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Understanding Connexin43 dependent Skeletal Morphogenesis - Progress made and Promises ahead

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Understanding Connexin43 Dependent Skeletal Morphogenesis – Progress Made and Promises Ahead

by

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Understanding Connexin43 Dependent Skeletal Morphogenesis – Progress Made and Promises Ahead

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LIST OF ABBREVIATIONS

Acan	Aggrecan
alf ^{dty86}	*another long fin*
And	Actinodin
BLE	Basal layer of epithelium
BMP	Bone morphogenetic protein
βME	Beta- mercaptoethanol (2- mercaptoethanol)
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
Col	collagen
Cx	Connexin
DAPI	4′, 6-diamidino-2-phenylindole
DIG	Digoxygenin
Dpa	Days post amputation
Dpe	Days post electroporation
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FGF	Fibroblast growth factor
FN	Fibronectin
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
GJIC	Gap junction intercellular communication
H3P	Histone-3-phosphate
HA	Hyaluronic acid
Hapln1a	Hyaluronan and proteoglycan link protein 1a
HAS	Hyaluronic acid synthase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hpa	Hours post amputation

HRP	Horseradish peroxidase
Hsp47	Heat shock protein 47
Hyb	Hybridization solution
ihh	*indian hedgehog*
KD	Knockdown
KDa	Kilo Daltons
LAM	Laminin
LP	Link protein
MABT	Maleic acid buffer with tween 20 solution
MAM	Meprin, Protein tyrosine phosphatase μ
MM	Mismatch
MMP	Matrix metallo protease
MO	Morpholino
mRNA	Messenger ribonucleic acid
μg	Microgram
μL	Microliter
μm	Micrometer
μM	Micromolar
NBT/BCIP	Nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate
Nrp2a	Neuropilin2a
ODDD	Occulodentaldigital dysplasia
OI	Osteogenesis imperfecta
PBS	Phosphate buffered saline
PBST	Phosphate buffered tween 20 saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PG	Proteoglycan
PI	Propidium iodide
PlxnA3	PlexinA3

qRT-PCR	Quantitative Real time polymerase chain reaction
RA	Retinoic acid
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
sema3d	*semaphorin3d*
serpinh1	*serine protease inhibitor 1*
shh	*sonic hedegehog*
sof ^{b123}	*short fin*
SPC	Skeletal precursor cell
SSC	Saline sodium citrate solution
tRNA	Transfer RNA
Vcan	Versican
wnt	*wingless*

ABSTRACT

Skeletal morphogenesis is a complex process through which bones grow to their correct size and shape and find their location in the body. A number of signaling pathways are involved in proper regulation of this process. Among them, Gap Junctional Intercellular Communication (GJIC) is important for proper bone growth and patterning. In our lab, we use regenerating caudal fin of a zebrafish to study skeletal morphogenesis. Our examination of a fin length mutant sof^{b123} has identified a gap junction protein called Connexin43 (Cx43). Cx43 regulates growth and patterning during regeneration by promoting cell proliferation and suppressing joint formation, respectively. The function of Cx43 is conserved in vertebrates. In humans, mutations in *Cx43* cause a malformation of craniofacial and limb skeleton, known as Occulodentodigital dysplasia (ODDD). In mice and chicks as well, similar defects in bone development are observed following mutations in the gene *Cx43*. What we do not understand, however, is how does communication through a gap junctional channel give rise to such tangible phenotypes? To answer this question, we need to identify how *cx43* regulates other genes during regeneration in zebrafish. We compared two fin length mutants with opposite phenotypes through a microarray. The mutant sof^{b123}, which shows reduced fin length, reduced segment length and reduced level of cell proliferation, demonstrates a lower level of *cx43* mRNA and protein. The other mutant alf^{dty86}, on the other hand, shows an elevated level of *cx43* expression and demonstrates increased fin length, longer segments due to stochastic joint placement and increased level of cell proliferation. Identification of genes that are upregulated in alf^{dty86} and downregulated in sof^{b123} are hypothesized to be part of the Cx43 dependent growth and patterning pathway during regeneration.

One of the genes identified and validated through this microarray is a gene called *serpinh1b*, which codes for a protein called Hsp47. The first part of my thesis demonstrates that *serpinh1b* is functionally downstream of *cx43*. Knockdown of Hsp47, recapitulates the *sof* phenotype. Furthermore, knockdown of Hsp47 results in disruption of collagen II dependent actinotrichia leading to defective growth and patterning events during regeneration.

Another gene previously validated from the microarray is *semaphorin3d* (*sema3d*). Sema3d mediates the downstream effect of Cx43 through interactions with its two putative receptors, Neuropilin2a (Nrp2a) and PlexinA3 (PlxnA3). The second part of my thesis characterizes the significance of Sema3d binding to its receptors. Our data suggest that Sema3d binding reduces homomeric interaction between Nrp2a receptors, and that PlxnA3 can act as a competitor for Nrp2a homo-oligomerization. We also hypothesize that the juxtamembrane MAM domain of Nrp2a receptor can act as an alternate Sema3d binding domain, besides its usual role as a facilitator of receptor oligomerization.

The third part of my thesis characterizes the AB9 fibroblast cell line, derived from regenerating caudal fin of an adult zebrafish as a complementary tool for pilot studies on gene regulation. Our data suggest that AB9 cells express major players of the Cx43 dependent growth and patterning pathway. We believe that this cell line can be a used as a simpler tool to do gene manipulation studies, as well as to complement our findings *in vivo*.

In conclusion, I believe that my thesis encompasses three different aspects of studying skeletal patterning events during regeneration. It characterizes one more member of the Cx43 dependent growth and patterning pathway during regeneration and identifies its significance in formation of an important fin structure, the actinotrichia. The findings suggest that actinotrichia co-ordinates between the dividing cells of the blastema and the differentiating cells in the lateral patterning compartment. My thesis also validates the functional significance of ligand-receptor interactions and suggests possibilities on how it might affect cell division and differentiation events. My thesis also introduces a new cell culture tool to be used in the future to perform pilot studies on genes involved in skeletal regeneration in zebrafish.

Chapter 1: Introduction

1.1 . Skeletal morphogenesis - Why do we look like what we look like?

The vertebrate skeleton provides a framework to support the body, maintain its shape and protect the vital organs. How an organism attains its exact shape and size is a question still unanswered. Skeletal morphogenesis or development of the vertebrate skeleton is a complex process; it requires the precise regulation of a number of signaling pathways controlling cell proliferation, differentiation, survival and organization. While observing limb development across various species, numerous similarities provide us with evidence that underlying molecular mechanisms are conserved during development (Iovine 2007). Drosophila wings and mouse limbs have evolved independently; however both of them use genes from the *Fgf*, *hedgehog* and *Wnt* families during limb or wing development. On the other hand, animals belonging to the same species often show differences in bone size and shape. For example, subtle difference in the development of craniofacial skeleton is observed in closely related species of teleost fish (He et al. 2009a). Why are we so similar? And what mechanisms give rise to these differences?

1.2. Understanding skeletal morphogenesis to treat skeletal diseases:

Skeletal development starts with the condensation of the mesenchymal cells of different lineages. The neural crest cells give rise to the craniofacial bone, the sclerotome compartment of the somites contribute to most axial skeletons, cells from the lateral plate mesoderm ultimately give rise to the limb skeleton. Each of these three cell lineages comes from different parts of the embryo and this positional identity is important during early patterning events. Needless to say, cell-cell communication is critical for proper patterning of the skeleton and identification of the signaling pathways involved in this process is of utmost interest. Understanding skeletal development in its own right, is a fascinating subject. But understanding it in order to be able to treat skeletal diseases makes it more motivating.

1.3. Zebrafish as a model organism to study development, disease and regeneration

Zebrafish are teleost fish that regenerate multiple structures including fins, optic nerves, scales, heart and spinal cord (Poss et al. 2003). For decades, zebrafish has been used as a model system to study limb growth and skeletal morphogenesis. It is easier and cheaper to maintain a population of zebrafish in the lab compared to other vertebrates. The zebrafish genome sequencing is near completion and genetic mutations can be easily generated and identified. Several disease phenotypes in humans can be recapitulated in zebrafish mutants. Regenerating caudal fin of an adult zebrafish is a rich source of bone and joints (Figure 1.1) and in our lab we use it as a tool to study how new bones and joints are formed.

1.4. Stages of epimorphic regeneration – formation of Blastema

Regeneration is the process by which damaged or lost parts are replaced in an organism. Epimorphosis is the regeneration of lost tissue through de-differentiation of existing adult tissue. The process of epimorphic regeneration can be divided into four parts (Figure 1.2), (i) Wound closure – Following amputation, within 1 to 3 hours, a thin layer of epidermal cells migrate to cover the wound. No cell proliferation occurs during this process, confirmed by BrdU assays (Poleo et al. 2001). An F-actin purse string is formed around the wound leading to its rapid contraction (Martin and Lewis 1992). (ii) Within 12 to 18 hours post amputation (hpa), the actin purse string disappears; epithelial cells migrate and cover the stump thus forming the wound epidermis (Poss et al. 2000; Kawakami et al. 2004). (iii) By 18 to 24 hpa, a specialized structure called the blastema is formed at the distal end of mesenchyma. The blastema contains fate restricted cell lines with distinct origins which coordinate with each other to regenerate the lost tissue (Gemberling et al. 2013; Tanaka and Reddien 2011). The formation of wound epidermis and blastema is absolutely necessary for epimorphic regeneration (Goss 1956). Within 24 hpa, the blastema is divided into two major compartments which are morphologically indistinct. The cells in the distal most blastema divide slowly, providing guidance cues for outgrowth to cells located in the proximal region. Cells in the proximal blastema express

proliferation markers like proliferating cell nuclear antigen (PCNA). They proliferate rapidly, migrate laterally and ultimately regenerate amputated tissue. No defined boundary exists between these two compartments; however there is a proliferation gradient that extends approximately 50µm. Immediately close to proximal blastema lies a zone containing moderately proliferating cells and is known as patterning zone (PZ). This zone contains scleroblasts (skeletal precursor cells) and most likely, differentiating mesenchymal cells. They migrate to their proper locations and differentiate to regenerate lost tissue. Bone formation in regenerating fins follows intramembranous ossification (direct ossification of bone matrix without forming a cartilaginous precursor) (Landis and Geraudie 1990).

The formation of blastema is an integral part of epimorphic regeneration. Regarding the origin of blastema, there are currently two major hypotheses. So far, most data point to de-differentiation of cells, but we cannot rule out presence of pluripotent stem cells either (Singh et al. 2012). In case of de-differentiation, mature cells lose their fully differentiated morphology, transiently convert into a more proliferative state and provide necessary cells in the newly formed blastema. Recent studies in salamander limbs and zebrafish fins have shown that most of the cells in the blastema are lineage restricted. For example, osteoblasts can only contribute to skeletal growth during regeneration, in both zebrafish and *axolotl* (Knopf et al. 2011; Kragl et al. 2009; Sousa et al. 2011; Tu and Johnson 2011). But this does not necessarily preclude the existence of pluripotent stem cells. In fact, fin ray regeneration is uninhibited in fins from which all osteoblasts have been genetically ablated (Singh et al. 2012). This observation partially supports the alternative hypothesis, which suggests that the blastema derives its cell population from dormant stem cells which become activated following amputation (Nakatani et al. 2008). However, the possibility of trans-differentiation cannot be ruled out either. Even though cells of the regenerating fin were observed to be fate restricted, in complete absence of one type of cells, closely related tissue might be able to provide required cells to regenerate lost tissue. However, more studies are required to prove if this is indeed true. A number of signaling pathways are involved in fin regeneration and include but are not limited to the Wnt/β catenin, FGF, IGF and RA signaling (Figure 1.2)(Blum and

Begemann 2012; Chablais and Jazwinska 2010; Poss et al. 2000; Stewart et al. 2014; Stoick-Cooper et al. 2007; Wehner et al. 2014; Whitehead et al. 2005). For example, both canonical Wnt signaling pathway and the FGF signaling pathway are involved in wound healing, blastema formation and regenerative outgrowth whereas Hedgehog, Activin-βA and RA signaling are involved in patterning (Tal et al. 2010). Blocking any of these pathways prevents regeneration either by inhibiting blastema formation or preventing the formation of a wound epithelium.

1.5. Fin growth in zebrafish – a model to study skeletal morphogenesis

The ray-fins of actinopterygian fish like zebrafish are composed of three different types of skeletal structures – the distal skeleton consists of lepidotrichia (calcified bone matrix) and the actinotrichia. The proximal skeleton contains the basal endochondral bone. It has been suggested that lepidotrichia is similar to both bone and cartilage (Avaron et al. 2006; Padhi et al. 2004). The fins grow indefinitely and are capable of regeneration within two weeks of amputation with the replacement of bone, blood vessels, nerve cells, pigments, fibroblast like cells and epidermis (Akimenko et al. 2003; Poss et al. 2003). The zebrafish fin skeleton has a dermal origin. The caudal fin is composed of 16-18 bony fin rays or lepidotrichia, each ray is comprised of a number of segments separated by fibrous joints (Figure 1.1). Each fin ray has an inner layer of mesenchymal cells like fibroblasts, nerves and blood vessels, surrounded by hemirays (Figure 1.3). So far, nine distinct lineage classes of cell line have been identified in the caudal fin, which include epidermis, melanocyte / xanthophore, iridophore, intraray glia, lateral line, osteoblast, dermal fibroblast, vascular endothelium and resident blood (Tu and Johnson 2011) (Figure 1.3, Bottom). A thin multilayered epithelium surrounds the fin ray. Each hemiray includes collagen like fibrils termed actinotrichia at the distal end, which acts as a substrate for osteoblast alignment and secretion of bone matrix (Becerra et al. 1983). Actinotrichia is synthesized by Actinotrichia Forming Cells (AFC) (Figure 1.3, Top). Besides playing a structural role, actinotrichia also plays an important morphogenic role. It has been suggested that actinotrichia regulates differentiation of the skeletal precursor

cells and thus is important in ray patterning (Santamaria and Becerra 1991). Mutants with defective actinotrichia develop abnormal lepidotrichia or bone matrix (Duran et al. 2011). Bone forming cells and joint forming cells, collectively known as skeletal precursors, are located laterally, adjacent to the bone matrix (Figure 1.3). This lateral compartment of blastema is highly organized (Figure 1.3, Top). At the distal end of this compartment we find *runx2* positive osteoblast precursors, which is followed by *sp7* (*osterix*) positive committed osteoblasts. At the most proximal region we find *osteocalcin*-positive mature osteoblasts (Brown et al. 2009; Wehner and Weidinger 2015). During fin regeneration, osteoblasts switch from a mature bone matrix producing, non-cycling state to a rapidly cycling less differentiated osteoblast precursor state and vice versa (Knopf et al. 2011; Sousa et al. 2011; Stewart and Stankunas 2012), thus allowing rapid replacement of lost bone. The actinotrichia forming cells (AFC) are located directly medial to the osteoblast progenitors (Zhang et al. 2010) (Figure 1.3, Top). Osteoblasts align themselves across the collagen like actinotrichia fibers and secrete bone matrix to form lepidotrichia. Bone growth occurs throughout life by the distal addition of bony segments (Goss and Stagg 1957; Haas 1962).

The joint forming cells are a subpopulation of the lateral skeletal precursor cells. They express the transcription factor *even-skipped 1* (*evx1*) (Borday et al. 2001), *distal-less homeobox 5a* (*dlx5a*) and *matrix metalloproteinase 9* (*mmp9)* (Ton and Iovine 2013). The joint forming cells condense on the surface of the uninterrupted bone matrix at its presumptive location. They exhibit an elongated morphology and are present in a single row. As they mature, they are divided into two rows and become more rounded (Sims et al. 2009). The mesenchymal compartment still remains continuous, the physical separation to form a segment only occurs in the bone matrix. Since the joints are connected by connective tissue, they are called 'fibrous' joints (Borday et al. 2001). Once formed, the segments do not increase in length; however the rays increase in diameter. The mechanism for addition of new segments and maintaining their size is however, still poorly understood.

1.6. Fin length mutants – A tool to identify important genes during skeletal morphogenesis

An excellent way to identify important regulators of skeletal morphogenesis is by examining fin length mutants. So far, a number of fin length mutants have been identified. For example, complete loss of fin rays is observed in *finless* and *nackt* mutants (Harris et al. 2008). The *long fin* (Iovine and Johnson 2000) and *rapunzel* (Goldsmith et al. 2003) mutants affect segment number whereas the *short fin* (sof^{b123}) (Iovine and Johnson 2000) and *another long fin* (alf^{dty86}) (van Eeden et al. 1996) mutant affects segment length (Figure 1.4). Since our lab is interested in understanding the mechanism underlying growth and patterning during regeneration, these mutants serve as excellent tools to study skeletal morphogenesis.

1.7. Connexin43 is required for skeletal development across species

Since regeneration appears to recapitulate ontogeny and is 3 to 5 times faster, our lab is interested in studying the underlying mechanisms for skeletal morphogenesis using zebrafish caudal fin regeneration as a tool. We have a number of fin length mutants. The sof^{b123} mutant exhibits short fins and short segments (Iovine and Johnson 2000) (Figure 1.4), and reduced cell proliferation compared to the *wild type* (WT) (Iovine et al. 2005). This phenotype has been attributed to a mutation in the non-coding region of the gap junctional gene *cx43* (Iovine et al. 2005). Gap junctions are proteinaceous channels mediating exchange of molecules (<1.2kDa) between neighboring cells. Six connexin subunits together form a connexon or hemichannel: two connexons from two neighboring cells come together to form one functional gap junction channel (Figure 1.5). It is still not clear how defects in this type of direct cell to cell communication can give rise to tangible defects in bone growth. Knockout mutation in the C*x43* gene in mice results in blockage of outflow tracts causing death several hours after birth (Reaume et al. 1995). Further examination of skeleton has shown delayed ossification of bone (Lecanda et al. 2000). In humans, lesions in *CX43* give rise to ODDD (occulodentodigital dysplasia), an autosomal dominant disease which leads to craniofacial and limb skeleton malformation (Flenniken

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et al. 2005; Paznekas et al. 2009). Targeted knockdown of Cx43 in chicks also causes ODDD like phenotypes (McGonnell et al. 2001). Morpholino mediated knockdown of Cx43 in zebrafish embryos causes defective heart folding, reduced hematopoiesis and sometimes, small eyes (Iovine et al. 2005). These studies suggest that loss of Cx43 can cause developmental heart and bone defects from zebrafish to human. Therefore, the function of Cx43 is conserved in vertebrates during skeletal development.

1.8. Cx43 mediates growth and patterning in regenerating fins

Another fin length mutant, known as *alf*^{dty86} (van Eeden et al. 1996) exhibits opposing phenotypes with increased cell proliferation, longer fins and longer segments due to stochastic joint failure (Figure 1.4). This overgrowth phenotype is due to a mutation in Kcnk5b, a two-pore domain potassium channel which leads to increased K+ conductance and hyperpolarization (Perathoner et al. 2014). Interestingly, *alf*^{dty86} overexpresses *cx43* (Sims et al. 2009). Knockdown of Cx43 in *alf*^{dty86} rescues joint formation providing us with evidence that overexpression of Cx43 leads to joint failure (Sims et al. 2009). Based on both loss and gain of Cx43 function analysis, we suggest that Cx43 is involved in two separate pathways during skeletal morphogenesis – promoting cell proliferation and inhibiting joint formation. One could suggest however that cell proliferation and segment length are dependent on each other, and reduced cell proliferation causes shorter segments. Segment length in similar sized fins is the same in young rapidly growing fish and old slower growing fish (Iovine and Johnson 2000). It has also been shown that blockage of Shh or Fgfr1 signaling causes reduced cell proliferation, but does not affect segment length (Lee et al. 2005; Quint et al. 2002), providing evidence that cell proliferation and joint formation, though concurrent, are independent events. Therefore, Cx43 is involved in two separate events during skeletal development; it promotes cell proliferation and inhibits joint formation.

Now the question is how does Cx43 regulate such events? The majority of *CX43* mutations linked to ODDD phenotypes generate defective ionic / dye coupling between cells (Lai et al. 2006; Roscoe et al. 2005; Seki et al. 2004; Shibayama et al. 2005). The mutant allele of *cx43* linked to zebrafish *sof*^{b123} phenotype shows low junctional

conductance and abnormal voltage-gating properties (Hoptak-Solga et al. 2007). These reports suggest that proper GJIC through Cx43 dependent gap junctional channels is very important for proper patterning events. But how does the exchange of these small molecules, which cannot be proteins or full length DNA, ultimately gives rise to differences in cell division and differentiation?

1.9. Identification of different players in Cx43 dependent skeletal morphogenesis

In order to answer the question, we utilized the two fin length mutants *sof* ^{b123} and *alf* ^{dty86}, which exhibit opposite phenotypes due to the presence of different levels of Cx43. We looked for genes upregulated in *alf* ^{dty86} and downregulated in *sof* ^{b123}, using a microarray. One of the genes we identified and validated is *semaphorin3d (sema3d),* a secretory signaling molecule (Ton and Iovine 2012). Semaphorins were originally identified as chemorepellent cues for axons in developing nervous system. In regenerating fins, *sema3d* is expressed in the skeletal precursor cells (Figure 1.6). Knocking down Sema3d recapitulates the *sof* ^{b123} phenotype. In *alf* ^{dty86}, knocking down Sema3d has been shown to rescue joints providing further proof that Sema3d is important in skeletal patterning (Ton and Iovine 2012). Two of the high affinity receptors for semaphorins are plexins and neuropilins. We have observed expression of *plexina1* (*plxna1*) and *plexina3* (*plxna3*) in the regenerating fin (Figure 1.6). The mRNA for *plxna1* is expressed mainly in distal-most blastema whereas the expression of *plxna3* is mainly localized in the skeletal precursor cells and the medial compartment of the regenerate (Ton and Iovine 2012). Among the different neuropilin receptors, we have seen expression of only *neuropilin2a* (*nrp2a*) in the regenerating fin, in particular in the distal blastema (Ton and Iovine 2012)(Figure 1.6). Morpholino mediated knockdown of PlexinA3 in regenerating fin caused reduced segment length, but did not affect cell proliferation. On the other hand, Nrp2a knockdown caused increased fin length and increased number of proliferating cells, but did not affect segment length (Ton and Iovine 2012). It can be deduced then that Sema3d is mediating Cx43 activity by interacting with PlexinA3 and inhibiting joint formation. Since Nrp2a acts as a negative signal for cell

proliferation, we believe that Sema3d binds to Nrp2a and represses its negative effect, thus promoting cell proliferation (Figure 1.7).

Another gene identified and validated from the microarray is *hyaluronan and proteoglycan link protein 1a* (*hapln1a*) (Govindan and Iovine 2014). The connective tissue is composed of three groups of proteins – collagens, proteoglycans (PG) and glycoproteins. Hapln1a, like the name suggests, 'links' hyaluronic acid (HA) and proteoglycans (PG) in the extracellular matrix (ECM). Knocking out Hapln1 in mice causes dwarfism and craniofacial abnormalities along with perinatal lethality (Watanabe and Yamada 1999). In humans, mutations in *HAPLN1* results in spinal osteoarthritis in aging Japanese women (Urano et al. 2011). During fin regeneration, *hapln1a* expression is *cx43* dependent and morpholino mediated knockdown of Hapln1a recapitulates *sof*[b123] phenotype of reduced fin length, reduced segment length and reduced level of cell proliferation. Moreover, knocking down Hapln1a destabilized HA in fins (Govindan and Iovine 2014). HA is synthesized by HA synthesizing enzymes (Has). In fins two different Has enzymes are expressed – Has1 and Has2. Has2 knockdown results in *sof*[b123] phenotype as well, though milder than Hapln1a knockdown, suggesting that other components of the Hapln1a dependent ECM are involved in the growth and patterning processes (Govindan and Iovine, *under review*). The PG components of Hapln1a dependent ECM that are expressed in the regenerating fin are Aggrecanb (Acanb) and Versicanb (Vcanb) (Govindan and Iovine 2015). Acanb knockdown results in reduced fin length, reduced segment length and reduced level of cell proliferation as well. Interestingly, the additive effects of knocking down both Has2 and Acanb is equivalent to Hapln1a knockdown indicating that both HA and Acanb are important components of Hapln1a mediated Cx43 pathway that regulates skeletal morphogenesis (Govindan and Iovine, *under review*). Among the glycoproteins, fibronectin and laminin are expressed during fin regeneration (Govindan and Iovine 2015).

1.10. Importance of Hsp47 – Collagen interaction in proper Cx43 dependent skeletal morphogenesis:

Collagens are one of the major components of ECM. They are found in all multicellular organisms and constitute of 25% of total body mass. There are more than 27 types of collagens, some are fibrillary – others are network forming or fibril associated. Every type of collagen contains three polypeptide chains (α chains) that form a triple helical structure, and can be homotrimeric or heterotrimeric (Viguet-Carrin et al. 2006). They often share a common Gly-X-Y repeat, where X can be any amino acid, but is generally a proline, and Y is often a hydroxyproline (Berg and Prockop 1973). The synthesis of collagen molecules is a tightly regulated process and a number of enzymes and molecular chaperones are involved in their proper folding and secretion (Figure 1.8). A precursor molecule called procollagen is produced initially. Procollagens have additional propeptide extensions at both the amino and carboxy terminal ends. Three procollagen chains wrap around each other through the formation of disulfide bonds at the amino terminal end that leads to the formation of a procollagen triple helix. This is followed by hydroxylation and glycosylation of specific proline and lysine amino acids. The procollagen molecules are then transported from ER to Golgi by binding to molecular chaperones and secreted. Specialized enzymes called procollagen proteinases then cleave the propeptide extensions from both ends of the molecule. The modified triple helical molecule is called collagen and undergoes further post translational modifications. Lysyl hydroxylases help in formation of crosslinks within and between different collagen molecules which ultimately align to form collagen fibrils and fibers (Figure 1.8). Among so many different types of collagens, zebrafish regenerating fins express Collagen (Col) I, II, IX and X (Avaron et al. 2006; Duran et al. 2011; Huang et al. 2009; Smith et al. 2006b). Col I and II are major components of both actinotrichia and lepidotrichia (Duran et al. 2011). Dominant mutations in gene encoding Col I gives rise to *chihuahua* (*chi*) mutant zebrafish which shows phenotypic similarities to human Osteogenesis imperfecta (OI) including abnormal bone growth and mineralization (Fisher et al. 2003b). Col IX is a non-fibrillar collagen that provides stability to the Col II dependent actinotrichia. Mutations in the *col9α1* gene cause the *prp* (*persistent plexus*)

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phenotype in zebrafish, characterized by persistent formation of vascular plexuses in the caudal fin which leads to defective patterning of the bony fin rays (Huang et al. 2009). The expression of *col10α1* has been observed in the basal layer of epithelium and skeletal precursor cells in the regenerating fin (Avaron et al. 2006; Smith et al. 2006b).

Cells like fibroblasts, osteoblasts and chondrocytes are responsible for collagen synthesis. The synthesis and proper folding of collagen molecules are complex and mutations in any of the critical biosynthesis and assembly steps can lead to aberrant collagen structure and functions. Mutations in *COL1A1* and *COL1A2*, genes encoding collagen I can give rise to Osteogenesis imperfecta (OI) or the 'brittle bone disease'. OI is characterized by multiple fractures in the bone, blue sclera, short stature, hearing loss and respiratory problems. Mutations in *COL2A1*, gene encoding collagen II, causes spondyloepiphyseal dysplasia (SED) which is characterized by short stature, myopia and hearing loss (Kannu et al. 2012).

More recently, mutations in other genes beside *Col I* have been identified in certain forms of OI (Baldridge et al. 2008; Barnes et al. 2006; Cabral et al. 2007; Morello et al. 2006; Van Dijk et al. 2009a; van Dijk et al. 2009b). For example, recessive mutations in *SERPINH1*, give rise to a severe form of OI in humans (Christiansen et al. 2010) and Dachshunds (Drogemuller et al. 2009). *SERPINH1*, belongs to the serpin (*ser*ine *p*rotease *in*hibitor) superfamily and codes for a protein called Heat Shock Protein 47 (Hsp47). Hsp47 is an ER resident protein that specifically binds to procollagen triple helix in the ER and transports it to the Golgi, thus preventing its premature aggregation and bundle formation in the ER (Figure 1.8). This transient association of Hsp47 to collagen molecules is pH dependent. Hsp47 binds to procollagen molecule in the pH-neutral ER and releases it in cis-Golgi which has a lower pH (Ishida and Nagata 2011; Satoh et al. 1996). *In vitro* assays have shown that Hsp47 can bind to collagen I to V with similar affinity (Natsume et al. 1994). In fibroblasts lacking Hsp47, triple helix formation of procollagen molecules are impaired and its secretion is delayed, leading to accumulation of procollagen in the ER (Ishida et al. 2006). Hsp47 knockout mice fail to survive beyond 11.5 days postcoitus (dpc) due to abnormal processing of collagen I and IV. The knockout embryos showed growth retardation and were very fragile. They also displayed

abnormally oriented epithelial tissues and ruptured blood vessels (Nagai et al. 2000). Chondrocyte specific knockout of Hsp47 in mice resulted in severe chondrodysplasia and abnormal endochondral bone formation due to decrease in collagen II and XI accumulation in the cartilage (Masago et al. 2012). The homozygous mice died just before or after birth due to cleft palate and breathing difficulties. They also displayed severely twisted limbs and deformed joints and exhibited bleeding in the joint region. These disease phenotypes make Hsp47 an interesting candidate responsible for bone and joint formation during skeletal morphogenesis. Indeed, *serpinh1b*, zebrafish homolog of *SERPINH1*, was identified through the microarray as a possible downstream effector of Cx43 dependent growth and patterning pathways during fin regeneration. In Chapter 2, I have discussed in detail the role of Hsp47 in skeletal morphogenesis. Hsp47 works in a Cx43 and Hapln1a dependent way and promotes cell proliferation and inhibits joint formation through the regulation of collagen II dependent actinotrichia (Bhadra and Iovine 2015).

1.11. Structural and Functional Significance of Sema3d – Nrp2a interaction:

Semaphorins are expressed widely and maintain a well-conserved structure across species. They are involved in cell migration and axonal growth cone guidance. There are eight classes of semaphorins discovered so far, they can be GPI-linked, transmembrane or secreted. Classes 1 and 2 are present in invertebrates only, classes 3 to 7 are found in vertebrates, and viruses contain class 8 semaphorins (Roth et al. 2009). Semaphorins in general contain a 500 amino acid long extracellular N-terminal sema domain, followed by a cysteine rich PSI (plexin, semaphorin, integrin) domain. Class 3 and Class 4 semaphorins undergo homodimerization which is necessary for their physical function(Koppel and Raper 1998). Semaphorins are involved in pattering of blood vessels, immune system, bone, heart and kidney development. Sema3A for example, is responsible for vascularization of bone and innervations of osteoblasts and osteoclasts during bone development (Roth et al. 2009). Sema7A on the other hand regulates osteoblast migration and osteoclast differentiation (Delorme et al. 2005).

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Class 3 semaphorins are secreted ligands and bind to the extracellular domains of receptors to exert their functions. Two of the high affinity receptors for semaphorins are plexins and neuropilins. Except class 2 semaphorins, all the others can bind to plexins directly (Negishi et al. 2005). Members of Class 3 semaphorins on the other hand, can bind to neuropilins (Huber et al. 2003).

Plexins are single transmembrane cell surface receptors. Semaphorin-plexin binding produces a repulsive signaling which is critical for proper axon guidance during development of neurons and also plays an important role in cell migration, patterning of blood vessels and immune response (Neufeld et al. 2012; Tran et al. 2007; Zhou et al. 2008). Defective signaling through plexins can give rise to cancer and neurological disorders and therefore plexins are emerging as new drug targets (Yazdani and Terman 2006). In vertebrates, nine plexins have been identified, divided in four classes (A,B,C,D) . All the four classes of plexins have very similar structural domains – an extracellular region that binds with semaphorins, which contains an N-terminal sema domain and multiple PSI and IPT (Ig domain shared by plexins and Transcription factors). This is followed by a single transmembrane spanning helix and an intracellular region, about 600 amino acids long (Figure 1.9). The intracellular region of plexin is composed of two domains – the GTPase activating domain (GAP) and the RhoGTPase binding domain (RBD) (Bos et al. 2007; Rohm et al. 2000; Vetter and Wittinghofer 2001). In order to activate the GAP domain, simultaneous binding of semaphorin on the extracellular side and RhoGTPase on the intracellular side is necessary (Oinuma et al. 2004; Turner et al. 2004). It is believed that semaphorins dimerize and bring together two plexin receptors as a step towards activation. Interestingly, the GAP domain in plexins exhibits an inactive conformation in absence of external stimuli, resulting in autoinhibition of the plexin intracellular region (He et al. 2009a).

Neuropilins are single transmembrane spanning proteins with a molecular weight of 120 kDa and are only found in vertebrates (Nakamura and Goshima 2002). Due to a short intracellular domain (Figure 1.9), this protein may need to bind to other molecules to

generate intracellular signaling. For signaling of secreted semaphorins, plexins from class A act as the signal transducers (Takahashi et al. 1999). In developing zebrafish embryos, Sema3d is required for early axon development and the two receptors that genetically interact with Sema3d are Nrp1a and Nrp2b (Wolman et al. 2004). Interestingly, in the same tissue system Sema3d can act as both an attractant and a repulsive guidance cue by interaction through different neuropilin receptors. Besides semaphorins, neuropilins can also interact with vascular endothelial growth factor (VEGF). Both Nrp1 and Nrp2 are essential for cardiovascular development as well. Nrp1 knockout is embryonically lethal due cardiovascular malformation (Kitsukawa et al. 1997). Nrp2 knockout mice on the other hand display abnormal development of the peripheral lymph vessels (Yuan et al. 2002). During embryogenesis in zebrafish, interaction between Nrp2a and VEGF receptor 2 (VEGFR2) are absolutely essential for proper intersegmental vessel (ISV) formation (Bovenkamp et al. 2004; Favier et al. 2006). Repulsive PlexinA3 signaling on the other hand is required for proper motor neuron growth cone guidance during early pathfinding events of motor axons (Palaisa and Granato 2007). From all these studies, we can infer that Sema-Nrp-Plxn signaling is essential for proper cell migration.

In regenerating fins, as mentioned previously; Nrp2a, PlxnA1 and PlxnA3 are three of the putative receptors of Sema3d that are expressed (Figure 1.6). Interestingly, the expression domains of *sema3d* and *plxna3* overlap in the lateral compartment containing skeletal precursor cells, but *nrp2a* is expressed away from them, in the distal most blastema (Ton and Iovine 2012). Whether their protein components interact is still not known. We suggest that Sema3d promotes cell proliferation through suppression of Nrp2a and inhibits joint formation through PlexinA3. However, whether these phenotypes are due to direct interaction between these receptors and their putative ligand is still unclear. Chapter 3 will discuss in detail about Sema3d and Nrp2a interaction and provide a hypothesis on how it might affect cell migration that is important for growth and patterning during regeneration.

1.12. A fibroblast cell line from Zebrafish caudal fin can be used to complement studies *in vivo*

Even though zebrafish caudal fin is an excellent tool to study genes regulating growth and patterning, it does have some limitations. It takes about 2 to 4 months to get mature zebrafish and manipulation of gene expression is not easy. Morpholino mediated knockdown of a gene requires well developed skills and off-target effects are an issue (Kok et al. 2015). Furthermore, manipulation of essential genes is difficult in adults. Chapter 4 will discuss in brief how AB9, a fibroblast cell line isolated from zebrafish caudal fin (Paw and Zon 1999), can be used for pilot studies on gene function that can then be tested in the regenerating fin (Bhadra et al, *manuscript accepted*).

The overall aim of this thesis is to characterize important regulators for skeletal morphogenesis. I believe this will, in time, help us identify proper targets for treating skeletal disease. Indeed, a number of skeletal diseases share several common phenotypes. It can only be assumed that some of these diseases share mutations in the same pathway as well. Our findings provide evidence that mutations in some of the identified downstream players of *cx43* during fin skeleton development produce similar disease phenotypes in other vertebrates and humans as well. In future, this knowledge can be utilized for drug designing in order to treat different skeletal diseases.
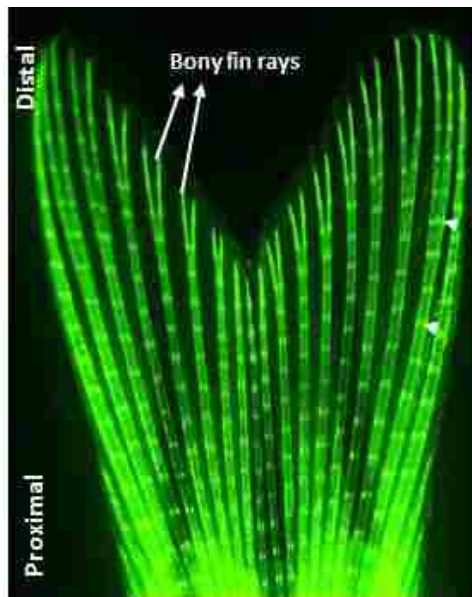
Figure 1.1: Zebrafish caudal fin contains bony fin rays and fibrous joints. Zebrafish caudal fin stained with calcein, a vital dye that binds to calcified matrix. Each bony fin ray contains a number of segments separated by joints (indicated by white arrow heads). New segments are added distally.
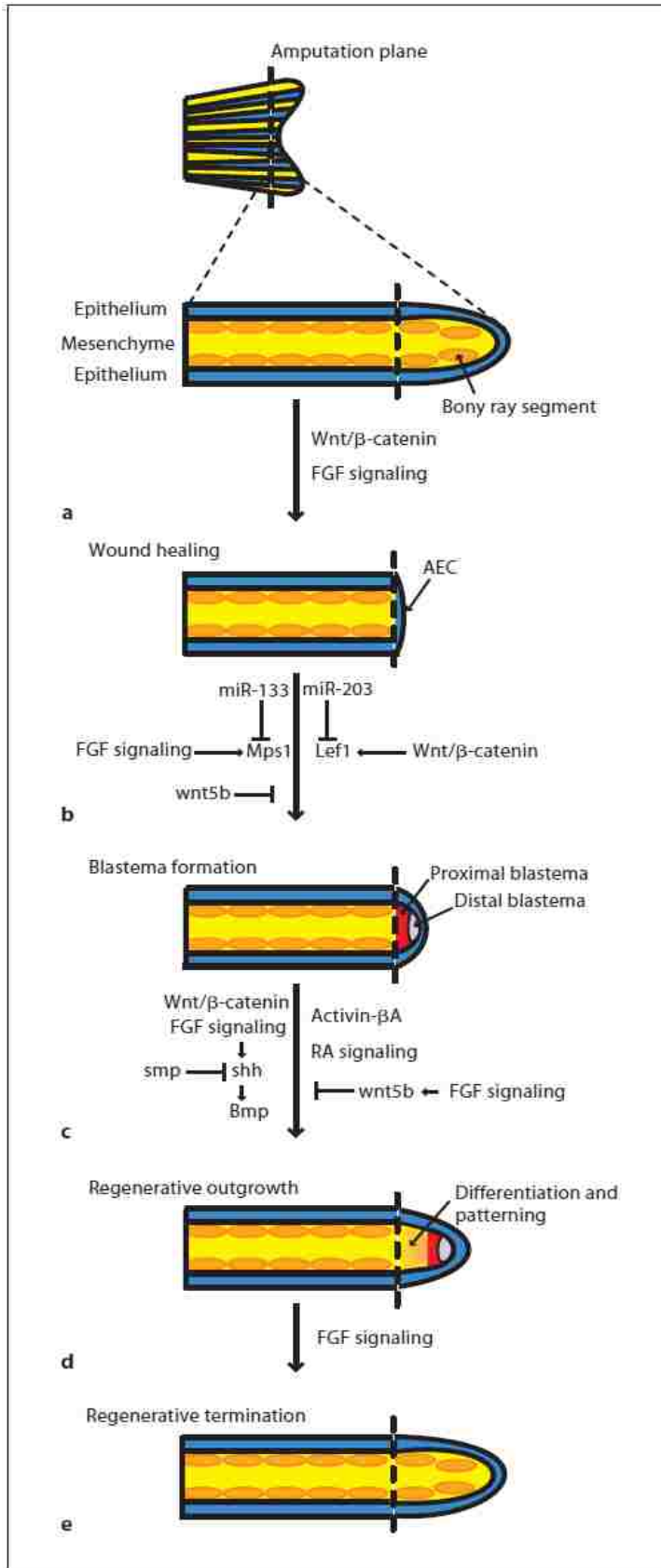
Figure 1.2: Multiple signaling pathways are involved in epimorphic regeneration of a caudal fin (Tal et al, 2009). (a) Each bony fin ray is covered by epithelium and encases a mesenchyme core. (b) Following amputation, the epithelial cells (blue) migrate to cover the wound and form AEC. (c) Blastema is divided into distal blastema (purple) and highly proliferative proximal blastema (red). (d) A moderately proliferative patterning zone is formed that contains skeletal precursor cells and fibroblasts. They migrate to their predetermine locations and regenerate lost tissue. (e) Regeneration occurs rapidly until the preamputation fin length is reached, and generally is completed within 14dpa.
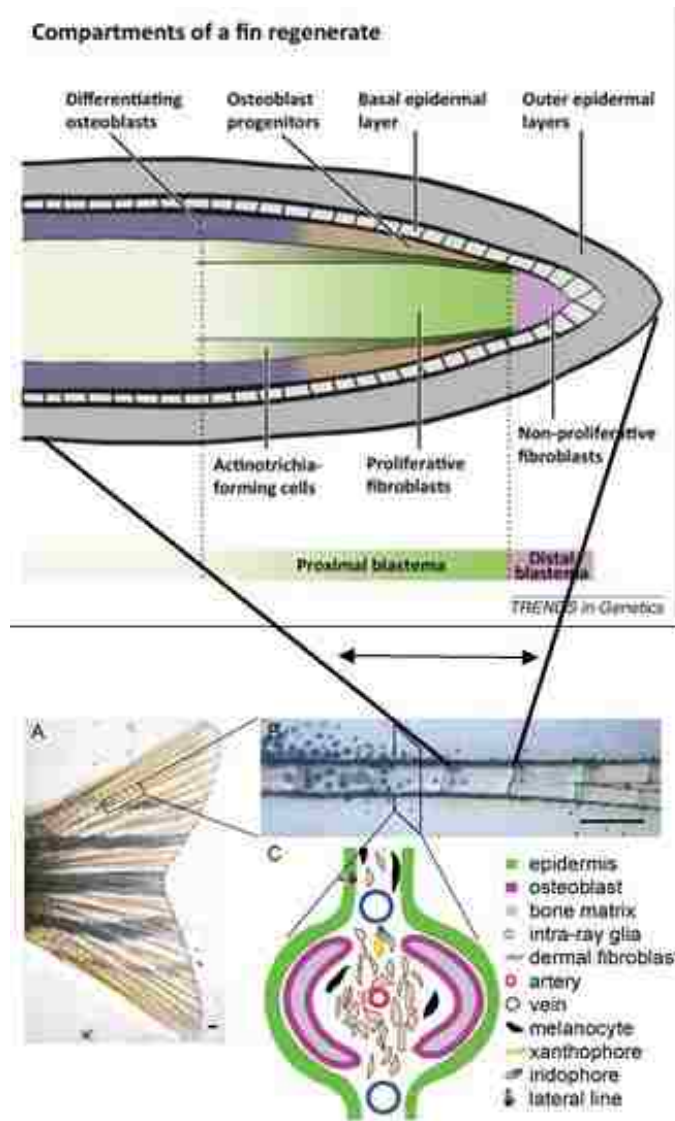
Figure 1.3: The longitudinal (Top) and transverse (bottom) section of a zebrafish caudal fin. Each fin ray is surrounded by an epithelial layer. Between the two hemirays, the mesenchymal compartment contains a diverse group of fate restricted cells. Top- from Wehner and Weidinger, 2015. Bottom – from Tu and Johnson, 2011.
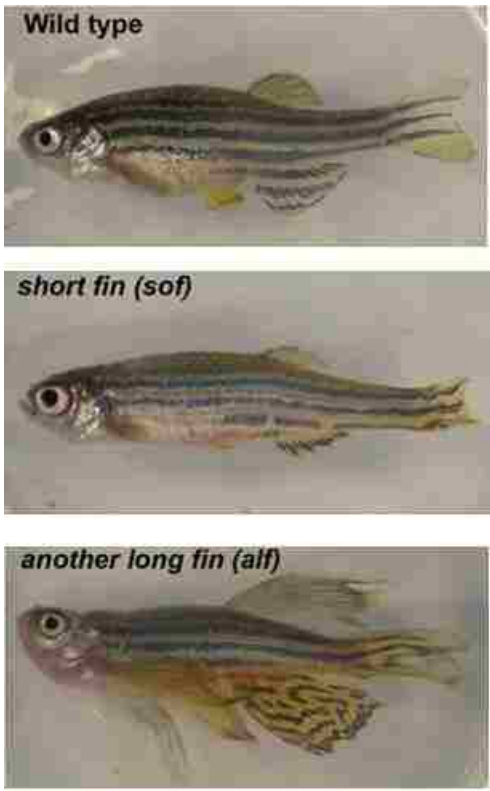
Figure 1.4: Fin length mutants show defects in growth and patterning.
The *short fin* (*sof^b123*) shows reduced fin length and segment length compared to the wild type (WT). The mutant *another long fin* (*alf^dty86*) shows longer fins and stochastic joint failure (reviewed in Ton & Iovine, 2013).
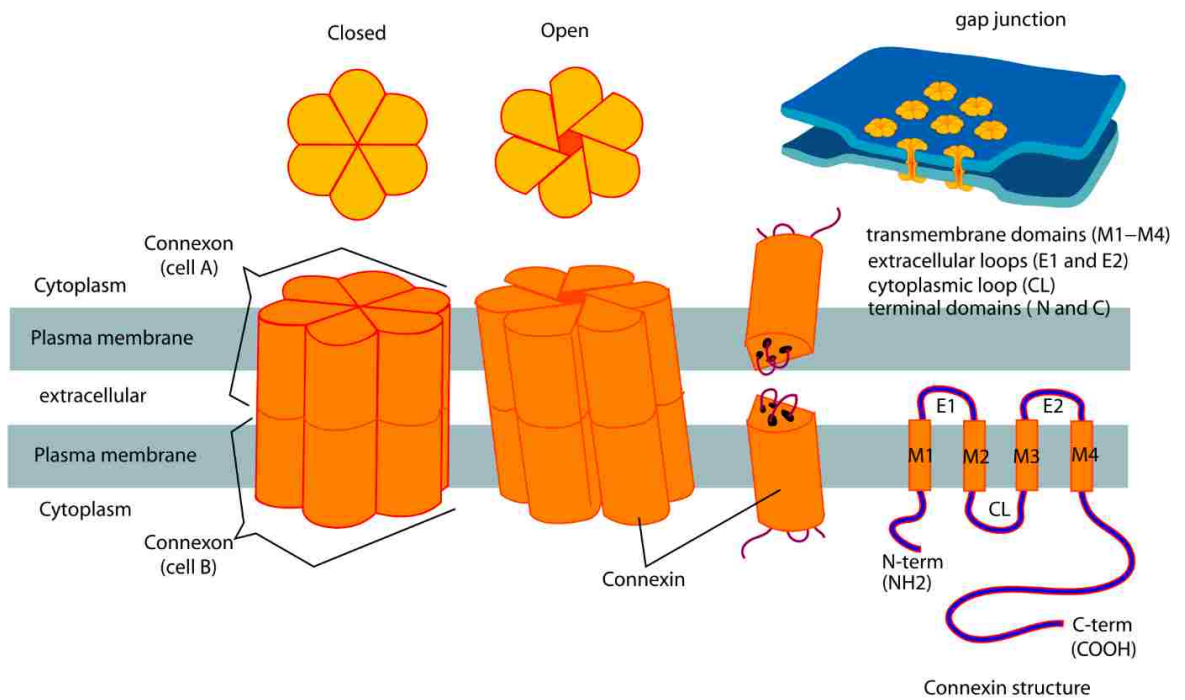
Figure 1.5. Connexins are subunits of gap junction channels. Each connexin is a four-pass transmembrane domain protein. Six connexins come together to form one connexon or hemi-channel. Two connexons from two neighboring cells dock together to form a gap junction channel. Image courtesy: Wikipedia.
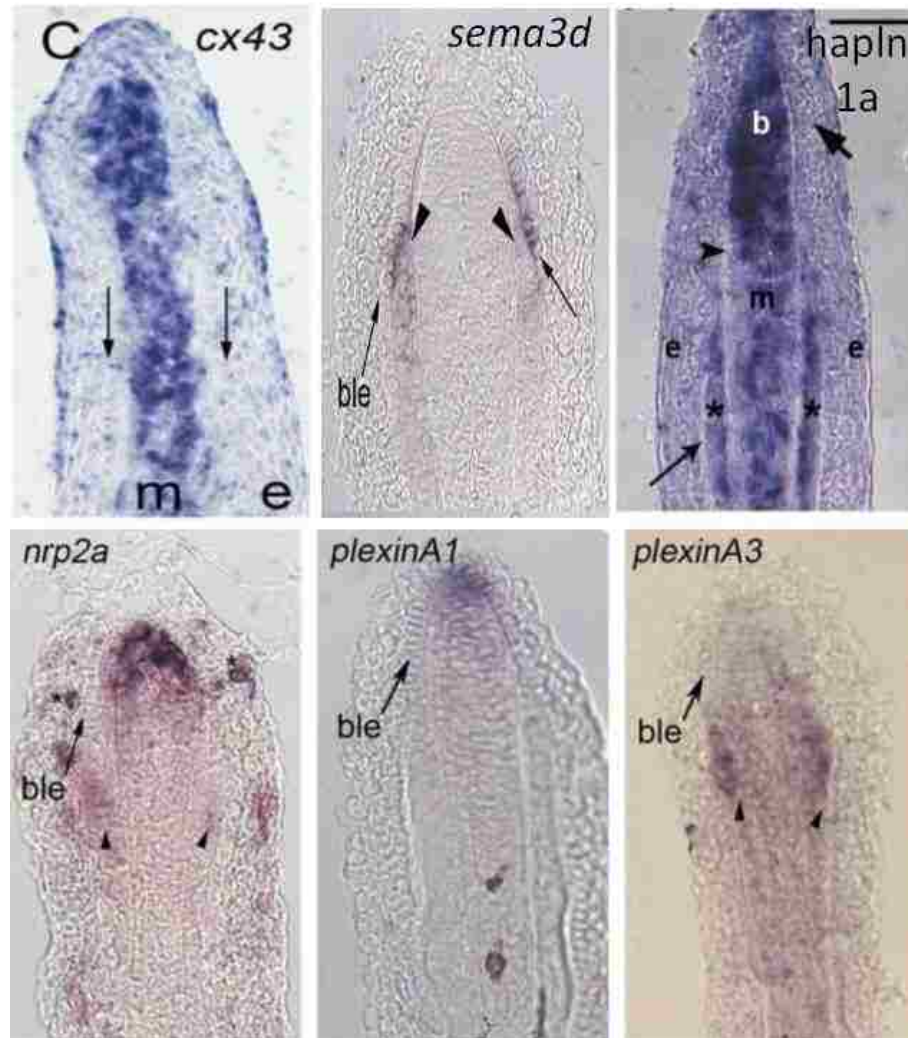
Figure 1.6: Expression pattern of different players in the *cx43* dependent skeletal morphogenesis pathway. *In situ hybridization* on longitudinal sections of WT 5dpa regenerating fins reveal the expression of important genes in the *cx43* dependent pathway for growth and patterning. (Top) *cx43* and *hapln1a* are expressed in the mesenchyme (m) including blastema (b), *sema3d* is expressed mainly in the skeletal precursor cells (arrow heads). *hapln1a* is also observed in the skeletal precursor cells (*). (Bottom) Both *nrp2a* and *plxna1* are expressed in the distal blastema. *plxna3* on the other hand is expressed in the skeletal precursor cells (arrow heads). 'e' is epidermis, 'ble' is basal layer of epidermis. (Ton & Iovine, 2012; Govindan & Iovine, 2014).
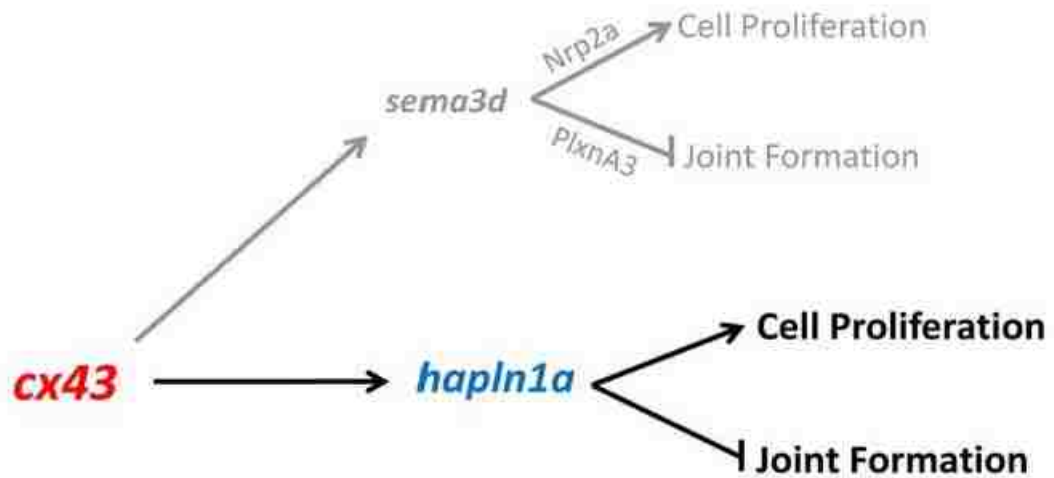
Figure 1.7: Regulators of cx43 dependent pathway in skeletal morphogenesis. Both
sema3d and *hapln1a* mediate downstream effects of *cx43*, presumably in a
transcriptionally independent manner (Ton & Iovine, 2012; Govindan & Iovine, 2014).
Both of them promote cell proliferation and suppress joint formation.
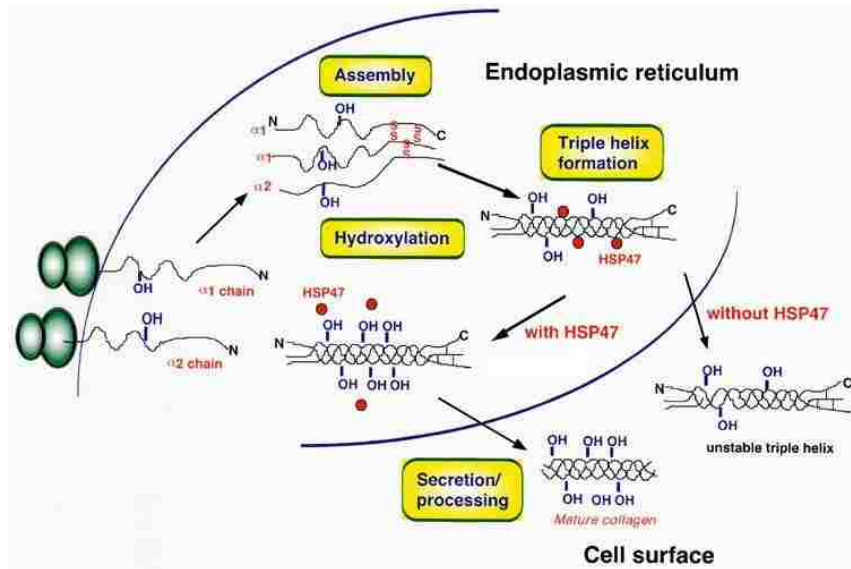
Figure 1.8: Hsp47 is important for proper collagen folding. Collagen molecules are synthesized in the ER. Hsp47 binds to the triple helical procollagen molecule and transports it to Golgi. In absence of Hsp47, procollagen triple helix becomes unstable. Image courtesy – www.cc.kyoto-su.ac.jp
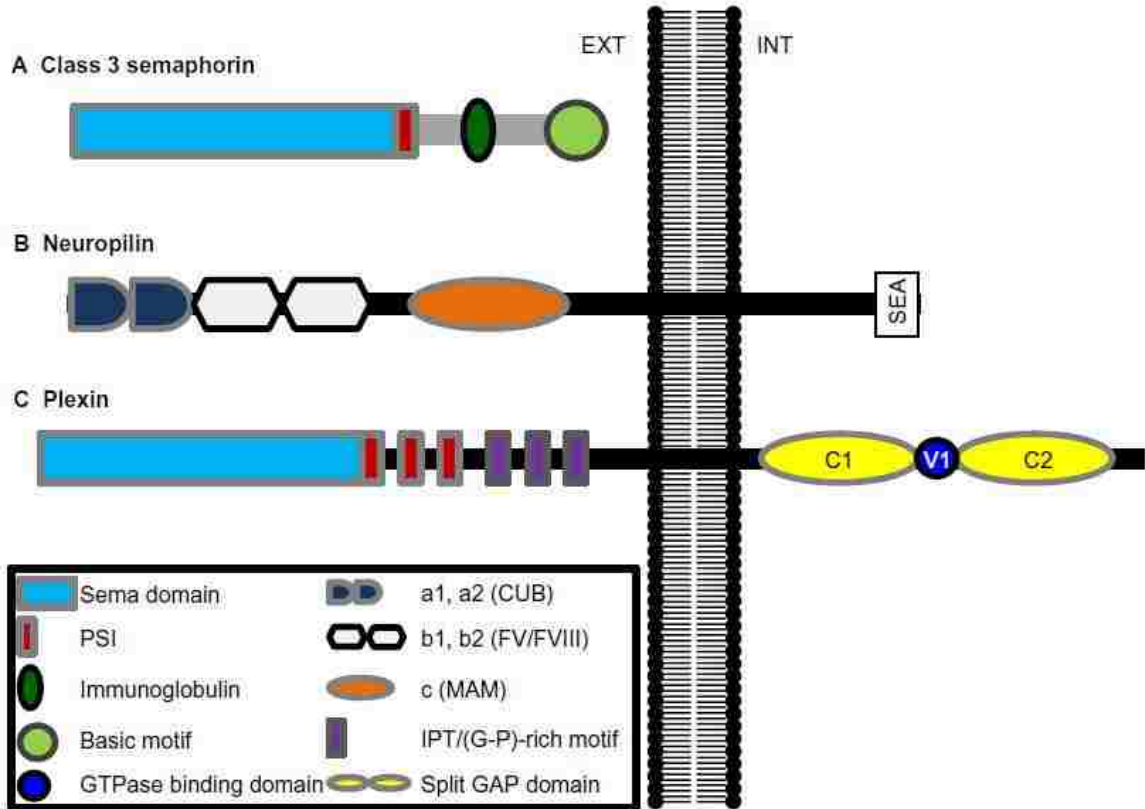
Figure 1.9: Structural domains of Sema3d, PlexinA3 and Nrp2a. Semaphorins contain an extracellular N-terminal sema domain, followed by a cysteine rich PSI (plexin, semaphorin, integrin) domain. The extracellular domain of PlexinA3 also contains a sema domain (which interacts with semaphorins) followed by 3 PSI and 4 IPT domains. The transmembrane domain has a conserved coiled-coil region followed by the intracellular domain. Neuropilins have a large extracellular region containing two CUB (complement binding factors), two Factor V/VIII homology domains, a MAM (meprin, A5 antigen, receptor tyrosine phosphatase μ) domain, a single transmembrane domain and a small cytoplasmic (44 amino acids) domain.

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Chapter 2: Hsp47 mediates Cx43-dependent skeletal growth and patterning in the regenerating fin

## 2.1.	Abstract

Skeletal morphogenesis describes how bones achieve their correct shape and size and appropriately position joints. We use the regenerating caudal fin of zebrafish to study this process. Our examination of the fin length mutant *short fin (sof ^{b123})* has revealed that the gap junction protein Cx43 is involved in skeletal morphogenesis by promoting cell proliferation and inhibiting joint formation, thereby coordinating skeletal growth and patterning. Here we demonstrate that *serpinh1b* is molecularly and functionally downstream of *cx43*. The gene s*erpinh1b* codes for a protein called Hsp47, a molecular chaperone responsible for proper folding of procollagen molecules. Knockdown of Hsp47 in regenerating fins recapitulates the *sof ^{b123}* phenotypes of reduced fin length, reduced segment length and reduced level of cell proliferation. Furthermore, Hsp47 knockdown affects the organization and localization of the collagen-based actinotrichia. Together, our findings reveal that *serpinh1b* acts in a *cx43* dependent manner to regulate cell proliferation and joint formation. We conclude that disruption of the collagen-based extracellular matrix influences signaling events required for cell proliferation as well as the patterning of skeletal precursor cells that influences segment length. Therefore, we suggest that Hsp47 function is necessary for skeletal growth and patterning during fin regeneration.

2.2. Introduction

Skeletal development in vertebrates is a tightly regulated process, although the underlying mechanisms that regulate the size and shape of bones remain largely unknown. Proper communication among cells of skeletal tissue is necessary in order to attain normal bone size and shape. One of the mechanisms by which cells communicate is through gap junctions, a group of channels between adjacent cells that allow the exchange of small molecules (< 1000 Daltons), such as ions and second messengers (Goodenough et al. 1996). Each gap junction channel is formed by docking of two hemichannels from neighboring cells, and each hemichannel is composed of six connexin molecules. Connexins are proteins that are comprised of four transmembrane domains. In humans, there are approximately 21 different connexin genes (Sohl and Willecke 2004). Among them, *CONNEXIN43* (*CX43*) is the most widely expressed connexin in bone cells (Jones et al. 1993). Gap junctional intercellular communication (GJIC) through Cx43 is of utmost importance during skeletal development. For example, mutations in human *CX43* cause occulodentodigital dysplasia (ODDD). ODDD is an autosomal dominant disease that causes craniofacial and limb abnormalities (Paznekas et al. 2009; Flenniken et al. 2005). Mice lacking Cx43 die perinatally due to cardiac malformations (Reaume et al. 1995). However, examination of the neonatal bones of the *CX43* knockout mice reveals delayed ossification of axial and craniofacial skeletons (Lecanda et al. 2000). Additionally, targeted gene knockdown of Cx43 in chicks also causes ODDD-like phenotypes (McGonnell et al. 2001). In zebrafish, recessive mutations in *cx43* cause the *short fin (sof^{b123})* phenotype, characterized by short fin length, short fin ray segments and reduced cell proliferation (Iovine et al. 2005). Together, these findings reveal that the role of Cx43 is conserved during skeletal development. However, how communication through GJIC brings about these tangible changes during skeletal development remains unclear.

We evaluate fin regeneration of the *sof^{b123}* mutant to provide insights into the role of Cx43 during skeletal morphogenesis. The *sof^{b123}* allele exhibits reduced levels of *cx43* mRNA and protein without a lesion in the coding sequence (Iovine et al. 2005; Hoptak-Solga et al. 2008). Importantly, morpholino-mediated knockdown of Cx43 in wild-type

(WT) fins recapitulates all of the *sof* ^{b123} phenotypes (Hoptak-Solga et al. 2008). In contrast to *sof*, the *another long fin* (*alf* ^{dty86}) mutant exhibits longer fins and stochastic joint failure (van Eeden et al. 1996). While the mutation is not in the *cx43* gene (Perathoner et al. 2014), this mutant shows an upregulation of *cx43* mRNA (Sims et al. 2009), suggesting that increased levels of *cx43* lead to joint failure. Indeed, Cx43 knockdown rescues joint formation in *alf* ^{dty86}. Together, these gain-of-function and the loss-of-function studies suggest that *cx43* is involved in skeletal morphogenesis in more than one way – by positively promoting cell proliferation and negatively regulating joint formation. Alternatively, one might speculate that reduced cell proliferation gives rise to shorter segments. However, it has been observed that reduced signaling via Shh or Fgfr1 dependent pathways causes reduced fin length and cell proliferation, but does not affect segment length (Lee et al. 2005; Quint et al. 2002). Therefore, cell proliferation is not sufficient to regulate segment length. We suggest that Cx43 coordinates skeletal growth (cell proliferation) and patterning (joint formation) during regeneration.

In order to address how Cx43 influences such tangible cellular outcomes like cell division and differentiation, we completed a microarray to identify downstream effectors of *cx43* that are downregulated in *sof* ^{b123} and upregulated in *alf* ^{dty86} (Ton and Iovine 2012).Two genes have been validated through this microarray: *semaphorin3d* (Ton and Iovine 2012) and *hapln1a* (Govindan and Iovine 2014). Here we provide molecular and functional validation of a third gene, *serpinh1b*. The gene *serpinh1b* belongs to the serpin (*ser*ine *p*rotease *in*hibitor) family of proteases and codes for Hsp47 (Ishida and Nagata 2011). Hsp47 is an ER resident protein that acts as a molecular chaperone for procollagen molecules, preventing their premature association into higher order collagen structure. Hsp47 binds to the procollagen triple helix in the pH-neutral ER and remains associated until reaching the Golgi, where the lower pH environment causes the procollagen to dissociate from Hsp47 (Nakai et al. 1992). Hsp47 is then recycled back to ER with the help of KDEL receptors (Satoh et al. 1996).

Recent studies have shown that autosomal recessive missense mutations in *SERPINH1* cause moderate to severe forms of Osteogenesis Imperfecta ('brittle bone' disease) in humans (Christiansen et al. 2010). The *SERPINH1* knockout mice do not

survive beyond 11.5 days post coitus and display abnormally oriented epithelial tissues and ruptured blood vessels due to severe deficiency in mature forms of type I and type IV collagen (Col) (Nagai et al. 2000). Chondrocyte specific knockout of Hsp47 causes severe chondrodysplasia and defective endochondral bone formation due to decreased level of type II and type XI Col (Masago et al. 2012). Therefore, Hsp47 function is critical for normal functions of collagen, including bone formation during skeletal development.

The teleost fin is supported by two types of skeletal elements –lepidotrichia and actinotrichia. The lepidotrichium is an individual bony fin ray composed of two hemirays of bone matrix which surrounds a mesenchyme consisting of fibroblasts, pigment cells, blood vessels and nerve cells. Several layers of epidermis surround the hemirays, where the basal layer of epidermis is the layer closest to the underlying lepidotrichium. Actinotrichia extend from the distal tips of growing fin rays during regeneration, providing a scaffold for migration for the osteoblast cells that will deposit the future bone matrix (Wood 1982). Actinotrichia are produced by actinotrichia forming cells (AFC), located distally and immediately beneath the basal layer of the epidermis (Duran et al. 2011). The composition of actinotricha includes elastoidin, which is a mix of collagen and non-collagenous proteins (Ramachandran 1962; Sastry and Ramachandran 1965). Recent studies have shown that the collagenous part of actinotrichia is composed mainly of Col I (expressed by the *col1a1a* gene) and Col II (expressed by the *col2a1b* gene (Duran et al. 2011). The non-collagenous component of actinotrichia is composed of the proteins Actinodin-1 and Actinodin-2 (And1 and And2) (Zhang et al. 2010).

Here we demonstrate that *serpinh1b* is a *cx43*-dependent gene required for growth (cell proliferation) and patterning (joint formation) during skeletal morphogenesis. We find that the Collagen II dependent actinotrichia are disturbed following Hsp47 knockdown. We suggest that aberrant deposition of Collagen could influence both cell proliferation (through disturbing Integrin-Collagen interactions) and joint formation (through disturbing patterning of osteoblasts and joint-forming cells). These findings provide new insights into the roles of Hsp47 in the vertebrate skeleton.

2.3. Experimental Procedures

2.3.1. Statement on the ethical treatment of animals

This study was carried out in strict accordance with the recommendations provided by the National Institute of Health in the Guide for the Care and Use of Laboratory animals. The protocols used for this manuscript were approved by Lehigh University's Institutional Animal Care and Use Committee (IACUC) (protocol identification # 128, approved on 11/16/2014). Lehigh University's Animal Welfare Assurance Number is A-3877-01. All experiments were designed to minimize pain and discomfort to the animals.

2.3.2. Housing and Husbandry

Zebrafish are housed in a recirculating system built by Aquatic Habitats (now Pentair). Both 3 L tanks (up to 12 fish/tank) and 10 L tanks (up to 30 fish/tank) are used. The fishroom has a 14:10 light:dark cycle and room temperature varies from 27-29°C (Westerfield 1993). Water quality is automatically monitored and dosed to maintain conductivity (400-600 μS) and pH (6.95-7.30). Nitrogen levels are maintained by a biofilter. A 10 % water change occurs daily. Recirculating water is filtered sequentially through pad filters, bag filters, and a carbon canister before circulating over UV lights for sterilization. Fish are fed three times daily, once with brine shrimp (hatched from INVE artemia cysts) and twice with flake food (Aquatox AX5) supplemented with 7.5 % micropellets (Hikari), 7.5 % Golden Pearl (300-500 micron, Brine Shrimp direct), and 5 % Cyclo-peeze (Argent).

2.3.3. In situ hybridization (ISH) on whole mount and cryosectioned fins

Antisense *serpinh1b* probe was generated from 250-500 ng of PCR amplified linear DNA in which the reverse primer contained the binding site for T7 RNA polymerase. The primer sequences for ISH is presented in Table 2.1. In situ hybridization

on whole mount regenerating and cryosectioned fins was performed following standard protocols (Govindan and Iovine 2014; Ton and Iovine 2012).

2.3.4. Morpholino mediated knockdown in regenerating fins

All fins were amputated at the 50 % level. Morpholinos (MO) used for this study were obtained from Gene Tools, LLC and were fluorescently tagged (Table 2.1). The working concentration was 1mM. The targeting MO blocks the ATG. Two different control MO were used – the standard control MO from Gene Tools or the five nucleotide mismatch MO. MOs were injected in half the fin at 3 dpa followed by electroporation for cellular uptake (injection and electroporation was carried out as described) (Ton and Iovine 2012). Fins were evaluated for uptake at 1 dpe (days post electroporation). For histone-3-phosphate (H3P) staining and qRT-PCR analysis, fins were harvested 1 dpe. For calcein staining or ZNS5 staining, fins were harvested at 4 dpe. Each MO knockdown was carried out in at least three independent experiments with n=7–10. Statistical significance was determined using Student's t-test (p<0.05).

2.3.5. Lysate preparation and Western blotting

Approximately 10 –15 fins were injected with target or control MO on 3 dpa and harvested the next day. For measuring antibody staining in WT, sof^{b123} and alf^{dty86} fins, approximately 10-12 WT fins, 20 sof^{b123} fins and 8-10 alf^{dty86} fins were harvested at 5dpa. Regenerating fins were harvested in 300 -500 µl of Incubation buffer (pH=7.5) supplemented with 200 µM Pefabloc, 1 mM DTT and protease inhibitor (Thermo Scientific, Halt^{TM} protease and phosphatase inhibitor cocktail). Fins were homogenized using a tissue homogenizer (Bio-Gen, PRO 200) at high speed (5X) for 5 seconds with a 10 seconds cooling time in between. Homogenized samples were centrifuged at 200 g for 10 minutes at 4 °C. The protein concentration in the supernatant was analyzed using a Bradford assay.

Immunoblotting was performed using previously described procedure (Hoptak-Solga et al. 2008). The following primary antibodies were used: Rabbit anti-Hsp47 (Abcam, 1:1000), Mouse anti-α-tubulin (Sigma, 1:2000). The following peroxidase

conjugated secondary antibodies were used: Goat anti-rabbit IgG (1:10,000) and Goat anti-mouse IgG (1:10,000). Signal detection was performed using ECL Prime western blotting detection reagent (Amersham^{TM} – GE Healthcare).

Relative pixel density of gel bands were measured using gel analysis tool in ImageJ software. The density of each gel band was obtained as the area under the curve using the gel analysis tool. In order to obtain the relative density, the density of the Hsp47 or tubulin bands for the experimental samples (i.e. Hsp47 MO, *sof*^{b123} or *alf*^{dty86}) was first normalized against the density of the Hsp47 or tubulin bands from the control sample (i.e. standard control MO or WT). Relative pixel density was calculated as the ratio of Hsp47 and Tubulin (loading control).

2.3.6. Immunofluorescence

Fins were harvested following MO knockdown. For H3P staining, fins were harvested 1 dpe, whereas for ZNS5 staining, fins were harvested 4 dpe. They were then fixed in 4% paraformaldehyde (PFA) overnight at 4°C and then dehydrated in methanol. H3P and ZNS5 staining were carried out following previously described procedures (Ton and Iovine, 2012). For calcein staining, MO injected fish were allowed to swim in 0.2% calcein solution (pH=7) for 10 minutes, followed by additional 10 minutes in fresh fish water (Du et al. 2001). Fluorescent fins were examined under a Nikon Eclipse 80i microscope at 4X or 10X magnification. Images were collected using a digital Nikon camera.

2.3.7. Measurement

The regenerate length, segment length and number of dividing cells were calculated as previously described (Ton and Iovine 2012). In brief, for regenerate length, segment length, number of dividing cells and regenerated bone matrix, all measurements were taken from the third fin ray. Regenerate length was measured from the amputation plane to the end of the fin. Segment length was measured as the distance between the first two joints formed following amputation. For regenerating bone matrix, bone growth was measured from the amputation plane to the distal tip of the calcein stained bone matrix

and was divided by the total regenerate length. H3P positive cells were counted from within the distal-most 250 μm of the third fin ray. For each experiment, at least seven fish were evaluated in triplicate and a Student's t-test was performed to indicate statistical significance ($p<0.05$). In order to evaluate the phenotypic effect following knockdown of Hsp47, the targeting or the control MO injected side was compared with the uninjected side and % similarity was calculated by dividing the value of injected side by uninjected side and multiplying by 100. Values close to 100% indicate no effect of the MO on the injected side, whereas values different from 100% indicate that the MO had an effect on injected side. Percent similarity of greater than 100 % reflects the fact that the experimental side can be measurably larger than the control uninjected side. Statistical significance was determined using Student's t-test ($p<0.05$).

2.3.8. Quantitative real time PCR analysis

For qRT-PCR analysis, RNA was extracted using TRIZOL from 5 dpa fins for WT, *sof ^{b123}* and *alf ^{dty86}* and 1 dpe for MO injected fins (targeting or control). A minimum of eight fins were used for total RNA extraction and for each sample, 1 μg of RNA was reverse transcribed using Superscript III Reverse transcriptase (Invitrogen) and oligo-dT primers. Primers for *serpinh1b, msxc* and *mps1* were designed using Primer express software (Table 1). For *msxb*, we used the primer sequence from (Kizil et al. 2009). Three independent RNA samples were used for each experiment, and each reaction was performed in duplicate. The samples were analyzed using Rotor Gene 6000 series software (Corbett Research) and the average cycle number (C_T) was calculated for each amplicon. Samples were then normalized with respect to the housekeeping gene *keratin* (Sims et al. 2009), and ΔC_T values were obtained. The relative level of gene expression or 'fold difference' was denoted by calculating $2^{-\Delta\Delta C_T}$. To obtain a range over which the fold-difference varies, we calculated a standard deviation for the $\Delta\Delta C_T$ values. We used the high and low $\Delta\Delta C_T$ values to calculate the high and low fold-difference values. When these values span a fold-difference of 1, it is interpreted as no change in expression from the control sample.

2.3.9. Immunofluorescence on sections

Fins were harvested at different timepoints following injections and fixed in 2% PFA for 30 minutes at room temperature. They were then washed in 1X PBS (3X, five minutes each), embedded in 1.5% agarose/5% sucrose dissolved in 1X PBS and equilibrated in 30% sucrose solution overnight. Fins were mounted in OCT and cryosectioned (18 μm) using a Reicherta Jung 2800 Frigocut cryostat. Sections were collected on Superfrost Plus slides (Fisher) and allowed to air dry overnight at room temperature. Sections were stored in -20 °C for future use. Before use, slides were brought to room temperature for an hour, then rehydrated in 1X PBS (2X, five minutes each). They were then transferred to coplin jars containing 10 mM sodium citrate (pH=6) and allowed to boil at 99.5 °C in the water bath for 10 minutes. The slides were allowed to cool down to room temperature and again washed in 1X PBS (2X, five minutes each). Permeabilization was achieved by treating the slides with 1% H_2O_2 solution in water for 20 minutes at room temperature followed by 2X wash in 1X PBS for five minutes each. The slides were then transferred to block (2% BSA, 0.1% Triton-X in 1X PBS). Two quick washes in block (10 minutes each) were followed by an hour incubation in block. The following primary antibodies were used: Rabbit anti-Hsp47 (Abcam, 1:100), Mouse anti-collagen type II (DSHB-II-II6B3, 1:10). Sections were circled using a PAP pen (Fisher or VWR laboratories). The slides were incubated in primary antibody overnight at 4 °C in a wet box followed by one quick wash and three 10 minutes washes in block. The following secondary antibodies were used: Anti-rabbit Alexa Fluor 488 or 568 (1:200), Anti-mouse Alexa Fluor 488 or 568 (1:200. Propidium iodide or To-Pro-3-iodide (Life technologies, 1:1000) was used to stain the nuclei. The slides were incubated with secondary antibody for either an hour at room temperature or overnight at 4 °C, protected from light. It was followed by three 15 minutes washes in block and one quick wash in distilled water. They were then mounted with Glycerol or Vectashield and were examined under LSM 510 Meta Confocal Microscope (Zeiss) at 40X, using Argon (488) and HeNe (543 and 633) lasers. Z-stacks were collected with a depth of 0.36 μm.

2.4. Results

2.4.1. *serpinh1b* acts downstream of *cx43*

One of the candidate genes identified through the microarray was *serpinh1b,* which intrigued us due to the finding that mutations in human *SERPINH1* cause Osteogenesis Imperfecta (OI) (Christiansen et al. 2010). Hsp47 is the protein product of the gene *serpinh1b*. The zebrafish Hsp47 protein is 404 amino acids long and shares 64% sequence similarity with rat and mouse Hsp47, 69% with human Hsp47, and 72% with chicken Hsp47 (Pearson et al. 1996). In order to validate *serpinh1b* as a downstream target of *cx43*, we performed a whole mount in situ hybridization to compare the expression level of *serpinh1b* in WT, *sof ^{b123}* and *alf ^{dty86}*. As predicted, *serpinh1b* mRNA expression was upregulated in *alf ^{dty86}* and downregulated in *sof ^{b123}* (Figure 2.1). Additional confirmation was provided by quantitative RT-PCR (qRT-PCR) to demonstrate reduced expression of *serpinh1b* in *sof ^{b123}* and increased expression of *serpinh1b* in *alf ^{dty86}* compared to WT (Figure 2.2 and Table 2.2). To further validate that *serpinh1b* expression is *cx43* dependent, we performed qRT-PCR on WT fins treated with *cx43* morpholino (MO). This is an independent test that *serpinh1b* expression depends on Cx43. Indeed, the expression level of *serpinh1b* is reduced following Cx43 knockdown (Figure 2.2, Table 2.2). Together, these data validate the microarray data and establish that *serpinh1b* is molecularly downstream of *cx43*.

In order to determine the tissue specific localization of *serpinh1b* mRNA we performed in situ hybridization on cryosections of 5 days post amputation (dpa) regenerating fins. Longitudinal sections of the regenerating caudal fin are constituted of outer layers of epidermis separated from the mesenchyme by a layer of cuboidal cells known as basal layer of epidermis. A group of dedifferentiated cells occupy the distal end of the mesenchymal compartment and establish a specialized structure called the blastema. The blastema contains the proliferating cells required for fin outgrowth during regeneration. The skeletal precursor cells are the cells that differentiate as either osteoblasts or joint-forming cells, and are located laterally, between the actinotrichia and basal layer of epidermis (Ton and Iovine 2013). The *serpinh1b* mRNA is most prominently expressed in the skeletal precursor cells, the overlying basal layer of

epidermis, and in the blastema (Figure 2.1D). These expression domains only partially overlap with Cx43 expression, which is found throughout the medial mesenchyme and in a discrete population of joint-forming cells in the lateral compartment (Hoptak-Solga et al. 2008; Sims et al. 2009). Therefore, we suggest that Cx43-dependent *serpinh1b* expression outside these domains requires an intermediate (such as a secreted growth factor) that acts between the medial mesenchyme and the lateral domains of *serpinh1b* expression (Ton and Iovine 2012).

To determine how the expression pattern of *serpinh1b* changes during regeneration, we performed in situ hybridization on fins and on fin cryosections harvested at different timepoints (Figure 2.3). We observed a similar pattern and similar levels of expression for 2.5, 3.5, 4 and 8 dpa regenerating fins by both whole mount in situ hybridization and in situ hybridization on cryosections. Staining of *serphinh1b* is not detected during ontogeny and is diminished at 14 dpa (Figure 2.4). Therefore, *serpinh1b* expression is upregulated during early stages of regeneration and is reduced in later stages of regeneration.

2.3.3. *serpinh1b* mediates downstream effects of *cx43*

In order to determine if *serpinh1b* mediates *cx43*-dependent growth and patterning, we evaluated regenerate length, cell proliferation, segment length following morpholino (MO) mediated gene knockdown. We used a gene specific MO targeting the start codon of *serpinh1b*. As a control, we used either a custom mismatch morpholino (5MM) containing five mismatches compared with the *serpinh1b* targeting MO (Table 2.1), or a 'standard control' MO from Gene Tools that does not recognize any zebrafish genes. All MOs are modified with fluorescein, permitting evaluation of cellular uptake. Positive fins were harvested one day post electroporation (1 dpe) to evaluate cell proliferation and regenerate length, or fins were allowed to regenerate for four days (4 dpe) to measure segment length. Knockdown of Hsp47 was demonstrated by immunofluorescence (Figure 2.5A) and by immunoblot (Figure 2.5B) on MO positive fins compared to control MO injected fins. Immunofluorescence on cryosections revealed

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the localization of Hsp47 protein in the regenerating fin. Hsp47 was observed in the basal layer of the epidermis as well as in the mesenchyme, similar to the expression pattern of the *serpinh1b* mRNA (Figures 2.1 and 2.3). Quantification of Hsp47 protein expression from the immunoblot reveals an approximately 90% reduction following MO knockdown. Hsp47 levels in *sof* [b123] and *alf* [dty86] are affected as predicted. We find that *sof* [b123] exhibits an approximately 25% decrease in expression level and that *alf* [dty86] has an approximately 30% increase compared to WT (Figure 2.5C). Differences in Hsp47 protein levels are therefore not as large in the mutants as observed in the Hsp47 knockdown. However, since Hsp47 is a chaperone, the more permanent differences in Hsp47 function of the mutants may have a stronger impact on collagen structure.

To evaluate the phenotypic effects following knockdown of Hsp47, we compared the target or control MO injected side with the respective uninjected side and calculated the percent similarity. Values with high similarity between the injected and noninjected side (i.e. close to 100%) indicate little effect of the MO, whereas values with low similarity (i.e. less than 100%) indicates that the MO had an effect on treatment side. This method minimizes fin-to-fin variation since the uninjected side serves as an internal control for each fin. We first evaluated changes in outgrowth, which are measured by regenerate length and cell proliferation. Fin length was measured as the distance between the amputation plane and the distal end of the regenerate. The level of cell proliferation was measured by counting cells in mitosis, labeled with an antibody against histone 3 phosphate (H3P). When using the control MO, we found high similarity between the treatment and control sides for regenerate length and cell proliferation, revealing no effect. In contrast, Hsp47 knockdown with the targeting MO exhibited reduced levels of similarity for cell proliferation and regenerate length; cell proliferation is approximately 25% reduced (Figure 2.6C) and regenerate length is about 20% reduced (Figure 2.6F) . We next evaluated segment length, measured in calcein stained fins as the distance between the first two joints distal to the amputation plane. We found that segment length was about 10 % reduced when using the targeting MO, and was not reduced when using the control MO (Figure 2.7A,C). Since Hsp47 knockdown has a milder effect on segment length, it may not play a major role in regulating joint formation. Further evidence of that

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is provided by the fact that knocking down Hsp47 fail to rescue *evx1* expression in *alf*
dty86. The gene *evx1*is required for joint formation (Schulte et al, 2011). Previous studies
from our lab have shown that expression of *evx1* is *cx43* dependent (Ton and Iovine,
2013). The gene *evx1* is expressed consistently in WT, but its expression is random in *alf*
dty86. Knocking down *cx43* rescues *evx1* expression in *alf* *dty86* (Ton and Iovine, 2013). In
order to see if Hsp47 is mediating patterning events in an *evx1* dependent way, fins were
injected with Hsp47-MM or MO and fin rays expressing *evx1* were counted. No
significant rescue of joints was observed in the MO treated fins (approx. 40%) when
compared to the control (approx. 30%) (data not shown) indicating that *serpinh1b* and
evx1 do not work in the same pathway in order to regulate joint formation. In summary,
we find that Hsp47 knockdown recapitulates all Cx43-knockdown phenotypes indicating
that *serpinh1b* is functionally downstream of *cx43*. We also suggest that Hsp47 plays a
minor role during joint formation and this event is *evx1* independent.

Since Hsp47 is a collagen chaperone, we further evaluated the length of
regenerated bone matrix. This was measured as the distance between the amputation
plane and the tip of the calcein stained bone matrix, and was normalized against the total
regenerate length. Following Hsp47 knockdown, we found a small but significant
difference in the bone regenerate length, about 4 % reduced (Figure 2.7A,D). This
finding suggests that bone-forming cells can secrete matrix. The quality of the secreted
bone matrix in Hsp47 knockdown fins will be evaluated in future studies.

Since mutations in *serpinh1b* gene are associated with a recessive form of OI, we
tested whether the short segment phenotype was caused by breaks in bone matrix (i.e.
caused by brittle lepidotrichia) or due to formation of premature joints (i.e. caused by
differences in skeletal patterning). ZNS5 staining identifies both joint-forming cells and
osteoblasts, and can be used to distinguish these possibilities (Sims et al. 2009). We
observed ZNS5 positive cells around the joints (Figure 2.8), revealing that shorter
segments were caused by formation of premature joints and not by breaks of the matrix
(which would not be associated with ZNS5 positive cells).

In order to confirm that markers of cell proliferation are reduced following Hsp47
knockdown, we evaluated expression levels of the transcription factors *msxb* and *msxc*,

and the intracellular kinase *mps1*. These genes are expressed in the blastema during regeneration and have been found to be required during cell proliferation (Akimenko et al. 1995; Nechiporuk and Keating 2002; Thummel et al. 2006; Poss et al. 2002; Smith et al. 2006a). Interestingly, knockdown of Hsp47 in WT 5 dpa fins resulted in reduced *msxb* and *msxc* expression, whereas the expression level of *mps1* remain unaffected (Figure 2.9 and Table 2.3). This finding supports the conclusion that cell proliferation is reduced following Hsp47 knockdown.

2.3.4. Role of *serpinh1b* on collagen structure

Hsp47 functions as a molecular chaperone for collagen by binding to the procollagen triple helix in ER. This interaction is believed to prevent premature higher order aggregation of procollagen (Widmer et al. 2012; Eyre and Weis 2013). In order to determine if Hsp47 influences collagen assembly and/or organization during fin regeneration, we evaluated the localization of Col II, a major component of actinotrichia following Hsp47 knockdown. In longitudinal sections of WT 5 dpa regenerating fins, Col II was observed prominently in the actinotrichia in its characteristic fibrillary pattern (Figure 2.10). As part of these studies, we realized that injections with standard control MO (Figure 2.11) or DMSO (data not shown) altered the longitudinal fibrillar structure of actinotrichia in a time-specific manner. The fibrillar association is disrupted immediately following injection, and recovers by 3-4 days post injection. In contrast, we did not observe changes in actinotrichia in transverse sections following either control treatment (Figure 2.11 for standard control MO; data not shown for DMSO control). As soon as one day post injection in transverse sections, actinotrichia are observed appropriately in two symmetric rows beneath the epidermis. Therefore, for continued functional studies we evaluated transverse sections. We also note that the antigen retrieval protocol used for immunofluorescence completely eliminated the fluorescein from the MO and therefore did not interfere with the immunofluorescence in the 488 channel (Figure 2.12).

Following standard control MO treatment Col II is localized in the two well-organized symmetric rows of actintrichia (Figure 2.11 and 2.13A). Strikingly, the organization of the actinotrichia is severely disrupted following Hsp47 knockdown

(Figure 2.13A). The two well-organized rows of actinotrichia are lost. Some rods of actinotrichia may be present, but are much smaller in diameter than actinotrichia from the control fins. To provide further evidence that Hsp47 functions in a common pathway with Cx43, we next evaluated actinotrichia in Cx43 knockdown, and in *sof*^{b123} and *alf*^{dty86} 5 dpa regenerating fins. Fins treated for Cx43 knockdown exhibit similar disruptions of actinotrichia as were observed for Hsp47 knockdown (Figure 2.13A). In the *sof*^{b123} and *alf*^{dty86} mutants, we observed variability in the severity of the actinotrichia morphology. We categorized the phenotypes as "mild," where the overall organization into two rows of actinotrichia is maintained but the individual actinotrichia may appear smaller; "disrupted," where the organization of actinotrichia into two discrete rows is not maintained; and "small," where there are more obvious reductions in the size of the actinotrichia (Figure 2.13B). Actinotrichia in *sof*^{b123} mutants were generally much smaller in diameter and were sometimes disorganized. Actinotrichia in *alf*^{dty86} were often both smaller and disorganized. Thus, the phenotypes of Cx43 knockdown, *sof*^{b123}, and *alf*^{dty86} are consistent with defects in Collagen maturation, and are similar with what is observed for Hsp47 knockdown. The longitudinal sections of both *sof*^{b123} and *alf*^{dty86} showed thinner and slightly broken actinotrichia fibril (Figure 2.14) unlike the thicker, more continuous fibrils in WT (Figure 2.10). These findings provide further support for the hypothesis that *cx43* and *serphin1b* function in a common molecular pathway.

2.3.5 *serpinh1b* functions in the *cx43-hapln1a* dependent pathway

 Prior studies have validated both *sema3d* and *hapln1a* as downstream effectors of *cx43* (Govindan and Iovine 2014; Ton and Iovine 2012) contributing to promotion of cell proliferation and inhibition of joint formation. As part of those studies, it was determined that neither *sema3d* nor *hapln1a* depend on each other for their transcription. Therefore, it was suggested that the *sema3d* and *hapln1a* molecular pathways act independently of each other (Govindan and Iovine 2014). In order to evaluate whether the transcription of *serpinh1b* is dependent on either *sema3d* or *hapln1a*, we evaluated *serpinh1b* expression following knockdown of Sema3d or Hapln1a. We find that expression of *serpinh1b* is reduced following Hapln1a knockdown, but not following Sema3d knockdown (Figure

2.2 and Table 2.2). These findings suggest that *serpinh1b* is transcriptionally downstream of *hapln1a* but not *sema3d* (Figure 2.15A).

2.5.　　　　Discussion

Here we demonstrate that *serpinh1b* is molecularly and functionally downstream of *cx43*. We found that the expression of *serpinh1b* is reduced in *sof* [b123], elevated in *alf* [dty86], and that Cx43 knockdown reduces *serpinh1b* expression. Further, we found that Hsp47 knockdown recapitulates the *cx43*-dependent phenotypes, namely reduced regenerate length, reduced segment length and a reduced level of cell proliferation. Moreover, we find that Hsp47 knockdown and changes in Cx43 activity lead to similar defects to actinotrichia, the collagen-based rods produced at the growing end of the fin. Together, these findings provide strong support for the hypothesis that *serpinh1b* functions in a common pathway with *cx43*. Since this is the third gene identified from a microarray to identify *cx43*-dependent genes, we also placed *serpinh1b* in the *cx43*-dependent gene network downstream of *hapln1a* (Figure 2.15A).

The *serpinh1b* gene codes for the collagen chaperone Hsp47. Hsp47 resides in the ER and binds procollagen trimers in order to prevent aggregation within the secretory pathway (Widmer et al. 2012; Eyre and Weis 2013). Once procollagen trimers are secreted, they are processed to mature forms and assembled into fibers. Remarkably, a recessive mutation in human *SERPINH1* causes a severe form of brittle bone disease, OI, due to unstable type I Collagen (Christiansen et al. 2010). Moreover, knockout of *SERPINH1* in mice is embryonically lethal due to defective type I and type IV Collagen (Nagai et al. 2000). Chondrocyte -specific knockout of *SERPINH1* caused substantially reduced secretion of type II and type XI Collagen in cartilage, resulting in faulty endochondral bone formation (Masago et al. 2012). Therefore, the function of Hsp47 is critical for collagenous structures, and in particular in the formation of bone matrix.

Mutations in two collagen genes have been found to influence collagen-based structures in the regenerating fin. Mutations in the *col9a1* gene (causing the *persistant plexus/prp* phenotypes) lead to disintegration of the actinotrichia and mispatterning of the osteoblasts that secrete the bony matrix of the lepidotrichia (Huang et al. 2009). The resulting lepidotrichia exhibit a wavy appearance, in stark contrast to straight wild-type fin rays. The Col IX protein serves to cross-link the fibrillar Col II (Diab et al. 1996), and may be responsible for the integrity of Col II fibrils. Mutations in the *col1a1a* gene cause

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the *chihuahua/chi* phenotypes, (Fisher et al. 2003a) and lead to stochastic failure of actinotrichia formation in the regenerating fin (Duran et al. 2011). Fin rays without actinotrichia exhibit a wavy appearance, while fin rays that produce actinotrichia are straight. Col I was identified as a component of the actinotrichia following proteomic analyses, although it remains uncertain how Col I and Col II interact to form fibrils (Duran et al. 2011). Defects in these collagen proteins likely have a direct impact on the structure of actinotrichia, but it is unknown if the concomitant effects on lepidotrichia are direct or indirect. Since Hsp47 is a collagen chaperone, it is not surprising that we also observe defects in actinotrichia following Hsp47 knockdown.

We focused on Col II in our studies to identify the effects of Hsp47 knockdown. We find that the structure of actinotrichia is highly perturbed following changes in Hsp47 expression in the regenerating fin, not unlike the appearance of the actinotrichia of the Col9a1 knockdown (Huang et al., 2009). It is not known how changes in the size and organization of the actinotrichia impact the Cx43 phenotypes of reduced cell proliferation and joint formation. However, the actinotrichia are located directly adjacent to both the proliferating cells of the blastema and to the skeletal precursor cells. Therefore, one possibility is that the Col II in the actinotrichia signals to the cells of both compartments (Figure 2.15B). For example, Integrins can serve as collagen receptors and transduce outside-in signaling that may influence changes in gene expression (Heino 2014). Thus, one possibility is that Hsp47-dependent cell proliferation is the result of aberrant Collagen-Integrin interactions, which could in turn influence the expression levels of genes required for cell proliferation. Similarly, Hsp47-dependent segment length may be the result of defective actinotrichia signaling inappropriate differentiation of osteoblasts and/or joint-forming cells. In turn, this may lead to the observed short segment phenotype in Hsp47-knockdown fins. Indeed, the *col9a1/prp* mutant exhibits both mispatterning of osteoblasts and short segments (Huang et al. 2009).

This study builds on our model of *cx43*-dependent gene interactions by validating a third gene identified in our microarray analysis. We not only demonstrate that *serpinh1b* is molecularly and functionally downstream of *cx43* and *hapln1a*, but we also extend the function of Hsp47 by demonstrating that Hsp47 can influence bone growth

and skeletal patterning. Hsp47 is believed to exert its function through the organization of Collagen proteins in the extracellular matrix. Future studies will further elucidate mechanistically how Collagen structure impacts cell proliferation and joint-formation.
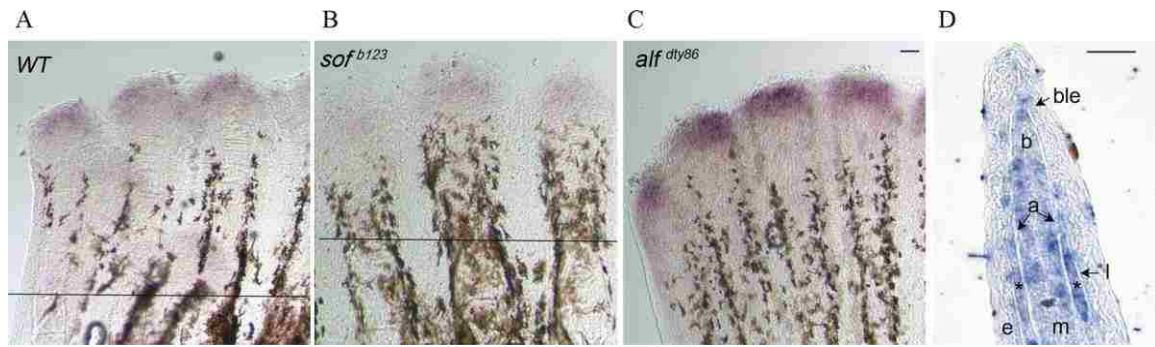
Figure 2.1: *serpinh1b* **is differentially expressed in wild-type,** *sof* [b123] **and** *alf* [dty86].
Whole mount in situ hybridization shows increased expression in *alf* [dty86] (C) and
decreased expression in *sof* [b123] (B) compared to wild-type (A). In situ hybridization on
cryosection of a WT-5dpa fin (D) reveals localization of *serpinh1b* in the blastema (b),
basal layer of epidermis (ble) and skeletal precursor cells (*). Epidermis is denoted by
'e', mesenchyme by 'm', lepidotrichia by 'l' and actinotrichia by 'a'. The amputation
plane is indicated in panels A and B. The amputation plane is beyond the field of view in
C. The scale bars in C and D are 50 µm; the scale bar in C applies to the images in A, B,
and C. and D.
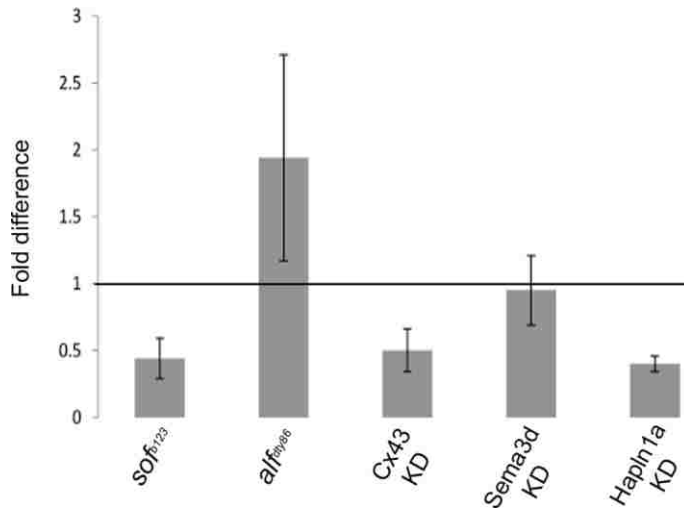
Figure 2.2: Changes in *serpinh1b* expression by quantitative RT-PCR. Expression of *serpinh1b* is down-regulated in *sof*[b123] and upregulated in *alf*[dty86] when compared to WT. *serpinh1b* expression level decreases following Cx43 and Hapln1a knockdown and remains unchanged following Sema3d knockdown when compared to standard control MO injected fins. The error bars represent the range in variation of the fold-difference based on the standard deviation. A fold-difference of one indicates no change in expression between experimental and control samples.
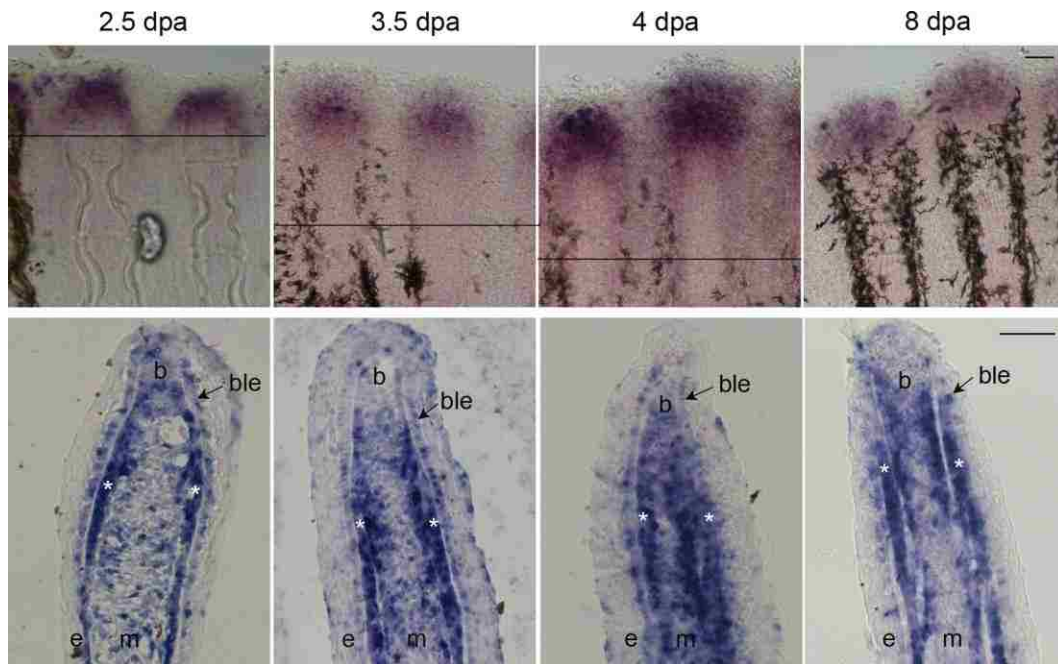
Figure2. 3: *serpinh1b* **expression over time in regenerating fins.** Whole mount in situ hybridization for *serpinh1b* at different time points on regenerating fins (top). The amputation plane is indicated in 2.5, 3.5 and 4 dpa fins. For 8 dpa fins, the amputation plane is outside the field of view. In situ hybridization on cryosectioned fins at different time points (bottom). Similar localization of *serpinh1b* gene in basal layer of epithelium (ble) and skeletal precursor cells (*) is observed. Blastema (b), basal layer of epidedrmis (ble), and skeletal precursor cells (*). The scale bars are 50 μm. The scale bar in the upper 8 dpa panel applies to all panels for whole mount images. The scale bar in the lower 8 dpa panel applies to all panels for cryosections.
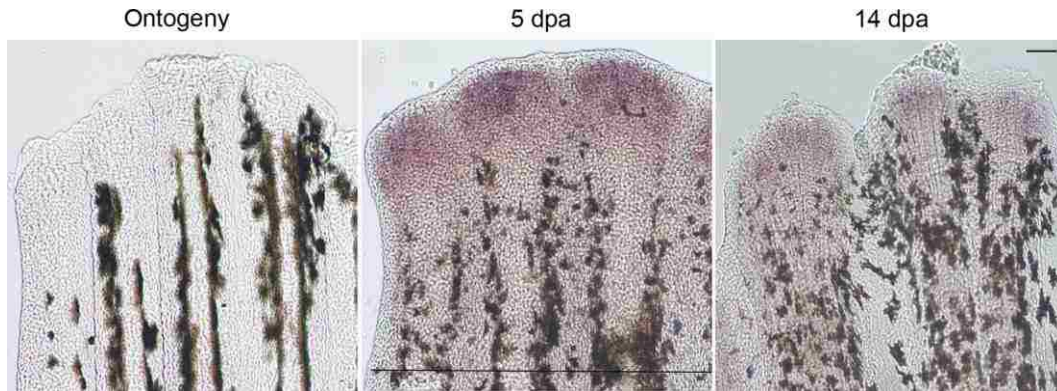
Figure 2.4: *serpinh1b* **expression level is upregulated during regeneration.** *serpinh1b* expression is undetectable during ontogeny. Expression of *serpinh1b* at 5 dpa is shown for comparison. The expression level is reduced at 14 dpa. The amputation plane is indicated in 5 dpa fin. The amputation plane is beyond the field of view in 14 dpa fin. Scale bar indicates 50 μm and applies to all panels.
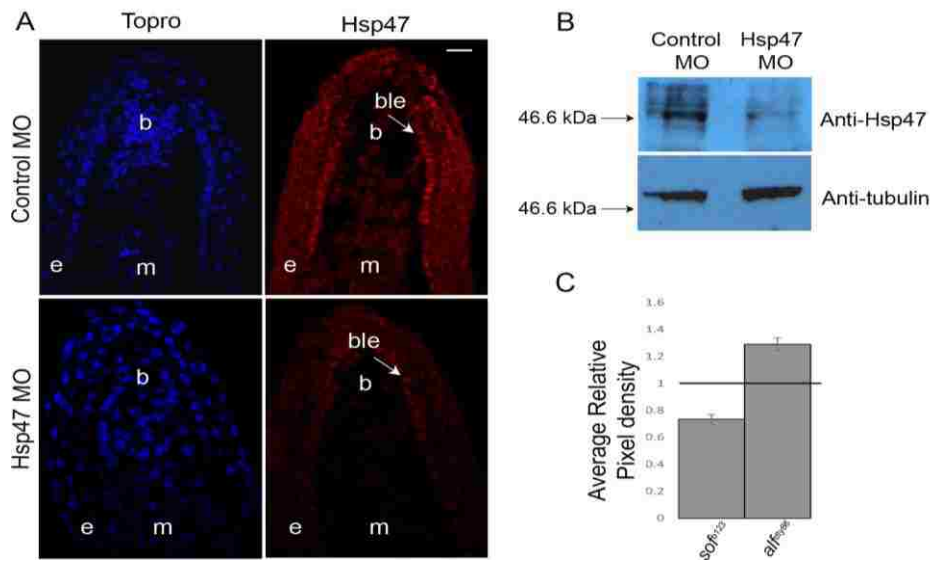
Figure 2.5: Morpholino mediated knockdown of Hsp47 results in reduced expression in WT regenerating fins. (A) Longitudinal sections of WT 5 dpa fins following treatment with standard control or targeting MO against *serpinh1b*. Hsp47-MO treated fins show reduced level of Hsp47 expression. Hsp47 (red), nuclei are stained with the far red dye To-Pro (blue). 'ble' indicates basal layer of epithelium; 'b' blastema; 'm' mesenchyme, and 'e' epidermis. Scale bar is 10 µm, and applies to all panels in A. (B) Immunoblots confirming reduced level of Hsp47 following Hsp47 knockdown. Hsp47 MO treated fins (MO) are compared to fins injected with standard control MO. Tubulin is used as loading control. (C) Bar graph depicting the ImageJ analysis of the ratio of Hsp47 levels between *sof* ^{b123} or *alf* ^{dty86} and wild-type (normalized by the tubulin loading control). Hsp47 protein levels are reduced in *sof* ^{b123} and upregulated in *alf* ^{dty86} relative to WT (represented as a relative density of 1).
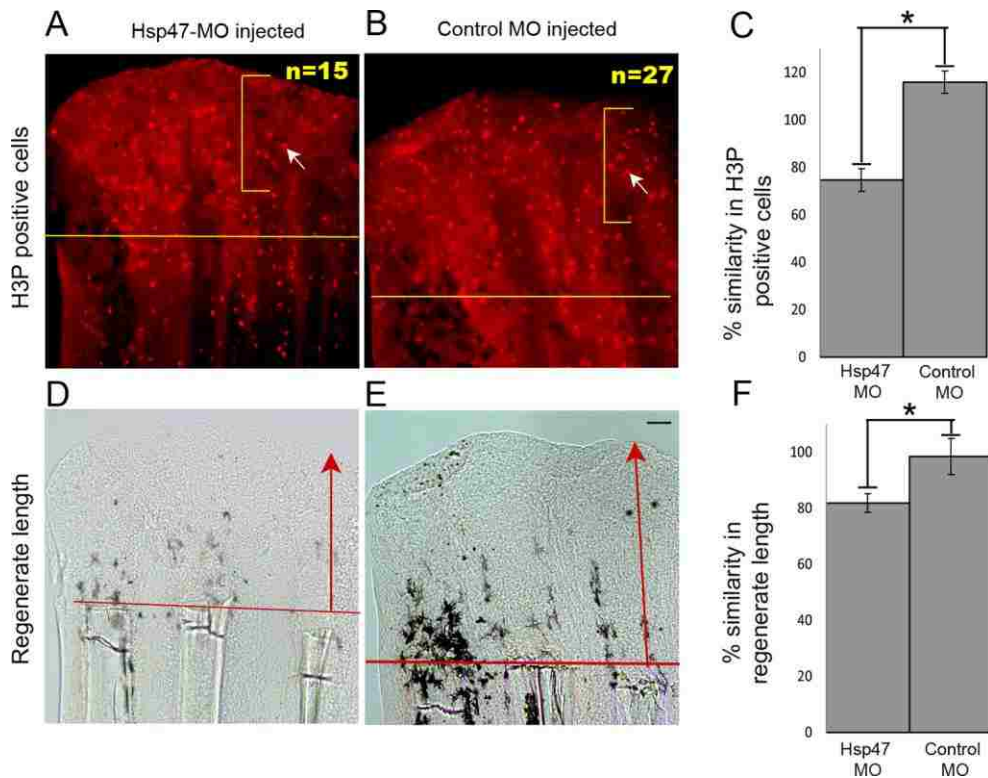
Figure 2.6: Morpholino mediated knockdown of Hsp47 causes reduced cell proliferation and fin length. All fins were amputated at the 50% level prior to injection and electroporation. Fin rays treated with targeting or control MO (injected) were measured and compared to their uninjected sides. The ratio of the injected side (targeting MO or control MO) and the control uninjected side, multiplied by 100, is the percent similarity. Percent similarity of greater than 100 % reflects the fact that the experimental side can be measurably larger than the control uninjected side. (A-C) Total number of H3P positive cells (white arrows identify one positive cell in each panel) were counted in injected and uninjected sides for Hsp47-MO (A) and Hsp47-5MM (B) treated fins. (C) The bar graph shows reduced level of H3P positive cells in Hsp47 MO injected fins compared to the control MO. (D-F) Total regenerate length in Hsp47-MO (D) and Hsp47-5MM (E) injected fins was measured by evaluating the distance between the distal tip of the fin and the amputation plane (indicated by red arrows) and normalized to the respective uninjected side. (F) Bar graph shows reduced regenerate length in Hsp47 MO treated fins. Scale bar is 50 μm and applies to all panels. The student's t-test was performed ($p < 0.05$) to determine significance.
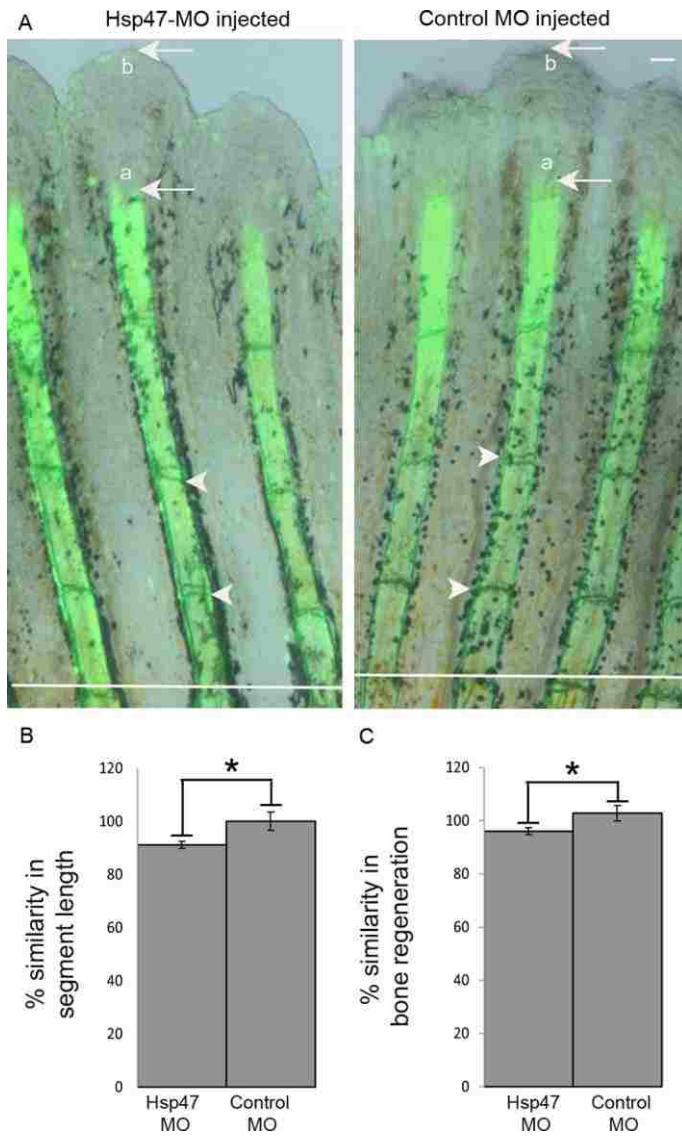
Figure 2.7: Morpholino mediated knockdown of Hsp47 causes reduced segment length and a small change in bone regenerate length. Calcein staining was used to measure segment length and bone regenerate length in the regenerate. The amputation plane is indicated by a white line. Fin rays treated with targeting or control MO (injected) were measured and compared to their uninjected sides. The ratio of the injected side (targeting MO or control MO) and the control uninjected side, multiplied by 100, is the percent similarity. Percent similarity of greater than 100 % reflects the fact that the experimental side can be measurably larger than the control uninjected side. (A) Segment length in Hsp47-MO and Hsp47-5MM injected fins was measured as the distance between the 1st and the 2nd joints (indicated by white arrow heads) distal to the amputation plane. Bone regenerate length was measured as the ratio of the distance of the regenerated length of the calcein stained bone matrix (marked as a) and the total regenerate length (marked as b). The treatment side was normalized against the uninjected side. (B) Bar graph shows reduced segment length following Hsp47 MO injection (p<0.05). (C) Bar graph shows small but significant reduction in bone regenerate length. The scale bar is 50 µm and applies to both panels in A. The student's t-test was performed (p<0.05) to determine significance.
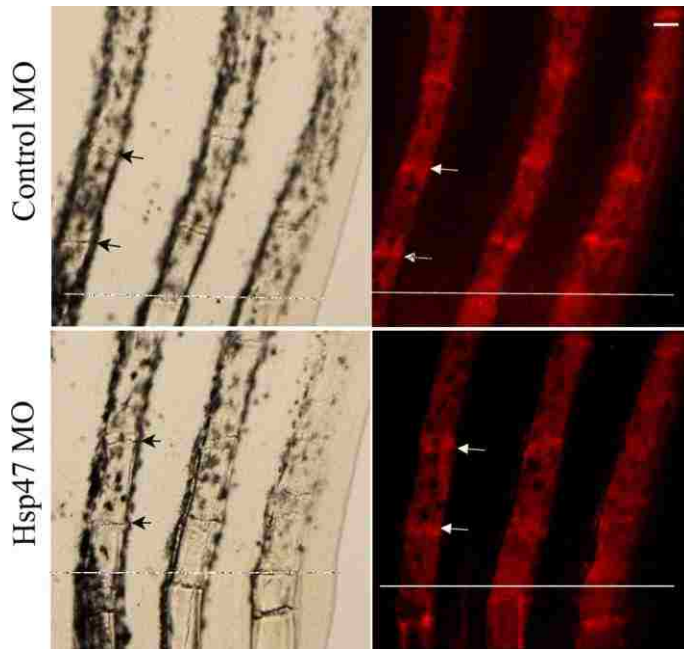
Figure 2.8: The short segment phenotype following Hsp47-MO treatment is due to formation of premature joints. ZNS5 immunostaining detects osteoblasts and joint-forming cells. Brightfield (left) and ZNS5 immunostaining (right) images are shown. ZNS5 positive joints (indicated by arrows) are observed in both standard control (top row) and targeting MO (bottom row) treated fins. Scale bar is 50 μm and applies to all panels.
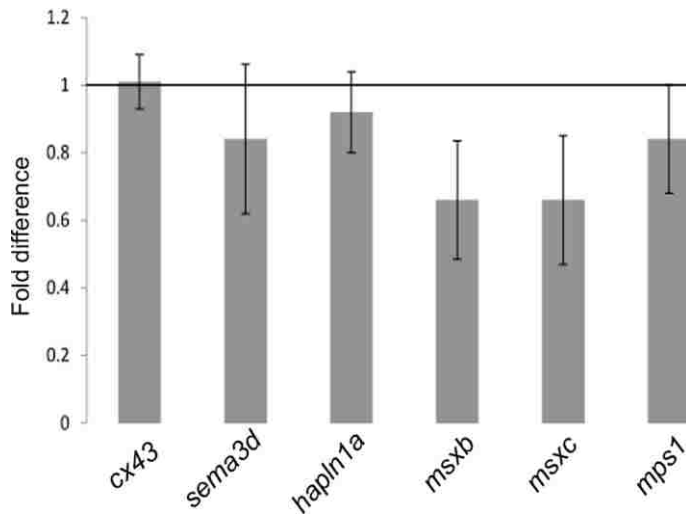
Figure 2.9: Identifying changes in gene expression following Hsp47 knockdown using qRT-PCR. Expression level of *msxb* and *msxc* decreases following Hsp47 knockdown. No change is observed in the expression levels of *cx43, sema3d, hapln1a* and *mps1*. Hsp47 knockdown samples are compared to standard control MO treated samples. The error bars represent the range in variation of the fold-difference based on the standard deviation. A fold-difference of one indicates no change in expression between experimental and control samples.
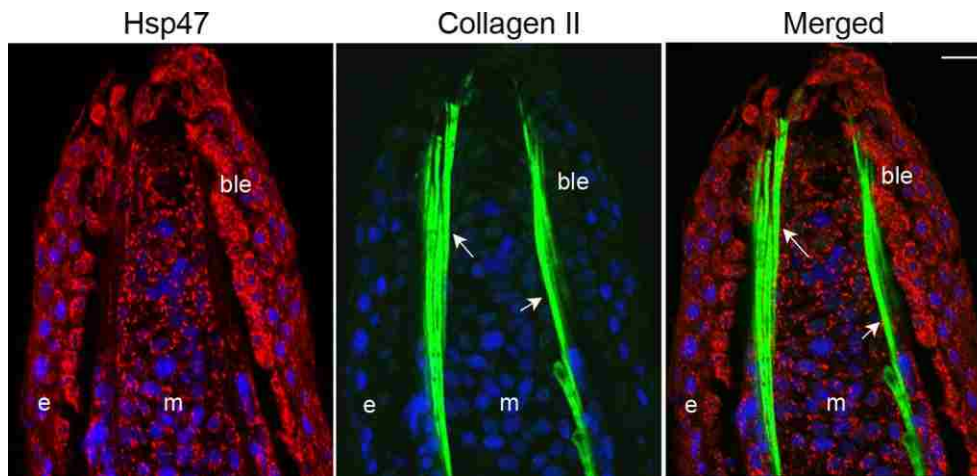
Figure 2.10: Localization of Hsp47 and Collagen type II in WT-5 dpa fins. Confocal images of a WT-5 dpa longitudinal fin section immunostained with Hsp47 (red) and collagen type II (green) and counterstained with To-pro to detect nuclei (blue). Arrows indicate actinotrichia. 'ble' is basal layer of epithelium, 'e' epidermis and 'm' mesenchyme. Scale bar is 10 µm and applies to all panels.
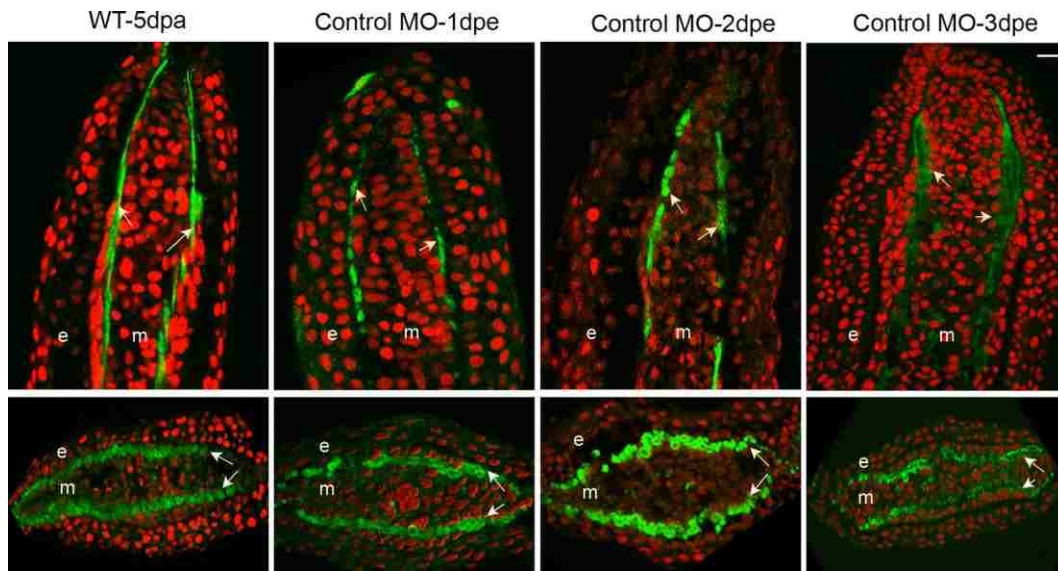
Figure 2.11: Examination of actinotrichia in fin sections following injection with standard control MO at different timepoints. Single plane confocal sections were obtained for longitudinal and transverse sections immunostained with Collagen type II (green) and counterstained with propidium iodide (red). Untreated fins are labeled as 'WT-5dpa'. Longitudinal (top) and transverse (bottom) sections show regeneration of actinotrichia at different time points following injection and electroporation with standard control MO. Arrows indicate actinotrichia, 'e' is epidermis and 'm' mesenchyme. Scale bar is 10 μm and applies to all panels.
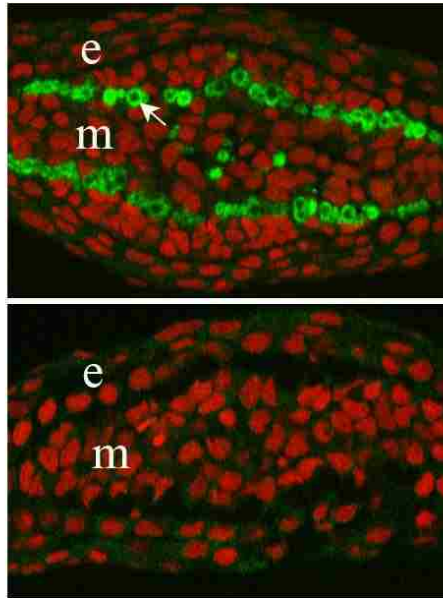
Figure 2.12: Antigen retrieval technique during Immunofluorescence gets rid of the fluorescence from MO injections. Single plane confocal sections were obtained for transverse sections from fins injected with standard control MO immunostained with Collagen type II (green) and counterstained with propidium iodide (red) (Top). Single plane confocal section from fins injected with standard control MO with no primary antibody and counterstained with propidium iodide (red) (Bottom). No green fluorescence for MO is observed in sections without primary antibody treatment (Bottom). Arrow indicates actinotrichia, 'e' is epidermis and 'm' mesenchyme.
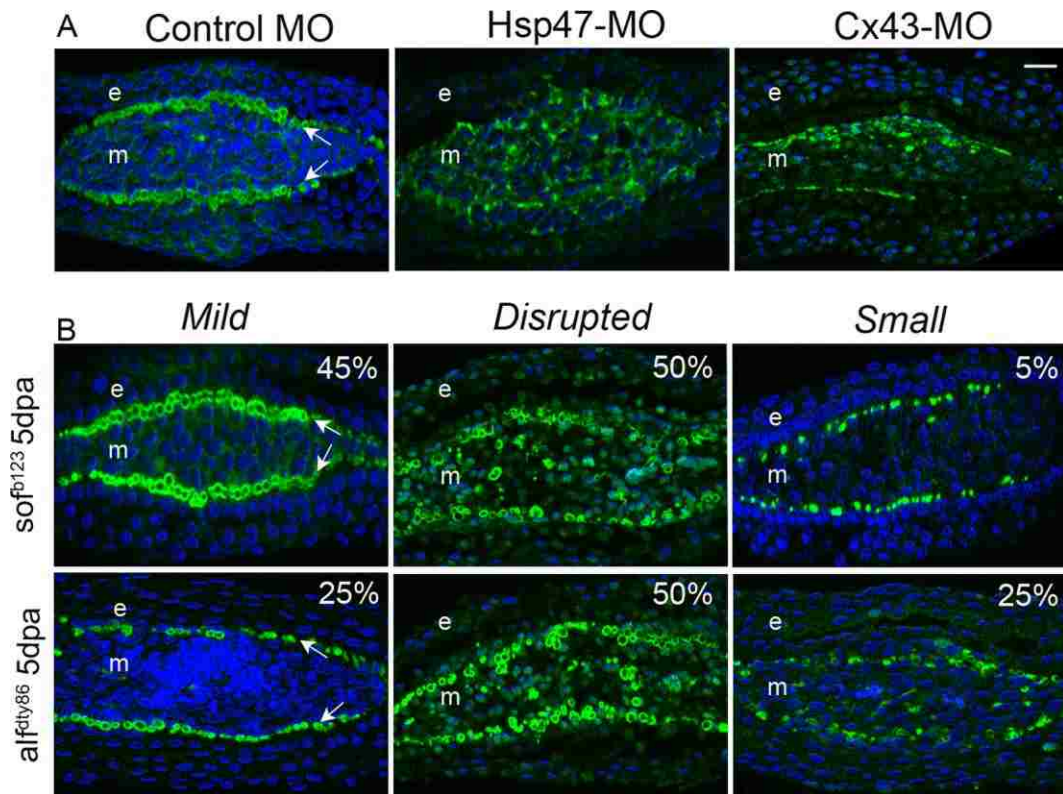
Figure 2.13: Disruption of actinotrichia following changes in Hsp47 or Cx43 expression Single plane confocal sections are obtained for transverse sections immunostained with Collagen type II (green) and counterstained for nuclei with To-pro (blue). (A) Fins are injected with standard control MO, Hsp47-MO or Cx43-MO and evaluated at 1 dpe. Col II expression is disrupted following Hsp47 and Cx43 knockdown. (B) Variable actinotrichia phenotypes observed in 5 dpa transverse sections of both *sof* ^{b123} and *alf* ^{dty86}. The defects were categorized as mild, disrupted organization, or small. Arrows indicate actinotrichia, 'e' is epidermis and 'm' mesenchyme. Scale bar is 10 µm and applies to all panels.
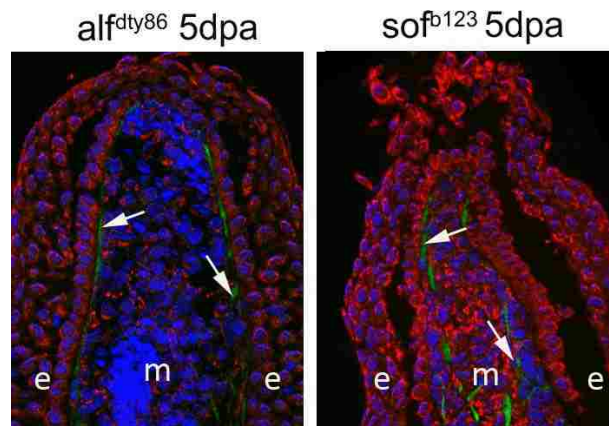
Figure 2.14: Thin, broken actinotrichia in longitudinal sections of *sof* ^{b123} **and** *alf* ^{dty86}**.** Single plane confocal sections are obtained for longitudinal sections of *sof* ^{b123} and *alf* ^{dty86} 5 dpa fins immunostained with Collagen type II (green) and counterstained for nuclei with To-pro (blue). Arrows indicate actinotrichia, 'e' is epidermis and 'm' mesenchyme.
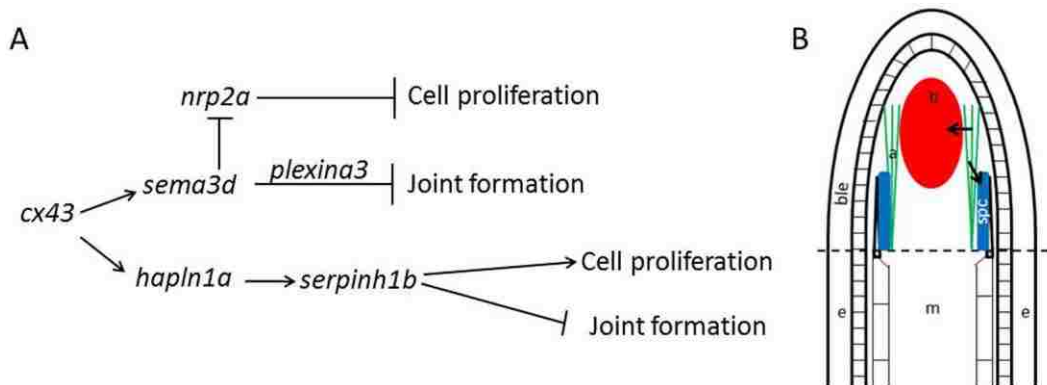
Figure 2.15: Model for *serpinh1b* and Hsp47 in regeneration. (A) Transcriptional
network of *cx43*-dependent genes. The *sema3d* and *hapln1a* genes were shown to be
transcriptionally regulated in independent *cx43* pathways (Ton and Iovine 2012;
Govindan and Iovine 2014). The *serpinh1b* gene is placed downstream of *hapln1a* based
on this study. (B) Speculative model for how Hsp47 may influence bone growth and
patterning. Defective Hsp47 leads to disturbed collagen-based actinotrichia (green).
Defects in collagen may signal to proliferating cells in the blastemal (red) and to skeletal
precursor cells (blue) in the lateral compartment. a, actinotrichia, b, blastemal, ble, basal
layer of the epidermis, m, mesenchyme, e, epidermis, spc, skeletal precursor cells. The
dotted line represents the amputation plane.

Table 2.1: Primers and Morpholinos

Genes	Primers for ISH	Primers for qRT-PCR	Morpholinos
serpin h1b	F= ATGTGGGTATCCAGCCTC RT7=**TAATACGACTCACTATAGGG**TCACCCTTAGGGCGAACTAGCC	F=AGGATGTGAAAAACACAGACG R=TGGAACTTCTCATCCCAGTGG	MO=GCAATGAGGCTGGATACCCACATTC 5MM=GCAtTcAGcCTGcATAgCCACATTC
serpin h1a	F=GCAACCACGCTAGCCGACA RT7=TAATACGACTCACTATAGGGGGTCGGCGTAGAAGAGTTTGGG		
msxb		F=GACCCGTTGAAACGACATCT R=GCCATCAGGGATTCAACACT	
msxc		F=CAACCTCTCCGACTGCAAGAGA R=TGAATGCCTTGGCGGAGAA	
mps1		F=TTCAAGTGTCCGCTCTGCA R=TCTGGACTGATAACAGGTGGCT	

The T7 RNA polymerase binding site in the reverse primer is in bold. F= Forward primer, RT7= Reverse primer, MO= Targeting morpholino, 5MM= Control morpholino with 5 mismatch pairs to target sequence. All primers and MOs are shown in 5' to 3' orientation.

Table 2.2: Changes in *serpinh1b* gene expression by Quantitative RT-PCR

Sample	Gene Average C_T	Keratin Average C_T	ΔC_T Gene-Keratin[a]	$\Delta\Delta C_T$ ΔC_T-$\Delta C_{T,WT/MM}$[b]	Fold Difference relative to WT/MM[c]
sof[b123] compared with WT	24.7± 0.11	17.38± 0.27	7.33± 0.29	1.19± 0.48	0.44 (0.31-0.61)
alf[dty86] compared with WT	23.98± 0.07	18.79± 0.4	5.19± 0.4	-0.955± 0.6	1.94 (1.32 – 2.85)
Cx43 KD compared with MM	24.56± 0.37	17.02± 0.22	7.53± 0.44	1.01± 0.47	0.5 (0.36-0.68)
Sema3d KD compared with MM	21.51 +/- 0.2	15.34 +/- 0.19	6.17 +/- 0.28	0.08 +/- 0.39	0.95 (0.72 - 1.24)
Hapln1a KD compared with MM	22.734 +/- 0.14	15.27 +/- 0.15	7.46 +/- 0.21	1.334 +/- 0.21	0.4 (0.34 - 0.46)

a. The ΔC_T value is determined by subtracting the average Keratin C_T value from the average Gene C_T value. The standard deviation of the difference is calculated from the standard deviations of the gene and Keratin values using the Comparative Method.
 b. The calculation of $\Delta\Delta C_T$ involves subtraction by the ΔC_T calibrator value. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value.
c. The range given for gene relative to WT/MM is determined by evaluating the expression: $2^\wedge -\Delta\Delta C_T$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s = the standard deviation of the $\Delta\Delta C_T$ value. MM refers to the five mismatch control morpholino.

Table 2.3: Quantitative RT-PCR identifies downstream effectors of *serpinh1b* following Hsp47 knockdown

Gene	Gene Average Ct	Keratin Average Ct	ΔCt (Gene-Keratin)	ΔΔCt	Fold difference compared to WT/MM
cx43	19.3 +/- 0.09	15.6 +/- 0.06	3.63 +/- 0.1	-0.02 +/- 0.1	1.01 (0.94 - 1.1)
sema3d	23.27 +/- 0.32	15.6 +/- 0.06	7.63 +/- 0.32	0.26 +/- 0.34	0.84 (0.66 - 1.1)
msxb	18.38 +/- 0.24	14.4 +/- 0.22	3.97 +/- 0.33	0.61 +/- 0.37	0.66 (0.5 - 0.85)
msxc	22.79 +/- 0.12	18.1 +/- 0.11	4.69 +/- 0.16	0.6 +/- 0.4	0.66 (0.5 - 0.88)
mps1	23.59 +/- 0.22	16.02 +/- 0.09	7.57 +/- 0.24	0.25 +/- 0.27	0.84 (0.7 - 1.02)

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Chapter 3: Characterization of Nrp2a- Sema3d-PlexinA3 signaling

3.1. Abstract:

Skeletal morphogenesis is a complex process that is regulated by a number of signaling pathways that interact with each other at a molecular level. Recently we identified Semaphorin3d (Sema3d) and its two putative receptors Neuropilin2a (Nrp2a) and PlexinA3 (PlxnA3) as important mediators of Connexin43 (Cx43) dependent skeletal growth and patterning during caudal fin regeneration in zebrafish. The goal of this study is to characterize the interaction between zebrafish Sema3d and its two putative receptors and to determine how it affects growth and patterning. Our findings suggest that Sema3d reduces homomeric interaction between Nrp2a receptors and also decreases heteromeric interaction between Nrp2a and PlexinA3. PlexinA3 itself acts as a competitor for binding to Nrp2a, thus reducing homo-oligomerization among Nrp2a molecules. We also suggest that the juxtamembrane MAM domain (meprin, A-5 protein, and protein tyrosine phosphatase μ) of Nrp2a can act as both a facilitator for oligomerization and an alternate ligand binding domain.

3.2. Introduction:

Skeletal patterning events are regulated by a number of signaling pathways and through cell-cell communication. Mutations in any of these events can give rise to various forms of skeletal dysplasias. In order to treat them, we need to first understand in detail the structural and functional significance of the individual players present in these pathways. Our lab has identified a number of genes required during skeletal patterning in vertebrates using zebrafish caudal fin regeneration as our model system. Gap junctional intercellular communication (GJIC) is important during normal tissue development. In particular, Connexin43 (Cx43) dependent GJIC is important for skeletal development among vertebrates. Mutations in human *Cx43* causes occulodentodigital dysplasia (ODDD) which is characterized by craniofacial and limb skeleton abnormalities (Flenniken et al. 2005; Paznekas et al. 2009). Knockout mutation in *cx43* in mice is embryonically lethal due to cardiac malformations (Reaume et al. 1995). Further examinations of the embryos also revealed delayed ossification of bones (Lecanda et al. 2000). In zebrafish, recessive mutations in *cx43* causes *short fin (sof ^{b123})* phenotype, which is characterized by short fin length, short segment length and reduced level of cell proliferation (Iovine et al. 2005). All of these events provide enough proof that Cx43 mediated gap junctional communication is important for development, and understanding how the exchange of such small molecules mediate such obvious phenotype is of utmost importance.

One of the genes identified in the *cx43* dependent growth and patterning pathway during skeletal regeneration is *semaphorin3d (sema3d)*, a secreted signaling molecule (Ton and Iovine 2012). Sema3d mediates Cx43 dependent growth and patterning events through two putative receptors – Neuropilin2a (Nrp2a) and PlexinA3 (PlxnA3). Sema3d promotes cell proliferation / growth through Nrp2a and suppresses joint formation / patterning through PlxnA3 (Ton and Iovine 2012). However, it is unclear how Sema3d interacts with either of these two receptors to mediate the process. Understanding how Sema3d signals through these receptors to mediate growth and patterning during skeletal morphogenesis will take us one step closer to understanding underlying mechanism of skeletal diseases.

Class 3 semaphorins are secreted signaling molecules, initially characterized in the developing nervous system and are known to regulate cell migration and axonal growth cone guidance. Recent studies have elucidated their roles in tumor progression, metastasis, angiogenesis and other important biological processes (Bielenberg and Klagsbrun 2007; Yazdani and Terman 2006). In zebrafish, during development, Sema3d is expressed in the neural crest cells and nervous system (Halloran et al. 1999) and is an important regulator for retinal axon guidance and myocardial development (Liu et al, 2004; Sato et al, 2006). Two of the high affinity receptors for semaphorins are plexins and neuropilins (He and Tessier-Lavigne 1997; Kolodkin et al. 1997; Tamagnone et al. 1999). Neuropilins are single transmembrane spanning proteins that are unique to vertebrates (Nakamura and Goshima 2002). Due to its short cytoplasmic domain, neuropilins binding requires interactions with other co-receptors in order to generate intracellular signaling. Generally for semaphorin mediated signaling events, neuropilins bind to PlexinA molecules, another class of transmembrane spanning receptors. During early development of axon pathways in zebrafish brain, Sema3d needs to interact with Nrp1a and Nrp2b (Wolman et al. 2004). PlxnA3, on the other hand, is required for proper motor neuron morphogenesis, possibly through the interaction with semaphorins (Palaisa and Granato 2007). Semaphorin dependent signaling through neuropilins and plexins has been identified in a number of cancers, making them an excellent target for drug treatment (Capparuccia and Tamagnone 2009). Therefore, it is important to understand how Sema3d regulates signaling events through its putative receptors Nrp2a and PlxnA3. Class 3 semaphorins contain a Sema domain, an immunoglobin domain and a basic amino acid rich domain (Nakamura and Goshima 2002) (Figure 1.9). It has been suggested that homodimerization of Class 3 semaphorins is required for proper biological activity (Klostermann et al. 1998). A full length transmembrane neuropilin contains four different domains, following a secretion signal sequence – two repeats of extracellular complement (CUB) domain, two repeats of coagulation factor (FV/VIII) domain, a MAM (meprin, A-5 protein, and protein tyrosine phosphatase µ) domain, a single transmembrane domain and a small cytosolic domain (Bagri et al. 2009; Caunt et al. 2008; Ellis 2006; Fukasawa et al. 2007; Rossignol et al. 2000; Schwarz and Ruhrberg

2010; Zachary et al. 2009; Nakamura et al. 1998). While the CUB and FV/VIII domains are considered to be major binding sites for class 3 semaphorins and other ligands (Chen et al. 1998; Nakamura et al. 1998), MAM domain is mostly involved in homo-oligomerization for neuropilins (Nakamura et al. 1998). MAM domain is also thought be involved in signal transduction, possibly through interaction with plexins as co-receptors (Takahashi et al. 1999). Plexins are characterized by an extracellular Sema domain, followed by three plexin-semaphorin-integrin (PSI) domains, three immunoglobulin-plexin-transcription factor (IPT) domains, a single transmembrane domain and a cytosolic Ras GTPase activating (GAP) domains with an inserted Rho GTPase binding (RBD) domain (He et al. 2009b; Tong et al. 2009). It has been suggested that plexin homo-oligimerization through interaction between their cytosolic domains is important for signal transduction (Barton et al. 2015b; Takahashi and Strittmatter 2001).

In this report, we have focused on identifying whether Sema3d physically binds to its two putative receptors Nrp2a and PlexinA3 and how does that binding affect receptor oligomerization. We also experimented with different cell based assays to determine the functional significance of ligand-receptor interaction.

3.3. EXPERIMENTAL PROCEDURES

3.3.1. Plasmids Preparation:

Cloning for the Nrp2a-GFP and Nrp2a-RLuc constructs used for BRET[2] measurements was previously described (Barton et al. 2015a). For maltose-binding protein (MBP)-fusion constructs, a 6-His tag, followed by MBP, a poly-glycine linker, and tobacco etch virus cleavage site was cloned into pET42 as an NdeI/BamHI insert. The pET28 multiple-cloning site between BamHI and XhoI was subsequently cloned into this plasmid at BamHI/XhoI. The Nrp2a MAM domain (residues 629-796 of NCB Accession # AI62118.1) and Sema3d were then cloned into this plasmid as EcoRI/XhoI inserts. The plasmid encoding AP-SEMA3F was a generous gift from Dr. Roman J. Giger (University of Michigan). The V5-His-TOPO tagged PlexinA3 plasmid was cloned as previously described (Barton et al. 2015b).

3.3.2. Expression and Purification of MBP, MBP-Sema and Nrp2a MBP-MAM:

An expression plasmid containing the Nrp2a MAM domain fused to MBP was transformed into BL21(DE3) cells (Invitrogen) and stored as a glycerol stock. For expression, cultures were started from glycerol stocks and grown to saturation overnight at 37°C in LB + 50 µg/mL kanamycin. Cells were then diluted to an optical density (600 nm) of 0.4 and allowed to grow an additional 3-6 hours at 20°C, at which point 1 mM IPTG was added to the culture. Cells were collected 16-24 hours post-IPTG induction, re-suspended in lysis buffer (100 mM HEPES, 500 mM sodium chloride, 10 mM imidazole, and 10% (v/v) glycerol) and lysed using freeze-thaw and tip-sonication. The soluble MBP-MAM fusion protein was subsequently purified by nickel affinity chromatography with chelating sepharose fast flow resin (GE Healthcare) and imidazole washes ranging from 10 mM to 500 mM in 20 mM HEPES, 500 mM sodium chloride, and 10% (v/v) glycerol. Purity of at least 90% was confirmed by 12% polyacrylamide gels run in NuPAGE MES SDS running buffer (Life Technologies) and stained using Coomassie blue solution.

3.3.3. BRET^2 Assay:

Transfections, generation of semaphorin-containing media, and measurements for the BRET^2 assay were performed as previously described (Barton et al, 2015). For treatment, wells were treated for 1.5 hours with 50% BRET^2 buffer (PBS + 0.1 g/L calcium chloride + 0.1 g/L magnesium chloride hexahydrate + 1 g/L glucose) with 0-0.17 mg/mL MBP-MAM and 50% spent media, containing AP-SEMA3F and Sema3d-FLAG as applicable.

3.3.4. Phalloidin Assay:

AB9 cells were incubated at 5% CO_2 and 28˚C and grown in tissue culture dishes with minimal essential media (DMEM) supplemented with 15% heat inactivated FBS and antibiotics-antimycotics (Gibco). The cells were seeded onto poly-L-lysine coverglasses and allowed to get 60-70% confluent. They were then either transfected with V5-His-TOPO tagged PlxnA3 through electroporation, or not transfected, depending on conditions required. 24 hours post transfection (hpt), AB9 cells were treated with appropriate peptides (MBP-Sema3d, AP-Sema3F, MBP-Nrp2a-MAM or MBP only) for 1.5 to 2 hours at 28˚C.They were then washed once with cold PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. This was followed by washes in 1X PBS (3X, 5 minutes each), permeabilization with 0.1% triton in PBS for 5 minutes, again multiple washes in 1X PBS. They were then stained with Phalloidin (1:50) and DAPI / TOPRO (1:1000) were used to counterstain the nuclei. It was followed by three 5 minutes washes in 1X PBS and one quick wash in dH2O. They were then mounted with Vectashield and were examined under Nikon Eclipse TE2000-U at 60X or a LSM 510 Meta Confocal Microscope (Zeiss) at 40X, using Argon (488) and HeNe (543 and 633) lasers.

3.4. Results:

Previous data from our lab have suggested cell signaling through the Nrp2a receptor suppresses cell proliferation during regeneration. Sema3d acts as a negative regulator for Nrp2a signaling and in turn promotes cell proliferation (Ton and Iovine 2012). In order to understand how Sema3d binding regulates Nrp2a signaling, we used the BRET^2 assay to determine whether Sema3d binding affects homo-oligomerization between Nrp2a receptors. The BRET^2 assay is used to determine if two proteins are closer than 10nm to each other or not. The proteins of interest are either tagged with the donor *Renilla* luciferase (RLuc) or the acceptor GFP^2. Upon addition of the substrate Deep Blue C, the RLuc catalyzes a reaction that causes emission at 395 nm. This in turn excites the neighboring GFP^2 tag, causing an emission at 510 nm. The level of interaction is characterized by the ratio of intensity of light emitted by GFP^2 (green fluorescence at 395 nm) to light emitted by RLuc (magenta luminescence at 395 nm) (Pfleger et al. 2006).

3.4.1. Nrp2a homo-oligomerization is reduced following Sema3d addition:

Full length zebrafish Nrp2a receptors form dimers (Barton et al, 2015). In order to determine if Sema3d affects homomeric interaction between Nrp2a receptors, we transfected Cos7 cells with full length wild type (WT) zebrafish Nrp2a tagged with either RLuc or GFP^2 (Nrp2a-Rluc, Nrp2a-GFP^2). 24 hours post transfection (hpt), we treated the cells with either media containing FLAG-tagged Sema3d (Sema3d-FLAG) or 17 nmol of recombinant human Sema3d (hSema3d). The level of oligomerization was determined as ratio of green to magenta emission. As a negative control, we transfected cells with GFP^2 and RLuc. Under normal conditions, free GFP^2 and RLuc should not interact with each other, which is supported by minimal green / magenta emission ratio for GFP and RLuc (Figure 3.1). The baseline in Figure 3.1 represents oligomerization among Nrp2a receptors under no treatment condition. A mild (approximately 5%) reduction in Nrp2a oligomerization was observed following addition of Sema3d (Figure 3.1). This suggests that Sema3d partially disrupts Nrp2a homo-oligomerization.

3.4.2. PlexinA3 acts as a competitor for Nrp2a-Nrp2a interaction:

In a number of systems, plexins need to interact with neuropilins to transduce signaling. In order to determine whether the presence of the PlexinA3 receptor affects Nrp2a homo-oligomerization, we transfected the Cos7 cells with Nrp2a-GFP[2], Nrp2a-RLuc and a full length V5-His-TOPO tagged PlxnA3 construct. 24 hpt, we treated the appropriate cells with media containing Sema3d-FlAG. The green/magenta emission ratio for all the conditions in Figure 3.2 is compared to the emission ratio of Nrp2a-GFP[2] and Nrp2a-RLuc under no treatment condition (indicated here by the baseline). Our data suggest that the presence of a full length PlexinA3 receptor was enough to reduce Nrp2a homo-oligomerization by approximately 15%. We also observed that addition of Sema3d-FLAG to cells containing all three receptors did not cause significant changes in receptor interactions when compared to cells without Sema3d treatment.

3.4.3. Nrp2a-PlexinA3 hetero-oligomerization is reduced following Sema3d addition:

In order to determine whether Sema3d impacts Nrp2a-PlexinA3 interactions, we transfected the Cos7 cells with full length WT Nrp2a-GFP[2] and PlexinA3-RLuc constructs. We then treated the cells with either media obtained from Sema3d-FLAG transfected cells or 17nmol recombinant hSema3d. In Figure 3.3, the baseline indicates hetero-oligomerization between Nrp2a and PlexinA3 receptors under no treatment condition. In this case however, we observed a lower level of oligomerization signal between Nrp2a and PlxnA3, when compared to cells transfected with no DNA. We did observe a reduction in receptor hetero-oligomerization, when compared to no Sema3d treatment (Figure 3.3). The reduction was moderate as well, approximately 5% for Sema3d-FLAG treatment and 3% for cells treated with human Sema3d. (Figure 3.3).

3.4.4. The juxtamembrane MAM domain can act as an enhancer for Nrp2a oligomerization and an alternate ligand binding domain for Sema3d:

MAM domains of meprins, protein tyrosine phosphatases and neuropilins have been known to facilitate receptor oligomerization (Marchand et al, 1996; Cismasiu et al, 2004, Chen et al, 1998; Giger et al, 1998; Nakamura et al, 1998). For zebrafish Nrp2a, a

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conserved cysteine (C711) in MAM regulates proper homomeric interactions (Barton et al. 2015b). In order to determine if addition of extra MAM will facilitate or inhibit similar interactions, we added a peptide containing Nrp2a-MAM domain fused to MBP to the cells transfected with Nrp2a-GFP[2] or Nrp2a-RLuc. We observed an increase in oligomerization following addition of MAM, when compared to the level of interaction between Nrp2a-GFP[2] and Nrp2a-RLuc under no treatment condition (indicated by the baseline) (Figure 3.4). In contrast, addition of Sema3d-FLAG together with MAM, resulted in completely opposite effect through reduction of homomeric interaction between Nrp2a receptors (Figure 3.4). These findings suggest that while exogenous MAM enhances Nrp2a homo-oligomerization, addition of Sema3d not only blocks this function of MAM, but further inhibits homo-oligomerization.

3.4.5. Sema3d does not affect cytoskeletal dynamics:

In order to determine the functional significance of change in receptor oligomerization, we decided to look at effects on intracellular structure, especially the actin cytoskeleton. Many of the class 3 semaphorins are known regulators for growth cones. For example, in sympathetic axons, Sema3F induces growth cone collapse through regulation of cytoskeletal structure (Atwal et al. 2003). In breast cancer cell lines, addition of Sema3F causes loss of lamellipodia extensions leading to reduced attachment and migration of cells (Nasarre et al. 2003). In order to evaluate whether addition of Sema3d elicited similar responses in regulating cytoskeletal dynamics, we added Sema3d tagged with MBP to a zebrafish fibroblast cell line (AB9) (Paw and Zon 1999). AB9 cells express both Nrp2a and PlexinA3 receptors (Bhadra et al, *accepted*). Cells were treated with MBP-Sema3d for 1.5 to 2 hours, using AP tagged Sema3F as our positive control. They were then fixed and stained with Phalloidin (to look at actin filaments). Cells treated with Sema3F showed growth cone collapse, as shown by a more rounded edge instead of microspikes (Figure 3.5A). We did not observe any obvious change in growth cones or lamellipodia / filopodia extensions in cells treated with MBP-Sema3d (Figure 3.5A). Similar results were observed for cells transfected with V5-His-TOPO tagged PlxnA3 (Figure 3.5B). We also measured the cell perimeter and cell area and calculated their

ratio, in order to evaluate any changes in the length of the filopodias formed. We did not observe any difference between control and treatment (data not shown).

3.4.6. Nrp2a-MAM addition does not affect cytoskeletal dynamics:

Since addition of Nrp2a-MAM increased receptor oligomerization, we decided to evaluate its effect on actin cytoskeleton. AB9 cells were treated with two different concentrations of Nrp2a-MAM – 0.085 mg/ml and 0.0425 mg/ml, both of which show increased receptor oligomerization, for 1.5 to 2 hours. As a control, we treated the cells with 0.085 mg/ml of MBP only. We then fixed the cells and stained them with Phalloidin. In this case also, we did not observe any differences in growth cones or lamellipodia / philopodia extensions between control and treatment (Figure 3.6).

3.4.7. Cell proliferation is not altered following Sema3d or Nrp2a-MAM treatment:

Both Sema3d and Nrp2a are regulators for cell proliferation during fin regeneration (Ton & Iovine, 2012). In order to correlate change in receptor oligomerization with change in cell proliferation, we used two different *in vivo* assays. First, we injected fins with different concentrations of Nrp2a-MAM or MBP-Sema3d. We failed to notice any significant difference in H3P positive cell proliferation or regenerate length between the control and treatment (data not shown). We then treated the AB9 cells with similar concentrations of Nrp2a-MAM and MBP-Sema3d for 24 hours and performed a BrdU assay to detect level of cell proliferation. In this case also, we did not see any observable difference in cell proliferation level between control and treatment (data not shown).

3.5. Discussion:

Semaphorins are most well characterized in the nervous system, but they are expressed by a wide array of other tissues including, but not limited to bone, cartilage, connective tissue and fibroblasts (Halloran et al. 1999; Lallier 2004; Puschel et al. 1995; Wright et al. 1995). Through interaction with its various receptors, semaphorins can regulate cell proliferation, migration, patterning and cytoskeletal organization (Yazdani and Terman 2006). It is therefore not surprising, that semaphorins play important regulatory roles in cancer (Neufeld and Kessler 2008). For example, Sema3B and Sema3F have been suggested to act as tumor suppressors (Potiron et al. 2009), whereas overexpression of Sema3C has been linked to metastasis in lung adenocarcinoma (Martin-Satue and Blanco 1999) and as a marker for poor patient survival for ovarian cancer (Galani et al. 2002). For these reasons, semaphorins and their receptors are studied as potential targets for drug treatment. Indeed, class 3 semaphorins, especially Sema3d and Sema3E significantly decrease tumor growth through inhibition of tumor angiogenesis (Sabag et al. 2012). However, even with its extensive role in regulating a wide variety of cellular events, the molecular mechanism through which semaphorins function is still not fully understood. In this chapter, we have tried to identify the molecular mechanisms through which Sema3d binding affects its putative receptor oligomerization and future work will try to determine the functional significance of Sema3d-Nrp2a-PlexinA3 interaction. Previous studies from our lab have identified genetic interactions between Sema3d and its two receptors, Nrp2a and PlexinA3 (Ton and Iovine 2012). Our findings suggest that Sema3d indeed can bind to Nrp2a and PlexinA3. Sema3d binding to Nrp2a, moderately decreases its oligomerization, thus potentially resulting in reduced signaling events. This confirms previous findings from our lab, which suggested that Sema3d is a negative regulator for Nrp2a mediated signaling during cell proliferation / growth. Nrp2a itself is a negative regulator of cell proliferation. Sema3d binds to Nrp2a, makes the oligomers come apart and thus reduces signaling, which in turn leads to promotion of cell proliferation.

The role of neuropilins during tumor angiogenesis is well established. Nrp2 expression for example is observed in osteosarcoma (Handa et al. 2000), melanoma (Lacal et al.

2000), myeloid leukemia (Vales et al. 2007) and many other types of cancer (Grandclement and Borg 2011). Full length neuropilin receptors are dimeric and this homomeric interaction is regulated by the juxtamembrane MAM domain (Chen et al. 1998; Giger et al. 1998; Nakamura et al. 1998; Takahashi et al. 1999; Takahashi et al. 1998). MAM domains in other protein families like meprins and protein tyrosine phosphatase are also associated with facilitating homomeric interactions (Marchand et al. 2006; Cismasiu et al. 2004). Recent studies have shown that the conserved cysteine residues in the MAM domain of full length zebrafish Nrp2a receptor play an important role in promoting oligomerization (Barton et al. 2015a). We saw an enhanced oligomerization of full length Nrp2a receptors following addition of MAM peptides through $BRET^2$ assay. Interestingly, upon addition of Sema3d at a concentration that does not affect oligomerization, we observed a marked decrease in homomeric interaction following addition of MAM peptides. This suggests an alternate ligand binding role for the MAM domain, besides its more common role as a facilitator for homomeric interaction.

Neuropilins have a short cytoplasmic tail and in order to transduce signals, they need to bind to other transmembrane receptors like plexins. It is suggested that class 3 semaphorins do not bind to plexins directly, instead they bind to neuropilins which in turn can form hetero-oligomers with plexins to transduce intracellular signaling (Takahashi et al. 1999). We observed a moderate decrease in hetero-oligomerization between Nrp2a and PlexinA3 receptors following Sema3d addition. Interestingly, PlexinA3 also seems to act as a competitor for Nrp2a-Nrp2a interaction. We saw a reduction in homomeric interaction between Nrp2a receptors when a full length PlexinA3 receptor is present as well. Interestingly, addition of Sema3d did not significantly change the level of homo-oligomerization in the presence of PlxnA3. This, in addition to the observed moderate decrease in receptor interaction suggests that Sema3d might not be the only ligand that is required to bring together a fully functional signaling complex. There might be other interacting transmembrane receptors present as well. Indeed, another common family members that interact with neuropilins include vascular endothelial growth factors (VEGF) and their receptors VEGFRs. It has also been proposed that class

3 semaphorins and VEGFs can compete for binding to neuropilins (Parker et al. 2010). Both *vegf-a* and *vegfr2* are expressed in regenerating fins and regulate blood vessel regeneration (Bayliss et al. 2006). In many cases, cell adhesion molecules (CAM) like L1CAM and NrCAM, and integrins also can interact with neuropilins and their co-receptors (Castellani et al. 2000; Falk et al. 2005; Fukasawa et al. 2007).

In this report, we failed to determine the functional significance of change in receptor oligomerization *in vivo*. There can be a number of reasons for that. It has been suggested that the effect of a ligand can be instant and temporary. For example, Sema3A induces growth cone collapse in dorsal root ganglia (DRG) neurons (Tessier-Lavigne and Goodman 1996). The actin filaments at the lamellipodia stopped protruding after only a 3 minute treatment with Sema3A (Fan et al. 1993). Phosphorylated cofilin accumulated at the growth cone at 3 minutes treatment and its level decreased within 10 minutes (Aizawa et al. 2001). Therefore, we might need to look at an earlier time point, preferably within 10 minutes to evaluate changes in actin cytoskeleton. This instant and temporary interaction can also explain why we did not observe any change in cell proliferation in regenerating fins or in AB9 cells. Future studies will attempt to look at actin cytoskeleton dynamics and its regulators following Sema3d or Nrp2a-MAM treatment.

In conclusion, we suggest that Sema3d binding negatively affect Nrp2a and PlexinA3 oligomerization. We also provide evidence that the juxtamembrane MAM domain is important for homomeric interaction between Nrp2a receptors and might also act as an alternate ligand binding domain for Sema3d. The signaling through Sema3d-Nrp2a-PlexinA3 complex is complicated and future work shall try to identify other possible interacting ligand / receptor complexes that help in transduction of intracellular signaling.
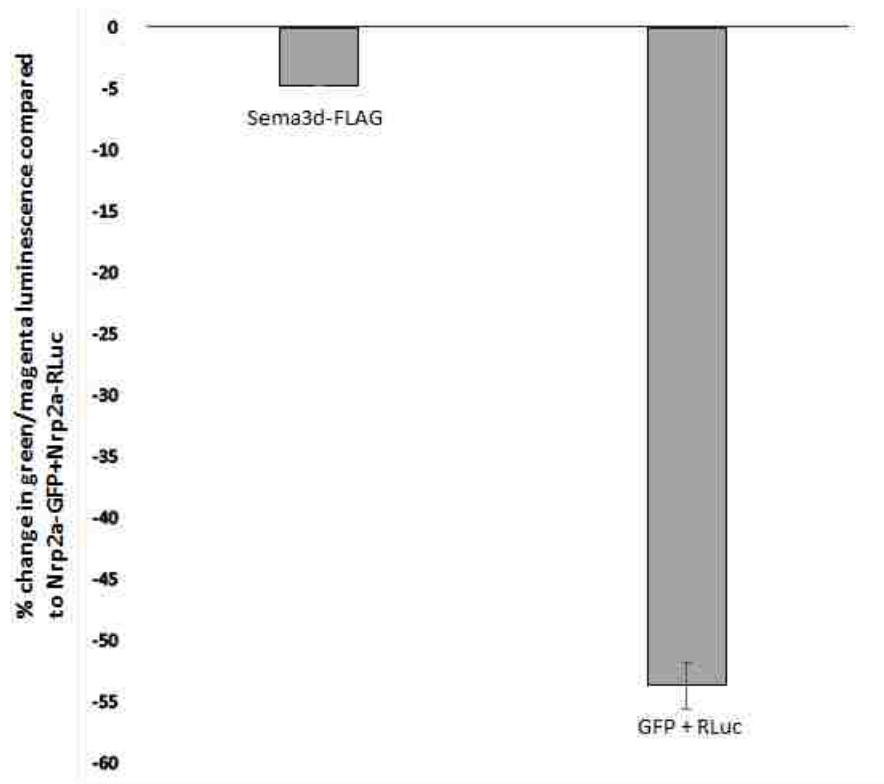
Figure 3.1: Addition of Sema3d-FLAG reduces Nrp2a homo-oligomerization. Results are collected from three different trials and error bars represent standard error. The baseline represents homomeric Nrp2a interaction under no treatment condition. Sema3d-FLAG was added to cells transfected with Nrp2a-GFP and Nrp2a-RLuc. Negative values indicate reduced GFP – Rluc interaction. As negative control, cells were transfected with only GFP and RLuc plasmids.
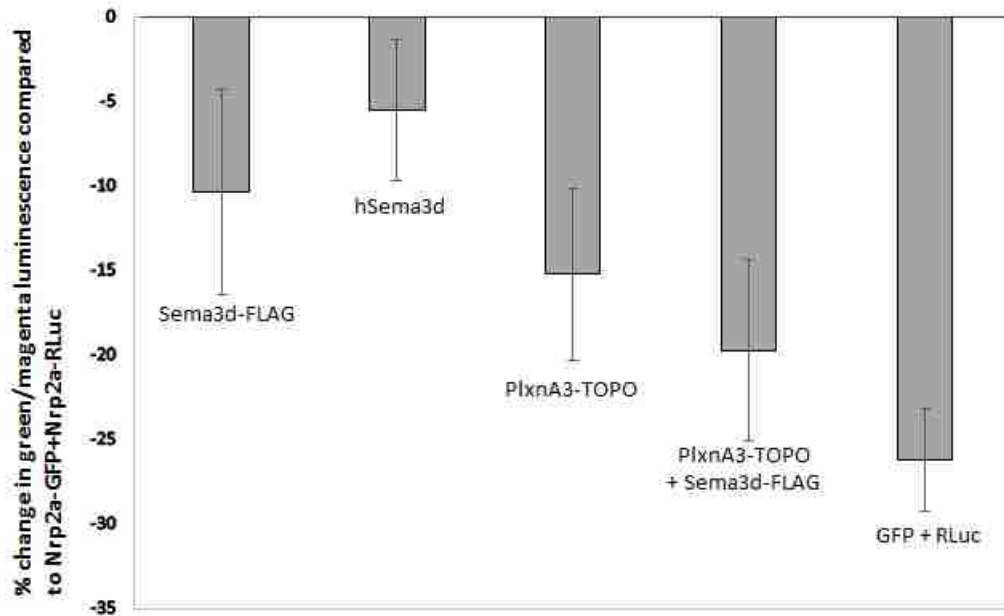
Figure 3.2: PlexinA3 acts as competitor for Nrp2a homomeric interaction. Results are collected from three different trials and error bars represent standard error. The baseline represents homomeric Nrp2a interaction under no treatment condition. Sema3d-FLAG or hSema3d was added to cells transfected with Nrp2a-GFP, Nrp2a-RLuc with or without PlxnA3-TOPO. Negative values indicate reduced GFP – Rluc interaction. As negative control, cells were transfected with only GFP and RLuc plasmids.
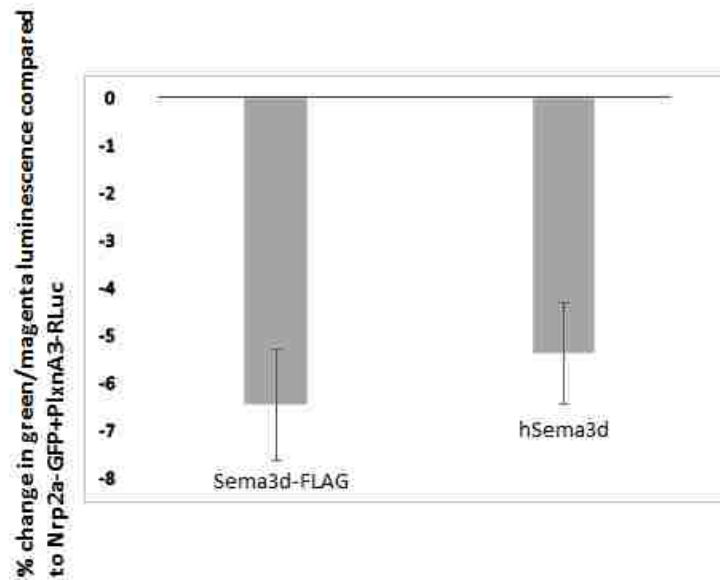
Figure 3.3: Sema3d reduces hetero-oligomerization between Nrp2a and PlxnA3.
Results are collected from two different trials and error bars represent standard error. The baseline represents heteromeric interaction between Nrp2a and PlexinA3 under no treatment condition. Sema3d-FLAG was added to cells transfected with Nrp2a-GFP and PlxnA3-RLuc. Negative values indicate reduced GFP – Rluc interaction. Same negative control from Figure 3.2 was used in this case.

Figure 3.4: Nrp2a MAM domain increases homomeric interaction between Nrp2a receptors and possibly interacts with Sema3d. Results are collected from three different trials and error bars represent standard error. The baseline represents homomeric Nrp2a interaction under no treatment condition. Nrp2a-MAM and/or Sema3d-FLAG was added to cells transfected with Nrp2a-GFP and Nrp2a-RLuc. Negative values indicate reduced GFP – Rluc interaction. As negative control, cells were transfected with only GFP and RLuc plasmids.
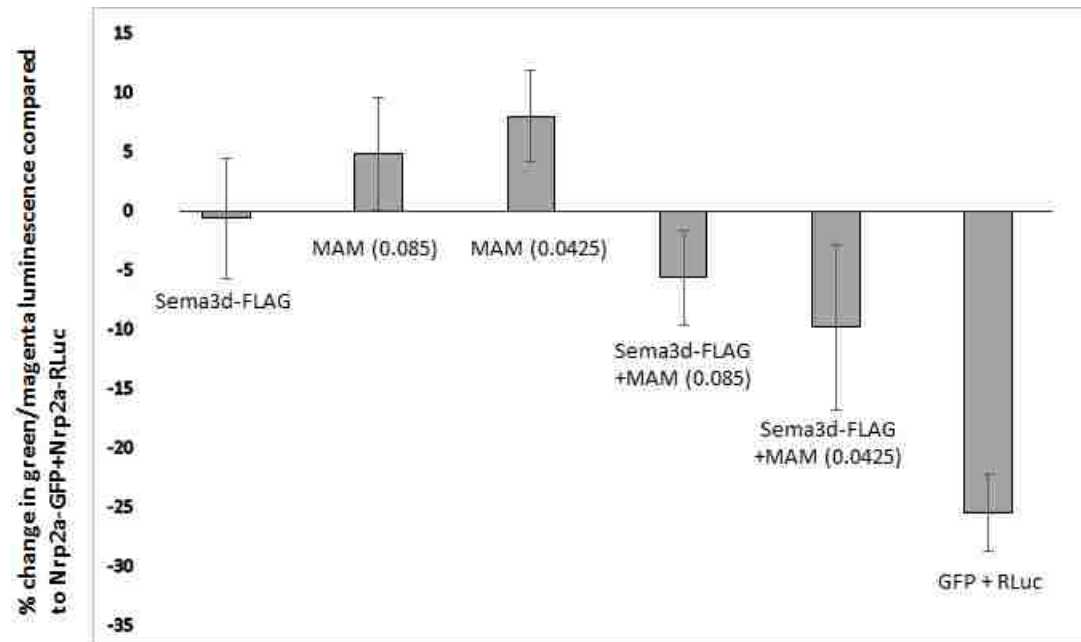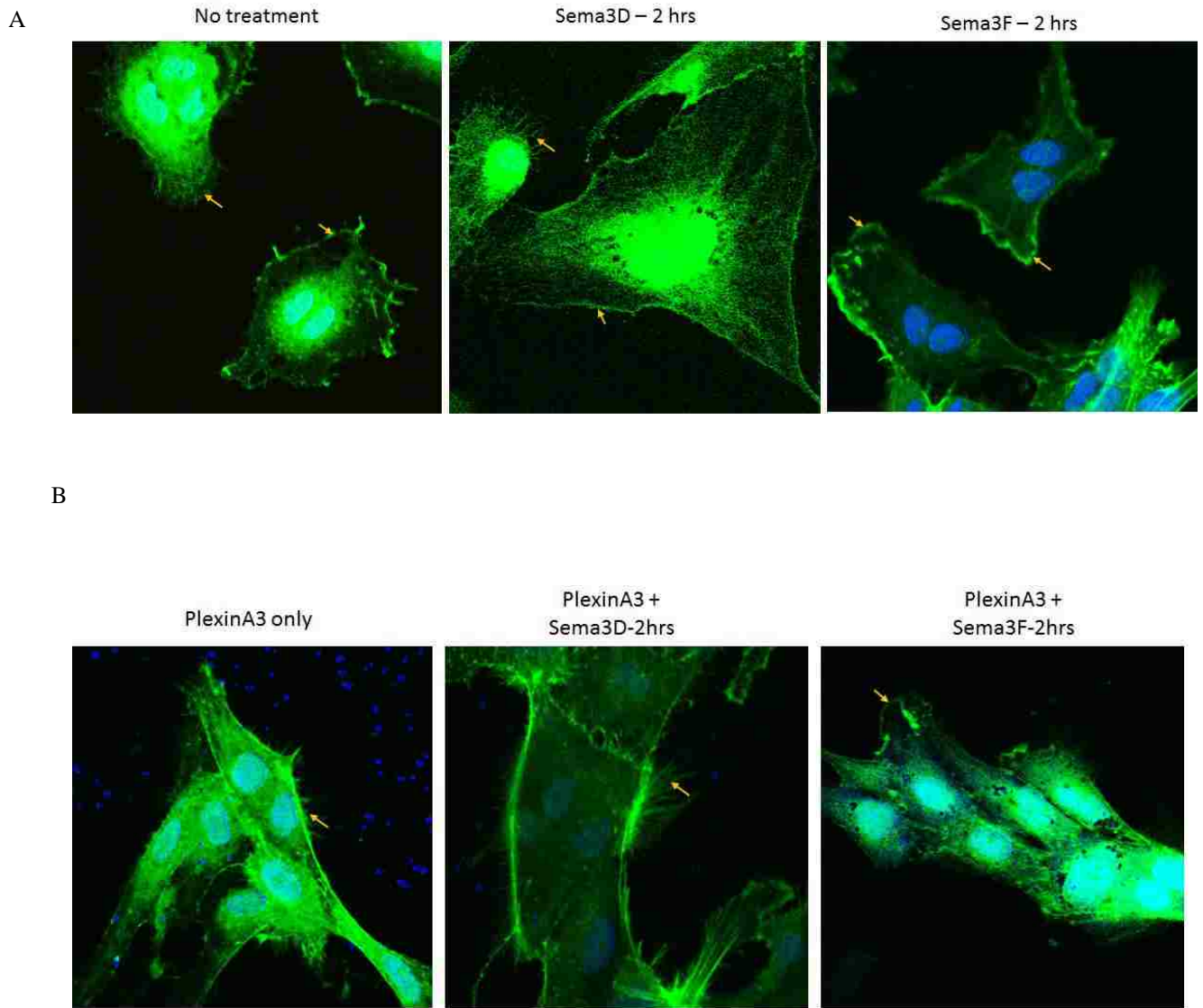
Figure 3.5: Addition of Sema3d and PlxnA3 do not affect actin cytoskeleton in AB9 cells. Cells were treated with MBP-Sema3d or AP-Sema3F for 1.5 – 2 hrs and stained with Phalloidin (green) (A). Cells were transfected with V5-His-TOPO PlxnA3 and after 24 hrs, were treated with MBP-Sema3d or AP-Sema3F and stained with Phalloidin (green) (B). Nuclei are stained with DAPI. Yellow arrows indicate lamellipodia or filopodia.
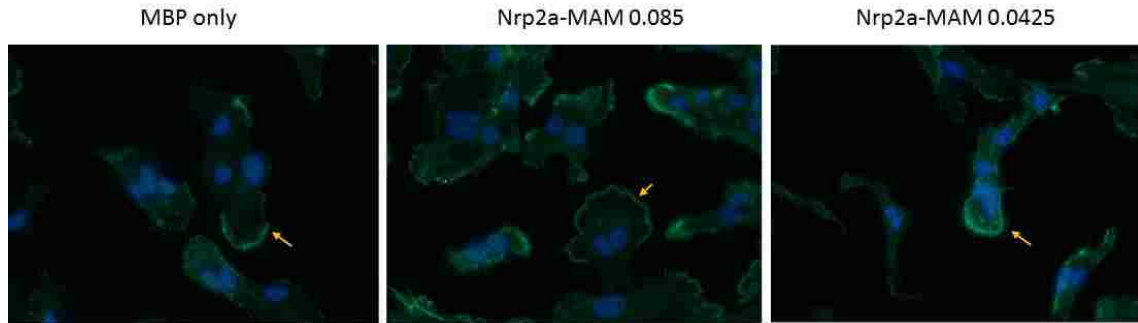
Figure 3.6: Addition of Nrp2a-MAM does not affect actin cytoskeleton in AB9 cells.
Cells were treated with two different concentrations of Nrp2a-MAM – 0.085 mg/ml and
0.0425 mg/ml. 0.085 mg/ml MBP was used as positive control. Treatment time was 1.5
to 2 hrs followed by Phalloidin staining (green). Nuclei are stained with DAPI. Yellow
arrows indicate lamellipodia or filopodia.

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Chapter 4: Zebrafish fibroblast cell line, AB9, is a simpler tool to complement gene regulation studies during fin regeneration

4.1. Abstract:

The regenerating caudal fin of an adult zebrafish is a great model system to study gene regulation and function. However, manipulation of essential genes in adult fins can be challenging. In this brief report, we suggest that a fibroblast cell line AB9, isolated from regenerating caudal fins of adult zebrafish, can be used to perform pilot studies for gene regulation. We also provide standardized protocols for morpholino and drug treatment of AB9 cells. Our findings suggest that the AB9 cell line is a simpler system that expresses major regulators of the Cx43 dependent growth and patterning pathway, identified in the regenerating fin. We also provide evidence that expression and function of a protein can be easily manipulated, either through targeted morpholino knockdown or by drug treatment.

4.2. Introduction:

Zebrafish has emerged as an important model system to study growth and development in the last few decades. Large scale mutagenesis studies in zebrafish have identified important genes during vertebrate development. The incredible regenerative capability of both embryonic and adult zebrafish has made it a great model system to examine vertebrate tissue regeneration. However, unlike human and mice, relatively few *in vitro* studies have been carried out in zebrafish to complement the *in vivo* findings.

In the past few decades, a number of stable cell lines from zebrafish embryos (ZEM2S – (Collodi et al. 1992); ZF4 – (Driever and Rangini 1993)) and adult caudal fins (AB9 and SJD.1 – (Paw and Zon 1999)) and liver (ZF-L - (Ghosh et al. 1994)) have been developed. Cell based assays are important since they can complement findings from *in vivo* studies. Moreover, they are also a simpler system to study gene functions. Zebrafish embryos have been used successfully in toxicology studies (reviewed in (Hill et al. 2005)) and characterizing drug treatment in a stable cell line derived from zebrafish can be an important first step. Here we provide evidence that the zebrafish fibroblast cell line, AB9, can be used to complement regeneration studies in adult fins. Though the zebrafish caudal fin is an excellent system to study regeneration, it has some limitations. It takes about 2 to 4 months to obtain mature zebrafish and manipulation of gene expression in adults can present challenges. Morpholinos are often used for gene knockdown, but off-target effects raise concerns (Kok et al. 2015). Examination of essential genes in mutants is not possible during adult stages. The zebrafish caudal fin cell line provides a less complex tissue for pilot studies on gene function that may ultimately be tested in regenerating fins.

Our lab is interested in the significance of Cx43 dependent gap junctional communication in bone growth and patterning during fin regeneration. We have previously shown that Cx43 promotes cell proliferation/growth and inhibits joint formation/patterning by regulating downstream effectors in regenerating caudal fin. Here we demonstrate that the AB9 cell line can be used to complement our *in vivo* findings. For example, we find that this cell line expresses most of the major players in our pathway such as Connexin43 (Cx43), Semaphorin3d (Sema3d), PlexinA3 (PlxnA3), Neuropilin2a (Nrp2a), Heat shock protein 47 (Hsp47) and Collagen type II (Col II). We also provide evidence that gene expression can be manipulated in this cell line using standard techniques for knock down and pharmacological inhibition.

4.3. Experimental Procedures:

4.3.1. Maintenance of AB9 cells:

AB.9 (ATCC® CRL-2298™) is a zebrafish caudal fin-specific fibroblast cell line which grows in Dulbecco's Modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal bovine serum (15%) and antibiotics-antimycotics (Gibco) at 28°C with 5% CO_2. Subculturing can be done once or twice a week depending upon the cell confluency. First, the cells were washed briefly with 1X PBS. To a 100mm culture dish, 2-3 ml of Trypsin-EDTA was added and incubated at 28˚C for approximately 5 minutes or until the cell layer was completely dispersed. To the trypsinized cells, approximately 2-3 ml of growth media was added to inactivate the trypsin. More media was added depending upon the subcultivation ratio (The recommended subcultivation ratio is 1:3 to 1:4) and the cells were aspirated gently with pipetting. Approximately 8-10ml of the culture media containing cells were seeded in 100mm culture dish and incubated for 24-48 hours at 28°C.

4.3.2. Immunofluorescence on AB9 cells:

AB9 cells were incubated at 5% CO_2 and 28˚C and grown in tissue culture dishes with minimal essential media (DMEM) supplemented with 15% heat inactivated FBS and antibiotics-antimycotics (Gibco). The cells were seeded onto poly-L-lysine coverglasses and allowed to grow to 80-90% confluency. They were then washed once with cold PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. This was followed by washes in 1X PBS (3X, 5 minutes each), permeabilization with 0.2%

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triton in PBS for 10 minutes, again multiple washes in 1X PBS and then blocked in room

temperature for an hour in 1% BSA in PBS. The following primary antibodies were used:

Rabbit anti-Cx43 ((Hoptak-Solga et al. 2008) – 1:200), Rabbit anti-Sema3d (Sigma,

1:200), Rabbit anti-Hsp47 (Abcam, 1:100), Mouse anti-Col II (DSHB-II-II6B3, 1:50),

Rabbit anti-Nrp2a (GenScript, 1:200) and Rabbit anti-PlxnA3 (GenScript, 1:200).The

coverslips were incubated in primary antibody overnight at 4°C in a wet box. The next

day, they were washed in 1X PBS (3X, 5 minutes) and were incubated with secondary

antibody for 1 hour at room temperature (RT), protected from light. The following

secondary antibodies were used: anti-Rabbit Alexa Fluor 488 or 568 (1:200), anti-Mouse

Alexa Fluor 488 or 568 (1:200). DAPI (1:1000) was used to stain the nuclei. It was

followed by three 5 minutes washes in 1X PBS and one quick wash in dH2O. They were

then mounted with Vectashield and were examined under Nikon Eclipse TE2000-U at

60X.

4.3.3. Morpholino-mediated protein knockdown via electroporation in AB9 cells:

AB9 cells were seeded on to 100mm culture dishes and allowed to grow till 80- 90%

confluency. On the day of electroporation, the adherent cells were washed briefly with 1x

PBS and trypsinized in 0.05% Trpsin-EDTA 1X (Gibco) for 5 min at 28°C. DMEM

media supplemented with 15% heat inactivated FBS, antibiotics-antimycotics (Gibco)

were added to inactivate the trypsin. The cells were harvested by centrifugation at 750

rpm for 5 minutes. The pellet was resuspended in 1-5ml of HEPES buffer (115mM NaCl,

1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 2.4mM K_2PO_4 and 20mM HEPES with pH adjusted to

7.4) and put on ice. Meanwhile, electroporation cuvettes (Cell Projects) were prechilled

on ice and morpholinos were heated at 65°C for 15 min. 5-10 µM of morpholinos were

added to 400µl of resuspended cells in the cuvettes on ice and incubated for 5 minutes. The cells were electroporated at 170V for 6-7 ms using an electroporater (BioRad Gene Pulser X Cell). Electroporated cells were added to 1ml of fresh media in 60mm culture dishes and incubated at 28˚C for 24 hours. The electroporated cells were then used to prepare cell lysates for western blots. Briefly, cells were washed in cold 1X PBS and 1X cold lysis buffer (5mM Tris-HCl, 5mM EDTA, 5mM EGTA with pH 7.5). 500µl of the lysis buffer, along with 100X Halt-TM protease and phosphatase inhibitor cocktail (Thermo Scientific) and 0.6% SDS buffer were added to the culture dishes and the cells were scraped using a sterile rubber cell scraper (Corning Incorporated, Costar). The cell lysate was collected using a 5ml syringe (Beckton Dickinson) with an 18G needle in a fresh tube and heated for 3 minutes at 100˚C. The lysate was passed through a 26G needle and cooled on benchtop. The cell lysates were stored in small aliquots in -80˚C. These cell lysates were used to run western blot as described in Hoptak-Solga et al, 2008. Cx43 and Tubulin were detected using anti-Cx43 (1:1000) and anti-α-Tubulin (1:1000) (Sigma) respectively. The primary antibody step was followed by incubation in peroxidase-conjugated Goat anti-Rabbit IgG (1:10,000) for Cx43 blot and peroxidase-conjugated Goat anti-Mouse IgG (1:10,000) for Tubulin. The ECL chemiluminescent reagent (SuperSignal West Femto Maximum Sentivity Substrate, Pierce Rockford, IL) and X-ray films were used for signal detection. Image J software was used for the calculation of relative band intensities (Bhadra and Iovine 2015). Stripping of the blot was done using stripping buffer (BioRad). The blots were incubated for 5 minutes in a rocker at room temperature and for 20 minutes at 65 ˚C in a water bath with agitation.

The blots were washed extensively with 1X TBST and processed for western blot as mentioned in Hoptak-Solga et al, 2008(Hoptak-Solga et al. 2008).

4.3.4. Drug treatment and BrdU staining on AB9 cells:

AB9 cells were seeded onto poly-L-lysine coverglasses and were allowed to get 60 – 70% confluent. They were then treated with the 100 µM Hsp47 inhibitor drug (RH01393, ThermoFisher Scientific) or DMSO as control in media for 24 hours. Bromodeoxyuridine (BrdU) staining was done following an immunoflurescence protocol from Cell Signaling Technology. In brief, BrdU (Roche) was diluted in pre-warmed growth media to a final concentration of 0.03 mg/ml. Cells were then incubated with this solution for 30 minutes at 28°C. They were then fixed in cold 70% ethanol for 5 minutes, followed by PBS washes (3X, 5 minutes each). After that, incubation in 1.5M HCl for 30 minutes at RT was followed by PBS washes (2X, 5 minutes each). The cells were then incubated in blocking buffer (5% Goat serum, 0.3% Triton X-100 in 1X PBS) for 1 hour at RT. The coverslips were incubated in Mouse monoclonal anti-BrdU (Roche, 1:50) diluted in antibody dilution buffer (1% BSA in 1X PBS) overnight at 4°C. It was followed by PBS washes (3X, 5 minutes each) the next day. The coverslips were then incubated in anti-Mouse Alexa Fluor 546 (1:200) and DAPI (1:1000) for 1.5 hours at RT protected from light. It was followed by three 5 minutes washes in 1X PBS and one quick wash in dH2O. They were then mounted with Vectashield and were examined with Nikon Eclipse TE2000-U at 40X.

4.4. Results:

For a cell line to be suitable for functional studies, at a minimum it must be easy to visualize protein expression, transfect plasmids, and inhibit protein function for functional assays. Efficient transfection of AB9 cells has been described previously using nucleofection (Badakov and Jazwinska 2006). For our studies, we began by evaluating protein expression through immunofluorescence. We especially focused on proteins related to the Cx43 dependent pathway identified in our lab ((Bhadra and Iovine 2015), (Ton and Iovine 2012)).

4.4.1. AB9 cells express major players in our current model pathway:

Immunofluorescence on AB9 cells showed the expression of a majority of proteins in our model pathway (Figure 4.1). For example, Cx43, a gap junction protein, was expressed as its characteristic punctate dots in the plasma membrane in the region where two cells abut one another (Hoptak-Solga et al. 2008) whereas Sema3d, a secreted signaling molecule, was observed primarily in secretory vesicles. PlexinA3 and Nrp2a are the two putative receptors of Sema3d (Ton and Iovine 2012), and were expected to be present on the membrane. PlxnA3 did show a membrane expression pattern, however we observed robust expression of Nrp2a in the nucleoli of AB9 cells. Interestingly, we have seen a similar expression pattern for Nrp2a in regenerating caudal fin as well (data unpublished). Hsp47, a molecular chaperone for collagens, has been recently identified in our lab to be a part of the Cx43 dependent pathway regulating growth and patterning (Bhadra and Iovine 2015). Hsp47 is an ER resident protein that binds to the collagen triple helix in the ER and transports collagen to the Golgi. In AB9 cells, we observe that

Hsp47 is present specifically around the nucleus in a vesicular pattern, where we expect to see ER and Golgi resident proteins. Col II, a secreted protein, is found in vesicles distributed across the cytoplasm. These findings reveal expression and localization of several proteins that function during fin regeneration.

4.4.2. Functional assays in AB9 cells:

Knocking down a protein is an important part in studying gene function during development. In our lab, we regularly use antisense morpholinos (MO) to knockdown a protein of interest and study the resulting phenotypes in regenerating caudal fins. To perform knockdown in AB9 cells, we first tested whether electroporation is a reliable method for cellular uptake of MO. We electroporated fluorescein labeled Cx43 MO into AB9 cells, using fluorescein labeled Standard Control MO as our control. In both cases, transfection efficiency was observed between 50% - 70% approximately 4 hours post electroporation (hpe) (Figure 4.2A). Next, we tested if MO delivery in AB9 cells led to protein knockdown. Lysates were prepared from Standard Control and Cx43 MO treated cells 24 hpe. Figure 4.2B confirms the efficient knockdown of Cx43 by immunoblot. The Cx43 protein levels were reduced (64%) in the knockdown sample (lane 2) compared to the control sample (lane 1). Tubulin was used as a loading control.

We next determined whether we can block protein function by treating AB9 cells with pharmacological agents. For these studies, we chose an inhibitor of Hsp47. In regenerating fins, Hsp47 knockdown causes reduced cell proliferation and growth (Bhadra and Iovine 2015). In order to determine whether we can replicate a similar downregulation of cell proliferation in AB9 cells, we treated the cells with 100 μM of a

chemical inhibitor for Hsp47, RH01393 (Thomson et al. 2005), using DMSO as control.

BrdU was used to label proliferating cells. Interestingly, we saw a decrease in cell

proliferation following treatment with 100 µM Hsp47 inhibitor (approximately 44%)

when compared to DMSO as control (approximately 65%) (Figure 4.3). Cell viability

was 85% following drug treatment (data not shown).

4.5. Discussion:

The AB9 cell line is an established stable primary fibroblast cell line cultured from the AB strain of adult zebrafish caudal fins (Paw and Zon 1999). Previous studies have shown high transfection efficiency in these cell lines using nucleofection, thus making it an excellent tool to generate a transgenic cell line (Badakov and Jazwinska 2006) or to study overexpression of a particular protein. In this brief report, we provide evidence that the AB9 cell line can also be used for gene knockdown and drug treatment studies. One of the ways to temporarily knockdown a protein in zebrafish is by injecting them with an antisense oligonucleotide targeted against a specific gene. Antisense oligonucleotides have been used to modify the splicing pathway in HeLa cells (Schmajuk et al. 1999). The uncharged phosphorodiamidate morpholino oligomers (PMO) are widely used in mammalian cell culture to inhibit RNA or DNA virus amplification ((Stein et al. 2001), (McCaffrey et al. 2003), (Neuman et al. 2004), (Neuman et al. 2005), (van den Born et al. 2005), (Deas et al. 2005), (Kinney et al. 2005), (Zhang et al. 2006)). Both PMOs and charged fluorescein tagged MOs have been extensively used in zebrafish to study gene function. MOs are especially critical in studying gene function in adult fins, where knocking out an essential gene can be embryonically lethal and generation of an adult specific conditional knockout is not yet possible. AB9 cells can be used to emulate growth and patterning phenotypes from regenerating fins. For example, growth may be evaluated using standard cell proliferation assays, such as BrdU labeling; patterning, on the other hand, may be evaluated by following differentiation markers. This will not only complement MO studies in regenerating fins, but will also help to identify 'on-target'

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effects in regenerating fins. To our knowledge, this is the first report describing MO uptake through electroporation by a cell line from zebrafish.

The AB9 cell line is also a simpler tool to study the preliminary efficacy of a drug or toxicity. These cells have been used to complement *in vivo* studies in embryos for screening of drugs affecting cell cycle (Murphey et al. 2006). Many of these compounds were shown to be active in both embryos and AB9 cell lines, thus complementing findings between *in vivo* and *in vitro* studies. We have shown that using the chemical inhibitor for Hsp47 in AB9 cells recapitulated the reduced cell proliferation phenotype observed in regenerating fins following MO mediated Hsp47 knockdown, providing additional evidence that inhibition of Hsp47 contributes to the regulation of cell proliferation. Continued studies in AB9 cells will reveal underlying mechanism of this regulation, which, in turn, may be tested during fin regeneration.

In conclusion, this report aims to promote the fibroblast cell line from zebrafish caudal fin, AB9, as an important tool to complement *in vivo* studies in adult regenerating fin. In particular, this cell line expresses several proteins identified in the Cx43 dependent growth and patterning pathways. Moreover, we believe this cell line will provide a simpler model to study regulation of cell signaling pathways during regeneration, as well as preliminary studies on the efficacy of a particular drug.

Acknowledgements
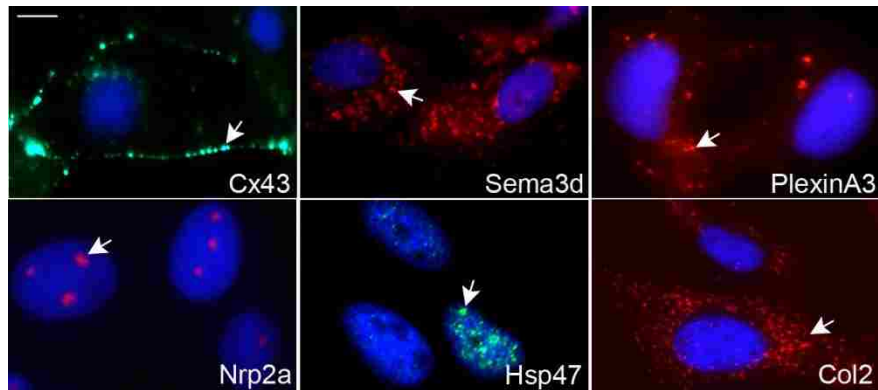
Figure 4.1: Immunofluorescence analysis on AB9 cells shows expression of various proteins. AB9 cells are stained using antibodies against Cx43, Sema3d, PlxnA3, Nrp2a, Hsp47 and Col II. DAPI (blue) is used for counter-staining nuclei. (Top left panel) Cx43 is expressed as punctate dots in the plasma membrane in the region where two cells are connected. (Top middle panel) Sema3d is observed to be distributed in the cytoplasm. (Top right panel) PlxnA3 is present on the membrane of the cells. (Bottom left panel) Nrp2a is present in the nucleolus of AB9 cells. (Bottom middle panel) Hsp47 is present around the nucleus. (Bottom right panel) Col II is distributed across the cytoplasm. The arrows indicate the specific staining of the respective antibodies. Scale bar is 10μm.
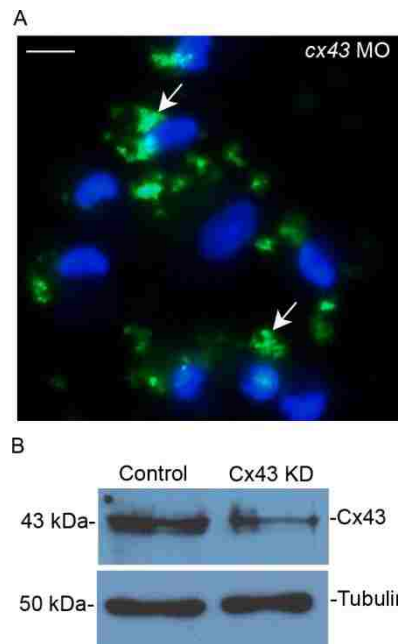
Figure 4.2: MO-mediated knockdown of Cx43 via electroporation results in reduction of Cx43 protein levels. (A) Representative image of *cx43* morpholino (MO) electroporated in AB9 cells. The arrows indicate the *cx43* MO (green) and was observed 4 hours after electroporation. DAPI (blue) was used for counter-staining nuclei. Scale bar is 10µm. (B) (Top) Western blot image showing a 64% reduction in Cx43 protein levels in Cx43 knockdown cell lysate (*cx43* MO electroporated in AB9 cells) compared to control cell lysate (standard-control morpholino electroporated in AB9 cells). The anti-Cx43 bands are observed at 43 kDa as expected. (Bottom) Western blot image showing the anti-tubulin bands at 50 kDa as the loading control.
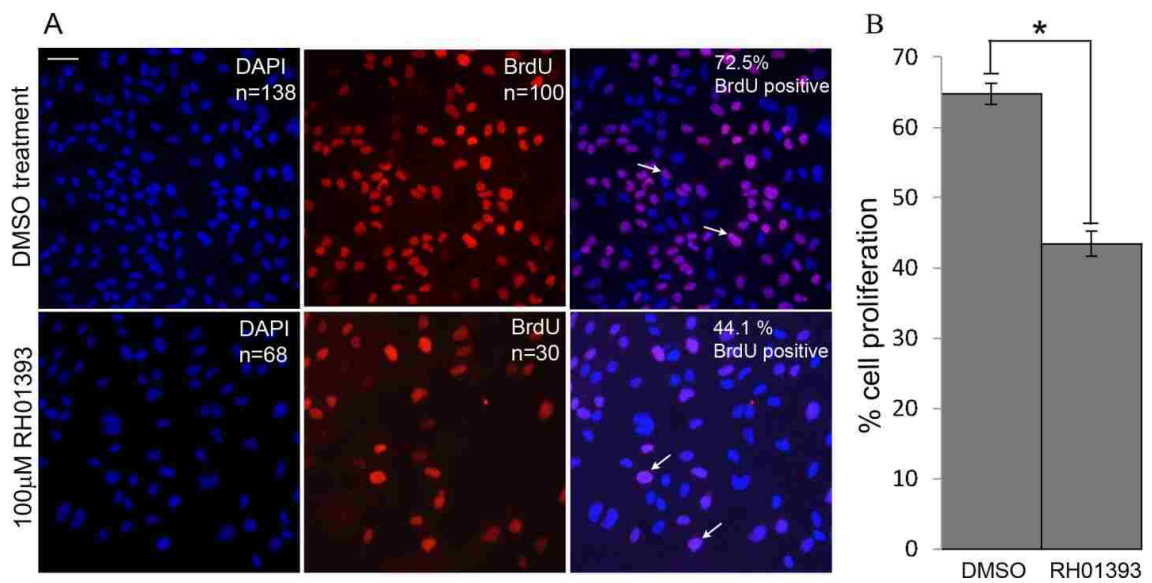
Figure 4.3: BrdU staining in drug treated AB9 cells results in significant reduction in cell proliferation. (A) Hsp47 inhibitor Drug (RH01393)/DMSO treatment of AB9 cells for 24 hours. DAPI (blue) is used for counter-staining nuclei indicating total number of cells (n). Top 3 panels represents DMSO treated cells as a mock treatment. Cells are stained with DAPI (n=138), BrdU (n=100) and merged showing 72. 5% of BrdU positive cells. Bottom 3 panels represents cells treated with Hsp47 inhibitor drug (RH01393). Cells are stained with DAPI (n=68), BrdU (n=30) and merged showing 44.1% of BrdU positive cells. Scale bar is 20μm. (B) Graph shows the significant reduction (*) in the percentage of BrdU positive cells in Hsp47 inhibitor drug (RH01393) treated cells compared to the DMSO treated control cells. Student's t-test was used for determining statistical significance where $p < 0.05$. Standard error is represented by error bars.

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Chapter 5: Summary and Future Direction

Summary:

The development of the skeleton is a complex process. The achievement of perfection in shape, size and positioning of each individual bone is the result of interaction among multiple signaling pathways that include proteins, ions, non-coding RNA molecules and other players, most of which are still currently unidentified. Cells need to talk among themselves for proper skeletal morphogenesis and one of the major type of cellular communication is through GJIC. As mentioned previously, our lab uses regenerating caudal fins of adult zebrafish to study new bone growth and patterning. Cx43 is an important regulator in this process. Mutations in this gene causes the *sof^{b123}* phenotype in zebrafish, characterized by shorter fin length, shorter segment length and reduced level of cell proliferation. Interestingly, the function of Cx43 is conserved in vertebrates during skeletal development. In humans, mutations in *CX43* causes ODDD (Flenniken et al. 2005; Paznekas et al. 2009). Knockout mutation in *CX43* in mice is embryonically lethal and show delayed ossification of bone (Lecanda et al. 2000). Therefore, it is important to understand how GJIC through Cx43 is regulating bone formation.

In this thesis, we have identified and characterized one important mediator of Cx43 dependent growth and patterning pathway, Hsp47, a molecular chaperone for collagen. We have shown that Hsp47 is functionally downstream of Cx43 and knockdown of Hsp47 recapitulates the *sof* phenotype (Bhadra and Iovine 2015). Hsp47 is essential for actinotrichia formation. Knockdown of Hsp47 severely disrupts the assembly of collagen II dependent actinotrichia spicules that ultimately leads to the disruption of cell proliferation and joint formation. We do not yet understand the mechanism through which actinotrichia mediates both growth and patterning. We suggest that integrins can act as possible regulators of collagen II mediated signaling (Heino 2014) that changes gene expression. This in turn can determine whether the cells should divide or differentiate and thus affect growth or patterning, respectively. Previous studies from our lab have elucidated the importance of ECM, especially the Hapln1a dependent ECM, in regulating growth and patterning in a Cx43 dependent manner (Govindan and Iovine 2014). Collagen is a major component of ECM and our studies reveal that Hsp47 acts

transcriptionally downstream of Hapln1a. Future studies will aim to characterize the interaction between Hsp47 dependent collagen molecules and Hapln1a dependent ECM. We have also aimed to elucidate the structural and functional significance of interaction between Sema3d and its two putative receptors – Nrp2a and PlxnA3. Our data suggest that full length zebrafish Nrp2a receptors prefer to form homo-oligomers and Sema3d binding reduces this homomeric interaction. This observation is supported by previously performed genetic studies in fins (Ton and Iovine 2012). We have seen that Sema3d knockdown reduces cell proliferation, indicating that it is a positive regulator for growth. Knockdown of Nrp2a on the other hand, increases cell proliferation, suggesting an inhibitory role of Nrp2a in growth. Since Nrp2a is a putative receptor for Sema3d, we hypothesized that binding of Sema3d to Nrp2a actually blocks its function. Our findings through BRET^2 assay suggest that Sema3d binding forces the Nrp2a oligomers to come apart, thus reducing intracellular signaling. Our findings also suggest that the juxtamembrane MAM domain of the Nrp2a receptor facilitates Nrp2a homo-oligomerization (Barton et al. 2015a) and can also act as a binding domain for Sema3d. We also found that PlxnA3 acts as a competitor for oligomerization between Nrp2a receptors, and Sema3d binding reduces heteromeric interaction between Nrp2a and PlxnA3. In fins, PlxnA3 suppresses joint formation, presumably through interaction with Sema3d (Ton and Iovine 2012). However, we do not know if this interaction requires the presence of Nrp2a receptors as well. It is possible that binding of Sema3d simultaneously reduces signaling through Nrp2a, thus reducing cell division and increases signaling through PlxnA3, thus increasing differentiation. We need to develop some *in vivo* assay to visualize the effect of receptor oligomerization on intracellular signaling in presence or absence of ligands. There is also the possibility of other ligands (e.g. VEGFs) and receptors (VEGFRs, CAMs, integrins) playing crucial roles in Sema3d-Nrp2a-PlxnA3 mediated signal transduction.

Finally, we have standardized a cell line obtained from regenerating caudal fin of an adult zebrafish, AB9 (Paw and Zon 1999) to complement our *in vivo* studies. Our data suggest that AB9 cells express most of the major regulators in the Cx43 dependent pathway. It is comparatively easy to manipulate protein expression in this system, either through

electroporation of a MO or through using an inhibitory drug (Bhadra et al, accepted). We believe AB9 cell line will provide a simpler model to study regulation of cell signaling pathways during regeneration as well as preliminary studies on the efficacy of a particular drug.

In order to understand more about Cx43 dependent skeletal morphogenesis, a lot more work need to be done. I have summarized some of the questions that need to be answered in order to elucidate underlying molecular mechanisms for Cx43 mediated growth and patterning.

Future Directions:

5.1. Does *serpinh1a* interact with *serpinh1b* during skeletal morphogenesis?

Zebrafish often contain multiple copies of a gene and *serpinh1* is no exception. A search through ZFIN revealed another copy of *serpinh1* called *serpinh1a* (NM_001110374) the mRNA of which is about 3829 nucleotides long. There is no significant similarity between the nucleotide sequences of *serpinh1a* and *serpinh1b*. However their protein products share a 76% sequence identity. We performed in situ hybridization to see if *serpinh1a* expression is dependent on *cx43* or not. Unlike *serpinh1b*, there was no significant difference in expression level of *serpinh1a* between WT and *sof*[b123] fins (Figure 5.1). In situ hybridization on cryosections of WT-5dpa fins revealed that *serpinh1a* was mostly expressed in the epithelial tissue (Figure 5.1), unlike *serpinh1b*, which is expressed in the skeletal precursor cells and blastema (Figure 2.1.).

However, even though the mRNA of *serpinh1a* and *serpinh1b* are expressed in different compartments of a regenerating fin section (Figure5.1 and Figure 2.1), there is still a possibility that their protein products interact. We observe Hsp47 protein expression in both epithelial and mesenchymal compartment indicating two different possibilities. First, the Hsp47 antibody recognizes both *serpinh1* protein products. Second, the protein products of these two copies interact with each other. Interestingly, *serpih1a* mRNA expression is not *cx43* dependent, as indicated by in situ hybridization (Figure 5.1). However, when we compared the epithelial Hsp47 staining via immunofluorescence on sections, we do see a decrease in *sof* and increase in *alf* (Figure 5.2). This suggests that the protein products of *serpih1* interact with each other.

The other way to answer this question will be to design a MO against *serpinh1a* and see if we can recapitulate the *sof* phenotype. It will also be interesting to know if knocking down both *serpinh1a* and *serpinh1b* causes any synergistic effect during growth and/or patterning which would be another way to answer whether *serpih1* products interact or not. I believe answering this question will provide further insight into how the epithelium and mesenchyme interact with each other during skeletal morphogenesis.

5.2. Identification of other collagens and actinodins in the Cx43 dependent pathway:

Hsp47 mediates downstream effects of Cx43 by promoting cell proliferation and suppressing joint formation. Hsp47 functions as a molecular chaperone for collagen. There are about 28 types of collagen described in literature, among which collagens I, II, IX and X are expressed in the regenerating fins (Avaron et al. 2006; Duran et al. 2011; Huang et al. 2009; Smith et al. 2006b). Collagen I and II are the two major fibrillary component of actinotrichia and collagen IX is a crosslinker (Duran et al. 2011; Huang et al. 2009). The non-collagenous components of actinotrichia include actinodin proteins And1 and And2 (Zhang et al. 2010). We have shown that knocking down Hsp47 affects the collagen II dependent actinotrichia structure which in turn leads to misregulation of growth and patterning. Interestingly, actinotrichia is deformed in both *sof* and *alf*, indicating how a proper level of *cx43* expression is important in maintaining fin morphology. Preliminary data from our lab indicate that Col I expression is also misregulated following Hsp47 knockdown (Figure 5.3). It will be interesting to know whether expression of Col IX, And1 and And 2, the other known components of actinotrichia is dependent on Hsp47 and / or Cx43. The other two collagens to look at will be Collagen X and XXII. Col 10a1a mRNA is expressed in the skeletal precursor cells, very close to the actinotrichia (Smith et al. 2008). We still do not understand how disruption of actinotrichia leads to defects in patterning. We also do not understand how in spite of being expressed only in the blastema, *cx43* can regulate patterning events through the skeletal precursor cells. I believe identification of collagens involved in *cx43* dependent pathway might provide us with further insights into the matter.

5.3. Generation of a conditional knockout for Hsp47 in order to generate a disease model for Osteogenesis imperfecta

Zebrafish fin regeneration can be useful in studying skeletal diseases.The lepidotrichia especially is very similar to bone and cartilage (Avaron et al. 2006; Padhi et al. 2004). Mutations in *SERPINH1* causes Osteogenesis imperfecta or the 'brittle bone disease' in humans. KO mutation for *serpinh1* is embryonically lethal in mice which also display

really fragile bones, among other conditions (Nagai et al. 2000). MO knockdown of Hsp47 in regenerating fins however did not generate brittle bones. It will be interesting to see if conditional knockout of Hsp47 in adult fin tissue can generate an OI disease model or whether it recapitulates the *sof* phenotype. Studies from our lab indicate that Hsp47 regulates Col II and possibly Col I (Bhadra and Iovine 2015) (Figure 5.3). Mutations in Col I in zebrafish lead to *chihuahua* mutants which display similar phenotypes as in OI patients (Fisher et al. 2003b). Whether knocking out Hsp47 will generate the same phenotype will be interesting to know. We can then use this as a disease model and see if expression of Hsp47 is enough to rescue the phenotype(s).

5.4.   Development of an *in vivo* assay to better understand the effect of Sema3d mediated change in oligomerization for Nrp2a and PlxnA3 in mediating intracellular signaling:

Semaphorins were originally identified as guidance cues on axonal growth cones (Figure 5.4). Growth cones facilitate axon growth through binding and polymerization of actin filaments into filopodia and microspikes (Figure 5.5). Semaphorin mediated growth cone collapse requires disruption of actin and tubulin cytoskeleton and suppression of integrin mediated adhesion. During morphogenesis in *Caenorhabditis elegans*, one of the targets for semaphorin (Sema3A in particular) is the actin-severing enzyme cofilin (Nukazuka et al. 2008). LIM-kinase, which phosphorylates, and therefore inactivates cofilin, also plays an important role in regulating Sema3A induced growth cone collapse (Aizawa et al. 2001). Interestingly, increased phosphorylation of cofilin was observed within 5 minutes of treatment with Sema3A, after which the level of phosphorylated cofilin gradually decreased. Using this information we can develop an assay to determine changes in cytoskeletal structure following Sema3d binding to Nrp2a or PlxnA3. For this, we will use the Cos7 cells which do not express zebrafish Sema3d, Nrp2a or PlxnA3. It will be interesting to see if addition of Sema3d in cells expressing Nrp2a or PlxnA3 or both show similar changes in the level of total active / inactive cofilin in a time dependent manner, through Western Blots. Understanding how Sema3d-PlxnA3-Nrp2a mediates cytoskeletal

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dynamics will take us one step closer in understanding how Sema3d guides the blastema in regulating growth and patterning during skeletal regeneration.

## 5.5.   Identification of other molecules involved in Sema3d-PlxnA3-Nrp2a mediated signaling events:

Besides Sema3d, the zebrafish genome contains other semaphorin genes as well. The same is true for the receptors - plexins and neuropilins. Neuropilins again can bind to both receptors and ligands from VEGF family. Integrins are another group of interesting candidates that might regulate neuropilin dependent cell adhesion and migration. Nrp2, for example, interacts with integrin α6β1 to regulate laminin dependent adhesion in breast carcinoma (Goel et al. 2012). The zebrafish genome has already been sequenced. Therefore, it will be simple enough to identify which other semaphorins / integrins are expressed in the regenerating fin and whether knocking them down affects regeneration. Sema-Nrp-Plxn signaling is a favored therapeutic target in a number of systems (Parker et al. 2012). Inhibition of Sema3A binding to Nrp1 increases recovery from spinal cord injury (Kaneko et al. 2006). Drugs targeting Nrp and its putatitive ligands VEGF / Sema mediated tumor angiogenesis have been extensively studied. I believe identification of other complexes that interact with Sema3d or its two receptors will help us understand more about skeletal growth and patterning and ultimately, how to treat skeletal diseases.

The function of Cx43 is conserved during skeletal morphogenesis. Cx43 plays important roles in proliferation, differentiation and survival of osteoblasts (Gramsch et al. 2001; Lecanda et al. 1998; Lecanda et al. 2000; Li et al. 2006; Plotkin et al. 2002). Loss of Cx43 results in decreased fracture healing in mice (Loiselle et al. 2013). Our lab has identified a number of genes functioning downstream of *cx43* during fin regeneration in zebrafish. However, we still do not understand fully how Cx43 dependent GJIC affects growth and patterning events during skeletal morphogenesis. Future studies will try to identify other players in this pathway to better understand how bone growth and patterning occurs, even in humans. This ultimately will take us one step closer in treating skeletal dysplasias.

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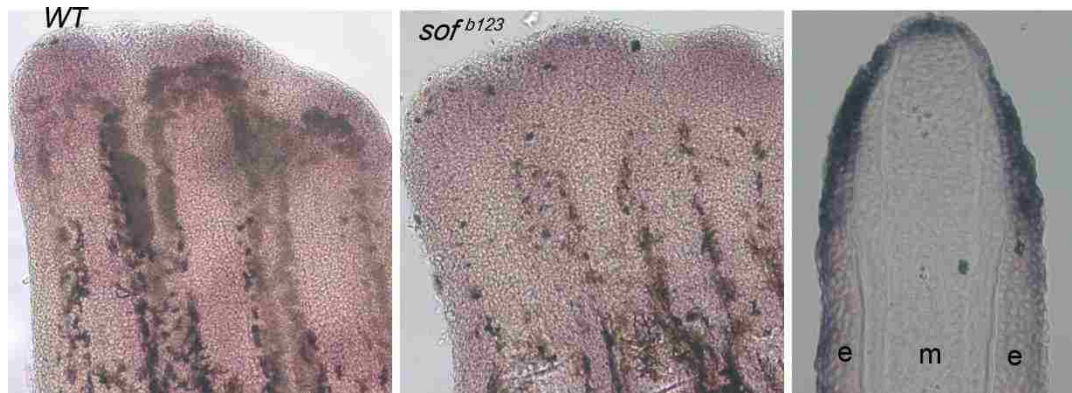
Figure 5.1: *serpinh1a* **expression is similar in wild-type and** *sof*[b123]. Whole mount in situ hybridization shows same level of expression in *sof*[b123] compared to wild-type. In situ hybridization on cryosection of a WT-5dpa fin reveals localization of *serpinh1a* mostly in the outer layer of epidermis . Epidermis is denoted by 'e', mesenchyme by 'm'.
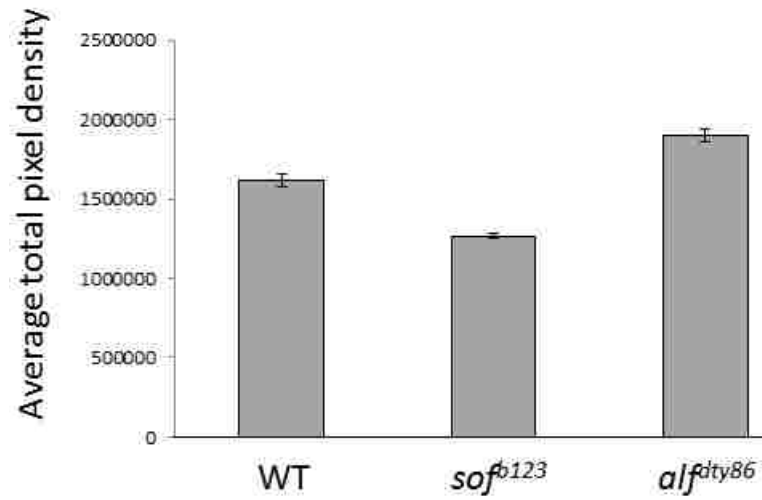
Figure 5.2: Differential expression pattern of Hsp47 in the epithelial tissue. The graph indicates total pixel density measured through ImageJ. The pixel density was measured from longitudinal sections immunostained with Hsp47 antibody.
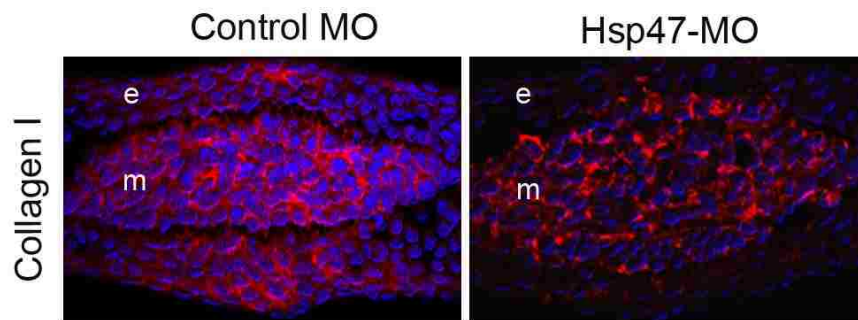
Figure 5.3: Collagen I expression is disrupted following Hsp47 knockdown. Single plane confocal image of a transverse section immunostained with Collagen type I (red) and counterstained with nuclei with To-pro (blue). Fins are injected with either Standard Control MO or Hsp47 MO. Col I distribution is disrupted following Hsp47 knockdown. 'e' is epidermis, 'm' is mesenchyme.
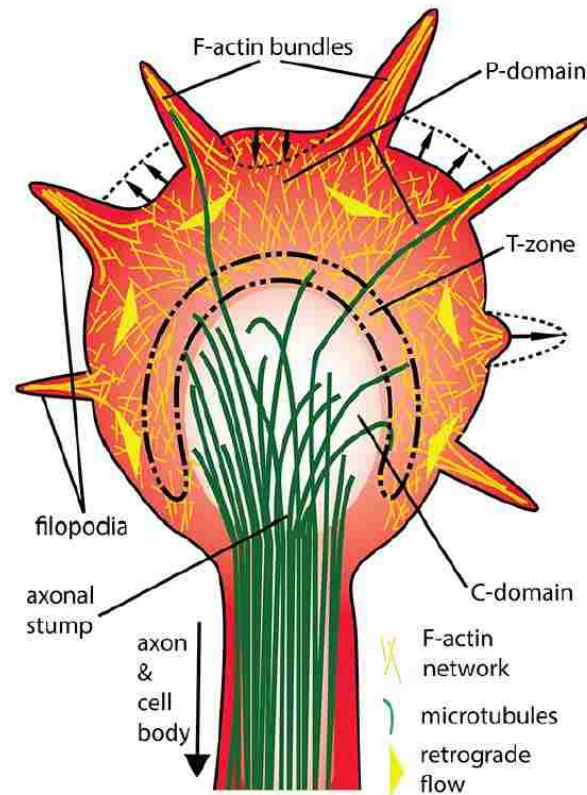
Figure 5.4: Structure of a Growth Cone. Growth cones are observed at the tip of the outgrowing axon. At the peripheral domain (P-domain), dynamically crosslinked actin filaments forms a dense sheet like structure, known as lamellipodia. This is interspersed with radially oriented bundles of aligned actin filaments known as filopodia. The microtubules are more rigid and are usually bundled within the neurite shaft. Image from Rauch et al, 2013.
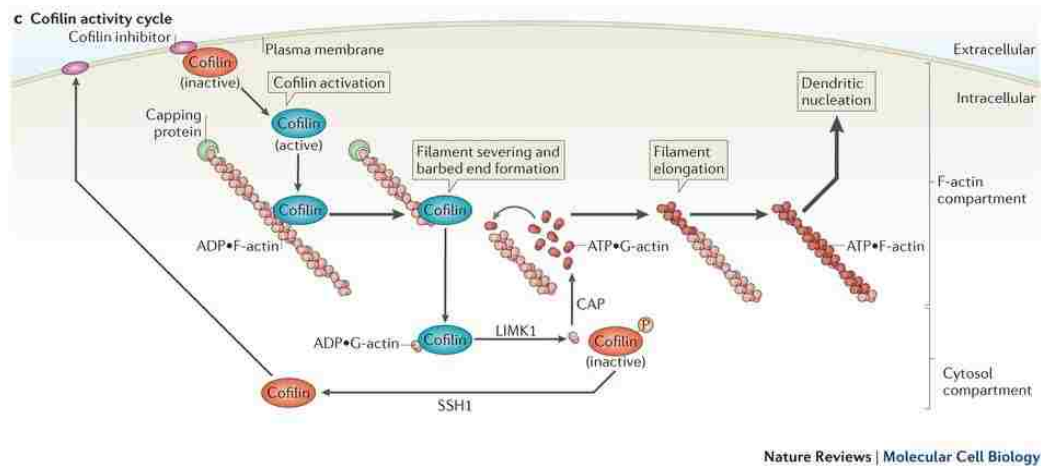
Figure 5.5: Cofilin severs and depolymerizes actin filaments at the base of the lamellipodium. Phosphorylated cofilin is inactive. Following dephosphorylation, cofilin binds to and severs actin filament, creating barbed end that are used for actin polymerization. LIM Kinase 1 accelerates the dissociation of ADP-G-Actin and cofilin complexes. LIMK1 phosphorylates and thus inactivates cofilin. (Bravo-Cordero et al, 2013).

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    Navarro, and M. A. Akimenko. 2010. 'Loss of fish actinotrichia proteins and the
    fin-to-limb transition', *Nature*, 466: 234-7.

<p align="center">**<u>Curriculum Vitae</u>**</p>

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Education

PhD in Department of Biological Sciences, Lehigh University (2009 – present)

Doctoral thesis: Understanding Connexin43 dependent skeletal morphogenesis: Progress made and Promises Ahead

Bachelor's (B.Tech): Haldia Institute of Technology (WBUT) (2004-2008)

Teaching Experience

Teaching Assistant for BIOS-41: Cellular and Molecular Biology (Spring 2010)

Teaching Assistant for BIOS-42: Biology core lab (Spring 2011, 2012)

Teaching Assistant for BIOS-377: Biochemistry lab (Fall 2011)

Teaching Assistant for BIOS-397: Developmental Biology Lab (Spring 2013, 2014)

Teaching Assistant for BIOS-10/90: Bioscience in the 21st Century (Fall 2014)

Took a two semester seminar course on innovating and improving teaching skills.

Mentoring Experience

Mentored undergraduate students in lab and in the interdisciplinary Biosystems Dynamics Summer Institute program (BDSI 2011-2014), most of them have gone to Medical Schools or are pursuing MS/PhD.

Mentees: Justine Nice, Silas Simotwo – BDSI, Summer 2011

   Dana DeSantis – Fall 2011 – Spring 2012

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Matt Wolfers – BDSI, Summer 2012

Sarah Kayode – BDSI, Summer 2013

Amanda Stillwell – Fall 2013 – Spring 2014

Nathanael Sallada, Jasmine Singh – BDSI, Summer 2014

Graduate Research Experience

Doctoral thesis titled "Understanding Connexin43 dependent skeletal morphogenesis: Progress made and Promises Ahead" under **Prof. M. Kathryn Iovine**, Department of Biological Sciences, Lehigh University.

Current Projects:

- Characterize the interaction between Sema3d and Nrp2a and how they affect bone morphogenesis during regeneration.
- Characterization of Hsp47, a molecular chaperone for collagen, involved in bone growth
- Characterization of the truncation allele for *connexin43* during development.

Undergraduate Research experience

- *"Size range of natural nanoparticles in plants and animals and their importance in evolution"*
  - One year internship under Prof. Arunava Goswami & Prof Ratan Lal Brahmachari, Indian Statistical Institute (2008-2009)
- *"Cost-effective media for good-quality pineapple"*
  - Final year undergraduate project under Dr. Siraj Datta, Haldia Institute of Technology (2006-2008)
- *"Consensus sequence in Mouse Olfactory Lobe"*

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- o Winter Internship under Prof. Arunava Goswami, Indian Statistical Institute (2007)

- *"Cytochrome P2D6 and its role in Cancer"*

  Summer Internship under Prof. Uma Dasgupta, Calcutta University (2007)

Publications:

1. **Joyita Bhadra** and M. Kathryn Iovine. (2015). "Hsp47 mediates Cx43-dependent skeletal growth and patterning in the regenerating fin". *Mechanisms of Development*, doi:10.1016/j.mod.2015.06.004

2. **Joyita Bhadra**, Rajeswari Banerji, Jasmine Singh, Nathanael Sallada, Diane M. Eble, M. Kathryn Iovine. (2015). "Zebrafish fibroblast cell line, AB9, is a simpler tool to complement gene regulation studies during fin regeneration". *Journal of Musculoskeletal Regeneration,* doi: 10.14800/mr.992

3. Ipsita Dutta, **Joyita Bhadra**, Pritha Ghosh, Babita Saha and Siraj Datta. "An Efficient and Cost Effective Protocol for *In-vitro* propagation of Pineapple". *Journal of Ornamental Plants,* 3 (4): 229-234, December, 2013.

4. **Joyita Bhadra** and Siraj Datta. "Do the birds have a compass?" Feature article in *Science Reporter*, November 2009, 43-45.

Manuscript in preparation:

Joyita Bhadra, Rachael Barton, Bryan Berger, M. Kathryn Iovine. "Characterization of interaction between Neuropilin2a and Semaphorin3d using BRET".

Joyita Bhadra and M. Kathryn Iovine. (2015) "Hsp47 mediates Cx43-dependent skeletal growth and patterning in the regenerating fin". 2015 Mid-Atlantic Society of Developmental Biology Regional Meeting, Princeton University, Princeton, NJ.

Conference Abstracts / Poster Presentation

1. *"Characterization of Hsp47, a collagen specific molecular chaperone involved in bone growth"* in the 11th International Conference on Zebrafish Development and Genetics, June, 2014 at Madison, Wisconsin.

2. *"Characterization of Hsp47, a collagen specific molecular chaperone involved in bone growth"* in the 7th International Young Researcher's Retreat, 2013, Osaka University, Japan.

3. *"Characterization of Hsp47, a collagen specific molecular chaperone involved in bone growth"* in the Mid-Atlantic Regional Meeting of the Society for Developmental Biology, 2013 at College of William & Mary, Williamsburg, Virginia.

4. *"Defining the Role of PlexinA3 receptor during Skeletal Morphogenesis"* in the Mid-Atlantic Regional Meeting of the Society for Developmental Biology, 2012, at Penn-State University.

5. *"Semaphorin Receptors and their Role in Bone Morphogenesis"* in the Graduate Student Open House, 2011, Department of Biological Science, Lehigh University.

6. *"Characterization of the Truncation Allele for connexin43: cx43[fh300]"* in the Graduate Student Open House, 2010, Department of Biological Science, Lehigh University.

7. *"Micropropagation of Pineapple: An attempt towards reducing the chemical cost during in vitro culture"* by Ipsita Dutta, Joyita Bhadra, Pritha Ghosh and Siraj Datta , 95th Indian Science Congress, January 2008.

Laboratory Skills

- *Molecular Biology Skills*: Plasmid preparation, Ligation, Transformation, Genomic DNA extraction, total RNA extraction, quantitative RT-PCR, PCR, Gel analysis.
- *Developmental Biology Skills*: Breeding and Caring for Zebrafish, Fin amputation, Microinjection in fins, Cryosection of fins, Preparation of RNA probe, In situ hybridization on whole mount and cryosections, Immunofluorescence.
- *Cell Biology Skills*: Culturing bacterial, mammalian and fish cell lines, Transfection, Drug / Peptide treatment, Cell proliferation assay, Fluorescent microscopy.
- *Protein Assay Skills*: Bacterial Protein Extraction, Protein purification, Column chromatography, Dialysis, SDS-PAGE, Coomasie staining, Western Blot, Bioluminescence Resonance Energy Transfer (BRET).
- *Microscope skills*: Brightfield, Fluorescent and Confocal.
- *Computer Knowledge*: MS Office, Adobe Photoshop, ImageJ, EndNote.

Fellowships and Awards

- College of Arts & Sciences Summer Research Fellowship, Lehigh University, 2015.
- Graduate Student Merit Award, Lehigh University, 2014.
- National Scholarship for Academic excellence and All State (West Bengal) Rank 61 in Madhyamik (Secondary) Examination.

Extracurricular activities

- Travel Grants Officer for Graduate Student Senate, Lehigh University (2013- 2014)
- Vice-President and Education Liaison of Biological Sciences Graduate Students Association (BOGS) (2012-2013)
- Student member of the Lehigh University Housing Survey Committee, (2011-2012)

References

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