Each of these inhibitory techniques was initiated 24 hours post surgery. Therefore, any effects caused by inhibiting FGF or Wnt signaling could only be observed after this time point. However, the first stages of regeneration, which are wound healing and epithelial thickening, begin immediately after amputation. These initial events result in the formation of a new epithelial cap that covers the wound, which is completed within the first 24 hours of regeneration. Thus, although both the FGF and Wnt pathways are involved in wound healing and epithelial thickening during
fin regeneration (Poss et al., 2000b; Whitehead et al., 2005), I was not able to determine if these pathways were also involved in the same stages of barbel regeneration.

Conversely, it appears that I was able to inhibit the FGF ligand, fgf20a, before these early regenerative events. I demonstrated that fin regeneration in temperature sensitive fgf20a mutants was blocked even at standard rearing temperature (28°C). Because all fgf20a mutants were raised at 28°C, I inferred that fgf20a signaling was inhibited throughout their entire lifespan, including the initial stages of regeneration. However, I was not able to analyze epithelialization in these fish because I collected barbels during a later stage (14 days post surgery). To effectively analyze wound healing and epithelial thickening, future experiments should inhibit FGF and Wnt signaling before barbel amputations and evaluate epithelial cell organization within the first few hours of regeneration.

_The initiation of a barbel blastema does not depend on FGF or Wnt signaling_

While it is unclear what roles the FGF and Wnt pathways play during barbel wound healing and epithelial thickening, it appears that these pathways are not essential for barbel blastema formation, the next stage of regeneration. A blastema is an accumulation of mesenchymal cells that are thought to be capable of proliferating and differentiating into the cell types that compose regenerating tissues. Blastema formation occurs after wound healing and is essential for the outgrowth and elongation of regenerating fins, limbs, and other appendages. Based on gross morphological analysis, it is presumed that barbels regenerate in the same order. To test if FGF and Wnt signaling are involved in blastema formation, I attempted to block each pathway before the initiation of this stage. I induced the expression of _dnfgfr1_ or administered IWR-1 starting at 24 hours post surgery, a time point when blastema cells start to accumulate and expression of blastema markers first appear in fins.

Like previous studies, I was able to completely prevent fin blastema formation by blocking FGF or Wnt signaling. Unexpectedly, inhibiting either pathway did not completely restrict the accumulation of
mesodermal cells past the amputation plane in regenerating barbels. This was evident in even the shortest regenerating samples. These barbels had a clearly distinguishable outer epithelium and inner mass of mesodermal tissue, resembling a typical blastema in size and shape. Therefore, in the absence of FGF and Wnt signaling, the newly formed barbel tissue cannot be attributed to wound healing and epithelial thickening only, but potentially to blastema formation.

Additionally, most heat-shocked dnfgr1 fish or IWR-1 treated wild types regenerated barbels past the typical size and length of a newly formed blastema. Intriguingly, this was also the case for barbels of fgf20a mutants, though I was able to completely inhibit blastema formation in fins of the same fish. The extent of regeneration in these barbels indicated that they had accomplished outgrowth. In fins, outgrowth is dependent on the formation of a functioning blastema. Because barbel regeneration is presumed to occur in the same sequential manner as fin regeneration, it can be inferred that these longer regenerates also formed a functioning blastema. Thus the FGF and Wnt signaling pathways do not appear to play a role in the initiation and formation of blastema cells during barbel regeneration, but they do affect cell proliferation during outgrowth. This indicates that there are appendage-specific differences between the barbel and fin in the molecular mechanisms controlling blastema formation.

My comparisons of blastema formation between appendages were entirely based on morphological observations. Our lab has yet to confirm that the mass of mesenchymal cells formed during barbel regeneration has the same characteristics as a blastema formed during fin regeneration. It is only presumed that barbels form a blastema because regenerating barbels follow the same sequence of morphological events as other regenerating appendages. To effectively compare blastema formation between barbels and fins, future experiments should analyze regenerating barbels around the time that the presumed blastema starts to form (1-3 days post surgery). Features of a fin blastema, such as mesodermal cell origin, proliferation and marker gene expression, should first be tested on wild type barbels. Tracing the origin of the cells found in the presumed blastema would indicate if these are truly
mesenchymal cells derived from mesodermal tissues. Mitotic assays examining the proliferative capabilities of these cells, as well as expression screens for known fin blastema marker genes, could confirm that barbels do form a functional blastema. These tests could then be used on regenerating barbels of heat-shocked dnfgfrl transgenic fish, fgf20a mutants or IWR-1 treated wild types to determine if these barbels also produce a blastema.

*Notum may be a critical regulator of many signaling pathways during blastema formation*

I was initially interested in investigating notum during regeneration because of its ability to regulate Wnt signaling. Notum is an enzyme that cleaves ligand trafficking molecules from the cell surface, including glypicans (Filmus et al., 2008). Glypicans are capable of facilitating ligand-receptor binding in multiple signaling pathways. In zebrafish development, notum1a (nom1a) acts as an antagonist to canonical Wnt signaling by interfering with glypican function (Flowers et al., 2010; J. Topczewski, pers. comm.). I previously demonstrated, using IWR-1, that canonical Wnt signaling is not essential for barbel blastema formation. However, my results also revealed a role for canonical Wnt signaling during a subsequent regenerative stage, which I will discuss later. Therefore, I transgenically overexpressed nom1a in hopes of inhibiting canonical Wnt signaling, expecting to affect regeneration in later stages. Interestingly, unlike phenotypes caused by IWR-1 treatments, nom1a overexpression completely blocked blastema formation in both barbels and fins. Thus, canonical Wnt signaling may actually play a role in blastema formation but only in conjunction with other pathways.

Interestingly, glypicans traffic ligands for a range of signaling pathways and it is not known in zebrafish adults which of these notum affects. Many of these pathways, including the non-canonical Wnt, FGF, hedgehog (Hh), and bone morphogenetic protein (BMP) pathways, are also required for fin regeneration (Filmus et al., 2008; Tal et al., 2009). My results indicate that non-canonical Wnts, including wnt5a, wnt5b, wnt11 and wnt11r, are expressed during barbel regeneration, and that FGF signaling is essential for later stages of barbel regeneration. It is possible then that nom1a overexpression
blocked a range of signaling pathways necessary for regeneration in both barbels and fins, thereby, causing complete inhibition. Furthermore, notum has only recently been identified as regulator of regeneration. In a recent Science article featuring planarian regeneration, a planarian notum was shown to negatively regulate Wnt signaling and control proper body-axis regrowth (Petersen and Reddien, 2011). This paper, which was not published when my experiments were designed, is the first to report that notum is important for regeneration in invertebrates. My experiments on the zebrafish nom1a are likely the first to test the regenerative function of this gene in a vertebrate and, if confirmed by future experiments, would establish that this pathway is likely conserved in all metazoans.

_FGF and Wnt signaling are highly conserved controllers of cell proliferation during regeneration_

After blastema formation, regenerating tissues continue to elongation during the outgrowth stage. Outgrowth is characterized by intense proliferation of cells within the blastema and the migration of these cells into more distal locations. To determine if FGF and Wnt pathways are also required for outgrowth in barbel regeneration, I measured the length of regenerating barbels in _dnfgfr1_ transgenic fish or IWR-1 treated wild types. As I previously described, each pathway was inhibited starting 24 hours post surgery, which is not only before blastema formation but well before blastema cells start to proliferate. Thus, fin regeneration was completely inhibited and I was unable to directly compare outgrowth between the fin and barbel within these fish. However, FGF and Wnt signaling have previously been described as essential regulators of proliferation during fin outgrowth (Tal _et al._, 2009). Not surprisingly, I concluded that FGF and Wnt signaling control barbel outgrowth as well. Blocking FGF or Wnt signaling caused decreases of regenerate length by 60% and 49%, respectively. A reduced regenerative length can be attributed to a reduced number of regenerated cells. Therefore, each pathway contributes to cell proliferation during barbel outgrowth. This is a characteristic of outgrowth that is shared between regenerating barbels and fins, indicating that FGF and Wnt signaling are highly conserved regulators of this stage of regeneration.
Unexpectedly, *dnfgfr1* transgenic fish and IWR-1 treated wild types produced a range of regenerate outgrowth lengths. However, if FGF and Wnt signaling are both necessary and sufficient for cell proliferation, I would have expected to completely prevent regeneration after blastema formation. These conclusions assume that FGF or Wnt signaling were completely inhibited during each respective experiment; however, I cannot exclude the possibility that signaling through each pathway persisted. For instance, I did not quantitatively analyze the expression of FGF receptors during regeneration in either barbels or fins and it is not known how the level of FGF signaling compares between these two appendages. It is possible that barbels express a greater quantity of FGF receptors and thus greater levels of FGF signaling. Therefore, the dosage of *dnfgfr1* that effectively inhibited FGF signaling and subsequent regeneration in fins may not have been enough to do the same in barbels.

Similarly, it is possible that canonical Wnt signaling persisted in IWR-1 treated barbels as well. IWR-1 inhibits canonical Wnt signaling by stabilizing the destruction complex that degrades β-catenin. However, I did not quantitatively compare β-catenin expression between regenerating barbels and fins. It is possible that regenerating barbels accumulate greater quantities β-catenin than regenerating fins. Therefore, the concentration of IWR-1 that sufficiently prevented β-catenin accumulation during fin regeneration may not be a high enough concentration to degrade the levels of β-catenin in regenerating barbels. In the future, quantitative real-time RT-PCR experiments and/or Western blots could emphasize differences in expression levels of β-catenin and FGF receptors between the two appendages. Increasing the peak temperature, length, and spacing of heat shocks could drive greater expression of *dnfgfr1* and completely prevent barbel outgrowth. Similarly, increasing the concentration of IWR-1 could more extensively inhibit accumulation of β-catenin in regenerating barbels. Thus, FGF and Wnt signaling expression may be greater during barbel regeneration than fin regeneration. This would indicate that there are tissue-specific set points for FGF and Wnt activity.
A second explanation for the partial outgrowth of barbels in the absence of FGF and Wnt signaling is that other signaling pathways are also necessary for cell proliferation. As I previously reviewed, other gene networks are involved in fin regeneration (Iovine, 2007; Tal et al., 2009). These pathways include the non-canonical Wnt, retinoic acid and hedgehog pathways, as well as microRNA regulation. In the fin, FGF and Wnt signaling seem to have indispensible roles upstream of many of these pathways. If FGF or Wnt signaling is blocked, these pathways are halted and regeneration does not occur. However, FGF and Wnt signaling may work in parallel with other pathways during barbel regeneration. Thus, inhibiting FGF and Wnt signaling would not affect other regulatory mechanisms, preserving the function of these other factors in regeneration and accounting for the partial outgrowth observed in *dnfgfr1* and IWR-1 treated barbels. Therefore, it is possible that FGF and Wnt signaling are only two of many regulatory pathways that are conserved between barbel and fin regeneration; many more likely exist.

*Fgf20a* is a fin-specific regeneration molecule

Previously, I described that *fgf20a* is not essential for blastema formation in barbels; additionally, it appears that this ligand is also dispensable for further regenerative stages. *Fgf20a* mutant barbels regenerated to lengths comparable to wild type controls. Thus, the *fgf20a* mutation did not inhibit cell proliferation during tissue outgrowth. Furthermore, *fgf20a* mutants appeared to regenerate all of the same tissue types that wild type barbels do. Therefore, *fgf20a* is not essential for differentiation in barbels. Future experiments analyzing the mitotic indices of proliferating cells and tracing their fate in *fgf20a* mutants would confirm that *fgf20a* is not essential to barbel regeneration. Conversely, it is not known what role *fgf20a* plays in outgrowth and differentiation in regenerating fins because *fgf20a* mutants do not reach these stages. Consequently, it is likely that *fgf20a* is specific only to blastema formation during fin regeneration.
Interestingly, I observed the expression of \textit{fgf20a} in 4 day regenerating barbels using RT-PCR (Figure 12A). This may indicate that this ligand does indeed play a role in barbel regeneration, one which I was unable to detect by analyzing gross morphology and anatomy. It was not determined, however, if this expression was caused by an upregulation of transcription compared to unamputated barbels. Real-time RT-PCR comparing amputated and unamputated wild type barbels would help to determine if this observation is due to normal \textit{fgf20a} expression in barbels. \textit{In situ} hybridization would also reveal if \textit{fgf20a} expression is localized during regeneration; however, this would be unexpected because \textit{fgf20a} does not appear to have a function during barbel regeneration. Furthermore, RT-PCR confirmed the expression of \textit{fgf20b} in regenerating barbels at this same time point (data not shown). It would be interesting to see where \textit{fgf20b} expression is localized during regeneration and if \textit{fgf20b} is involved in any aspects of barbel regeneration. It is possible that \textit{fgf20} signaling regulates both barbel and fin regeneration, but by different analogues specific to each appendage.

\textit{The importance of comparing regeneration within zebrafish}

Zebrafish barbels and caudal fins differ in size, shape, function and certain cell types. Despite these differences, these two structures regenerate through similar morphological sequences, and restore many of the same tissues, including nerves, blood vessels, connective tissue and pigment cells. For these reasons, barbels and fins are excellent models to examine the molecular context of regeneration within the same species. Further comparative study of these appendages could give insights as to how and why zebrafish are capable of regenerating a variety of structures and organs, and how these processes have evolved.

It has been hypothesized that regenerative processes within the same species share related molecular mechanisms because the ability to regenerate is a primitive evolutionary adaptation that did not arise independently for each organ (Bely and Nyberg, 2010). My results support the model that a set of common molecular mechanisms, including FGF and Wnt signaling, are conserved among regenerating
zebrafish tissues. These sets of genes may control the basic cellular events, including wound healing, proliferation and differentiation, which are shared among all regenerating processes. In this model, metazoans most likely evolved the mechanisms to regenerate once, and these mechanisms are then “reused” in multiple structures. This would explain why the same pathways are used for regeneration across organs. For instance, the FGF and Wnt signaling pathways are important regulators of not only barbels and fins but heart (Lepilina et al., 2006), cerebellum (Köster and Fraser, 2006) and liver (Goessling et al., 2009; Kan et al., 2009) regeneration in zebrafish. Interestingly, other researchers have discovered similar results with other genes and gene pathways. Qin et al. (2009) found that heat-shock 60-kDa protein 1 (hspd1) and monopolar spindle 1 (mps1), which are required for fin and heart regeneration, are also necessary for retinal regeneration. Discovering conserved regulators of regeneration across different tissues within zebrafish may indicate that mechanisms for regeneration evolved only once and were then applied to each tissue.

It has also been hypothesized that regeneration is an epiphenomenon of development and has evolved independently, with modifications, in each structure, organ or tissue (Bely and Nyberg, 2010). My results may also support this model. Despite the importance of FGF and Wnt signaling to both barbel and fin regeneration, I observed distinct differences in the function of each pathway. Both FGF and Wnt signaling are important for the initiation and formation of blastema in the fin, but are not necessary for this process in barbels. Furthermore, it seems that the function of specific ligands, like fgf20a, may also be different between structures. It would then appear that regeneration is more complex than anticipated and that each structure, organ or tissue may regenerate using a specific combination of molecular mechanisms. This could mean that the ability to regenerate has evolved independently multiple times within the same species. In this model, differences in FGF and Wnt signaling would indicate that the mechanisms controlling regeneration in each appendage are more closely related to development of each appendage respectively. However, barbel development has received little research attention and comparisons between development and regeneration cannot be thoroughly made.
Finally, it appears that barbels are more resilient than fins at regenerating. In each experiment, I was able to completely inhibit fin regeneration by inhibiting a single pathway or even a single gene. In contrast, I was only able to completely inhibit barbel regeneration when overexpressing nom1a, a coreceptor that is known to bind ligands from multiple pathways. This suggests that the molecular networks controlling barbel regeneration are more expansive than those of fins, and that, in the barbel, these networks have functionally redundant roles. The complexity and redundancy of the molecular basis for barbel regeneration may suggest that barbels are more ecologically important, and that losing a barbel may have more significance than losing part of a fin. Unlike fins, barbels are a sense organ primarily used for taste. In the absence of barbels, catfish become disoriented and have an extremely difficult time locating food (Bardach 1967). Although the functionality of zebrafish barbels has not been tested, regenerating barbels may be an excellent model to study the molecular mechanisms of resilient regenerating tissue, as well as the behavioral and ecological consequences of losing and regenerating certain structures.

*Regeneration among different species*

Understanding molecular signaling during zebrafish regeneration may play a pivotal role in understanding why only certain species can regenerate and others cannot. Interestingly, diverse taxa share regenerative characteristics such as the mechanisms controlling re-epithelialization, activation of the immune response, re-innervation and the dependence on re-innervation by the surrounding regenerating tissues (Bely and Nyberg, 2010). As previously stated, FGF and Wnt signaling are two of the most highly conserved and necessary pathways among regenerating species (Sánchez Alvarado and Tsonis, 2006). Despite these highly conserved mechanisms of regeneration, many species have either not evolved or have lost regenerative capabilities.

The fact that the ability to regenerate is highly variable could be explained by the organization of regenerative mechanisms. It has been hypothesized that regeneration depends on largely conserved
molecular mechanisms that are present in all taxa, even those that cannot regenerate, but that these mechanisms are regulated by taxon-specific DNA regulatory elements (Garza-Garcia et al., 2010). Therefore, evolutionarily conserved proteins may be controlled by taxon-specific mechanisms in such a way that only promotes regeneration in certain species. My results can expand this hypothesis by indicating that there may not only be taxon-specific, but tissue-specific regulatory mechanisms as well. Studying simple, accessible and novel structures such as the barbel helps to highlight specific features of appendage regeneration that are lacking in animals that cannot regenerate, and may potentially lead to tissue-specific therapies that compensate for the absence of such features.

**Conclusion**

This study has demonstrated that the FGF and Wnt signaling pathways regulate zebrafish barbel regeneration. However, these pathways may control regeneration differently between the barbel and caudal fin. Experiments that inhibited FGF signaling by expressing the dominant negative FGF receptor (*dnfgfr1*) reduced the length of barbel regenerates by 60% but completely prevented fin regeneration in the same fish. Additionally, zebrafish homozygous recessive for a point mutation in *fgf20a* regenerated barbels normally, while caudal fin regeneration in the same fish was completely inhibited. Furthermore, experiments that blocked Wnt signaling by the application of the inhibitor drug IWR-1 reduced the length of barbel regenerates by 50% but also completely prevented fin regeneration. Finally, overexpressing the zebrafish homolog of the Wnt antagonist *nom1a* dramatically prevented regeneration in both the barbel and fin. These results are the first to highlight the molecular mechanisms controlling zebrafish barbel regeneration and demonstrate that these mechanisms may be different than other appendages. This could indicate that some pathways controlling regeneration are tissue-specific. Uncovering further regulators of barbel regeneration can help us to determine how the ability to regenerate has evolved and if any conserved regenerative themes exist at the taxon, tissue and molecular levels.
References


Köster, R.W., Fraser, S.E., 2006. FGF signaling mediates regeneration of the differentiating cerebellum through repatterning of the anterior hindbrain and reinitiation of neuronal migration. J Neurosci 26, 7293-7304.


Tables and Figures
Table 1. Reverse transcription polymerase chain reaction (RT-PCR) primers for regenerating barbel and fin RNA transcripts.

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<td>ACCCACAGGTGAAACAGGAG (Dorsky et al., 1999)</td>
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<tr>
<td></td>
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Figure 1. An adult zebrafish (*Danio rerio*). The Penn State University Zebrafish Atlas, 2007, http://zfatlas.psu.edu/index.php. Image is not to scale.
Figure 2. Anatomy of the ZMB on a wildtype adult zebrafish. A) The location of the nasal and maxillary barbel (nb and mb). B) Whole-mount image of a ZMB shaft using differential interference contrast (DIC). All central tissue types are visible through the transparent outer epithelium, including nerves, blood vessels, taste buds, pigment cells (melanophores and xanthophores), the central rod and putative lymph vessel (*). Scale bar = 100 µm. Modified from LeClair et al., 2010.
Figure 3. Proximal amputation of the ZMB induces regeneration. **A**) Procedure for unilateral barbectomy. **B-D**) Fixed, unstained ZMB regenerates immediately after amputation (**B**) and 24-72 hours post surgery (hps) forming an epithelial cover (**C**) and blastema (**D**). Dotted lines indicate the amputation plane. **E**) Four matched pairs of ZMB regenerates 7-28 days post surgery (dps). **nb** = nasal barbel; **mb** = maxillary barbel; **e** = epithelium; **cr** = central rod; **R** = right barbel (control); **L** = left barbel (regenerate). Modified from LeClair *et al.*, 2010.
Figure 4. Major morphological stages and molecular signaling events during fin regeneration.  

**A)** A mature fin ray contains a mesenchymal core surrounded by bony segments and an epithelial layer. **B)** After amputation, epithelial cells (blue) cover the wound. **C)** A group of undifferentiated cells called the blastema (red and purple) start to form distal to the amputation plane. **D)** Blastema cells differentiate and regenerative outgrowth occurs. **E)** Regeneration is terminated at the original fin length. The involvement of different molecular pathways is indicated as arrows (activation) or dead ends (inhibition). Modified from Tal et al., 2009.
Figure 5. A simplified representation of the fibroblast growth factor (FGF) signaling pathway. FGF signaling occurs when extracellular ligands (blue) bind to FGF receptors (FGFR1-4). Major downstream pathways illustrated here are (from left to right) Protein kinase B (PKB)/Akt, mitogen-activated protein kinase (MapK), and Phospholipase Cγ (PLCγ) pathways. These pathways lead to changes in gene transcription, cell proliferation, cell differentiation and cell survival (red arrows). Adapted from Iwata et al., 2009.
Figure 6. A simplified representation of Wnt/β-catenin (canonical) and β-catenin-independent (non-canonical) signaling pathways. In the absence of Wnt ligand (far left), a “destruction complex” composed of Axin, glycogen synthase kinase 3 (GSK3) and adenomatous polyposis coli (APC) causes the degradation of β-catenin. Binding of Wnt ligands (blue) to Frizzled (FZD) causes dishevelled (DVL) to inhibit the formation of the “destruction complex,” allowing β-catenin to activate transcription factors. Ligand binding is facilitated by glypicans co-receptors and low-density lipoprotein receptor-related proteins (LRP). The extracellular lipase Notum can cleave glypicans and inhibit Wnt/β-catenin signaling. The β-catenin-independent signaling pathways (far right) include the planar cell polarity (PCP) and Wnt/Calcium pathways, which act through different Wnt ligands than Wnt/β-catenin signaling. The effect of different molecules is indicated as arrows (activation) or dead ends (inhibition). Adapted from Chien et al. 2009.
Figure 7. Thermal cycle parameters for polymerase chain reactions (PCR). A-B) Parameters specific for enhanced green fluorescent protein (EGFP) (A) and fgf20a (B) genotyping primers. C) “Touchdown” parameters for reverse transcription PCR (RT-PCR) of tail and barbel RNA for primers listed in Table 1. Vertical dashed lines mark the beginning and end of each repeated cycle. Asterisks indicate changes in time or temperature per cycle.
Figure 8. A schematic of the regeneration experiment using heat-shock inducible transgenic *dnfgfr1* zebrafish. Tg(*hsp70l:dnfgfr1-EGFP*) fish were bought from ZIRC as embryos and reared until adulthood. ZIRC fish were genotyped by outcrossing adult males to wild type females (A) and observing offspring for transgenic GFP expression after heat-shock treatment (B). Clutches of 50:50::transgenic:wildtype fish were reared for 3-6 months, after which barbel surgeries and fin clips were performed (C). After waiting 24 hours for wound healing to occur, fish were haphazardly placed in heat-shock (HS) or non-heat shock (NHS) tanks (D). Once daily heat shocks were automatically applied to the experimental animals for 14 days; control animals were kept at a constant temperature. Barbels and caudal fins were then collected for analysis (E).
Figure 9. A schematic of the regeneration experiment using the temperature sensitive $fgf20a^{zp3}$ mutant fish line. $Fgf20a^{zp3}$ fish were bought from ZIRC as embryos and reared until adulthood. ZIRC fish were genotyped by performing caudal fin amputations and collecting DNA. Polymerase chain reaction (PCR) was performed using primers designed to produce a restriction enzyme digestion site in the presence of the mutation. After restriction enzyme digestion, fragments were analyzed by gel electrophoresis (A). Homozygous recessives were crossed to wild types (B) generating 100% heterozygotes. Heterozygous female offspring were then back crossed to their original ZIRC homozygous recessive fathers (C) to produce a clutch of 50:50:homozygous:heterozygous fish. Offspring were reared 3-6 months, after which barbel and caudal fin surgeries were performed (D). After 24 hours at 28°C to allow for wound healing, fish were haphazardly split into tanks at a constant 33°C or 28°C (E). Barbels and caudal fins were allowed to regenerate for 14 days and then collected for analysis (F).
Figure 10. A schematic of the regeneration experiment using the small-molecule Wnt inhibitor IWR-1. 
A) Wild type fish were crossed to produce a new clutch. Offspring were reared for 3-6 months, after which barbel and caudal fin surgeries were performed (B). After 24 hours to allow for wound healing, fish were split into tanks of 10 mM IWR-1 dissolved in 0.15% DMSO, or 0.15% DMSO (C). Solutions were changed daily. After 10 days, tissue was collected for analysis (D).
Figure 11. Schematic of appendage measurements used to quantify ZMB regeneration.  
A) Representation of dissected barbels while being analyzed in ImageJ.  Red-orange indicates original tissue on the control barbel and stump, while green-blue indicates regenerated tissue.  Barbel lengths were measured using the segmented line tool in ImageJ to plot manually selected points (white squares) along the midline of each barbel.  Total length (TL) was measured on the control barbel from the proximal end of the central rod to the tip of the barbel.  Stump length (SL) was measured on the regenerated barbel from the proximal end of the central rod to the amputation plane (black arrowhead).  Regeneration length (RL) was measured on the regenerated barbel from the amputation plane to the tip of the barbel.  
B) An example of paired barbels imaged in a Petri dish of saline.  White arrowheads indicate the proximal end of the central rod.  The black arrowhead indicates the amputation plane.  
C) The equation for finding percent regeneration using the measurements from the barbel segments in B.

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\text{% regeneration} = \left( \frac{\text{RL}}{\text{TL} - \text{SL}} \right) \times 100
\]

\[
= \left( \frac{1.78}{3.66 - 0.33} \right) \times 100 = 53.5\%
\]
**Figure 12.** FGF and Wnt signaling members are expressed during barbel regeneration. Reverse-transcription polymerase chain reaction (RT-PCR) analysis comparing 4 days post surgery (dps) regenerating barbel and 3 days post surgery regenerating tail tissue (control) using primers for FGF receptors, ligands and downstream targets (A) and the canonical Wnt signaling target, *lef1* (B). β-actin served as a positive control. All samples produced a band of the expected size. C) Our lab has previously identified many non-canonical Wnt pathway members expressed during barbel regeneration. RT-PCR of 3 and 7 dps regenerating barbels were compared to a sample of adult barbel shaft (control).
Figure 13. FGFR1 inhibition reduces ZMB regeneration. White light (A) and green fluorescent light (B) images show inhibition of fin regeneration and enhanced green fluorescent protein (EGFP) expression, indicating activation and expression of dnfgfr1. B' EGFP expression was observed in barbels as well. C) Comparison of regenerate percentages among heat-shock wild type (red circle), heat-shock dnfgfr1 (red squares) and non-heat shock wild type and transgenic zebrafish (black triangles). Bars = median values. Paired wild type (D) and dnfgfr1 (E) barbels after 14 days of heat-shock treatment. These barbels were dissected from fish of the same standard length. L = left, amputated barbel and R = right, unamputated barbel. Black dotted lines are amputation planes. In the absence of heat shock, wild type and dnfgfr1 fish cannot be distinguished and are grouped together. F) Magnification of the dnfgfr1 regenerated barbel shown in E, showing an epithelium and mesenchyme past the amputation plane. e = epithelium, m = putative mesenchymal cells and cr = central rod of connective tissue.
Figure 14. Heat-shock treatments do not inhibit barbel regeneration in wild types. Wild types were treated with heat shocks of >37°C for 1 hour/day (red) or without heat-shock cycles (black). Barbels were collected after 14 days (circles) or 21 days (diamonds). Bars = median values.
Figure 15. The temperature sensitive mutation $fgf20a^{zp3}$ has no effect on ZMB regeneration. Wild type (A) and $fgf20a^{zp3}$ (B) caudal fin amputations demonstrate that wild types regenerate normally at 33°C, while $fgf20a^{zp3}$ homozygotes are inhibited. The white dotted line and white arrow show the amputation planes. C) Comparison of barbel regeneration among wild types (circles) and $fgf20a^{zp3}$ mutants (squares) for two different 14-day temperature treatments: 33°C (red) or 28°C (black). Bars = median values. Regeneration is expressed as a percent (see Methods). D-E) Paired wild type (D) and $fgf20a^{zp3}$ (E) barbels treated at 33°C for 14 days. These barbels were dissected from the same fish shown in (A) and (B) respectively, which were of the same standard length. L = left, amputated barbel and R = right, unamputated barbel. Black dotted lines are amputation planes. F) Magnification of the $fgf20a^{zp3}$ regenerated barbel in E, showing regenerated taste buds, melanophores, blood vessels and connective tissue.
Figure 16. The small-molecule Wnt inhibitor IWR-1 causes decreased ZMB regeneration. Control caudal fin (A) and barbel (B) of the same fish demonstrate that fish held in DMSO can regenerate. IWR-1 treated caudal fins (C) and barbels (D) of the same fish show inhibited regeneration. E) Comparison of wild types treated with IWR-1 (blue circles) or DMSO (black squares) after 10 days post surgery. Dotted lines and whit arrowhead are amputation planes. Bars = median values. Regeneration is expressed as a percent (see Methods).
Figure 17. Overexpression of notum1a inhibits tail and barbel regeneration. A) Heat shocks caused complete inhibition of fin regeneration in some individuals. B) All non-heat shock fish regenerated fins normally. C) Paired barbels of the fish shown in (A) indicate that barbel regeneration was dramatically inhibited. D) All non-heat shock fish regenerated barbels normally. L = left, amputated barbel and R = right, unamputated barbel. White arrowheads and dotted lines are amputation planes. Scale bars = 1 mm.