The Roles of Semaphorin3d (Sema3d) Signaling in Fin Regeneration

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Abstract

The zebrafish fin is composed of multiple bony fin rays. Each fin ray is comprised of multiple segments separated by joints. Regulatory mechanisms that control joint morphogenesis and ray segment length in zebrafish fins are not fully understood. We found that the *semaphorin3d* (*sema3d*) gene is expressed during fin regeneration in a *cx43*-dependent manner. Semaphorins are signaling molecules involved in different signaling pathways, especially in axon guidance cues during pattering of the central nervous system. Interestingly, s*ema3d* is found at the lateral compartment of the regenerating fin consistent with the location of precursor cells for bones and joints. Moreover, *sema3d* knock-down recapitulates the *cx43* phenotype including reduced in growth and reduced in segments. Studies show that depending on the cell-type, Sema3s interact with neurophilin receptors (Nrps) either physically or indirectly via other receptor complexes. During fin regeneration, we found expression of only *nrp2a*. Interestingly, *nrp2a* is expressed in the skeletal precursors where *sema3d* expression was identified. We hypothesize that *sema3d* signaling through *nrp2a* regulates cell proliferation and inhibits joint formation.

Introduction

Cx43 activity promotes cell division and suppresses joint formation:

Mutations in the gap junction gene *connexin43* cause the *short fin* phenotype. The *short fin* mutant exhibits reduced levels of cell proliferation, less Connexin43 activity, which leads to shorter bony fin ray segments and short fins.

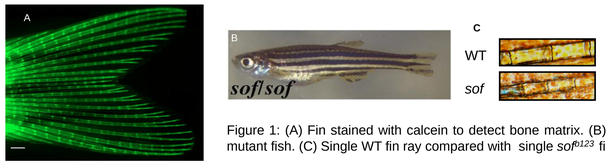


Figure 1: (A) Fin stained with calcein to detect bone matrix. (B) *sof*^{b123} mutant fish. (C) Single WT fin ray compared with single *sof*^{b123} fin ray.

In contrast, *alf*^{dty86} mutant fish shows over-expression *cx43* mRNA and exhibits essentially the opposite segment length phenotype as *sof*^{b123} mutants.

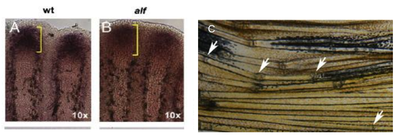
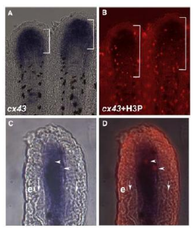


Figure 2 (A,B) *cx43* mRNA expression level between WT and *alf*^{dty86}. (C) The *alf*^{dty86} mutant is showing irregular joint formation and segment length

Interestingly, *cx43* mRNA is expressed in the blastema and co-localizes with cells in mitosis



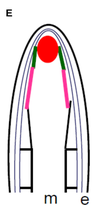


Figure 3: (A) *In situ* hybridization shows the expression of *cx43*. (B) Fin stained with H3P marker to detect mitotic cells. (C) Cryosectioning of the (A) fin shows the expression of *cx43* in the middle compartment called the blastema. (D) Cryosectioning of the (B) fin shows *cx43* mRNA colocalizes with the mitotic cells. (E) Cartoon illustrates the location of *cx43* expression adjacent to the lateral compartment where skeletal precursor cells are found.

e: epithelium m: mesenchyme

Knock-down *cx43* rescues joints and reduces segment length in *alf* ^{dty86} mutant.

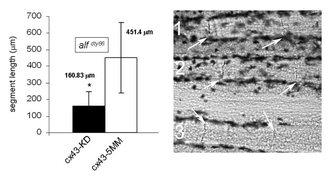


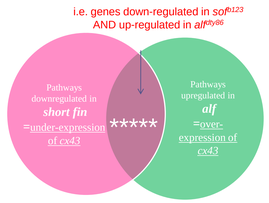
Figure 4: Injection/electroporation of *cx43* morpholino in *alf* ^{dty86} regenerating fins. Image of the *cx43*-knockdown *alf*^{dty86} fin rays (1,2, and 3) showing the formation of regular joints. Figure modified from Sims et al., 2009.

Next question: What are the genes that function downstream of Cx43?

The Discovery of *sema3d* Gene

Identify *sema3d*-dependent functions:

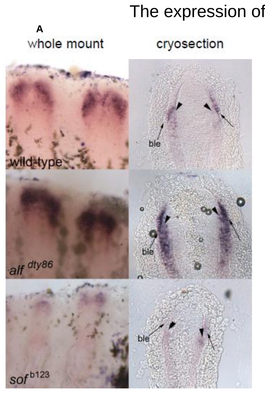
To find genes mediated by *cx43*, we look at the microarray analysis to search for genes that are both down-regulated in *sof*^{b123} and up-regulated in *alf*^{dty86}



gene name	reason for interest
cx43	**validates microarray**
igf1	*igf2b* important during regeneration
itg1b	integrins are often downstream of semas)
sema3d	**secreted signaling molecule**
igfbp2a	Igf-binding proteins regulate Igf activity
delta d	Deltas are involved in body segmentation
rx3	acts downstream of Igf-signaling

Figure 5: (Left) Venn diagram identifies 180 genes overlap that are downstream of *cx43*. (Right) Result from the microarray

The expression of *sema3d* is influenced by Cx43 activity



B

Expression of *sema3d* via qRT-PCR	
strain	**fold-difference**
wild-type	1
sof ^{b123}	0.6
alf ^{dty86}	1.95
cx43-KD fins	0.7

Figure 6: (A) Whole mount *in situ* hybridization shows *sema3d* expression is up-regulated in *alf*^{dty86} and down-regulated in *sof*^{b123} compared with the expression in wild type. Cryosectioning shows the location of *sema3d* expression that is at the ble and in the skeletal precursor cells. (B) Results from qRT-PCR confirms the level of *sema3d* expression among wild type, *alf*^{dty86} and *sof*^{b123}

ble: basal layer of epithelium

Morpholino knockdown of *sema3d* recapitulates the *short fin* phenotype

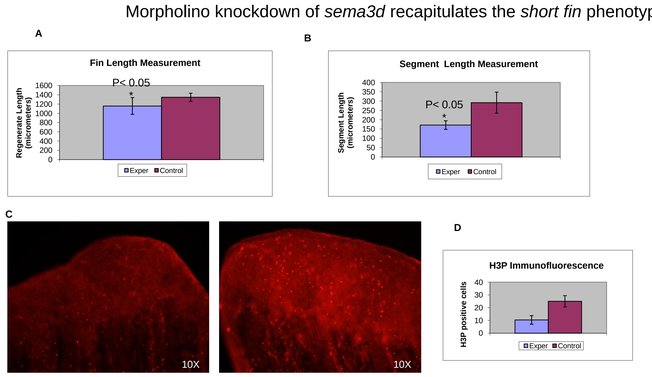


Figure 7: Morpholino of *sema3d* in wild type shows reduced fin length (A), reduced segment length (B) recapitulating the *short fin* phenotypes. (C) H3P immunofluorescence result shows reduced number of H3P positive cells when Sema3d is knock-downed. Left panel : injected side of the fin. Right panel: control side of the fin. (D) Graph shows in average number of H3P positive cells is reduced in Sema3d knock-down compared with the control side .

***cx43* and *sema3d* function in a common molecular pathway**

Our Proposed Model

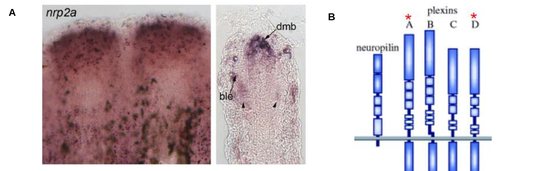


Figure 8: (A) Sema3d receptor, *nrp2a*, is expressed in the two population of cells: distal most blastema (dmb) and skeletal precursors. (ble) basal layer of epithelium. (B) Cartoon illustrates semaphorins interacting with neurophilin receptors and varied co-receptors.

Adapted in Takegahara et al., 2005

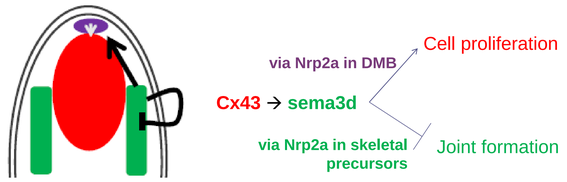


Figure 9: Our proposal mechanism illustrates how Sema3d signaling regulates cell proliferation and joint formation

Predictions

If our model is correct, we will expect:

1) Inhibition of *nrp2a* causes reduced cell proliferation and short segments.
2) Over-expression of *cx43* causes increased cell proliferation and lost/over-long segments.
3) Over-expression of Sema3d causes increased cell proliferation and lost/over-long segments.
4) Over-expression of Nrp2a causes reduced cell proliferation and short segments.

Future Directions

Semaphorins are recorded to have association with co-receptors plexins forming a complex with a specific Nrp to transmit signals inside the cells (review in Zhou et al, 2008). Our lab identified *plexin A1* expression in the dmb suggesting that Sema3d signlaing interacts with Nrp2a and PlexinA1 complex in the dmb regulates cell proliferation. Next, we will look at *plexinA3*, a candidate for expression in skeletal precursor cells and perform additional experiments to verify our proposed model:

1) Inhibition of PlexinA1 influences ONLY cell proliferation
2) Inhibition of PlexinA3 influences ONLY joint formation.

Credits and References

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Zhou, Y., R. Gunput, and R.J. Pasterkamp. 2008. Semaphorin signaling: progress made and promises ahead. *Cell Press*. 33(2008). 161-170.