Bios90: Defining the mechanisms that regulate skeletal morphogenesis
The skeleton has important functions

- Flexibility
- Protection of vital organs
- Strength
Skeletal defects impact human health

Developmental diseases

Degenerative diseases

Normal joint

Damaged cartilage
Advantages to examining growth mechanisms in the zebrafish fin

The fin and body are easy to measure

The fin grows throughout the lifetime of the fish

When amputated, the fin grows back (regeneration)

The fin is comprised of relatively few tissues

Zebrafish are easy to manipulate genetically
Fin length mutants provide opportunities to evaluate abnormal growth
Identification of the mutation causing the sof phenotype reveals a gene required for normal bone growth in fins.

Mutations in the gap junction gene *connexin43* cause the *short fin* phenotype.

The *short fin* mutant exhibits less *connexin43* activity, which leads to less growth and shorter bony segments.
100 Trillion = $10^{14}$ cells/human body; >200 different cell types
Cells are Organized in Tissues and Organs

- Outer layers of cells in close contact
- Basement membrane
Cells in tissues communicate by the exchange of small molecules via gap junction channels.

“gap junctions”

Development

Differentiation

Cell and Tissue Function

- Heart beat
- Onset of labor
- Conduct neuronal signals through electrical synapses
Appearance of Gap Junctions

Thin Section

Negative Stain

Immunofluorescence
Gap junctions facilitate the exchange of small molecules between cells.

Mammals: 20-21 different genes coding for connexin proteins.
Zebrafish: 37 different genes coding for connexin proteins.
Loss of Gap Junction Proteins Leads to Disease

- cx43-/- leads to heart malformation
- cx46-/- leads to cataracts
- cx37-/-;cx40-/- leads to defects in vasculature
Mutations in mammalian CX43 also cause skeletal malformations.

The function of Cx43 is conserved from zebrafish to mammals.
Fin growth requires cell proliferation and cell differentiation.

**Transverse section**
- bone forming cells
- collagen-like fibers
- dividing/undifferentiated cells

**Longitudinal section**
- bone forming cells
- collagen-like fibers
- dividing/undifferentiated cells
Different cells use different sets of genes

Expressed genes generate a “message” or mRNA, that gets translated into protein.
Detecting gene expression – in situ hybridization

Permits localization of gene-specific mRNAs in a complex tissue
cx43 is expressed in dividing cells and in bone-forming cells at segment boundaries
All methods that *reduce* Cx43 activity causes *reduced* fin growth, *reduced* cell proliferation, and *reduced* segment length.

We predicted that methods that *increase* Cx43 would lead to *increased* fin growth, *increased* cell proliferation, and *increased* segment length.
The *another long fin* (*alf*) mutant exhibits phenotypes opposite of the *short fin* mutant overlong bony segments increased *cx43* mRNA
**sof** and **alf** exhibit opposing fin phenotypes and opposing expression levels of **cx43**

<table>
<thead>
<tr>
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<th>Fin length</th>
<th>Segment length</th>
<th>Cell division</th>
<th>cx43 mRNA</th>
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</thead>
<tbody>
<tr>
<td>sof&lt;sup&gt;b123&lt;/sup&gt;</td>
<td>decreased</td>
<td>decreased</td>
<td>decreased</td>
<td>decreased</td>
</tr>
<tr>
<td>alf&lt;sup&gt;dty86&lt;/sup&gt;</td>
<td>increased</td>
<td>increased</td>
<td>increased</td>
<td>increased</td>
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Hypothesis: Cx43 promotes cell proliferation and inhibits joint formation

HOW does this work? What happens after Cx43?
Microarray analysis detects differences in gene expression from whole tissues.

Different genes are spotted on "chips" – glass slides the size of a postage stamp.

Method:

1. Sample 1 mRNA (control)
2. Sample 2 mRNA (experiment)
3. Green fluorescent nucleotide
4. Reverse transcriptase
5. Red fluorescent nucleotide
6. Mix probes
7. Hybridization and washing
8. Confocal microscope fluorescence scanning

In sample 2, relative to sample 1,
- Gene D moderately overexpressed
- Gene E equally expressed
- Gene F moderately underexpressed
To find genes regulated by Cx43, identify genes regulated in opposing manners in short fin and alf

i.e. genes down-regulated in short fin AND up-regulated in alf

Pathways downregulated in short fin

= under-expression of cx43

Pathways upregulated in alf

= over-expression of cx43

Total genes on zebrafish microarray: 43,000 genes

Identified by overlap

180 genes

Genes discussed today

1 gene (sema3d)
Genes identified by microarray analysis must be “validated”

<table>
<thead>
<tr>
<th>Expression of <em>sema3d</em> via qRT-PCR</th>
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<tbody>
<tr>
<td><strong>strain</strong></td>
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<tr>
<td>wild-type</td>
</tr>
<tr>
<td><em>sof</em> (^{b123})</td>
</tr>
<tr>
<td><em>alf</em> (^{dty86})</td>
</tr>
<tr>
<td><em>cx43-KD fins</em></td>
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Thus, the expression of *sema3d* is influenced by Cx43 activity
Morpholino-mediated knockdown of *sema3d* recapitulates all of the *short fin* phenotypes.

Reduced fin length

Reduced segment length

Thus, the *sema3d* is functionally related to Cx43 activity.
Cx43 activities are mediated by Sema3d:

cx43 \rightarrow \textit{sema3d}

- growth/
- proliferation
- joint formation/
- patterning
Semaphorins are ligands that mediate signal transduction pathways for growth cone extension or collapse, cell proliferation, gene expression, migration, etc.
What is signal transduction?

Signal from outside to inside

Signal = button

Electrical transmission across the door/wall

Sound inside
Electrical transmission of the signal requires multiple events before the chime sounds.
Receptors are analogous to doorbells.

Changes inside the cell that influence cell division, gene expression, etc.

outside
inside
The putative Sema3d receptor, *nrp2a*, is expressed in two populations of cells in the regenerating fin.

Knockdown of *nrp2a* influences cell division, but not joint formation.

Conclusion: Nrp2a is a Sema3d receptor for growth.
PlexinA3 is expressed during fin regeneration

Knockdown of plxna3 influences joint formation, but not cell division.

Conclusion: PlexinA3 is a Sema3d receptor for bone patterning
PlexinA1 is expressed in the distal-most blastema

Knockdown of *plxna1* does not influence joint formation or cell division.

**Conclusion:** PlexinA1 is not a Sema3d receptor
Cx43 coordinates bone growth and bone pattern formation via divergent Sema3d pathways.

Diagram:

- Cx43 → Sema3d
- Sema3d → Nrp2a (Growth/proliferation)
- Sema3d → PlexinA3 (Patterning/joint formation)
How does Sema3d-binding to the receptor initiate changes across the membrane?

Identified Nrp2a and PlexinA3 as likely receptors for Sema3d.

These receptors typically interact with a second receptor.

Sema binding causes structural changes in the receptors that lead to changes inside the cell.

Changes inside the cell that influence cell division, gene expression, etc.
Sema binding causes structural changes in the receptors that lead to changes inside the cell.
Skeletal morphology is regulated by mechanisms that coordinate bone growth and patterning.

Misexpression of Cx43 leads to defects in cell proliferation and joint formation.

Using a microarray strategy, we found that Sema3d functions downstream of Cx43.

Continued studies will reveal how the Sema3d-signal is transduced and other Cx43-dependent genes.
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