The role of Ewing's sarcoma protein EWS in

endochondral ossification and angiogenesis

By

Seth Jason Lewin

Submitted to the graduate degree program in Molecular Biosciences and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Arts.

_____

Chairperson: Dr. Mizuki Azuma

_____

Dr. Erik Lundquist

_____

Dr. Stuart Macdonald

Date Defended: 09-December, 2015

The Thesis Committee for Seth Jason Lewin

certifies that this is the approved version of the following thesis:

The role of Ewing's sarcoma protein EWS in

endochondral ossification and angiogenesis

_____

Chairperson: Dr. Mizuki Azuma

Date approved: 16-December, 2015

ABSTRACT

Ewing sarcoma is a bone and cartilage cancer affecting children and adolescents. Up to 90% of Ewing sarcoma patients express a chimeric EWS/FLI1 protein which is formed by a chromosomal translocation, and is known to induce aberrant transcription, bind with endogenous EWS and cause mitotic defects similar to knockdown of EWS. In EWS knockout studies, $EWS^{-/-}$ mice produce smaller pups with shorter, brittle bones compared to wild-type littermates suggesting EWS has a role in skeletal and overall development. Since Ewing's sarcoma develops in the bone, the role of endogenous EWS is of interest as it may provide an insight into the parthenogenesis of this disease. To investigate this we generated a maternal zygotic (MZ) *ewsa* null mutant zebrafish, produced by insertional mutagenesis. Chondrocyte maturation begins after mesenchymal condensation at about 3 days past fertilization (dpf), then cells undergo morphological changes by 4dpf to become prehypertrophic chondrocytes with most fully differentiated into hypertrophic chondrocytes by 6dpf. Previously, we reported that Ewsa modulates the mRNA expression of target genes of transcription factor Sox9, the master transcriptional regulator of chondrocyte differentiation. Given the regulatory role of Sox9 in chondrogenesis, an important question is whether Ewsa directly regulates the expression level of Sox9 proteins. Thus, we generated antibodies against Sox9a and Sox9b, and performed immunohistochemistry in 3-6dpf of *wt/wt* and MZ *ewsa/ewsa* zebrafish mutants. As a result, Sox9a localized in the nucleus, and the expression levels of Sox9a proteins were unchanged among 3-6dpf *wt/wt* and MZ *ewsa/ewsa* zebrafish mutants. This suggests that Sox9a protein is not regulated by Ewsa during chondrocyte maturation. One upregulated Sox9 target gene was

connective tissue growth factor (*ctgf*). Ctgf is known to have a critical role in endochondral ossification, furthermore the overexpression of Ctgf regulation results in skeletal defects and reduced hypertrophy. We suspect that Ews regulates Ctgf during endochonral ossification and aim to confirm this in this study. We discovered that Ctgf is overexpressed in craniofacial cartilage of mutant embryos at 4dpf and 6dpf. Also measurements of cartilage structures show evidence of skeletal defects in these embryos as early as 4dpf. This is evidence that the regulation of Ctgf by Ewsa is essential for normal chondrocyte maturation and skeletal development. In addition, a reduction of *ctgfa* signal in situ and Ctgf in immunohistochemistry assays was observed in the vascular structures in mutant embryos at 3dpf and 6dpf suggesting Ewsa may regulate *ctgf* expression in vascular tissue. Which is important since vascular invasion into mature chondrocytes is necessary in skeletal development. In summary, we discovered that Ewsa may regulate stage-specific, and tissue specific expression of Ctgf during embryonic development. Understanding the pathway of Ewsa-dependent skeletogenesis may supply a platform for the molecular pathogenesis of Ewing sarcoma.

ACKNOWLEDGMENTS

I would like to thank Dr. Mizuki Azuma, Dr. Erik Lundquist, and DR. Stuart Macdonald, for their support and direction as members of my committee. I would like to thank Dr. Yoshiaki Azuma for use of reagents in my research. I also would like to thank Hyewon Park for designing and obtaining zebrafish-specific Sox9 antibodies as well as Luke Wenger, and Raeann Whitney for their support. Finally, I would like to thank The Massman Family Ewing Sarcoma Research Fund and the National Institutes of Health (grants no. P20GM103418, P20GM103638) for support of this research.

Table of Contents

INTRODUCTION

Ewing's sarcoma (ES) is a bone cancer usually affecting children and adolescents. One hallmark of Ewing's tumors is that patients express an aberrant oncogenic fusion protein which consists of the N-terminal domain of the Ewing's sarcoma protein (EWS) and the C-terminal domain of an ETS transcription factor [1]. The fusion protein is produced due to a translocation event which involves specific breakpoints in genetic coding regions for both of the proteins [2]. The most common fusion protein, which is reported in 90% of Ewing's sarcoma patients is EWS/FLI1 [3]. EWS/FLI1 can act as a transcription factor and can interact with the spliceosome machinery to produce altered splicing events [4]. Also EWF/FLI1 can interact with endogenous EWS thus possibly interfering with normal endogenous function [5]. Lastly, the knockdown of EWS causes mitotic defects and genomic instability in human cells similar to the phenotype seen in EWS/FLI1 expressing cells, thus suggesting a reduction of endogenous EWS function in the presence of EWS/FLI1 [5, 6]. The study of the fusion protein demonstrates a gain of function within the context of this disease but there still remains the possibility of a loss of function in terms of EWS haploinsufficiency, or inhibition of endogenous EWS function.

Given that EWS function could be effected by EWS/FLI1 which is found in ES patients, and since the tumors arise in the developing bone, the role of EWS in skeletal development must be investigated. Knockout mice for EWS exhibited 90% postnatal lethality, pups were smaller, and had less bone

density than wild-type littermates [7]. Although the mechanisms for this phenotype were not explored, this suggests that EWS is involved in bone development. Long bones develop through endochondral ossification; the process of chondrogenesis lays down cartilage with later calcification of the tissue giving rise to mature bone. Chondrogenesis begins with mesenchymal condensations at about 2dpf (days past fertilization) in zebrafish. The cells then undergo highly regulated transcriptional and morphological changes that promote progression from a proliferative and undifferentiated state into an intermediate or prehypertophic state by about 3dpf, [8], and eventually they further differentiate into hypertrophic chondrocytes by 6dpf [9]. A recent report from our group shows that in a zebrafish model, MZ *ewsa/ewsa* mutant fish had significant facial cartilage deformations at 4dpf indicating that ewsa regulates chondrocyte maturation. This same report also elucidated a biochemical interaction between EWS and SRY (Sex Determining Region Y)-Box 9 (SOX9)[10]. The transcription factor SOX9 has been dubbed the “master regulator” of chondrogenesis due to its essential role in the process and since the transcriptional targets of SOX9 are involved in chondrocyte maturation [11]. In vivo knockdown or knockout of SOX9 results in mice with severe skeletal defects and elimination of SOX9 target gene expression [12]. In our previous study, we reported 8 of the 30 SOX9 target genes tested were modulated in MZ ewsa/ewsa mutant embryos at 27hpf, thus suggesting that EWS regulates skeletal development by modulating the targets of SOX9

[10]. Yet it is not known if Sox9 expression is being affected by Ewsa, which in turn would explain the changes in targets of Sox9.

One of the target genes of Sox9 upregulated with the loss of ewsa is connective tissue growth factor (ctgf). CTGF is of interest as it is involved in many processes including extracellular matrix (ECM) production, chondrogenesis, proliferation, apoptosis, and angiogenesis [13]. The ECM is essential for normal development by serving as scaffolding and signaling. Mutations in genes for ECM components can lead to severe skeletal defects and fibrosis [14]. Transgeneic mice overexpressing CTGF exhibited smaller stature, increased expression of ECM components, increased chondrocyte proliferation, and shorter hypertrophic zones in the bones of embryos as compared to wild type littermates [15-17]. Indeed, in our previous study MZ ewsa/ewsa embryos had shorter hypertrophic zones in the facial cartilage, which also develops through endochondral ossification, and a higher number of chondrocytes when compared to the wild type [10]. Given the importance of CTGF in this process, and given the phenotype observed in our mutants, the possibility of EWS regulation of CTGF during normal endochondral ossification warrants investigation. Furthermore, the final stages of endochondral ossification require vascular invasion into the mature cartilage before calcification. CTGF has been shown to induce proliferation and migration of endothelial cells, and to promote production of vascular endothelial growth factor (VEGF) [18], a key signaling molecule promoting vascular endothelial growth, but at higher expression levels, CTGF binds to

and inhibits VEGF [19]. Therefore strict control of CTGF expression levels is imperative for normal angiogenesis, yet there have been no reports describing EWS as the regulatory protein in this process.

In this report, to further investigate the regulatory role of EWS in chondrogenesis we focus on the temporal effect of EWS regulation of SOX9 first of all, then focus on EWS regulation of Ctgf, both over the course of chondrocyte maturation. This will elucidate the role of EWS in the regulation of CTGF during chondrocyte maturation and to answer the questions; is CTGF regulation accomplished though regulation of SOX9 expression? Also, is the protein expression of CTGF regulated by EWS during chondrogenesis? Furthermore we hope to elucidate a regulatory role of EWS in endothelial expression of Sox9 and Ctgf. Since CTGF is involved in chondrogenesis and ECM production as well as angiogenesis, it would be expected that the misregulation of CTGF would lead to skeletal defects and inhibited vascular invasion into bone. Therefore in this report we use MZ ewsa/ewsa zebrafish to demonstrate the regulation of CTGF by EWS in chondrocyte maturation. Furthermore this report is the first to implicate EWS regulation of CTGF in vascular endothelial cells, and this may help in illuminating the mechanism by which misregulation of chondrocyte maturation proteins could contribute to the formation of Ewing’s sarcoma.

MATERIALS AND METHODS

Aquaculture

Zebrafish families were bred and maintained at 28.5^{o}C using an automatic filtration system from Aquatic Eco-Systems Inc, and embryos were staged as previously described [20]. The wild type line used was the Oregon AB line. The ewsa/ewsa line was generated by insertional mutagenesis by Znomics Inc, and is maintained as a Maternal Zygotic (MZ) line in our system.

Immunohistochemistry

Embryos were visualized as previously described [6]. Fixed embryos were permeabilized with methanol at -20^{o}C overnight, digested with 0.01% trypsin (5min for 3dpf, 6min for 4dpf, 8min for 5dpf, 10min for 6dpf), and then blocked with blocking solution (10% fetal bovine serum, 1% dimethyl sulfoxide, 0.1% triton x- 100) for 1 hour. Primary antibodies were applied overnight at 4^{o}C; embryos were washed thoroughly; and secondary antibodies were applied overnight at 4^{o}C. Embryos were then washed thoroughly again, equilibrated to 50% glycerol, and stored in 100% glycerol. We used rabbit polyclonal antibodies for Sox9a or Sox9b (Pacific Immunology, CA) diluted 1:250 (1µL antibody into 250µL blocking solution) or 1:500 (0.5µL antibody into 250µL blocking solution) and to visualize we used anti-rabbit Alexa 488 secondary antibody diluted 1:250 (1µL antibody into 250µL blocking solution). We used a rabbit polyclonal anti-CTGF primary

antibody (ab6992 from abcam) diluted 1:1000 (0.5µL antibody into 500µL blocking solution) and anti-rabbit Alexa 488 secondary antibody diluted 1:250 (1µL antibody into 250µL blocking solution) to visualize Ctgf. We also tested a rabbit polyclonal anti-CTGF primary antibody (07-1386 Millipore) diluted to 1:1000 (0.5µL antibody into 500µL blocking solution) and anti-goat Alexa 594 secondary antibody diluted 1:250 (1µL antibody into 250µL blocking solution) to visualize Ctgf. Images were taken with an Exi Aqua camera (Q Imaging) mounted on an Eclipse Ti microscope (Nikon).

In situ hybridization

In situ hybridization was performed as previously described [21]. Fixed embryos were bleached for 1 hour in a solution of 3%H_2O_2 / 0.5% KOH, washed in PBS, permeabilized in methanol at -20^{o}C overnight, and then washed in PBS again. Embryos were digested with 0.01% trypsin (5min for 3dpf, 6min for 4dpf, 8min for 5dpf, 10min for 6dpf) then equilibrated in HYB buffer at 60oC for 3 hours. Embryos were then hybridized overnight at 60oC with antisense RNA for *ctgfa* generated with T7 RNA polymerase and DIG-labeled dUTP. Embryos were then treated with anti-DIG antibodies conjugated with alkaline phosphatase (AP) overnight at 4oC. After equilibration with AP buffer, signal was developed with BM Purple, washed with PBS, and then embryos were fixed again in 4% paraformaldehyde at room temperature for 30 minutes.

Statistics

Statistical analysis was performed using Prism, version 5.03 (GraphPad Software Inc.). Welch's t-test was used to compare ceratohyal cartilage dimensions between wild type and MZ *ewsa/ewsa* embryos. An unpaired t-test was used to compare the number of Ctgf expressing cells in wild-type cartilage to MZ *ewsa/ewsa* cartilage.

RESULTS

Zebrafish specific antibodies recognize Sox9a and Sox9b. Previous studies showed that *sox9a* mRNAs are expressed in cranial cartilages and somites [22], and *sox9b* mRNAs in notochord at early stages [23]. However, the local expression of zebrafish Sox9a and Sox9b proteins remains unknown. Thus, we generated antibodies specific to both zebrafish Sox9a and Sox9b. Recombinant proteins containing C-terminus of Sox9a (304-462 amino acid), and of Sox9b (290-408 amino acid) were purified from *E. coli*, and was used as antigens for synthesis of polyclonal antibody in rabbit (fig. 1A). The C-terminus of Sox9a and Sox9b were specifically chosen to avoid cross-reactivity against the HMG-box in the N-terminus, which is universally conserved among Sox proteins. First, the reactivity of antibodies were optimized. Wild-type (*wt/wt*) zebrafish embryos at 3dpf were subjected to immunohistochemistry using either 1:250 or 1:500 dilutions of Sox9a and

Sox9b antibody (fig. 1B). Embryos stained with a 1:250 dilution of antibody had a high background when compared with embryos strained with a 1:500 antibody dilution. The embryos stained with 1:250 dilution of antibodies displayed no coinciding tissue specific signals compared to the previous report, suggesting that there are high background signals in both samples. Therefore, wild-type embryos at 1dpf were stained with 1:500 dilution of either Sox9a or Sox9b antibodies (fig. 2). Sox9a signal was detected in the somites (fig. 2A, white arrows) with Sox9b being expressed only in the posterior of the notochord (fig. 2B). This data correlates with published *sox9a* and *sox9b* mRNA expression data suggesting a lack of post-transcriptional regulation in the tissues where *sox9a* and *sox9b* are being expressed at 1dpf.

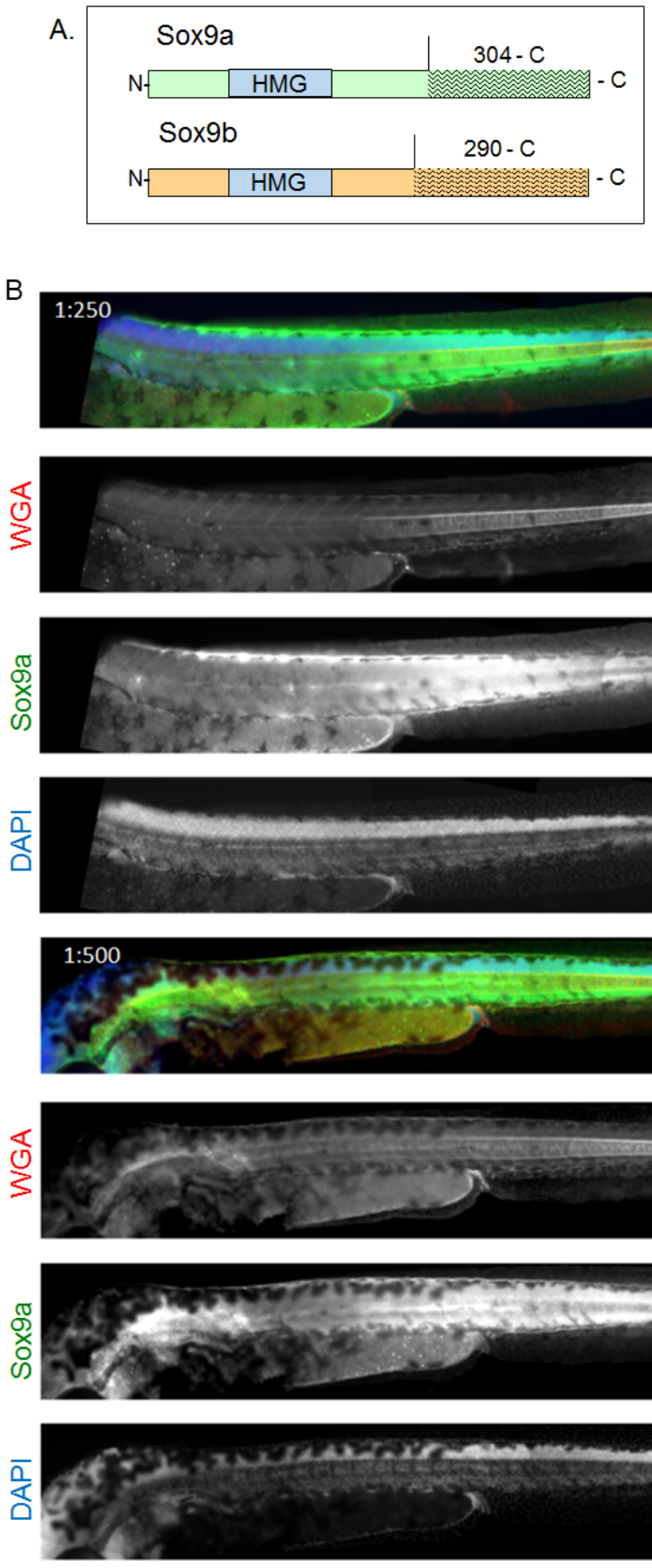


Figure 1. Zebrafish specific antibodies produced for Sox9a and Sox9b. (A) The diagram shows zebrafish-specific antibodies against Sox9a and Sox9b designed to recognize the C-terminal domains. (B) Immunohistochemestry of wild-type (WT) zebrafish embryos at 3dpf with 1:250 and 1:500 dilutions of Sox9a primary antibody show a more intense background signal in the 1:250 dilution than in the embryos stained using the 1:500 dilution.

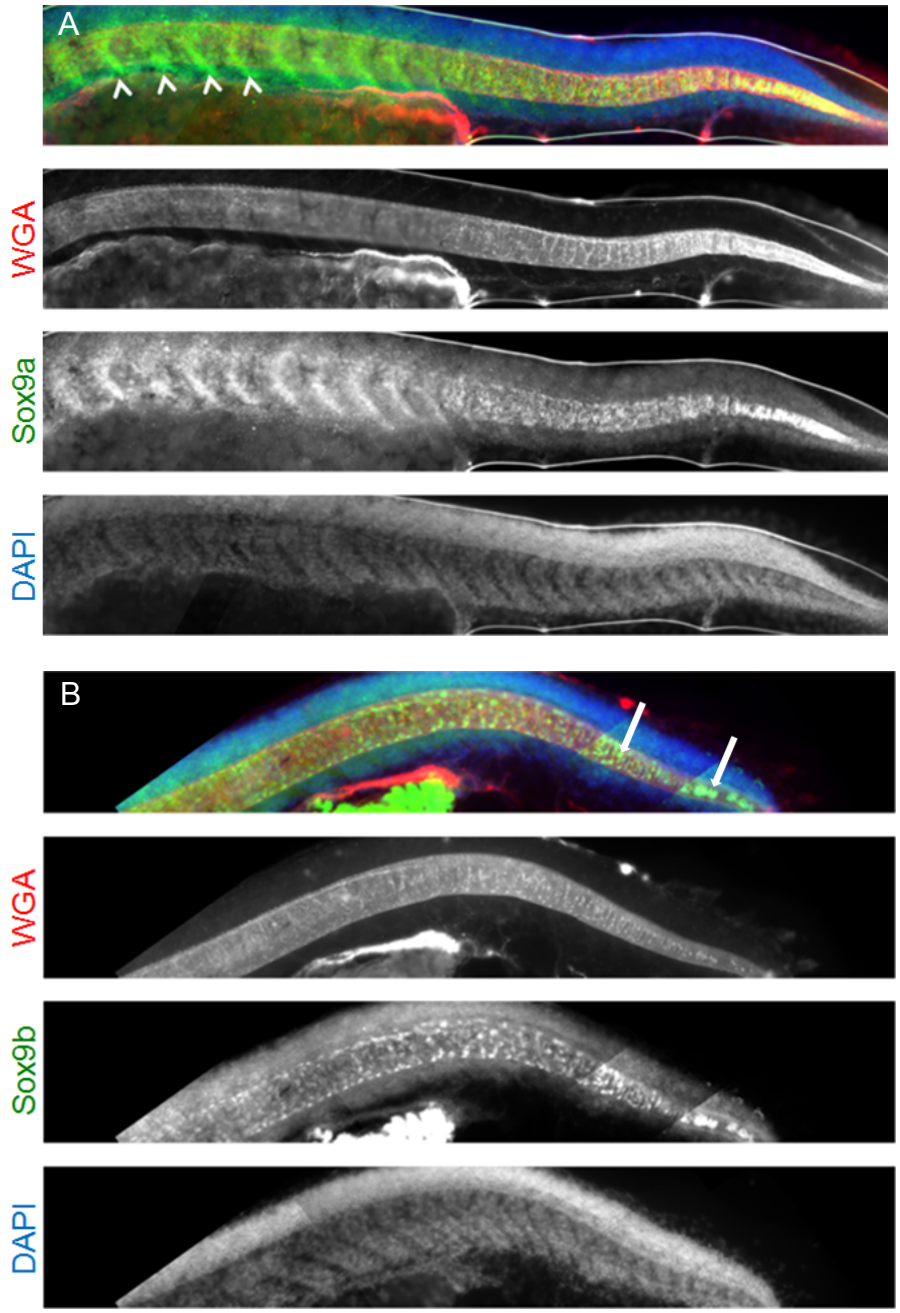


Figure 2. Sox9a and Sox9b specific expression. Immunohistochemistry of wild-type embryos at 1dpf with and Sox9b. White arrows show Sox9a (A.) expressing regions in the anterior tail section somites. Staining by Sox9b (B.) did not produce similar signal in the somite region yet both Sox9a and Sox9b showed expression in most posterior section of the notochord (arrows). Antibody dilution 1:500.

Ewsa does not regulate the expression levels of Sox9a and Sox9b proteins in facial cartilage of zebrafish. Since Ewing Sarcoma arises in bone and Sox9 is essential for chondrocyte maturation [12], Sox9 expression and the expression of the genes it regulates is of interest. We have previously reported that Ewsa binds to Sox9 and Sox9 target genes were modulated in the *ewsa* MZ *ewsa*/*ewsa* embryos at 27hpf by qPCR [10]. Being that these are targets of Sox9, it is possible that any modulation of these targets are the result of expression modulation of their transcriptional regulator Sox9 during this stage of development. Furthermore, given the evolutionary genome duplication in the zebrafish which produced two homologs of many genes including *sox9a* and *sox9b*, the question is raised if Sox9b is involved in chondrocyte maturation as a redundant or compensatory mechanism to counteract any change in Sox9a expression. Therefore, we investigated whether the expression Sox9a and Sox9b proteins is altered in MZ *ewsa/ewsa* mutant compared to *wt/wt* zebrafish. By 2dpf neural crest derived mesenchymal cells condense to form the pharyngeal arches, the proliferative precursors to chondrocytes. At 3dpf, these cells start to differentiate into pre-hypertophic chondrocytes. These cells further undergo morphological changes from small and round cells to long columnar cells called hypertrophic cells, and these cells align in the cartilage structure through intercalation (conversion extension) [24]. To determine whether the expression of Sox9a and Sox9b proteins are regulated by Ewsa in facial cartilage, *wt/wt* and MZ *ewsa/ewsa* zebrafish ~~e~~mbryos were harvested at 3-

6dpf, and subjected to immunohistochemistry using Sox9a or Sox9b antibodies (figs. 3,4,15-19). At 3dpf (fig. 3), there was no difference in the percentage of embryos expressing Sox9a signal between *wt/wt* and MZ *ewsa/ewsa* in both the Meckel's cartilage and ceratohyal cartilage. Furthermore, the Sox9a signal was localized to the nucleus. In addition, consistent with a previous report [22], there was no Sox9b (fig.4) signal in both *wt/wt* and MZ *ewsa/ewsa* embryos. This data suggests that Sox9b does not play a redundant role of Sox9a in the Meckel's cartilage and ceratohyal cartilage. This trend continued 4dpf to 6dpf (figs.18,19). Overall, Ewsa does not regulate the expression level of Sox9a and Sox9b expression during chondrocyte maturation.

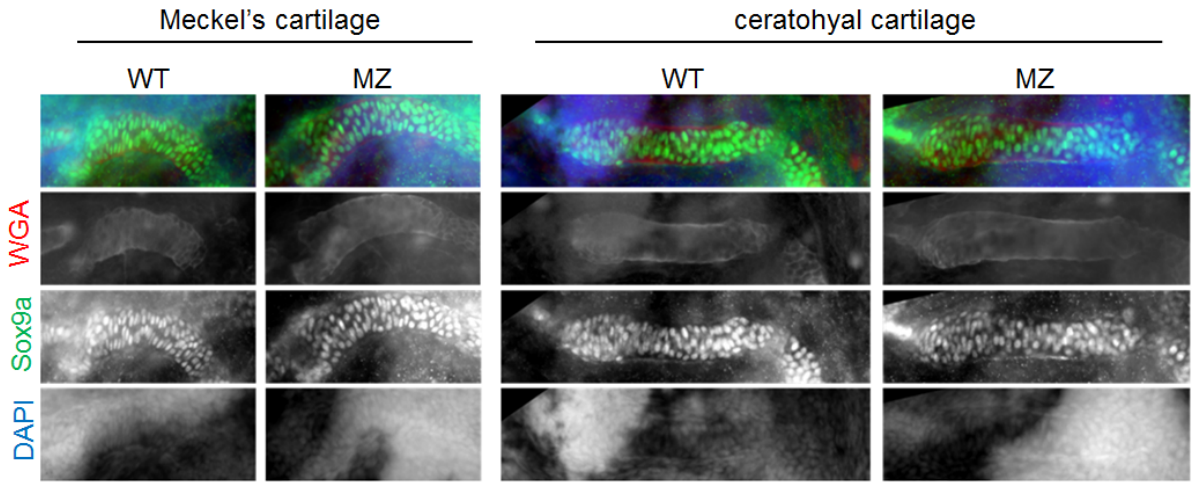


Figure 3. Sox9a expressed in wild-type and mutant embryo facial cartilage. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage and ceratohyal cartilage at 3dpf with anti-Sox9a antibodies show that 100% of the WT and 100% of the MZ embryos tested expressed Sox9a in both the Meckel's cartilage and ceratohyal cartilage. 6 WT and 3 MZ embryos total. Antibody dilution 1:500.

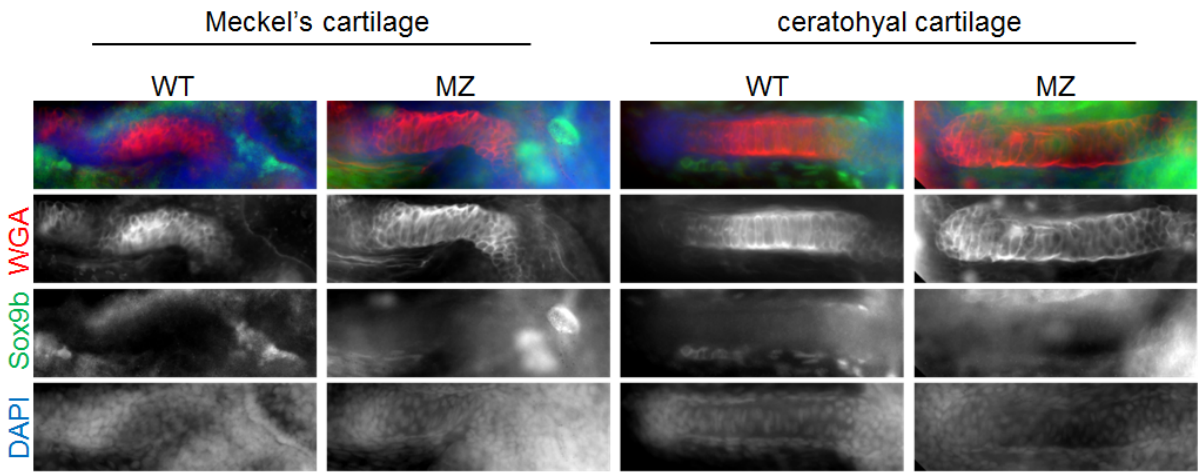


Figure 4. Sox9b not expressed in wild-type or mutant embryo facial cartilage. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage and ceratohyal cartilage at 3dpf with anti-Sox9b antibodies show that in 100% of both WT and MZ embryos lack expression of Sox9b. 5 WT and 5 MZ embryos total Antibody dilution 1:500.

Cartilage deformation in MZ *ewsa*/*ewsa* embryos occurs during chondrocyte maturation. Previously we have described several craniofacial deformations which occur in adult MZ *ewsa/ewsa* zebrafish. The exact time in embryonic development when these deformations begin to appear is not known. To answer this we began by measuring the length and width of the ceratohyal cartilage in MZ *ewsa*/*ewsa* embryos and compared to those taken from *wt/wt* embryos that have been stained with wheat germ agglutinin (WGA) as a marker for ECM (fig. 5A). At 4dpf, when compared to the *wt/wt* embryos the ceratohyal of the MZ *ewsa/ewsa* embryos did not significantly differ in length but were slightly wider (fig. 5B). At 6dpf the width of the MZ *ewsa*/*ewsa* remained significantly shorter yet the degree of deformation severity was increased due to the *wt/wt* cartilage being significantly lengthened whereas the MZ *ewsa*/*ewsa* cartilage remained short (fig. 5C).

The timing of this deformation is significant since it is occurring during a critical time period of chondrocyte maturation. Yet since the cartilage was already significantly wider at 4dpf, the beginning stages of the deformation must begin earlier with the mechanism causing the deformation occurring at an even earlier stage of development.

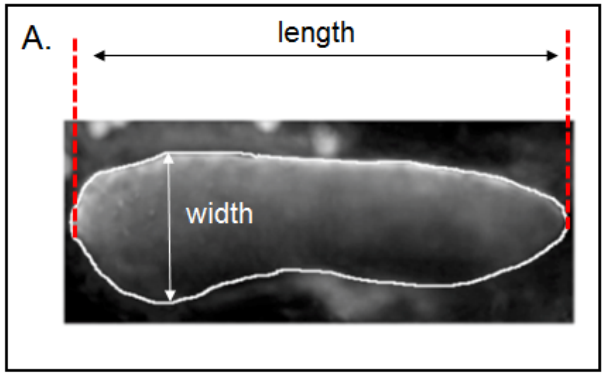
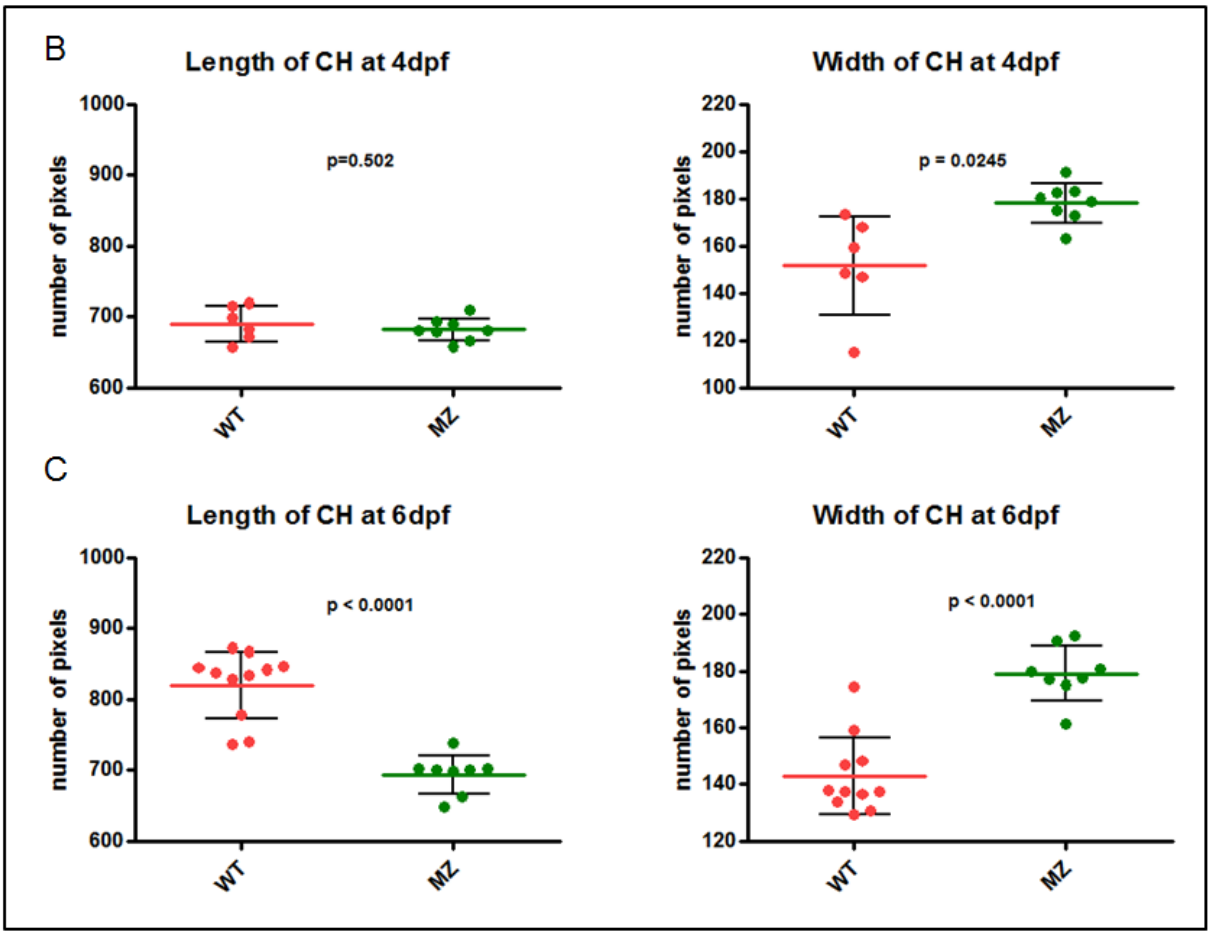


Figure 5. Cartilage deformation seen in MZ *ewsa* mutant cartilage. The length and width of ceratohyal cartilages taken from WT and MZ embryos were measured at their widest and longest points as shown in A. At 4dpf (B), there was no difference in the length of the ceratohyal between the WT and MZ tissues however, the width of the MZ ceratohyal was significantly greater than that of the WT. The deformation became more severe at 6dpf (C) as the width of the MZ remained significantly greater yet the length of the MZ ceratohyal did not increase as did the WT ceratohyal thus making it signifigantly shorter than WT. Measured with Image J, units are in pixels.



Optimization of commercial CTGF antibodies in zebrafish. As we previously reported, the loss of *ewsa* induces the upregulation of *ctgfa* at 27hpf but the protein expression pattern for Ctgf remains unclear. To monitor Ctgf expression in zebrafish embryos during development, we tested commercially available antibodies against human CTGF. The epitope sequence of the Abcam anti-CTGF antibody (ab6992) produced in rabbit corresponded to amino acids 223-348 of mouse CTGF and had an 83% identity with zebrafish Ctgfa and 62% with Ctgfb. The second antibody tested from Milipore (07-1386) was produced in goat and was against human CTGF with the epitope sequence corresponding to amino acids 27-349 and had a 78% identity with zebrafish Ctgfa and 58% with Ctgfb. *wt/wt* embryos at 5dpf were stained with both antibodies. Both the goat and rabbit antibodies stained chondrocytes within the Meckel's cartilage (fig. 6A). Full length Ctgf is approximately 40 kDa but it has been reported that Ctgf is rapidly cleaved by plasmin into either a 21 kDa or a 28 kDa fragments [25]. In a western blot analysis, both of the α-CTGF antibodies recognized zebrafish Ctgf in lysate from *wt/wt* zebrafish 6dpf (fig.9B) at approximately 40 kDa with the Abcam antibody and with the Millipore antibody two fragments were found at 28 kDa and 21 kDa possibly recognizing a cleaved version of Ctgf. To validate whether the signals obtained from immunohistochemistry are specific to Ctgfa, in situ hybridization using antisense RNA probes for *ctgfa* mRNA was performed using 3dpf *wt/wt* embryos (fig.7). In situ hybridization revealed that *ctgfa* mRNA expresses in facial cartilage. This correlates with the signal

detected in the α-CTGF IHC assay above. The co-localization of antibody signal on structures that also expresses *ctgfa* mRNA helps to validate both of these antibodies for use in detecting Ctgf in zebrafish.

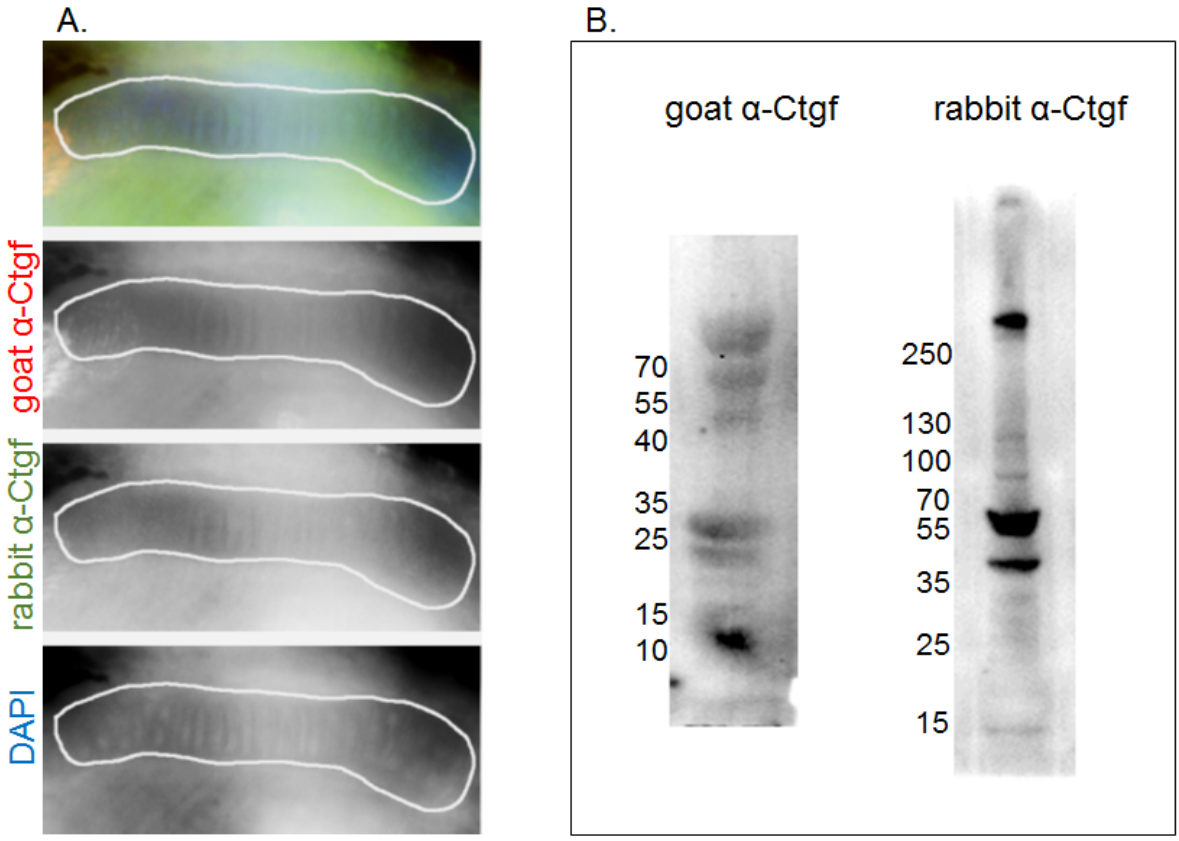


Figure 6. Anti-Human Ctgf antibodies recognize ZF Ctgf. (A) Immunohistochemistry of wild-type embryos at 5dpf using rabbit α-Ctgf and goat α-Ctgf antibodies, signal colocalization shown in Meckel's cartilage,(B) Western blot analysis of 6dpf zebrafish embryo lysate probed with the antibody from Millipore (goat α-ctgf) against Ctgf gives signal at ~40Kda where full-length Ctgf is expected as well as two bands at 28Kda and 21Kda where cleaved Ctgf would be expected. The antibody from ABcam (rabbit α-ctgf) provided a band at the expected size as well as a prominent non-specific band. IHC; both antibodies 1:1000 dillution. WB; 1° Ab 1:200, 2° Ab 1:100,000.

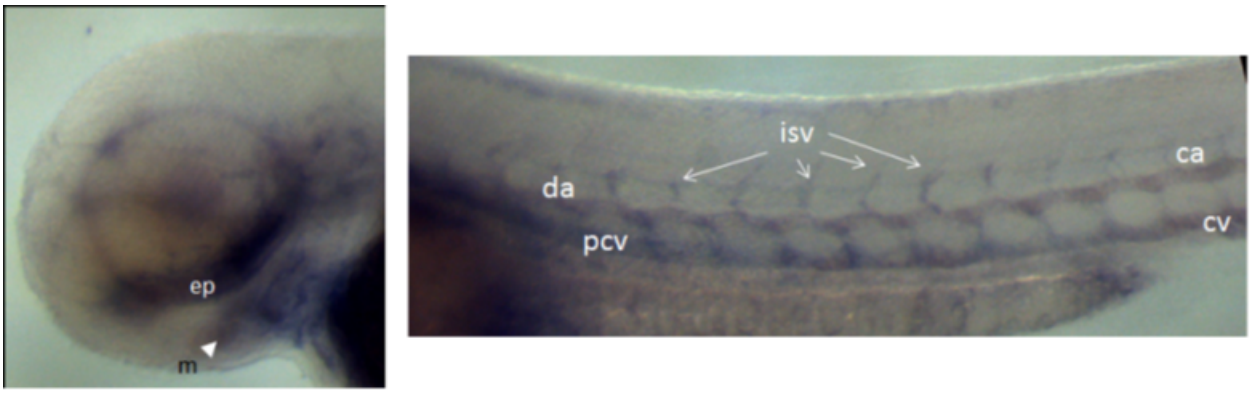


Figure 7. Ctgf expression confirmed in wild-type vasculature and facial cartilage. In situ hybridization with probes for zebrafish *ctgfa* show expression of transcripts in vasculature and facial cartilage in WT embryos at 3dpf. ep, emthmoid plate; m, Meckel's cartilage; isv, intersegmental vessels; da, dorsal aorta; pcv, posterior cardinal vein; ca, caudal artery; cv caudal vein.

Ewsa inhibits the expression of Ctgfa protein in prehypertrophic chondrocytes at 4dpf and 6dpf. A previous study showed that in transgenic mice, overexpression of CTGF leads to shorter hypertrophic zones [15] suggesting that these chondrocytes undergo a delay of differentiation into the hypertrophic state. Because our previous study showed that the expression level of *ctgfa* mRNA is increased in MZ *ewsa/ewsa* compared to *wt/wt* at 27hpf [10], we further extended our study to investigate how Ctgfa protein is regulated during chondorgenesis. Immunohistochemistry was conducted on *wt/wt* and MZ *ewsa/ewsa* embryos at 4dpf and at 6dpf; the time span between 4dpf and 6dpf is when chondrocytes mature from prehypertophic into hypertophic chondrocytes. At 4dpf the number of chondrocytes in the MZ *ewsa*/*ewsa* Meckel's cartilages expressing Ctgf were significantly higher than chondrocytes in *wt/wt* Meckel's cartilage (fig. 8A), this was also the case with the ceratohyal cartilage (fig. 8B). Although a

similar trend was observed in the hyomandibular and palatoquadrate cartilages (fig. 20), the 3-D dimensionality of the structure made quantification technically challenging. Likewise, at 6dpf the number of chondrocytes in the MZ Meckel's cartilages expressing Ctgf were significantly higher than chondrocytes in *wt/wt* Meckel's cartilage (fig. 9A), again this was also the case with the ceratohyal cartilage (fig. 9B). Similar to the previous data, the trend for greater number of chondrocyte Ctgf expression was observed in the hyomandibular and palatoquadrate cartilages (fig .21). This data suggests that Ewsa inhibits Ctgf in chondrocytes during a critical period of differentiation and this negative regulation is essential for proper cartilage formation.

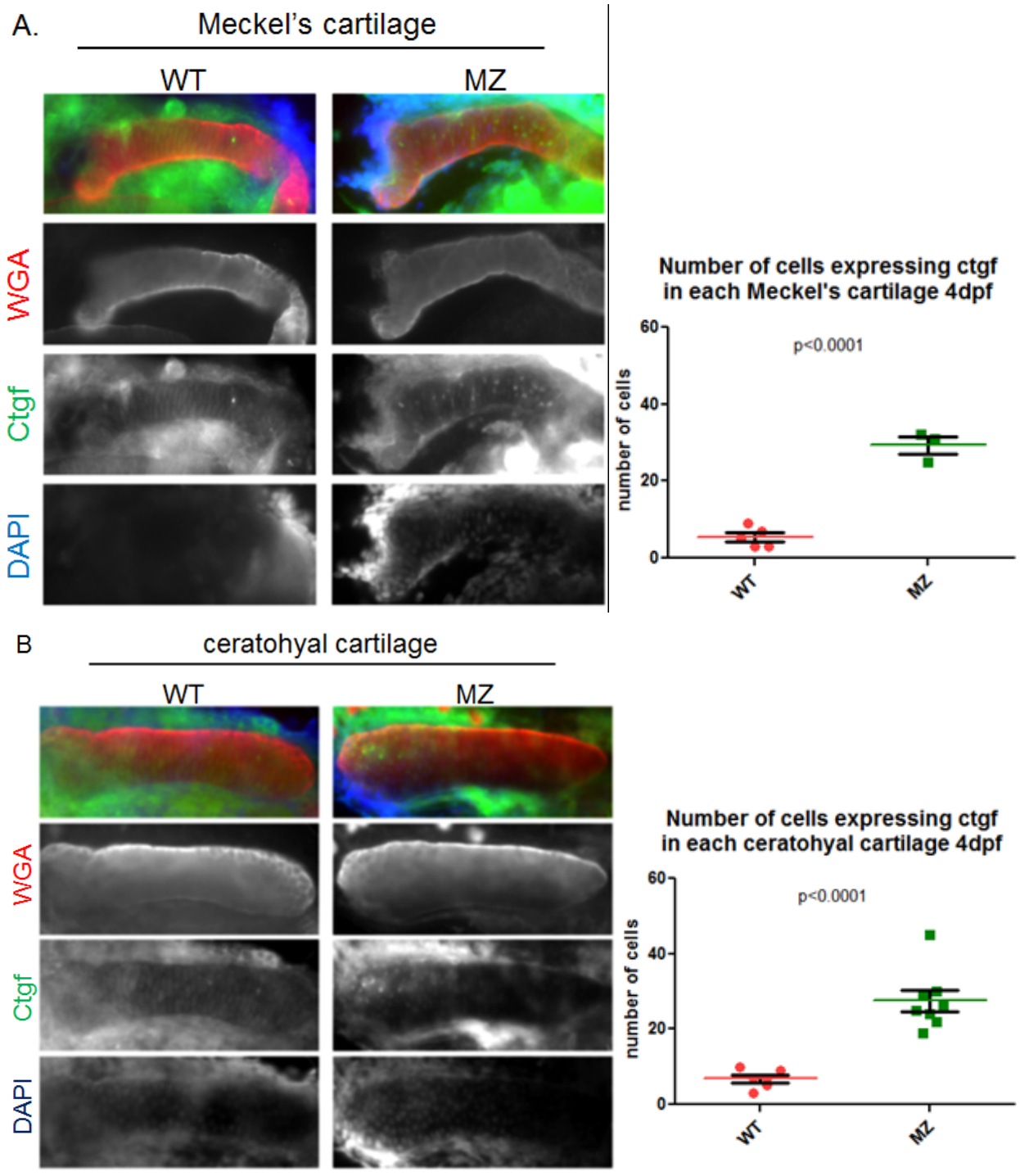


Figure 8. Ctgf is overexpressed in MZ *ewsa* mutant facial cartilage at 4dpf. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage (A) and ceratohyal cartilage (B) at 4dpf show a significantly increased number of chondrocytes expressing Ctgf in MZ cartilage than in WT tissue. Antibody dilution 1:1000.

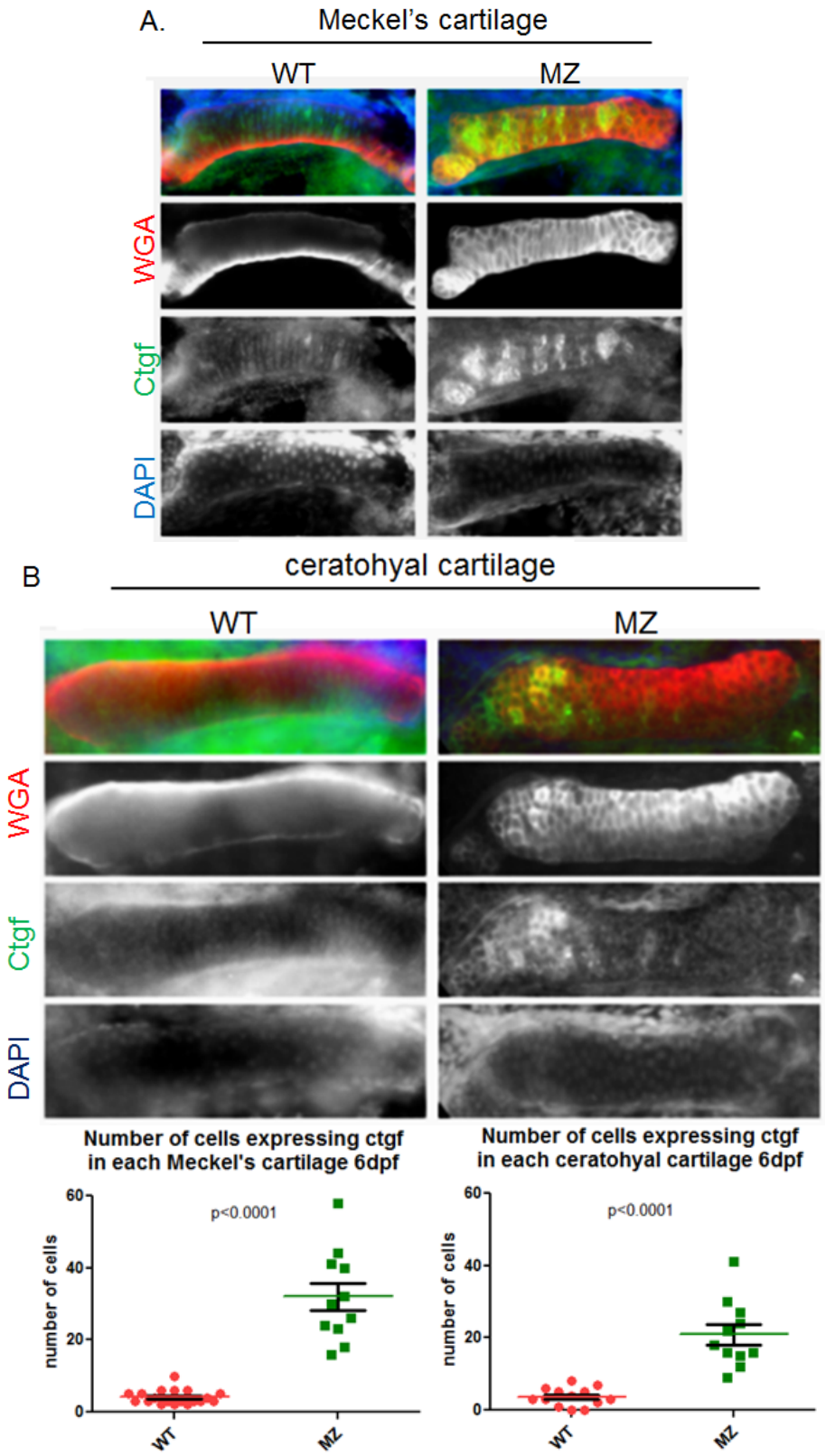
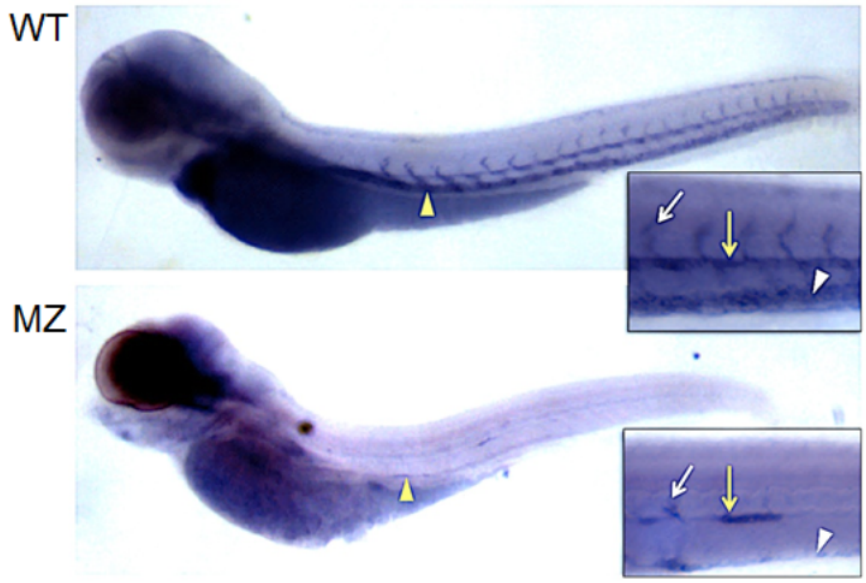

A.
Meckel's cartilage
WT
MZ
WGA
Ctgf
DAPI
B
ceratohyal cartilage
WT
MZ
WGA
Ctgf
DAPI
Number of cells expressing ctgf in each Meckel's cartilage 6dpf
p<0.0001
number of cells
60
40
20
0
WT
MZ
Number of cells expressing ctgf in each ceratohyal cartilage 6dpf
p<0.0001
number of cells
60
40
20
0
WT
MZ

Figure 9. Ctgf is overexpressed in MZ *ewsa* mutant facial cartilage at 6dpf. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage (A) and ceratohyal cartilage (B) at 6dpf show continued increase in the number of chondrocytes expressing Ctgf in MZ cartilage over WT tissue. Antibody dilution 1:1000.

Ewsa regulates the expression of Ctgfa at vascular endothelial tissue in 4dpf zebrafish embryos. Recently CTGF has been shown to play a role in basal lamia production in endothelium with severe vascular defects and embryonic lethality in CTGF knockout mice [26]. Studies of *ctgfa* expression in wild-type zebrafish embryonic vasculature at 90hpf showed *ctgfa* transcription occurring in the dorsal aorta, caudal artery and caudal veins [27]. As shown previously, *ctgfa* is also expressed in the intersegmental vessels (ISV) (fig. 7). Based on our previous report, it is highly possible that Ewsa-dependent regulation of Ctgf is required for angiogenesis. To elucidate this possibility, in situ hybridization was performed on *wt/wt* and MZ *ewsa*/*ewsa* embryos at 4dpf. In the results above, *ctgfa* mRNA was overexpressed in craniofacial cartilage 4dpf of MZ *ewsa/ewsa* MZ *ewsa*/*ewsa*. However unexpectedly in MZ *ewsa/ewsa* vasculature, there is not any evidence of *ctgfa* overexpression (fig. 10A) and was marked by either a loss of signal or possibly a loss of vascular structure. Therefore the changes were measured by counting the number of ISV with the similar degree of signal loss (fig. 10B). Normal ISVs consisted of approximately 100% of the vessel expressing *ctgfa*, ISVs designated as "I" had approximately 75% of the vessel showing signal, "II" had approximately 25% -50%, "III" were less than approximately 25% of the vessel with a signal, and

“IV” were vessels that were not showing any signal. Wild-type embryos had significantly higher normal ISVs on average than the mutants which had a significantly higher number of ISVs of the type IV, or “missing” designation. Immunohistochemistry was performed on *wt/wt* and MZ *ewsa*/*ewsa* embryos also at 4dpf using α-Ctgf antibodies and ISVs were scored in the same manner as above (fig. 11). Similar to the above, *wt/wt* embryos averaged significantly more “normal” Ctgf signal in ISVs than did the mutants and likewise the mutants showed significant abnormal Ctgf expression in the ISV. Although the signal is modulated in MZ *ewsa*/*ewsa* vasculature, it is unclear whether this indicates a reduction in *ctgfa* mRNA and Ctgf protein being expressed in the axial vasculature or if there is an absence of vasculature all together. Regardless, this is evidence that Ewsa is critical for normal vascular endothelial signaling and possibly angiogenesis.

A.



B.

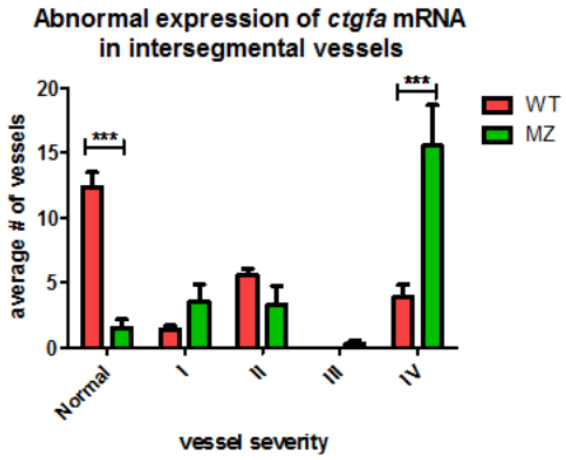


Figure 10. Disruption of *ctgfa* mRNA expression patterning in MZ *ewsa* vasculature evident at 4dpf. In situ hybridization with probes for zebrafish *ctgfa* show expression of *ctgfa* transcripts in vasculature at 4dpf (A). In the Meckel's cartilage (red arrowhead), *ctgfa* expression is upregulated in the mutant embryos. However, MZ mutant *ctgfa* transcription is reduced in the posterior cardinal vein (yellow arrowhead), the intersegmental vessels (white arrow), the caudal artery (yellow arrow), and the caudal vein (white arrowhead). There are a significantly greater number of severely incomplete (IV) ISV in the MZ embryos, by comparison there are a significantly greater average number of normal ISV in the WT embryos (B). Score is average ISV per embryo, WT and MZ n=5 embryos, $p \leq 0.001$.

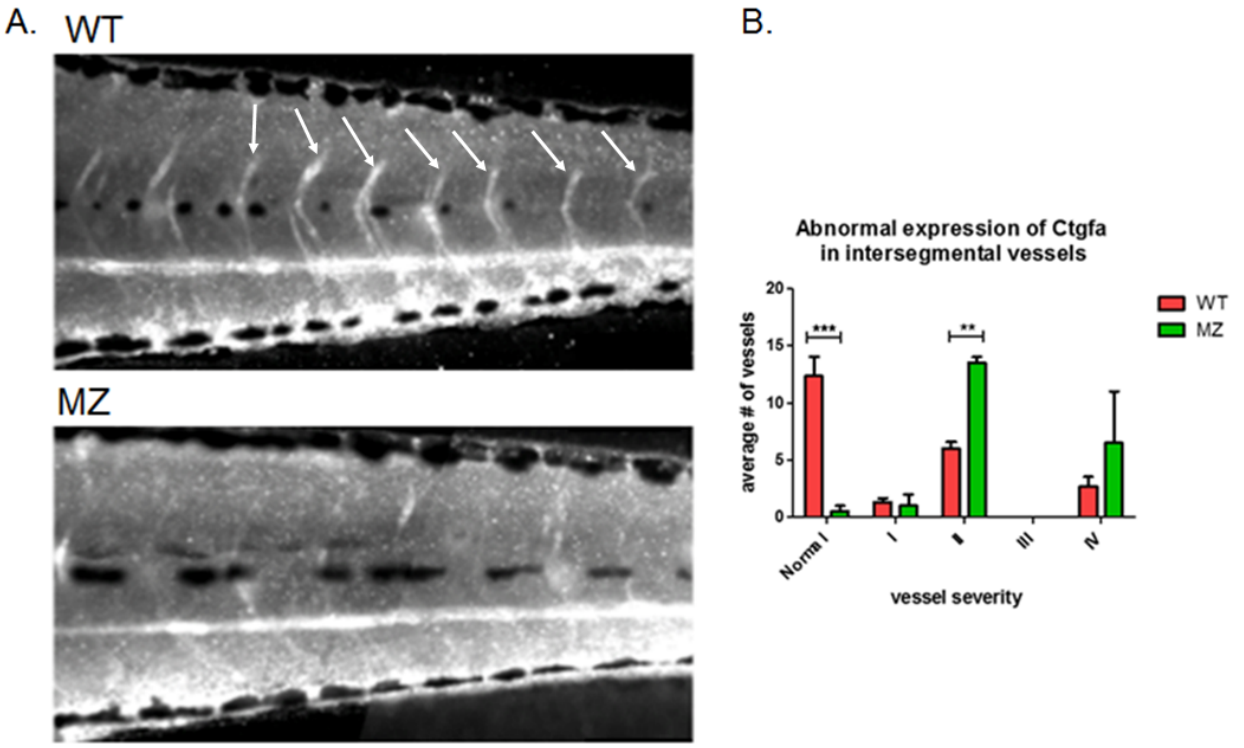


Figure 11. Expression pattern of Ctgf is perturbed in MZ *ewsa* mutant vasculature at 4dpf. Immunohistochemistry of WT and MZ embryos at 4dpf with anti-Ctgf antibodies (A). When compared to WT, MZ ebryos stained with anti-Ctgf showed a significant shift in the expression pattern of Ctgf in intersegmental vessels (B). White arrows show "normal" expression pattern in ISV of wild-type embryos.

Ewsa is essential for normal Sox9 and Ctgf expression in axial vasculature. Whereas ISVs develop by angiogenesis, the primary axial vasculature (dorsal aorta for example) are derived from mesodermal tissue via vasculogenesis [28, 29]. The role of SOX9 in this process is unclear but SOX9 regulation is essential for cartilage vascularization due to its ability to inhibit VEGF [29]. Since EWS is able to bind to SOX9 [10], the loss of EWS could lead to increased bioavailable SOX9, the inhibitor of VEGF and therefore inhibited angiogenesis. To test any modulations of Sox9 expression, immunohistochemistry on *wt/wt* and MZ *ewsa*/*ewsa* embryos at

3dpf and 6dpf was conducted with zebrafish Sox9a-specific antibodies (fig. 12). Each embryo then had the dorsal aorta (DA) and posterior cardinal vein (PCV) scored for continuity starting with “cont” for a contiguous signal throughout the structure and “I” for one or two minor breaks in the continuity, “II” and “III” for increasing amount of breaks or severely large breaks, and “IV” for the structure missing any signal. At 3dpf the loss of *ewsa* yields a disruption in the Sox9a expression pattern of the MZ *ewsa*/*ewsa* embryonic DA in comparison to *wt/wt*, but this pattern disruption is not present in the PCV (fig. 12B). All of the *wt/wt* DA had continuous signal throughout the structure whereas by comparison the MZ *ewsa*/*ewsa* DA at 3dpf had breaks in the structure (Fig. 12B). Yet in mutants at 6dpf (fig. 13) both the DA and the PCV showed more severe breaks in Sox9a signal by comparison to *wt/wt*. This data suggests that Sox9a regulation in vascular development is stage and tissue specific, and is regulated by Ewsa. Likewise, to test if Ewsa modulates Ctgf expression in both the DA and PCV, embryos at 3dpf and 6dpf were subjected to immunohistochemistry with anti-Ctgf antibodies (figs. 12, 13). The Ctgf signal of MZ *ewsa*/*ewsa* embryos at 3dpf was reduced compared to *wt/wt*. This suggests that as a target of Sox9, Ctgf expression might be affected by a Ewsa-Sox9a interaction in these structures. Given that the response is not complete leaves the possibility of another mechanism regulating Ctgf expression. Given that Ctgf expression is nearly missing in *wt/wt* vasculature at 6dpf, and that in MZ *ewsa*/*ewsa* it seems to be maintained, wild type vasculature at 6dpf may be experiencing a

downregulation of Ctgf [30] in this tissue type. With *ewsa* as a regulatory element missing in mutants, the misregulation of Ctgfa could signal a developmental delay in the clearance of Ctgfa, therefore Ewsa may function to negative inhibition of Ctgfa in a tissue specific and temporal manner.

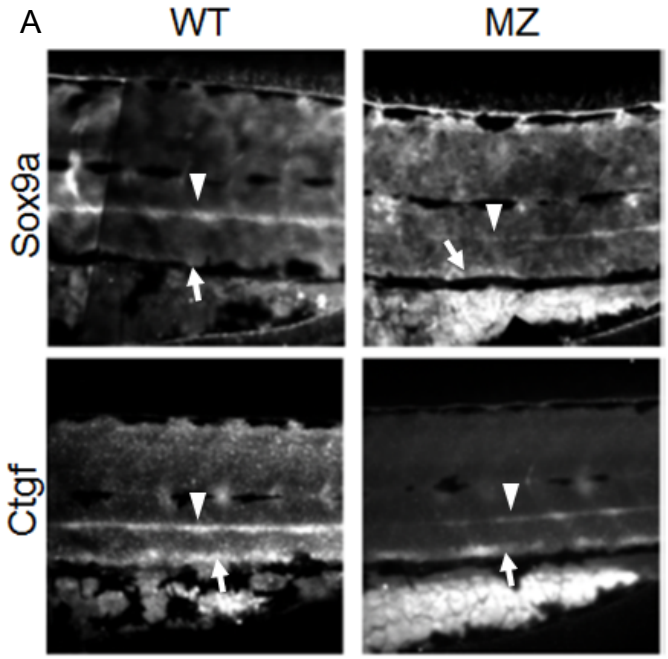
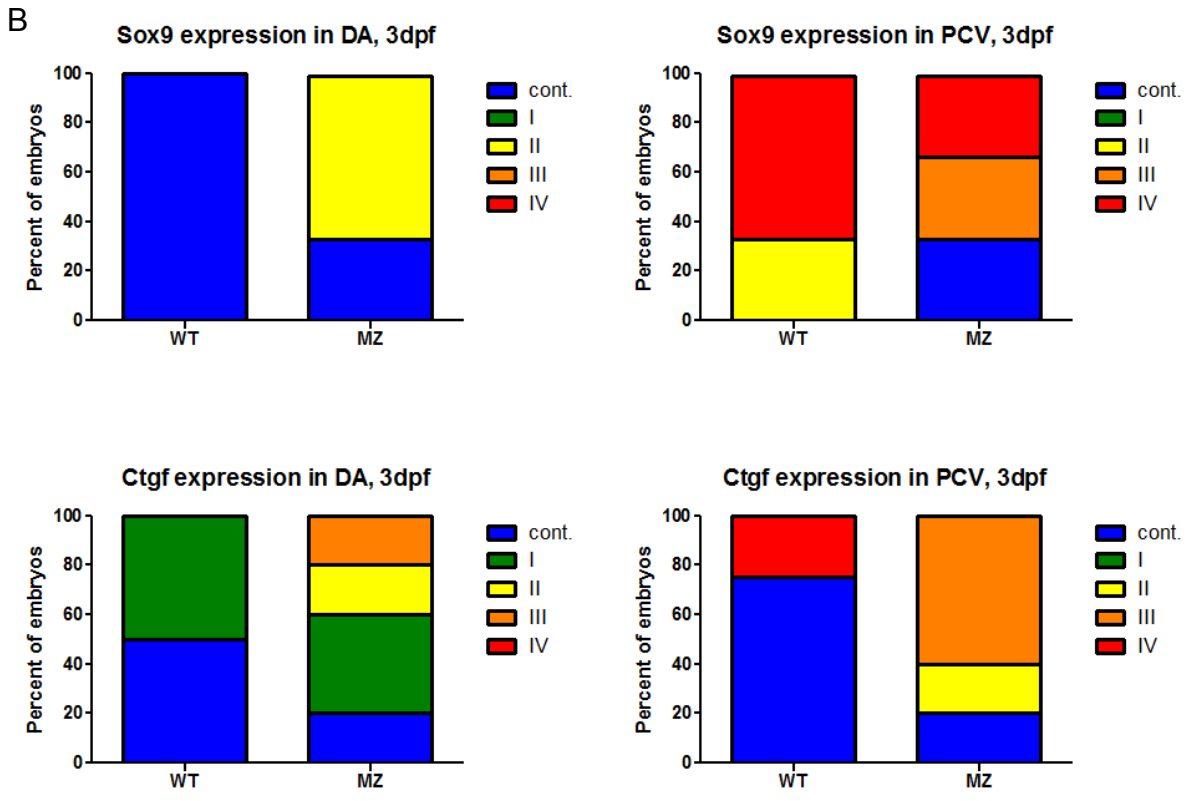


Figure 12. Sox9a and Ctgf expression reduction in MZ *ewsa* mutant vascular endothelial tissue at 3dpf. Immunohistochemistry of WT and MZ embryos at 3dpf with anti-Sox9a and anti-Ctgf antibodies (A) revealed that staining in the dorsal aorta (arrowhead) and posterior cardinal vein (arrow) of the MZ embryo tails contained breaks in signal. The embryos were rated for severity of discontinuity in signal from continuous (cont.) to missing (IV) (B). The loss of *ewsa* resulted in a reduction of endothelial expression of both Sox9 and Ctgf in the DA tissue, and loss of Ctgf in the PCV tissue. The exception to this reduction was Sox9 expression in the PCV which was increased. Sox9 WT and MZ; n=3. Ctgf WT, n=4; MZ, n=5.



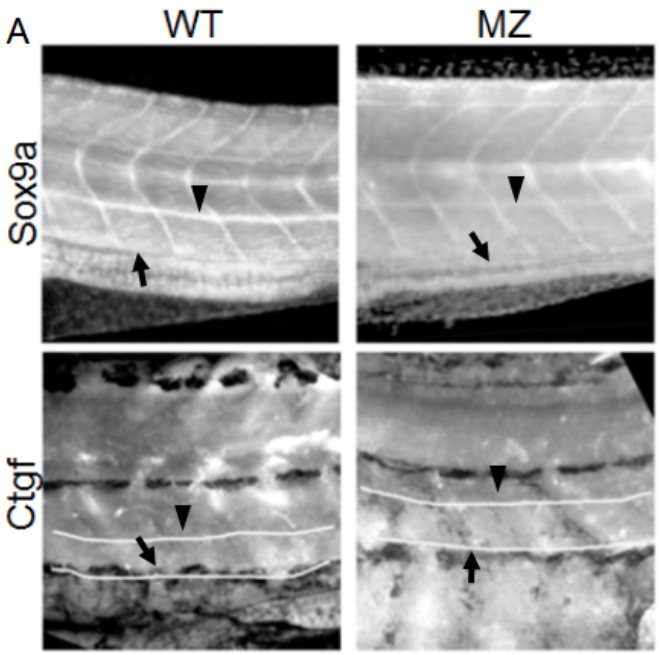
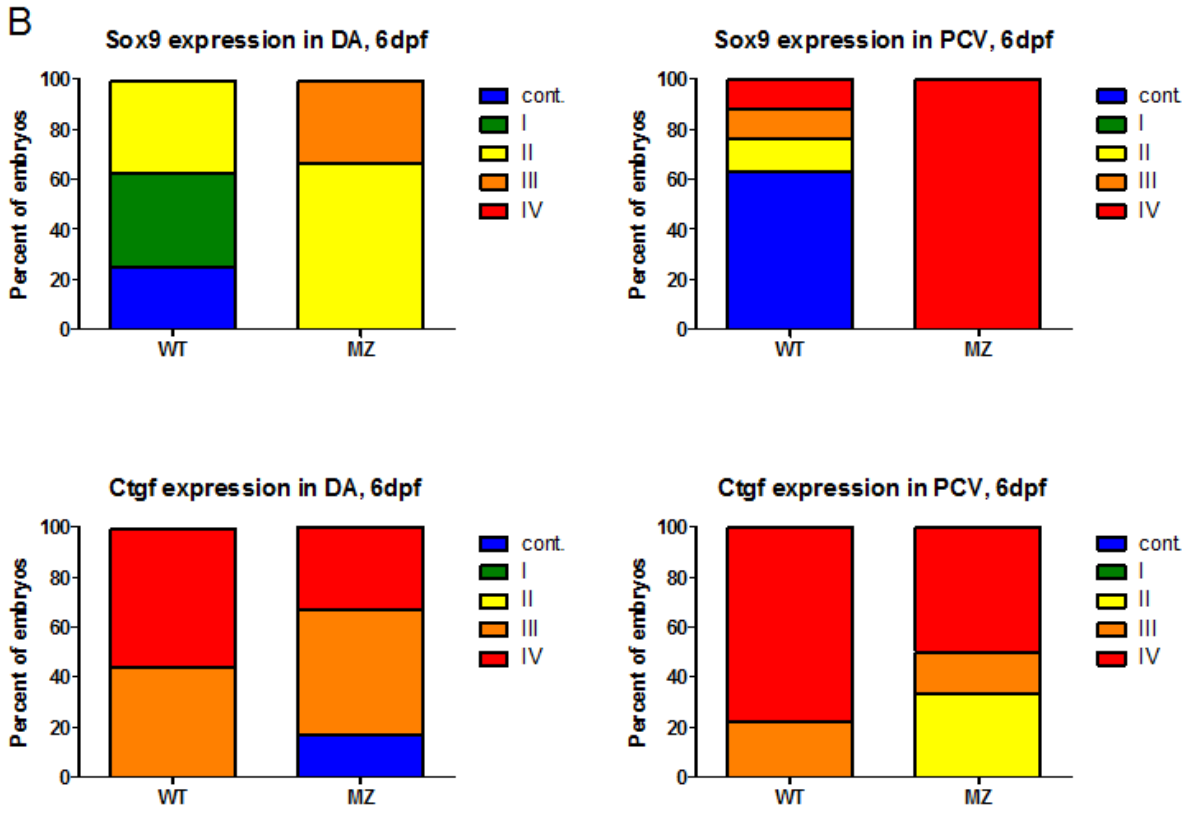


Figure 14. Sox9a expression reduced in MZ ewsa/ewsa mutants at 6dpf. (A), Torso sections of anti-Sox9a or Ctgf stained 6dpf embryos showing the dorsal aorta (arrowhead) and posterior cardinal vein (arrow) in both wild-type and MZ *ewsa* mutant embryos. The embryos were rated for severity of discontinuity of each structure from continuous (cont.) to missing (IV) (B). The Sox9a signal in DAs and PCVs of WT embryos were mostly continuous whereas in the same structures in the MZ embryos the signal was reduced. Ctgf signal was reduced in both WT and MZ structures. Sox9; WT n=8, MZ n=3. Ctgf; WT n=9, MZ n=6.



DISCUSSION

The understanding of the role of EWS in early development has only recently begun to be explored. Previous studies in this lab have confirmed EWS involvement in skeletal development, yet the mechanisms of that role remain undiscovered. To investigate if EWS regulates chondrogenesis by regulating SOX9 transcription we looked at SOX9 protein levels through this process. Since Sox9 levels remained unchanged we next investigated the protein levels of Ctgf, a SOX9 target gene that is involved in chondrocyte maturation. Indeed we have found that both *ctgf* mRNA and Ctgf protein levels are higher in the cartilage of MZ *ewsa/ewsa*. Furthermore, skeletal defects in the MZ *ewsa/ewsa* mutant cartilage was observed. This suggests one way in which EWS regulates the process of endochondrial ossification is by regulating the expression of CTGF.

In a previous report, the transcriptional targets of SOX9 were upregulated at 27hpf in MZ ewsa/ewsa [10], thus presenting the possibility that EWS is in fact regulating SOX9 expression and therefore the targets of SOX9. In this study we have found that, expression levels of Sox9a and Sox9b protein remained constant in the craniofacial cartilage of zebrafish embryos during the chondrocyte differentiation period and that Sox9b does not play a major role in this process. In this report, we discovered an increase in the number of cells expressing one of these transcriptional targets, Ctgf, in the craniofacial cartilage with the loss of *ewsa.* Although in immunohistochemistry experiments Sox9a was localized to the nucleus, and

in data not shown, Ewsa also was seen in the nucleus, both experiments were conducted separately therefore it remains unclear if Ewsa and Sox9 are co-localizing on a specific region on the chromatin. Furthermore immunohistochemistry would not be able to elucidate if co-localization occurred on a regulatory locus of a Sox9 transcriptional target. Given that SOX9 requires dimerization for transcriptional activation [31], and that EWS biochemically binds to SOX9 [10], it is possible that upstream modifications or the presence of other protein factors can act as a switch to allow an EWS-SOX9 dimerization event which inhibits target gene transcriptional activation on the regulatory locus of the target gene. This could explain why there were an increased number of cells expressing Ctgfa in MZ *ewsa/ewsa* rather than an increase in protein levels. The role of Ewsa may be to simply inhibit Ctgf production during a critical period of chondrocyte differentiation and not necessarily to end all normal Sox9-activated transcription of *ctgfa*.

We previously reported deformations in the craniofacial cartilage of MZ *ewsa*/*ewsa* mutant zebrafish[10]. These were described as positional shifts in structures compared to the same structure in both *wt/wt* adult fish and in embryos at 4dpf. The time of the start of these deformation remains unknown. In this report we measured skeletal deformations as dimensional changes in an individual structure at 4dpf and noted the increase in severity by 6dpf (fig. 3). These deformations appear to me separate from the positional shifts described before. Even so, we have discovered that these deformations take place during endochondrial ossification. CTGF is involved

in ECM production and skeletal deformations occur with the overexpression of CTGF [14, 32]. Furthermore CTGF has been reported to be involved in chondrocyte proliferation [33]. Indeed, we have previously shown the craniofacial cartilage of MZ *ewsa/ewsa* mutants which contained a higher number of chondrocytes at 4dpf than *wt/wt* individuals [10]. As mentioned above, MZ *ewsa/ewsa* mutant Ctgf is increased in the craniofacial cartilage, therefore we propose that the misregulation of Ctgf is leading to these deformations through two ways; ECM overproduction inhibiting normal chondrocyte stacking, which then would affect the length of a structure, and the over-proliferation of chondrocytes which then could affect the width of a structure. Studies tracking ECM production in both *wt/wt* and MZ *ewsa/ewsa* would help to clarify this process.

In late stages of bone formation, the proteins expressed to promote chondrocytes mature to the hypertrophic state, must cease and express high levels of VEGF, HIF1 and other factors to induce angiogenesis for vascular invasion into the bone tissue [34]. Since EWS is involved in regulating chondrocyte maturation, EWS may play a role in vascular invasion as well. To elucidate the role of EWS in this process we have turned to the axial vasculature of the zebrafish as a model of vasculogenesis. The development of intersegmental vessels (ISVs) is an angiogenic process arising from preexisting dorsal aortal (DA) cells while the development of the DA, posterior cardinal vein (PCV), caudal vein (CV) and caudal artery (CA) represent vasculogenesis. In the MZ *ewsa/*ewsa mutant fish we discovered

perturbations in both processes suggesting that similarly, the process in bone vascular invasion is also perturbed. This is the first report to suggest that endogenous EWS has a role in normal vascular endothelial expression of Sox9 and Ctgf . Yet since it is still unclear if the loss of *ctgfa* signal in our MZ *ewsa/ewsa* mutants (figs. 16, 17) is due to loss of expression or loss of vascular structures, the immunohistochemistry experiment must be repeated with staining for a known vascular marker. WGA and DAPI staining produce some evidence of tube-like structures where there is a loss of Sox9 or Ctgf signal .Also blood flow can be observed in live MZ *ewsa*/*ewsa* embryos, giving evidence that vascular structure exists, but it is possible that the endothelial cells of the mutant are not expressing Ctgf or Sox9. It is known the CTGF promotes vascular endothelial cell migration and proliferation in vitro, and promotes angiogenesis in vivo [18], but at high doses CTGF is found to inhibit VEGF and inhibit angiogenesis [19]. We have shown in this and our previous report that loss of *ewsa* leads to Ctgf overexpression therefore we propose that this overexpression could be the inhibiting mechanism not only for angiogenesis but also for vasculogenesis as well. Interestingly, the CTGF/VEGF angiogenic inhibitory complex can be rescued by the cleavage of CTGF by matrix metalloproteinase 9 (MMP9)[19] which is antagonized by thrombospondin 2 (TSP2) [35]. Furthermore, it has been shown that EWS/FLI1 inhibits the expression of thrombospondins. Therefore it is plausible that during the human pathology of Ewing's sarcoma, the loss of function of endogenous EWS may lead to CTGF overexpression which in

turn inhibits VEGF, which cannot be rescued due to the inhibition of TSPs by the fusion protein EWS/FLI1. There are a multitude of pathways to regulate both VEGF and CTGF so the ramifications of CTGF overexpression in the human condition remains to be explored.

LITERATURE CITED

1. Jedlicka, P., *Ewing Sarcoma, an enigmatic malignancy of likely progenitor cell origin, driven by transcription factor oncogenic fusions.* Int J Clin Exp Pathol, 2010. **3**(4): p. 338-47.
2. Delattre, O., et al., *Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours.* Nature, 1992. **359**(6391): p. 162-5.
3. Riggi, N. and I. Stamenkovic, *The Biology of Ewing sarcoma.* Cancer Lett, 2007. **254**(1): p. 1-10.
4. Erkizan, H.V., V.N. Uversky, and J.A. Toretsky, *Oncogenic partnerships: EWS-FLI1 protein interactions initiate key pathways of Ewing's sarcoma.* Clin Cancer Res, 2010. **16**(16): p. 4077-83.
5. Embree, L.J., M. Azuma, and D.D. Hickstein, *Ewing sarcoma fusion protein EWSR1/FLI1 interacts with EWSR1 leading to mitotic defects in zebrafish embryos and human cell lines.* Cancer Res, 2009. **69**(10): p. 4363-71.
6. Azuma, M., et al., *Ewing sarcoma protein ewsr1 maintains mitotic integrity and proneural cell survival in the zebrafish embryo.* PLoS One, 2007. **2**(10): p. e979.
7. Li, H., et al., *Ewing sarcoma gene EWS is essential for meiosis and B lymphocyte development.* J Clin Invest, 2007. **117**(5): p. 1314-23.

8. Bird, N.C. and P.M. Mabee, *Developmental morphology of the axial skeleton of the zebrafish, Danio rerio (Ostariophysi: Cyprinidae).* Dev Dyn, 2003. **228**(3): p. 337-57.

9. Eames, B.F., et al., *Mutations in fam20b and xylt1 reveal that cartilage matrix controls timing of endochondral ossification by inhibiting chondrocyte maturation.* PLoS Genet, 2011. **7**(8): p. e1002246.

10. Merkes, C., et al., *Ewing sarcoma ewsa protein regulates chondrogenesis of Meckel's cartilage through modulation of Sox9 in zebrafish.* PLoS One, 2015. **10**(1): p. e0116627.

11. Wehrli, B.M., et al., *Sox9, a master regulator of chondrogenesis, distinguishes mesenchymal chondrosarcoma from other small blue round cell tumors.* Hum Pathol, 2003. **34**(3): p. 263-9.

12. Akiyama, H., et al., *The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6.* Genes Dev, 2002. **16**(21): p. 2813-28.

13. Hall-Glenn, F. and K.M. Lyons, *Roles for CCN2 in normal physiological processes.* Cell Mol Life Sci, 2011. **68**(19): p. 3209-17.

14. Bateman, J.F., R.P. Boot-Handford, and S.R. Lamande, *Genetic diseases of connective tissues: cellular and extracellular effects of ECM mutations.* Nat Rev Genet, 2009. **10**(3): p. 173-83.

15. Tomita, N., et al., *Cartilage-specific over-expression of CCN family member 2/connective tissue growth factor (CCN2/CTGF) stimulates insulin-like growth factor expression and bone growth.* PLoS One, 2013. **8**(3): p. e59226.

16. Takigawa, M., et al., *Role of CTGF/HCS24/ecogenin in skeletal growth control.* J Cell Physiol, 2003. **194**(3): p. 256-66.

17. Ivkovic, S., et al., *Connective tissue growth factor coordinates chondrogenesis and angiogenesis during skeletal development.* Development, 2003. **130**(12): p. 2779-91.

18. Shimo, T., et al., *Connective tissue growth factor induces the proliferation, migration, and tube formation of vascular endothelial cells in vitro, and angiogenesis in vivo.* J Biochem, 1999. **126**(1): p. 137-45.

19. Inoki, I., et al., *Connective tissue growth factor binds vascular endothelial growth factor (VEGF) and inhibits VEGF-induced angiogenesis.* FASEB J, 2002. **16**(2): p. 219-21.

20. Kimmel, C.B., et al., *Stages of embryonic development of the zebrafish.* Dev Dyn, 1995. **203**(3): p. 253-310.

21. Azuma, M., et al., *Perturbation of rRNA synthesis in the bap28 mutation leads to apoptosis mediated by p53 in the zebrafish central nervous system.* J Biol Chem, 2006. **281**(19): p. 13309-16.

22. Yan, Y.L., et al., *A pair of Sox: distinct and overlapping functions of zebrafish sox9 co-orthologs in craniofacial and pectoral fin development.* Development, 2005. **132**(5): p. 1069-83.

23. Greenhill, E.R., et al., *An iterative genetic and dynamical modelling approach identifies novel features of the gene regulatory network underlying melanocyte development.* PLoS Genet, 2011. **7**(9): p. e1002265.

24. Eames, B.F., et al., *FishFace: interactive atlas of zebrafish craniofacial development at cellular resolution.* BMC Dev Biol, 2013. **13**: p. 23.

25. Pendurthi, U.R., et al., *Proteolysis of CCN1 by plasmin: functional implications.* Cancer Res, 2005. **65**(21): p. 9705-11.

26. Chaqour, B., *Molecular control of vascular development by the matricellular proteins () and ().* Trends Dev Biol, 2013. **7**: p. 59-72.

27. Fernando, C.A., et al., *Temporal and spatial expression of CCN genes in zebrafish.* Dev Dyn, 2010. **239**(6): p. 1755-67.

28. Gore, A.V., et al., *Vascular development in the zebrafish.* Cold Spring Harb Perspect Med, 2012. **2**(5): p. a006684.

29. Hattori, T., et al., *SOX9 is a major negative regulator of cartilage vascularization, bone marrow formation and endochondral ossification.* Development, 2010. **137**(6): p. 901-11.

30. Chiou, M.J., et al., *The physiological role of CTGF/CCN2 in zebrafish notochond development and biological analysis of the proximal promoter region.* Biochem Biophys Res Commun, 2006. **349**(2): p. 750-8.

31. Coustry, F., et al., *The dimerization domain of SOX9 is required for transcription activation of a chondrocyte-specific chromatin DNA template.* Nucleic Acids Res, 2010. **38**(18): p. 6018-28.

32. Hall-Glenn, F., et al., *CCN2/CTGF is required for matrix organization and to protect growth plate chondrocytes from cellular stress.* J Cell Commun Signal, 2013. **7**(3): p. 219-30.

33. Nakanishi, T., et al., *Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture.* Endocrinology, 2000. **141**(1): p. 264-73.

34. Gerber, H.P., et al., *VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation.* Nat Med, 1999. **5**(6): p. 623-8.

35. Potikyan, G., et al., *EWS/FLI1 regulates tumor angiogenesis in Ewing's sarcoma via suppression of thrombospondins.* Cancer Res, 2007. **67**(14): p. 6675-84.

Supplemental Figures

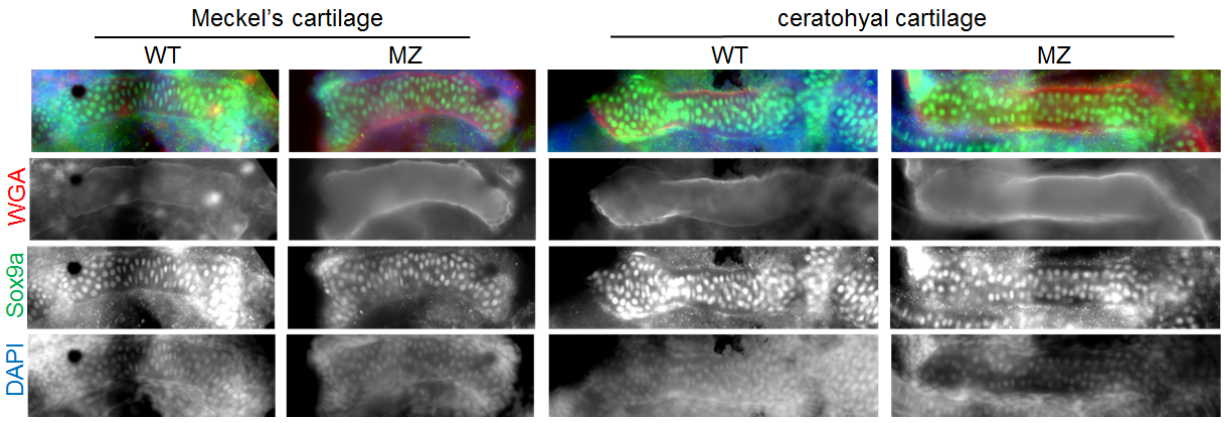


Figure 15. Sox9a is expressed in 4dpf embryo facial cartilage.
Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage and ceratohyal cartilage at 4dpf with Sox9a showing no change in Sox9a expression between WT and MT facial cartilage. α-Sox9a dilution 1:500.

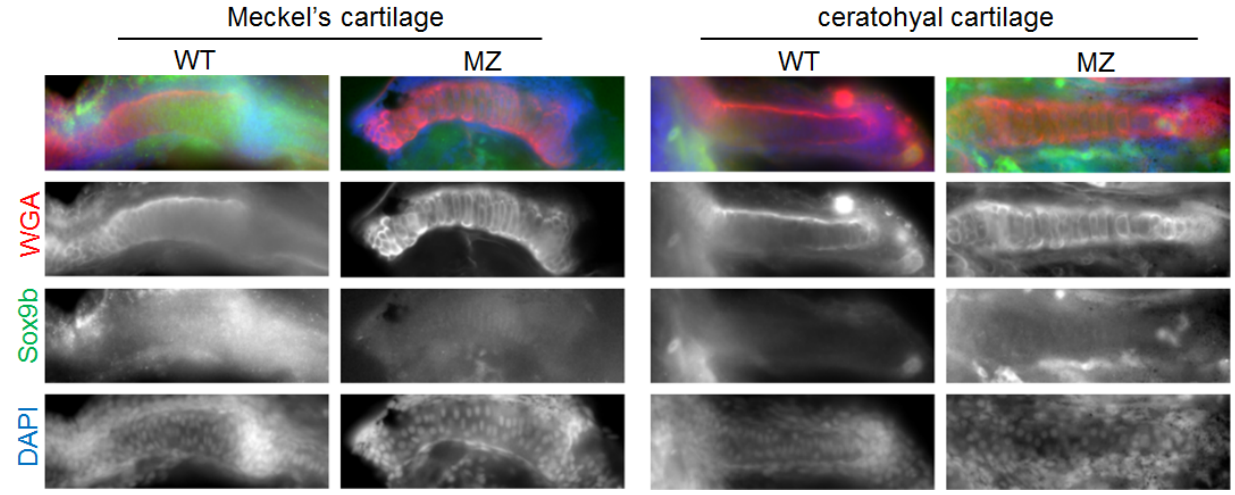


Figure 16. Sox9b not expressed in wild-type or mutant embryo facial cartilage. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage and ceratohyal cartilage at 4dpf with anti-Sox9b antibodies show that there is no change in the lack of Sox9b expression between WT and MT. Antibody dilution 1:500.

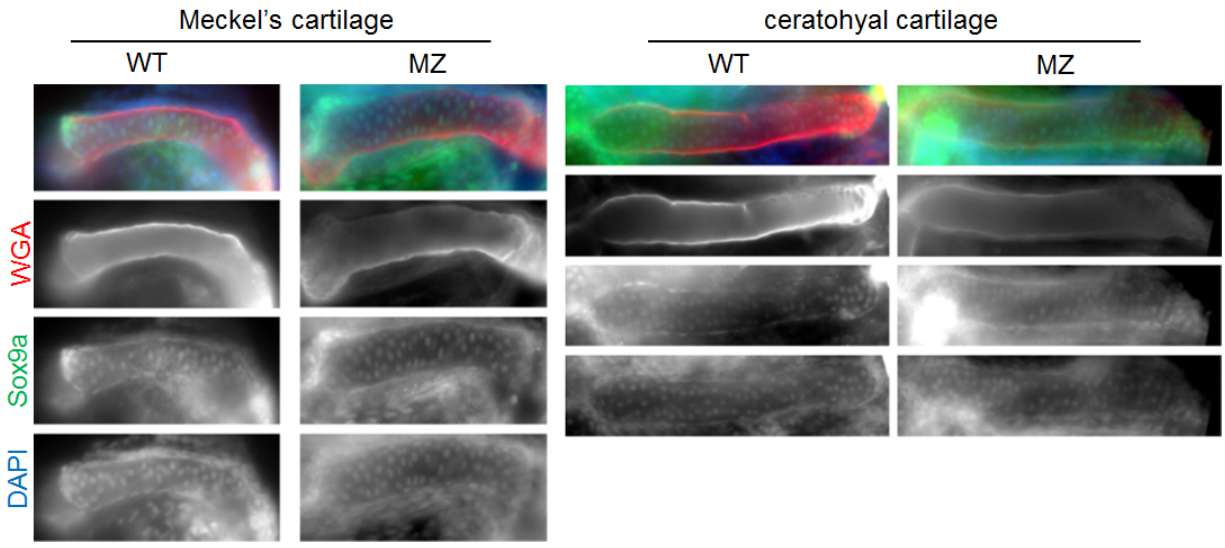


Figure 17. Sox9a is expressed in 5dpf embryo facial cartilage. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage and ceratohyal cartilage at 5dpf with Sox9a showing no change in Sox9a expression between WT and MT facial cartilage. α-Sox9a dilution 1:500

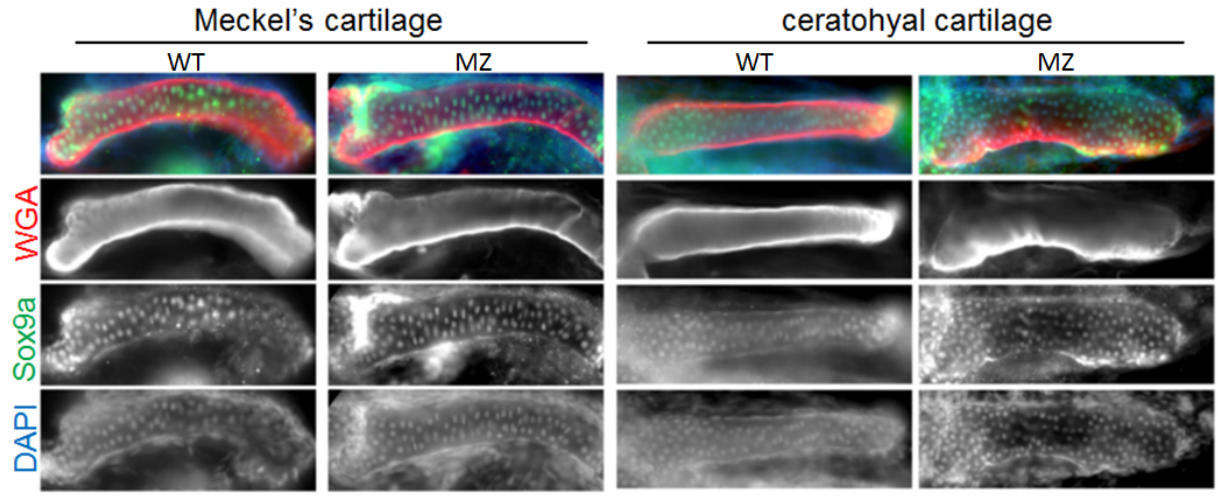


Figure 18. Sox9a expressed in 6dpf facial cartilage. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage and ceratohyal cartilage at 6dpf with Sox9a (A.) and Sox9b (B.) show that there is no change in Sox9a expression between WT and MT. Sox9b lacks expression in both WT and MT Meckel's cartilage, ceratohyal cartilage (B), hyomandibular cartilage, and palatoquadrate cartilage (C). α-Sox9a and α-Sox9b dilutions 1:500.

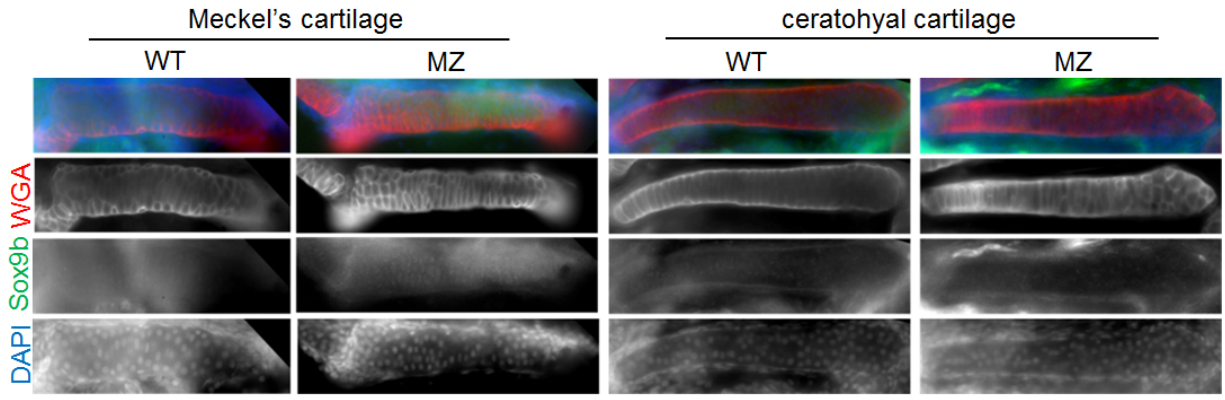


Figure 19. Sox9b lacks expression in 6dpf facial cartilage. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) mutant Meckel's cartilage and ceratohyal cartilage at 6dpf with antibodies against Sox9b show that there is no change in expression between WT and MT. Sox9b lacks expression in both WT and MT Meckel's cartilage and ceratohyal cartilage. α-Sox9b dilutions 1:500.

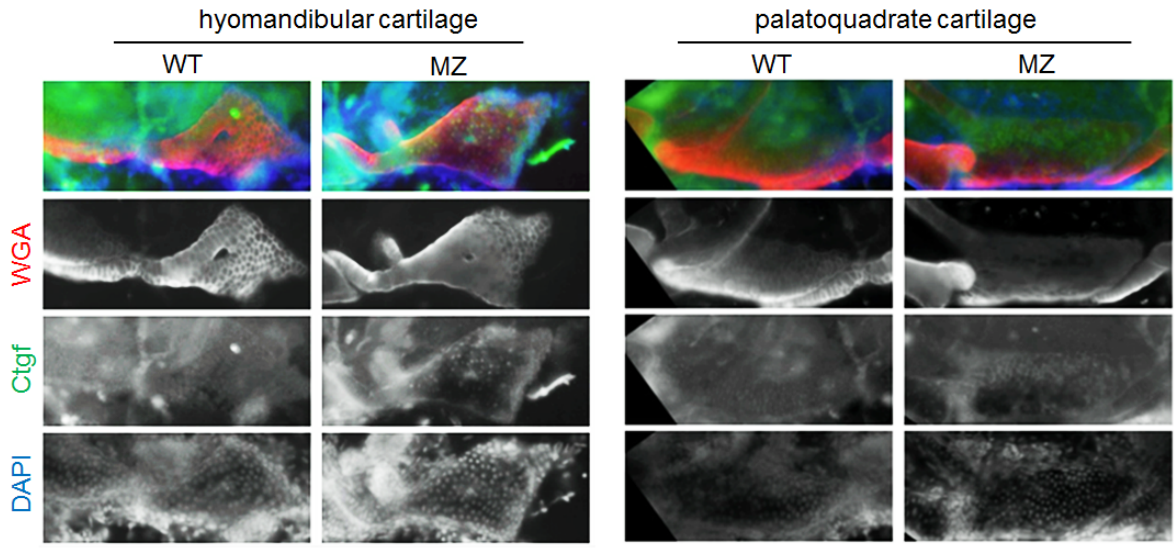


Figure 20. Ctgf is overexpressed in MZ *ewsa* mutant facial cartilage at 4dpf. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) at 4dpf show a significantly increased number of chondrocytes expressing Ctgf as seen in the hyomandibular and palatoquadrate cartilages hm, hyomandibular cartilage; pq, palatoquadrate cartilage. Antibody dilution 1:1000.

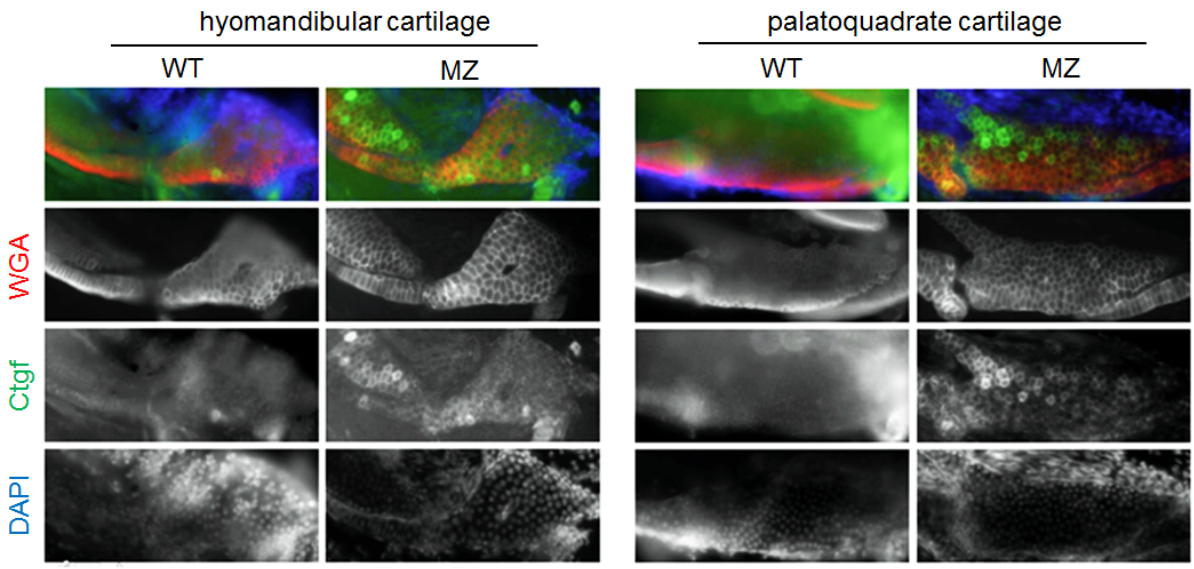


Figure 21. Ctgf is overexpressed in MZ *ewsa* mutant facial cartilage at 6dpf. Immunohistochemistry of wild-type (WT) and maternal zygotic (MZ) at 6dpf show continued increase in the number of chondrocytes expressing Ctgf as seen in the hyomandibular and palatoquadrate cartilages. hm, hyomandibular cartilage; pq, palatoquadrate cartilage. Antibody dilution 1:1000.