The Role of Collagen 11a2 in Zebrafish Vertebral Development

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Collagen 11a2 is a minor collagen in cartilage, which maintains spacing and fibrillar integrity of the major cartilaginous collagen. Work in the zebrafish shows robust expression of *col11a2* in the craniofacial cartilage, the hearing organs, and in the notochord, which is the precursor to the spine. It has recently been shown that *col11a2* mutant zebrafish have craniofacial defects[1], but no other phenotypes have been reported. Morpholino knockdown of collagen 11 in the zebrafish shows disruptions in axial morphogenesis along the notochord[2], which suggests that loss of function in this gene would disrupt not only craniofacial development, but also spinal and vertebral development. Here, I aim to examine the role of *col11a2* in zebrafish vertebral development.

I have created a zebrafish hypomorphic indel mutant for *col11a2*, and characterized a mild vertebral fusion phenotype in these animals. I have also created a transcriptless allele of this gene which avoids a genetic compensation response by deleting the *col11a2* locus. These mutants exhibit a more severe vertebral fusion phenotype. In this project, I have visualized these fusion defects across development using *in vivo* staining techniques to show when fusions first arise and how they progress over time. I have quantified the position of these fusions and found that the caudal vertebrae are most affected in *col11a2* mutants. I have ongoing work to explore the role of the intervertebral disc in the formation of vertebral fusions using fluorescent reporter lines, and elucidate the mechanism by which these malformations develop. Using the zebrafish as a model, I have shown that *col11a2* is necessary for proper vertebral development, and created a new model of congenital scoliosis which I will be using for further exploration of vertebral fusion formation, as well as characterizing the involvement of genetic compensation mechanisms in this phenotype.

col11a2 is expressed in the face and notochord

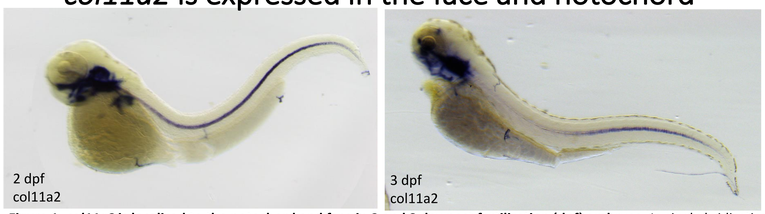


Figure 1. *col11a2* is localized to the notochord and face in 2 and 3 day post fertilization (dpf) embryos. *In situ* hybridization with a probe for *col11a2* shows early expression in the notochord. Staining is also visible in the head, in the otic vesicles, the jaw, and the eyes.

col11a2 knockdown impairs axial morphogenesis

- *col11a2* homozygous mutant embryos shown to have cartilage defects in the jaw[1]
  - No vertebral phenotypes examined
- Morpholino knockdown of *col11a2* resulted in disrupted notochord structure[2]

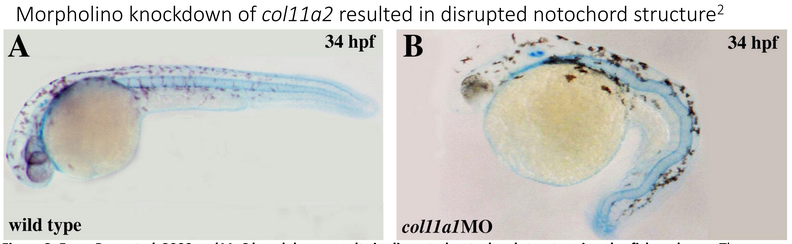


Figure 2. From Bass *et al.* 2009, *col11a2* knockdown results in disrupted notochord structure in zebrafish embryos. The morpholino used here is labelled as *col11a1*, but due to an annotation error, actually targets *col11a2*. The notochord is stained with alcian blue.

Suggests that loss of function in *col11a2* would disrupt spinal development

What is the role of *col11a2* in zebrafish spinal development?

CRISPR/Cas9 mediated mutagenesis of *col11a2*

- gRNA selected for highest cleavage efficiency (88%), used to create indels

col11a2 alleles
5 bp deletion - G428*
4 bp deletion - G641*
1 bp deletion - L642*

→ Premature termination codon → Predicted loss-of-function

Figure 3. Schematic of the *Col11a2* protein structure. The dominant functional domain of all collagens is the triple helix domain, which allows the protein to trimerize with other collagens. The other protein domains are important for glycosylation and secretion of *col11a2* into the extracellular matrix. Black marks along the bottom of the diagram denote area to which gRNAs were targeted. 35 gRNAs were tested in total. Black star indicates gRNA which was used for generation of indel mutations.

col11a2 mutagenesis results in vertebral fusions in F0 fish

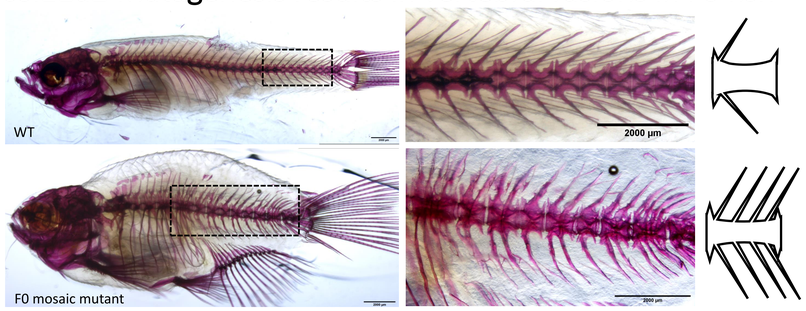


Figure 4. Mutagenesis of *col11a2* using CRISPR-Cas9 results in F0 mosaic mutant fish with vertebral fusions. Fish pictured 6 months post fertilization, stained with alizarin red. Wildtype fish exhibits even patterning of the vertebrae and ribs. Fish injected with gRNA at the one cell stage, the F0 mosaic mutant, exhibits irregular vertebral spacing and patterning.

col11a2$^{G641*/G641*}$ fish develop vertebral fusions

- *In vivo* stains show progression of vertebral fusions over development
- Homozygous mutants develop vertebral fusions with 74% penetrance

# of fusions	% of fish
0	26%
1	52%
2	9%
3	9%
4	4%

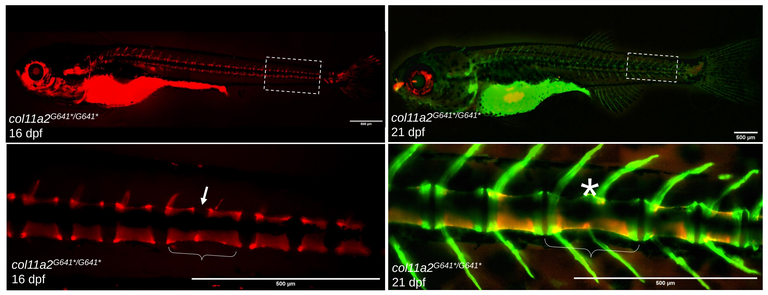


Figure 5. Individual *col11a2*$^{G641*/G641*}$ mutant stained *in vivo* with vital dyes across development. Alizarin red staining at 16dpf shows vertebrae beginning to fuse along the ventral edge, with space still present on the dorsal edge (arrow). 21 dpf calcein stains show that vertebral fusion has progressed fully, leaving no space between adjacent vertebral bodies (asterisk).

Why are fusions more severe in F0 fish than in mutants?

Genetic compensation masks *col11a2*^{G641*} phenotype

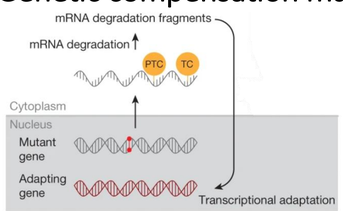


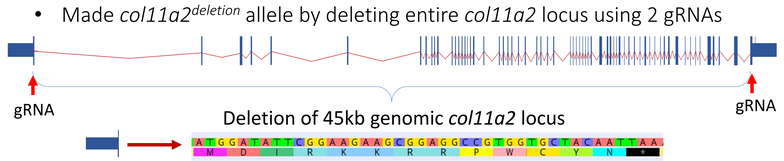
Figure from El-Brolosy *et al.* 2019

- Premature termination codons cause upregulation of similar genes[3, 4]
- Functionally compensates for loss of mutant protein

Is genetic compensation masking *col11a2*^{G641*} phenotype?

Transcriptless allele circumvents genetic compensation

- Made *col11a2*^{deletion} allele by deleting entire *col11a2* locus using 2 gRNAs



- Resulting *col11a2*^{deletion} allele encodes 13 amino acids and stop codon
  - Predicted not to trigger genetic compensation

What is the phenotype of *col11a2*^{deletion} mutant zebrafish?

col11a2^{deletion} fish develop severe vertebral fusions

- *col11a2*^{deletion} homozygotes develop fusions with 90% penetrance

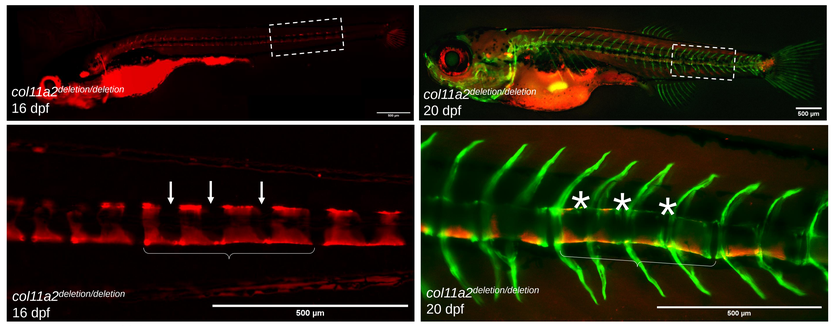
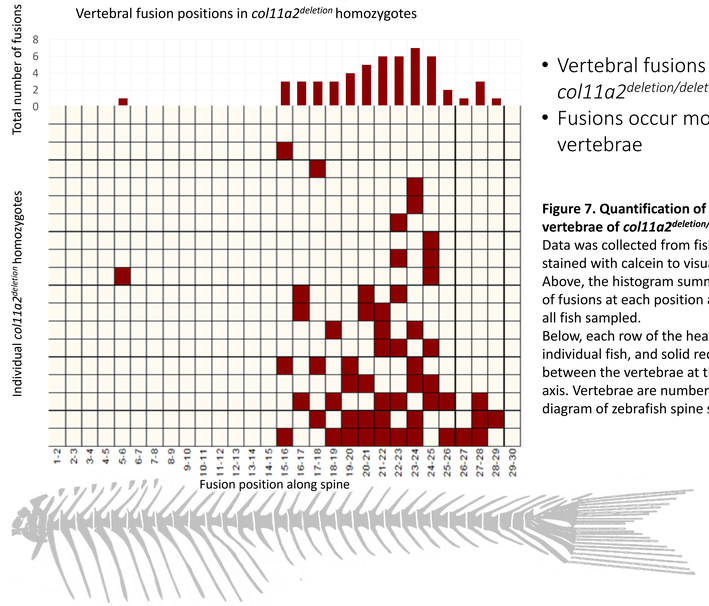


Figure 6. *col11a2*^{deletion} homozygous fish develop severe vertebral fusions at larval stages. This fish was initially stained with alizarin red at 16 dpf, when fusions are just beginning to form in the caudal vertebrae. By 20dpf, the fusions have progressed significantly, such that there is little or no remaining space between adjacent vertebrae. White bracket indicates four fused vertebrae.

col11a2^{deletion} homozygote have fused caudal vertebrae



- Vertebral fusions in *col11a2*$^{deletion/deletion}$ 90% penetrant
- Fusions occur mostly in the caudal vertebrae

Figure 7. Quantification of position of fusions in vertebrae of *col11a2*$^{deletion/deletion}$ fish.
Data was collected from fish imaged at 20 or 21 dpf, stained with calcein to visualize vertebrae.
Above, the histogram summarizes the total number of fusions at each position along the spine, among all fish sampled.
Below, each row of the heatmap represents an individual fish, and solid red blocks indicate a fusion between the vertebrae at that position on the x-axis. Vertebrae are numbered 1-30, corresponding diagram of zebrafish spine shown below.

Future directions:

- Test the ability of a *col11a2* transgene to rescue the fusion phenotype
- RT-qPCR to quantify the effects of genetic compensation on *col11a2*^{deletion}, *col11a2*^{G431*}, and *col11a2* mosaic mutants
- Assess the effects of vertebral fusions on growth and body length
- Create transcriptional *col11a2* reporter to assess expression pattern *in vivo*
- Use *col2a1aBAC::mCherryNTR*[5] to elucidate the role of intervertebral discs in fusion formation

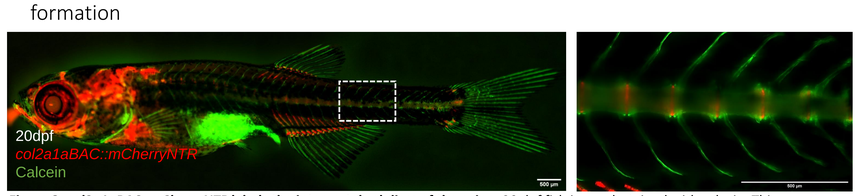


Figure 8. *col2a1aBAC::mCherryNTR* labels the intervertebral discs of the spine. 20 dpf fish imaged, stained with calcein. This reporter will be used to assess how fusions develop in the *col11a2*^{deletion} mutant by allowing for visualization of the intervertebral discs. It will be used to distinguish if intervertebral discs are not forming in these mutants or if they form normally and deteriorate later in development.

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References:
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