

Hdac4 mediates perichondral ossification and pharyngeal skeleton development in the zebrafish (#29625)

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Hdac4 mediates perichondral ossification and pharyngeal skeleton development in the zebrafish

April DeLaurier ^{Corresp.}, Cynthia Lizzet Alvarez ¹, Kali J Wiggins ¹

¹ Department of Biology and Geology, University of South Carolina - Aiken, Aiken, South Carolina, United States

Corresponding Author: April DeLaurier
Email address: april@usca.edu

Background. Histone deacetylases (Hdacs) are epigenetic factors that function to repress gene transcription by removing acetyl groups from the N-terminal of histone lysines. Histone deacetylase 4 (HDAC4), a class IIa HDAC, has previously been shown to regulate the process of endochondral ossification in mice via repression of Myocyte enhancing factor 2c (MEF2C), a transcriptional activator of Runx2, which in turn promotes chondrocyte maturation and production of bone by osteoblasts.

Methods & Materials. In this study we generated two zebrafish lines with mutations in *hdac4* using CRISPR/Cas9 and analyzed mutants for skeletal phenotypes and expression of genes known to be affected by Hdac4/Mef2c interactions.



Results: Lines have insertions causing a frameshift in a proximal exon of *hdac4* and a premature stop codon. Mutations are predicted to result in aberrant protein sequence and a truncated protein, eliminating the Mef2c binding domain and Hdac domain. Zygotic mutants show a mild to moderate increase in ossification of pharyngeal ceratohyal and to some extent hyosymplectic cartilages at 7 days post fertilization (dpf). **mRNA in situ hybridization** at 72 hpf shows that *hdac4* is normally expressed in regions of the wild-type ceratohyal, hyosymplectic, and posterior pharyngeal arch cartilages, indicating that loss of function may lead to premature or excessive ossification at these sites. At 4 dpf, mutant larvae have an increase of expression of *runx2a* and *sp7* in the ceratohyal cartilage, which we hypothesize is due to the de-repression of Mef2c through loss of Hdac4. Expression of *runx2b* was unchanged in mutants compared to wild types. A subset of maternal-zygotic mutant and heterozygote larvae show a dramatically increased level of ossification at 7 dpf compared to zygotic mutants, including formation of a precocious anguloarticular bone and mineralization of the ceratobranchial and symplectic cartilages, which normally does not occur until fish are approximately 12 dpf. Some maternal-zygotic mutants and heterozygotes also **show loss of pharyngeal first arch elements, suggesting loss of Hdac4 may affect neural crest development.**

Discussion. The results of this study demonstrate a role for Hdac4 in zebrafish cartilage ossification consistent with the function of this protein in mice, indicating that the function of Hdac4 in skeletal development is conserved among vertebrates. Furthermore, we have identified a potentially novel role for maternal Hdac4 in zebrafish cartilage ossification and neural crest development.

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April DeLaurier¹, Cynthia Lizzet Alvarez¹, Kali J Wiggins¹

¹ Department of Biology and Geology, University of South Carolina Aiken, Aiken, South Carolina, USA

Corresponding Author:

April DeLaurier¹

Dept. of Biology and Geology, University of South Carolina Aiken, 471 University Parkway, Aiken, South Carolina, 29801, USA

Email address: aprild@usca.edu

Abstract

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of a precocious anguloarticular bone and mineralization of the ceratobranchial and symplectic cartilages, which normally does not occur until fish are approximately 12 dpf. Some maternal-zygotic mutants and heterozygotes also show loss of pharyngeal first arch elements and neurocranium defects, suggesting loss of Hdac4 may affect neural crest development.

Discussion. The results of this study demonstrate a role for Hdac4 in zebrafish cartilage ossification consistent with the function of this protein in mice, indicating that the function of Hdac4 in skeletal development is conserved among vertebrates. Furthermore, we have identified a potential role for maternal Hdac4 in zebrafish cartilage ossification and neural crest development.

Introduction

The majority of the vertebrate skeleton including the axial, limb, and pharyngeal elements form as cartilaginous elements that grow rapidly during early development through the proliferation of matrix-secreting chondrocytes. At specific stages of development, chondrocytes cease their rapid proliferation and matrix secretion, become hypertrophic, and signal to nearby cells to commence endochondral ossification (Karsenty & Wagner, 2002). In zebrafish, ossification involves the recruitment of osteoblasts to the surface of cartilage to secrete a perichondral collar of mineralized bone. In amniotes, endochondral ossification involves both perichondral ossification and the invasion of the cartilage by blood vessels to deliver osteoblasts which deposit bone (Hall, 2014). In all vertebrates, the timing of the transition between endochondral growth and ossification is important in the determining size and shape of skeletal elements.

This process is precisely regulated by expression of factors in chondrocytes and osteoblasts including *Ihh*, *Pthrp*, *Runx2* (*Cbfa1*), and *Sp7* (*osterix*) (Vortkamp et al., 1996; Komori et al., 1997; Nakashima et al., 2002; Maeda et al., 2007). Loss of function of these factors can cause ~~insufficient ossification~~, resulting in severe growth and patterning defects of the skeleton (Quack et al., 1999; Wysolmerski et al., 2001; Gao et al., 2001; Valadares et al., 2014).

Histone deacetylase 4 (HDAC4), a member of the class IIa group of HDACs (including HDAC 4, 5, 7, and 9), has previously been demonstrated to be an important regulator of chondrocyte maturation and initiation of endochondral ossification in mice (Vega et al., 2004). HDACs lack the ability to bind to DNA directly, but associate with other proteins to remove acetyl groups from the N-terminal of histone lysines, causing histones to condense, blocking access of transcription factors to DNA, resulting in transcriptional repression (Haberland, Montgomery & Olson, 2009). Class IIa HDACs are characterized by a carboxyl-terminal binding protein domain (CtBP), a MEF2 binding domain for binding the transcription factor myocyte ~~enhancing~~ factor 2c (MEF2C), sites for binding of the chaperone protein 14-3-3, and an HDAC domain (Haberland, Montgomery & Olson, 2009). MEF2C is a transcription factor which controls chondrocyte hypertrophy and bone formation by activating ~~transcription of~~ target genes such as *Runx2* (Arnold et al., 2007). When class IIa HDACs are unphosphorylated, they localize to the nucleus where they bind to MEF2C, and function to repress transcription of MEF2C-target genes (Lu et al., 2000; Passier et al., 2000; McKinsey et al., 2000; Arnold et al., 2007). When calcium/calmodulin protein kinase (CaMKII) and protein kinase D (PKD) phosphorylate 14-3-3 and shuttle the HDAC into the cytoplasm, MEF2C

becomes unbound to HDAC, and can activate transcription of target genes (Lu et al., 2000; Passier et al., 2000; McKinsey et al., 2000). Through this process of interaction with MEF2C, HDAC4 delays the hypertrophy of chondrocytes within cartilage, controlling the timing and extent of ossification of endochondral bone by osteoblasts (Vega et al., 2004).

Zebrafish represent a useful model for studying mechanisms of chondrocyte maturation and the initiation of the cartilage ossification process as they develop cartilaginous elements as early as 60-72 hours post-fertilization and commence perichondral ossification of these elements as early as 96 hpf (Eames et al., 2013). Compared with other vertebrates, zebrafish undergo the same cellular and genetic signaling pathways associated with skeletal ossification including chondrocyte hypertrophy, differentiation and matrix secretion by osteoblasts, including expression of factors associated with ossification such as *ihha*, *runx2a*, *runx2b*, *sp7*, *col1a2*, *col10a1*, and *osteonectin* (Flores et al., 2004; Avaron et al., 2006; Li et al., 2009). These similarities make zebrafish a useful model to study genetic pathways associated with skeletal development and disease.

In this study, we describe two zebrafish lines with early frameshift mutations in *hdac4*. Mutant larvae from heterozygote in-crosses show a mild to moderate increase in ossification of certain pharyngeal cartilage elements, and an up-regulation of markers of ossification of the skeleton including *runx2a* and *sp7*. A further enhancement of the excessive ossification defect is observed in maternal-zygotic mutants, indicating an early maternal contribution to regulation of skeletal patterning in zebrafish. Previously, we identified a potential role for Hdac4 in neural crest development and neurocranium

formation in zebrafish (DeLaurier et al., 2012). Although this phenotype was not reproduced in any zygotic mutants, a profound loss of anterior facial structures was observed in a subset of ~~maternal-zygotic mutants~~, indicating a function for maternal Hdac4 in neural crest development or formation of other anterior structures of the head or face. In conclusion, *hdac4* mutants reproduce aspects of the mouse HDAC4 mutant and may be a useful model, especially along with other reverse-genetic mutants for other class IIa Hdacs, to study the function of these factors in skeletal patterning and other developmental pathways.

Methods

Zebrafish husbandry

AB strain wild-type (WT) zebrafish were originally obtained from the Zebrafish International Resource Center (ZIRC, Eugene, OR). Fish were reared and maintained at 28.5°C on a 14 hour on/10 hour off light cycle. Fish were fed as previously described (Wasden, Roberts & DeLaurier, 2017). Maintenance and use of zebrafish followed guidelines from ZIRC, the Zebrafish book (Westerfield, 2007), and the Institutional Animal Care and Use Committee (IACUC) of the University of South Carolina Aiken (approval number 010317-BIO-01).

Generation of CRISPR lines

The CRISPR/Cas9 procedure was based on previously described methodologies (Hwang et al., 2013). CHOPCHOP (<http://chopchop.cbu.uib.no/>) was used to design a

guide RNA (gRNA) sequence targeting exon 5 of *hdac4* (ensembl
 ENSDART00000165238.3), 5' of the Mef2c binding site (located in exon 6) and histone
 deacetylase domain, with minimal potential off-target binding (Montague et al., 2014;
 Labun et al., 2016). An *hdac4*-specific oligonucleotide was designed containing a 20 bp
 T7 promoter sequence, 20bp of target sequence (GGAGCGTCATCGACAGGAGC),
 followed by a 20 bp scaffold overlap sequence as described (Bassett et al., 2013). This
 oligonucleotide was annealed to a scaffold oligonucleotide containing the tracrRNA
 stem loop sequence using Phusion PCR (New England Biolabs, Ipswich, MA) to
 produce a 120 bp template. Template DNA was column purified (DNA Clean &
 Concentrator kit, Zymo Research, Irvine, CA) and was used to synthesize RNA using
 T7 polymerase (MAXIscript T7 Transcription kit, Thermo Fisher, Vitnus, Lithuania).
 Cas9 mRNA was synthesized from pCS2-nCas9n (Addgene, Cambridge, MA). Plasmid
 was linearized with *NotI*-*HF* (New England Biolabs, Ipswich, MA), column purified
 (Zyppy Plasmid Miniprep kit, Zymo Research, Irvine, CA), and mRNA was synthesized
 (mMessenger mMachine SP6 kit, Thermo Fisher, Vitnus, Lithuania). Column-purified
hdac4 gRNA and nCas9n mRNA (RNA Clean & Concentrator kit, Zymo Research,
 Irvine, CA) were co-injected into one cell-stage embryos. Each embryo was injected
 with approximately 3nl of a 5ul mix containing *hdac4* gRNA (~60ng/microliter), nCas9n
 mRNA (~160ng/microliter), and phenol red as a marker. Unfertilized or dead embryos
 were removed from the dish at the end of the first day of injection and on subsequent
 days.

Identification of founders and generation of mutant lines

At 36 hpf, approximately 20% of injected embryos (abnormal and normal-looking) were pooled into groups of 5 fish per tube, lysed using HotShot (Truett et al., 2000), and PCR was performed using genomic primers flanking the site of potential mutation. PCR products were gel-purified and digested using T7 endonuclease (New England Biolabs, Ipswich, MA) to identify mismatched DNA indicating potential founder lines (Hwang et al., 2013). Siblings of fish (approximately 40 fish) with positive T7 results were reared to adulthood and used as founder (F_0) lines for subsequent experiments. Three F_0 fish demonstrated germ line transmission to offspring, and F_1 lines were generated from these founders by out-crossing founders to AB wild types. Adult F_1 fish were identified as heterozygous carriers of potential mutations using PCR and T7 endonuclease digest on fin clip DNA. Heterozygous F_1 fish were outcrossed to generate F_2 lines, and F_2 lines were in-crossed to produce homozygous mutants. PCR products from potential mutants and wild type siblings were sequenced (Eurofins, Louisville, KY) and genomic sequences were compared to wild-type siblings to identify mutations (Geneious, version 8, <http://www.geneious.com>) (Kearse et al., 2012). Mutations were confirmed by RT-PCR of cDNA (RevertAid First Strand cDNA Synthesis kit, Thermo Fisher, Vitnus, Lithuania) using an exon 3 forward primer 5'-gccactggaacttctcaagc-3' and an exon 6 reverse primer 5'-gcagtgggtgagactcctct-3' ($T_m = 58^\circ\text{C}$ x40 cycles or Touchdown PCR, $T_m = 72-65^\circ\text{C}$ x15 cycles followed by $T_m = 64.5^\circ\text{C}$ x20 cycles). RT-PCR products were column purified as described above and sequenced to confirm the mutation. Heterozygous F_2 and F_3 carriers of mutant alleles were in-crossed to produce wild type, heterozygote, and mutant offspring. Maternal-zygotic mutants were generated by crossing a homozygote mutant female with a heterozygote male.

Genotyping adults and larvae

Fin clips from adult fish and fin clips or whole larvae used for histological stains were genotyped using Hotshot lysis as described above. An 822bp region of exon 5 spanning the site of mutation in *hdac4* was PCR-amplified using an intron 4-5 forward primer 5'-atgttctccctgtgttggtg-3' and an intron 5-6 reverse primer 5'-gctgtatttccgctcatgtg-3' (Tm = 58°C, x40 cycles). PCR products were run on a 2% agarose gel at 60V for 5-6 hours to produce band separation sufficient to distinguish heterozygote (2 bands) fish from wild type and mutant fish (both 1 lower and upper band, respectively).

Histological stains and mRNA in situ hybridization

Alcian Blue and Alizarin Red staining to label cartilage and bone was performed as described (Walker & Kimmel, 2007). Double fluorescent mRNA *in situ* hybridization was performed as described (Talbot, Johnson & Kimmel, 2010), using probes for *hdac4*, *runx2a*, *runx2b*, *sp7*, and *sox9a* (DeLaurier et al., 2010; Huycke, Eames & Kimmel, 2012). For each mRNA *in situ* hybridization experiment, 5-10 individual wild type and mutant larvae were imaged and analyzed. Representative examples of expression patterns are shown in this paper.

Imaging and image analysis and statistics

Alcian Blue and Alizarin Red stained specimens were dissected and flat mounted on microscope slides and imaged on a compound microscope. The right pharyngeal skeleton was flat mounted for each specimen unless it was damaged or defective, in

which case the left side was used. The areas of flat mounted ceratohyal and hyomandibula cartilages were measured using pixel area of cartilage and bone using ImageJ (NIH). The area of bone as a ratio of cartilage area was used as a measure of ossification in statistical analysis. In total, 93 fish were measured for the *aik2* line (wild type = 38, heterozygote = 23, mutant = 32) and 61 fish were measured for the *aik3* line (wild type = 17, heterozygote = 28, mutant = 16). Statistical analyses were performed in SAS 9.4 (SAS Institute, Cary, NC). Fluorescent confocal microscopy was performed using a Leica SPEII confocal microscope (Leica Microsystems, Buffalo Grove, IL).

Results

Hdac4 mutants have a frameshift lesion

Using CRISPR/Cas9 to target exon 5 of *hdac4* (Fig. 1A), we induced frameshift mutations in two individual fish that were used to generate mutant lines. Line *hdac4^{aik2/aik2}* has a 19 bp insertion three bases upstream of the protospacer adjacent motif (PAM) site associated with Cas9 binding and cleaving of DNA. Line *hdac4^{aik3/aik3}* has a 2 bp insertion, followed by retention of 7 bp of the wild-type sequence, followed by a 27 bp insertion one base pair upstream of the PAM site (Fig. 1B). In both cases, frameshifts were induced by insertion of nucleotides into exon 5, resulting in aberrant amino acids being added to the protein sequence (Fig. 1C). In both mutant lines the frameshift is predicted to cause the loss of the Mef2c binding domain and premature stop codons resulting in truncated proteins 174 aa (*hdac4^{aik2/aik2}*) and 181 aa (*hdac4^{aik3/aik3}*) in length (Fig. 1D). Frameshifts were detected in mutant cDNA compared

to wild type cDNA using primers spanning exons 3-6, and there was no evidence of splice variants or exon skipping detected on agarose gels for either mutant (Figs. 1E and F). In the case of *hdac4^{aik3/aik3}*, a larger band was detected along with the band of expected size (Fig. 1F, indicated by asterisk). This band was excised and sequenced and was found to have an identical sequence to the band of the expected size. We interpret that this larger band is the product of heterodimers of our PCR product or a slower running single-stranded DNA product and not a splice variant or other genomic feature within the mutant. In both the *hdac4^{aik2/aik2}* and *hdac4^{aik3/aik3}* lines, adult fish and embryos were genotyped using intronic primers spanning exon 5. The larger mutant band (841 bp *hdac4^{aik2/aik2}*, 858 bp *hdac4^{aik3/aik3}*) can be distinguished from the wild type band (822bp), and heterozygotes show both bands (Figs. 1G and H). At 7dpf and earlier, mutant embryonic and larval fish have no discernable abnormalities compared to wild type siblings (Figs. 1I and J). Mutant fish form swim bladders, feed, and grow normally into adult fish. Mutant fish from the *hdac4^{aik2/aik2}* line also showed no overt abnormalities compared to wild type siblings (data not shown).

Mutants have increased ossification of pharyngeal cartilage

Heterozygous in-crosses were used to generate wild type, heterozygous, and mutant larvae for skeletal preparations and analysis. At 7 dpf, mutants from the *aik2* and *aik3* lines showed a greater extent of ossification of the ceratohyal cartilage compared to wild-type siblings (Fig. 2B-F, *aik2* only shown in D-F, *aik3* not shown). Wild type fish typically had a small area of bone stain localized to the mid-shaft of the ceratohyal, usually appearing first at the dorsal margin of the cartilage and spreading ventrally (Fig.

2D and D'). Among mutants, excess bone was observed at the mid-shaft of the ceratohyal, either as a mild (Fig. 2E and E') or moderate (Fig. 2F and F') increase compared to wild types. In mutants, the extent of bone growth appeared to have irregular boundaries compared to the wild type pattern (Fig. 2E indicated by arrow in E') and a greater extent of formation of a perichondral bone collar around the shaft of the ceratohyal (Figs. 2E', F', indicated by asterisks). No other defects were detected in the pharyngeal skeleton in mutants analyzed at this stage.

For both the ceratohyal and hyosymplectic, the area of bone and the area of cartilage were measured, and the amount of ossification was calculated as the ratio of bone to cartilage present (Figs. 2G-J). For the *aik2* line, ANCOVA revealed that the effect of genotype on the area of ossification of the ceratohyal was significant ($F=4.01$; $df=2,89$; $p=0.0215$). Tukey's multiple comparisons showed that mutants had significantly more bone than wild types ($p=0.0057$), but heterozygotes were not significantly different from either mutants ($p=0.1751$) or wild types ($p=0.2488$) (Fig. 2G). For the *aik3* line, ANOVA revealed that the effect of genotype on the area of ossification of the ceratohyal was also significant ($F=7.77$; $df=2,58$; $p=0.001$). Tukey's multiple comparisons showed that mutants had significantly more bone than wild types ($p=0.0002$), that heterozygotes also had significantly more bone than wild types ($p=0.0134$), and that there was no significant difference between heterozygotes and mutants ($p=0.0697$) (Fig. 2H). For the *aik2* line, ANOVA revealed no significant effect of genotype on area of ossification of the hyosymplectic ($F=2.3$; $df=2,90$; $p=10.62$) (Fig. 2I). For the *aik3* line, ANCOVA also revealed no significant effect of genotype on area of ossification of the hyosymplectic ($F=2.48$; $df=1,56$; $p=0.0928$) (Fig. 2J).

***hdac4* is expressed in regions of the pharyngeal skeleton consistent with a role in cartilage maturation**

At 60 hpf, *hdac4* expression is broadly expressed throughout the head, with no apparent strong colocalization with *sox9a*-expressing cartilages (not shown). Previously, we described the expression of *hdac4* in the ventral region of the developing pharyngeal skeleton at 72 hpf (DeLaurier et al., 2012), and here we show how specific regions of expression are associated with sites of ossification of cartilage. By 72 hpf, expression is localized to regions of *sox9a*-expressing cartilage as well as in tissue surrounding cartilage and dermal bone elements. Co-expression of *hdac4* and *sox9a* is detected in the hyosymplectic, ceratohyal, and palatoquadrate cartilages (Figs. 3A-H, indicated by arrows). In the case of the hyosymplectic and ceratohyal, co-expression of *hdac4* and *sox9a* is in regions that will undergo ossification at later stages. *hdac4* is strongly expressed in the posterior pharyngeal arches, overlapping in the mid-region of each arch with a domain of *sox9a* expression in the ceratobranchial cartilage within each arch (Figs. 3I-L).

Mutants have changes in expression of factors associated with onset of ossification

MEF2C is known to activate transcription of *Runx2*, a transcription factor that activates chondrocyte maturation (Arnold et al., 2007). *Runx2* in turn activates *Sp7*, a transcription factor associated with osteoblast differentiation (Nishio et al., 2006). We examined how loss of *Hdac4*, which would lead to over-activity of *Mef2c*, affects levels

of mRNA expression of these factors. Among teleost fish, a whole genome duplication event generated two copies of vertebrate *runx2*, *runx2a* and *runx2b* (van der Meulen et al., 2005). As in previous studies, we observed differential patterns of expression of both genes, indicating that these genes have unique functions in skeletal development in zebrafish (Fig. 4B and F). At 4 dpf, *runx2a* and *runx2b* are expressed in the mid-shaft region of the ceratohyal cartilage and branchiostegal ray (Fig. 4B and F), and also in the opercle (not shown). *runx2b* was also expressed in the hyosymplectic at this stage, but *runx2a* was not (not shown). In *hdac4^{aik3/aik3}* mutants, there is an increase in *runx2a* expression in the ceratohyal compared to wild type siblings (Fig. 4B-D, indicated by arrows, M, N). In mutants *runx2a* expression in the ceratohyal cartilage is broader, encompassing more length of the ceratohyal compared to wild type siblings, and expression is stronger (Fig. 4B-D, indicated by arrows, M, N). Expression of *runx2b* in *hdac4^{aik3/aik3}* mutants appears the same as in wild type siblings (Fig. 4E-H, indicated by arrows, M, N). At 4 dpf, in both wild types and mutants, *sp7* expression is located as a small region of expression on the lateral aspect of the mid-shaft of the ceratohyal near the branchiostegal ray in both wild type and mutant larvae. Compared to wild types, expression of *sp7* is increased in *hdac4^{aik3/aik3}* mutants (Fig. 4I-L, indicated by arrows, M, N).

Maternal-zygotic mutants have increased ossification of the pharyngeal skeleton and defects in the anterior facial region

At 7 dpf, a subset of maternal zygotic mutants and heterozygotes showed a prominent increase in cartilage ossification compared to wild types (non-sibling controls) (Fig. 5A-

G). In total, 40.0% (16/40) of maternal zygotic mutants and 40.0% (16/40) heterozygotes had precocious ossification, including formation of an anguloarticular (aa) bone associated with the Meckel's cartilage (Figs. 5B-D, G), an enlarged quadrate (see Fig. 2A for reference, Fig. 5B), a dorsal hypohyal element (hhd) associated with the ceratohyal (Figs. 5B and D), ossification of the **symplectic of the hyosymplectic** (Fig. 5B, indicated by asterisk), and ossification of the mid-shaft of the first or first and second ceratobranchial cartilages (Figs. 5C, D, F). Among maternal zygotic mutants, 25.9% (15/58) had defects associated with loss of the first pharyngeal arch, including a shortened face (Figs. 5G-J) and loss of one or both Meckel's cartilages and the anterior portion of the palatoquadrate cartilage, along with retention of a **small remnant of the entopterygoid and quadrate** (Fig. 5J, see Fig. 2A for reference). Among maternal zygotic heterozygotes, 10.2% (6/59, Fig. 5G) also had loss of first arch structures. In the case of larvae with a loss of first arch cartilages, posterior second arch and more posterior arch structures including the ceratohyal, branchiostegal ray, and opercle were present (Fig. 5J). Maternal zygotic mutants and heterozygotes also had defects in the neurocranium cartilage (the primary palate in fish), including clefts, holes, and shortening of the anterior portion of the element (Fig. 5K representative heterozygote with normal neurocranium, L-M heterozygote and mutant with neurocranium defects). In total, 30.8% (16/52) of maternal zygotic mutants, and 15.2% (7/46) of maternal zygotic heterozygotes had neurocranium defects (Fig. 5G). Most neurocranium defects occurred in fish which also had loss of first arch structures (11/16, 68.8% of maternal zygotic mutants, and 5/7, 71.4% of maternal zygotic heterozygotes).

Discussion

In this study, we generated two novel zebrafish lines with germ line mutations near the start of *hdac4*. Both lines have insertions that cause frameshifts and premature stop codons, which we predict causes truncated proteins with no functional Mef2c binding domain or HDAC domain. Although a CtBP domain may exist in a truncated form of the mutant protein, we do not think this is sufficient to preserve function of the Hdac4 protein as a repressor of ossification in the absence of the Mef2c and HDAC domains. RT-PCR and cDNA analysis does not reveal any alternative splice forms of the transcript, so we do not believe there is evidence for alternate versions of this protein caused by exon skipping or alternative splicing (Sharpe & Cooper, 2017). The presence of RT-PCR bands of the predicted size in mutants also indicates that transcripts are not subject to nonsense-mediated decay, and so it is unlikely that there is a trigger for a genetic compensatory response in mutants that could result in a less severe phenotype (El-Brolosy & Stainier, 2017).

Analysis of zygotic mutants revealed a mild to moderate but statistically significant increase in ossification of the ceratohyal cartilage in both lines examined. One line showed a trend towards increased ossification of the hyosymplectic (*hdac4^{aik2/aik2}*), although the increase in bone was not significant in mutants compared to wild type siblings. Normally, the hyosymplectic is the first element to commence ossification 4-5 dpf, followed by the ceratohyal by 6 dpf (Eames et al., 2013). In the case of the ceratohyal, ossification normally begins on the anterior margin of the mid-shaft of the element and extends posteriorly and along the length of the cartilage element until around 12 dpf when the proximal end of the ceratohyal ossifies to form the

epihyal and the distal end of the ceratohyal ossifies to form the dorsal hypohyal (Cubbage & Mabee, 1996; Eames et al., 2013). Increased ossification of the ceratohyal in zygotic mutants resembles the pattern observed in 12-14 dpf wild type zebrafish (Eames et al., 2013), indicating that the ossification program in mutants is either precocious or is over-activated due to loss of *Hdac4*. Previous studies in the *Hdac4* mutant mouse show precocious ossification of the endochondral skeleton, with particular enhancement of ossification of chondral rib elements and limb cartilages in newborns (Vega et al., 2004). In both the mouse and the zebrafish, ossification of elements is not ectopic, but rather appears to reveal a premature onset of the ossification process.

In mouse *Hdac4* mutants, *Runx2* expression is increased in cartilage, and is associated with the increase of endochondral ossification of elements (Vega et al., 2004). In zebrafish, orthologs of *runx2*, *runx2a* and *runx2b* are both expressed in embryonic and larval cartilage and bone, consistent with a function in ossification (Flores et al., 2004; Li et al., 2009; van der Velden et al., 2013). At 48-96 hpf, *runx2a* is expressed in the maxilla, dentary, ceratohyal, opercle, and branchiostegal ray elements of the pharyngeal skeleton (Flores et al., 2004; Li et al., 2009; van der Velden et al., 2013). At 48-96 hpf, *runx2b* is expressed in the ceratohyal, hyosymplectic, parasphenoid, entopterygoid, opercle, branchiostegal ray, and ceratobranchial cartilages of the third to seventh pharyngeal arches (Flores et al., 2004; Li et al., 2009; van der Velden et al., 2013). These subtly different expression patterns indicate that following the teleost whole genome duplication approximately 300 million years ago (Amores et al., 1998; Hoegg et al., 2004), orthologs have similar but divergent functions

in zebrafish development. We observed increased expression of *runx2a* in *hdac4*-mutant zebrafish compared to wild types, which we hypothesize is due to a de-repression of Mef2c function leading to increased transcription of *runx2a*. We did not observe any change in *runx2b* expression in mutants compared to wild types, potentially indicating that *runx2b* expression is not affected by loss of Hdac4 and increased function of Mef2c. Phylogenetic analysis indicates that *runx2a* and *runx2b* are divergent paralogs, and thus may be regulated by different factors in zebrafish (van der Meulen et al., 2005). The observation of a differential response in *runx2* paralog expression in *hdac4* mutants may reveal that *runx2a* and *runx2b* are regulated differently by Mef2c. We demonstrated an increase in *sp7* expression in the ceratohyal of *hdac4* mutants, which we hypothesize underlies the increased bone formation we observed in mutants, and may be up-regulated as a consequence of the increase in *runx2a* observed in mutants.

Maternal-zygotic mutants and heterozygotes showed an enhancement of the ossification phenotype observed in zygotic mutants. In addition to the increased ossification of the ceratohyal observed in zygotic mutants, 40% of maternal-zygotic mutants and heterozygotes had additional premature ossification of other pharyngeal skeletal elements. At 7 dpf, these larvae had evidence of a dorsal hypohyal at the distal portion of the ceratohyal, ossification of the symplectic cartilage of the hyosymplectic element, and ossification of the anterior ceratobranchial cartilages. Ossification of the dorsal hypohyal and symplectic normally occurs around 12 dpf, and ceratobranchial around 13 dpf (Cubbage & Mabee, 1996; Eames et al., 2013), indicating that the ossification program in maternal-zygotic mutants and heterozygotes is accelerated.

Among maternal-zygotic mutants and heterozygotes, the presence of an anguloarticular bone associated with the Meckel's cartilage was also observed. This element is normally first observed at around 12 dpf in wild type zebrafish. Unlike the other elements showing premature ossification, this element is a dermal bone that forms without a cartilaginous precursor (Cubbage & Mabee, 1996). Other dermal bones appear to be unaffected in zygotic or maternal-zygotic *hdac4* mutants, and dermal bones are reported to be unaffected in *Hdac4* mutant mice (Vega et al., 2004). We cannot explain why this particular dermal bone appears precociously in *hdac4* mutants; however, as it forms on the surface of the Meckel's cartilage, anguloarticular precursor cells may be responding to signals from the underlying Meckel's cartilage to commence formation of bone. As an aside, it was noted that the cartilage of maternal-zygotic mutants and heterozygotes with excessive ossification generally had weaker Alcian Blue stain, and chondrocytes appeared rounder and less well organized compared to wild type controls (Figs. 5C and D). These differences in chondrocyte morphology and cartilage matrix indicate that loss of *Hdac4* may also affect chondrocytes or chondrogenesis.

The profound increase in ossification in a subset of maternal-zygotic mutants and heterozygotes compared to zygotic mutants suggests that there is a maternal influence on the chondral ossification program in zebrafish through *Hdac4*. In zebrafish, the maternal-zygotic transition (MZT) commences around 2 hpf (128 cells) where maternal transcripts are degraded and the first waves of zygotic transcripts are generated (Tadros & Lipshitz, 2009). Using the RNA-seq expression atlas data for zebrafish (<https://www.ebi.ac.uk/gxa/home>), we found that the highest level of *hdac4* mRNA

expression in zebrafish is between cleavage (2 cell) and blastula dome (over 1K cell stage), followed by very low expression of transcripts up to 3 dpf (larval protruding mouth), after which transcript levels increase (Fig. 6). Since the high levels of *hdac4* mRNA in embryos prior to 128-cell stage can only be maternal transcripts, we believe this shows that there is a role for maternal Hdac4 in early development, and that only several days later does zygotic Hdac4 function in development. From our experiments, it is unclear how early maternal *hdac4* may be affecting ossification of cartilage several days post-fertilization. However, it is possible that maternal Hdac4 protein is still present in cells several days following the MZT and can influence ossification, or alternately, maternal Hdac4 may establish an epigenetic environment or signaling cascade in very early embryos which has consequences on downstream skeletogenesis. Future experiments will establish the levels of maternal Hdac4 in zebrafish embryos and we will examine the function of maternal transcripts or proteins on skeletal development in larvae.

Previously, we reported that morpholino knockdown of *hdac4* causes loss of neural crest and neurocranium defects in zebrafish (DeLaurier et al., 2012). Our zygotic mutants do not show any evidence of this phenotype. However, approximately one quarter of *hdac4* maternal-zygotic mutants show neurocranium defects and a loss of first arch cartilages consistent with a role for Hdac4 in neural crest development. Given the proportion of approximately one quarter of maternal-zygotic mutants showing this effect, we believe there may be a dihybrid effect of another unknown gene influencing the phenotypic outcome of loss or reductions of Hdac4 on neural crest. Future experiments will establish the levels of specifically maternal Hdac4 in zebrafish embryos

and we will examine the function of maternal transcripts or proteins on neural crest patterning and skeletal development in embryos and larvae.

In mouse, loss of MEF2C causes impaired chondrocyte hypertrophy and loss of endochondral ossification due to the failure to activate Runx2 and Ihh targets (Arnold et al., 2007). In zebrafish, the pharyngeal development of *mef2ca* and *mef2cb* mutants has been previously described (Miller et al., 2007; DeLaurier et al., 2014). *Mef2ca* mutants have a profound fusion defect of dorsal and ventral first and second arch cartilages and an expanded opercle bone. *Mef2ca* regulates expression of *dlx* genes, *hand2*, and *nkx3.2 (bapx1)* targets downstream of **Edn1**, and it is proposed that changes to expression of these factors through loss of *mef2ca* results in the joint fusions and dorsal-ventral patterning defects in mutants (Miller et al., 2007). **Single *mef2cb* mutants have normal pharyngeal patterning, with no evidence of fusions or expansions of skeletal elements, indicating that Mef2cb does not function in the same capacity as Mef2ca in the Edn1 pathway during pharyngeal development (Miller et al., 2007; DeLaurier et al., 2014). If the function of zebrafish Mef2ca and Mef2cb is conserved with MEF2C function in the mouse, it is predicted that loss of either or both genes combined would result in a loss of perichondral ossification of cartilage. Previous studies do not report a perichondral ossification defect in single homozygous mutants or in *mef2ca*^{+/-}; *mef2cb*^{-/-} or *mef2c*^{-/-}; *mef2c*^{+/-} fish, although in all cases, larvae were studied at 6dpf, not 7dpf, and cartilage ossification was not quantitatively measured (Miller et al., 2007; DeLaurier et al., 2014). It is possible that the reported absence of a cartilage ossification defect in either single mutant is due to functional redundancy of the Mef2c paralogs to induce perichondral ossification, and that complete loss of both genes could result in**

ossification defects in mutants. Unfortunately, the combined double homozygous mutant has a heart malformation by 48 hpf (Hinits et al., 2012) resulting in severe developmental defects by larval stages (DeLaurier et al., 2014). Future studies should reexamine the function of *mef2c* genes in cartilage ossification. For the *mef2ca* mutant, it would be difficult to compare cartilage ossification patterns with that of wild type larvae, due to the nature of joint fusions and patterning defects in cartilage. However, future studies could quantitatively study ossification in *mef2cb* single mutants, or in combined mutants where one *mef2ca* allele is heterozygous (i.e. *mef2ca*^{+/-};*mef2cb*^{-/-}) and fish develop without cardiac abnormalities, as previously reported (DeLaurier et al., 2014).

489

490 Conclusions

In conclusion, this study shows that mutation of *hdac4* in zebrafish causes excessive ossification of the pharyngeal skeleton, consistent with previous findings in the mouse (Vega et al., 2004), indicating a conserved function for Hdac4 among vertebrates. Mutants have increased expression of the transcription factors *runx2a* and *sp7*, activators of the skeletal ossification program, which we hypothesize are upregulated in response to increased activity of Mef2c through loss of Hdac4. Maternal-zygotic crosses, along with RNA-seq analysis indicate that maternal Hdac4 is an important contributor to embryonic and larval development. Zebrafish *hdac4* mutant lines may be useful resources for future study of the function of *hdac4* in development of the skeleton or other tissues, potentially along with other class IIa *hdac* mutants (i.e. *hdac5*, *hdac7*, and *hdac9*) which may be compensating in part for loss of Hdac4. Maternal-zygotic

502 *hdac4* mutants and heterozygotes further offer novel insights into the role of maternal
503 transcripts or proteins on late-stage larval tissue patterning.

504

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510

Figure legends

Figure 1: Overview of CRISPR strategy and generation of *hdac4* mutant lines. A: Genomic structure of *hdac4* showing gRNA target associated with the protospacer adjacent motif (5'-NGG, PAM) upstream of the Mef2c binding domain sequence. The histone deacetylase domain sequence is at the 3' end of the gene. Green arrows indicate forward (exon 3F) and reverse (exon 6R) primers for RT-PCR and sequencing of cDNA. Red arrows indicate intronic genotyping primers (*hdac4* F6, *hdac4* R6) flanking exon 5. Intron 2/3 and 3/4 not to scale, indicated by hash marks. B: Alignment of wild type (*hdac4*^{+/+}) with mutant cDNA sequence showing nucleotide insertions (blue) in *hdac4*^{aik2/aik2} and *hdac4*^{aik3/aik3} mutants in exon 5. C and D: Insertion of nucleotides results in reading frame shifts causing aberrant protein sequences (magenta in C, grey boxes in D), loss of the Mef2c binding domain (indicated in yellow in C), and premature termination of the protein sequence (asterisk in C indicates stop codon). E and F: RT-PCR showing *hdac4* cDNA is spliced correctly in mutants and there is no evidence of splice variants. The wild type cDNA product is expected to be 535 bp and mutant bands are 554 bp (*hdac4*^{aik2/aik2} in E) and 571 bp (*hdac4*^{aik3/aik3} in F). The larger band in *hdac4*^{aik3/aik3} mutants (indicated by white asterisk in F) was sequenced and determined to be identical to the lower band. G and H: Genomic DNA samples were genotyped by PCR and show differences in band sizes indicating mutant (841 bp *hdac4*^{aik2/aik2}, 858 bp *hdac4*^{aik3/aik3}), wild type (822bp), and heterozygous fish (mutant and wild type bands). I and J: At 7dpf, mutant *hdac4*^{aik3/aik3} fish (J) appear normal compared to wild type siblings (I). H₂O = negative control. 100 bp ladder.

534

535 Figure 2: Analysis of ossification of the pharyngeal skeleton of *hdac4* zygotic mutants.

536 A: Schematic showing elements of the 7 dpf larvae pharyngeal skeleton. Cartilage is

537 indicated in blue, bone is indicated in red. B and C: Total scores assigned to 7 dpf

538 larvae scored for *aik2* and *aik3* lines, respectively. D and D': wild type larval pharyngeal

539 skeleton and enlarged view of ceratohyal. E and E': *hdac4^{aik2/aik2}* mutant larvae showing

540 "mild" increase of ossification of ceratohyal. Arrow indicates irregular border of

541 ossification and asterisk indicates bone collar on dorsal surface of ceratohyal. Lower

542 arrow indicates spread of ossification to ventral aspect of ceratohyal. F and F':

543 *hdac4^{aik2/aik2}* mutant showing "moderate" increase of ossification of ceratohyal. Asterisks

544 indicate bone collar on dorsal and ventral surfaces of the ceratohyal. G and H: Bar

545 graphs comparing ratios of bone to total area of the ceratohyal for *aik2* and *aik3*. I and

546 J: Bar graphs comparing ratios of bone to total area of the hyosymplectic for *aik2* and

547 *aik3*. For G-J, *aik2* total fish = 93, including WT = 38, heterozygote = 23, and mutant =

548 32; *aik3* total fish = 61, including WT = 17, heterozygote = 28, and mutant = 16.

549 Abbreviations: ch = ceratohyal, bsr = branchiostegal ray, de = dentary, en =

550 entopterygoid, hs = hyosymplectic, ih = interhyal, m = Meckel's cartilage, op = opercle,

551 pq = palatoquadrate, q = quadrate. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bar = 100

552 microns. Cartilage is stained blue (Alcian Blue), bone is stained red (Alizarin Red).

553

554 Figure 3: Expression of *hdac4* and *sox9a* mRNA in the pharyngeal skeleton of wild type

555 embryos at 72 hpf. A: Schematic of skeletal elements, lateral view for B-D, blue

556 indicates cartilage, red indicates bone. B-D: expression of *hdac4* and *sox9a*, arrows

indicate expression in hyosymplectic, ceratohyal, and palatoquadrate cartilages. E: Schematic of skeletal elements, lateral view for F-H. F-H: expression of *hdac4* and *sox9a*, arrows indicate expression in ceratohyal and hyosymplectic cartilages. I: Schematic of skeletal elements, ventral view for J-L. J-L: expression of *hdac4* and *sox9a*, arrows indicate expression in the posterior pharyngeal arches. Abbreviations: bb = basibranchial, ch = ceratohyal, hs = hyosymplectic, ih = interhyal, m = Meckel's cartilage, op = opercle, pa3-7 = posterior pharyngeal arches, pq = palatoquadrate. Scale bar = 50 microns.

Figure 4: Expression of *runx2a*, *runx2b*, and *sp7* mRNA in the pharyngeal skeleton of wild-type and *hdac4^{aik3/aik3}* mutant larvae at 4 dpf. A, C, E, G, I, K: Schematic view of skeletal elements, ventral view, blue indicates cartilage, red indicates bone. B and D: expression of *runx2a* in wild type (B) and mutant (D), arrows indicate expression in the ceratohyal. F and H: expression of *runx2b* in wild type (F) and mutant (H), arrows indicate expression in the ceratohyal. J and L: expression of *sp7* in wild type (J) and mutant (L), arrows indicate expression in the ceratohyal. M: schematic showing overlapping domains of expression of *runx2a*, *runx2b*, and *sp7* in wild type larvae. N: schematic showing expanded *runx2a* and *sp7* domains in mutant larvae. Abbreviations: ch = ceratohyal, bsr = branchiostegal ray, pa3 = pharyngeal arch 3, pq = palatoquadrate. Scale bar = 50 microns.

Figure 5: Analysis of maternal-zygote mutant and heterozygote skeletal patterning. A: Wild type (non-sibling) larval pharyngeal skeleton at 7 dpf. B-D: maternal-zygotic mutant

(B and C) and heterozygote (D) pharyngeal skeletons at 7 dpf showing precocious angular articular bone (aa, indicated in B and C), dorsal hypohyal (hhd, indicated in B and D), ossification of the symplectic of the hyosymplectic (indicated by asterisk in B). E: Wild type posterior pharyngeal arches 3 and 4 showing first and second ceratobranchial cartilages (cb1, cb2), ventral view, 7 dpf. F: maternal-zygotic mutant showing ossification of the ceratobranchial cartilages, indicated by arrows (also in C). G: Total scores of maternal zygotic mutants and heterozygotes for first arch (M, Pq defect), neurocranium, and early ossification defects. H: Maternal-zygotic heterozygote at 72 hpf, lateral view. I: Maternal-zygotic mutant at 72 hpf, lateral view. J: Maternal-zygotic mutant showing loss of first arch cartilage, a small remnant entopterygoid bone (en) and quadrate (q) associated with the palatoquadrate are present (see Fig. 2A for reference). K and L: Maternal-zygotic heterozygote neurocrania, ventral view, 7 dpf. L: Maternal-zygotic mutant neurocranium, ventral view, 7 dpf. A-D, H-M: Scale bar = 200 microns, E and F: Scale bar = 50 microns. Cartilage is stained blue (Alcian Blue), bone is stained red (Alizarin Red).

Figure 6: RNA-seq mRNA levels of *hdac4* transcripts from zygotic to larval day 5 stage. Bars indicate numbers of *hdac4* transcripts per million transcripts read at each stage. Between the 128 and 256-cell stage, maternal transcripts degrade and zygotic transcripts become predominant. See <http://www.ebi.ac.uk/gxa/experiments/E-ERAD-475> for Expression Atlas data.

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Figure 1

Overview of CRISPR strategy and generation of *hdac4* mutant lines.

A: Genomic structure of *hdac4* showing gRNA target associated with the protospacer adjacent motif (5'-NGG, PAM) upstream of the Mef2c binding domain sequence. The histone deacetylase domain sequence is at the 3' end of the gene. Green arrows indicate forward (exon 3F) and reverse (exon 6R) primers for RT-PCR and sequencing of cDNA. Red arrows indicate intronic genotyping primers (*hdac4* F6, *hdac4* R6) flanking exon 5. Intron 2/3 and 3/4 not to scale, indicated by hash marks. B: Alignment of wild type (*hdac4*^{+/+}) with mutant cDNA sequence showing nucleotide insertions (blue) in *hdac4*^{aik2/aik2} and *hdac4*^{aik3/aik3} mutants in exon 5. C and D: Insertion of nucleotides results in reading frame shifts causing aberrant protein sequences (magenta in C, grey boxes in D), loss of the Mef2c binding domain (indicated in yellow in C), and premature termination of the protein sequence (asterisk in C indicates stop codon). E and F: RT-PCR showing *hdac4* cDNA is spliced correctly in mutants and there is no evidence of splice variants. The wild type cDNA product is expected to be 535 bp and mutant bands are 554 bp (*hdac4*^{aik2/aik2} in E) and 571 bp (*hdac4*^{aik3/aik3} in F). The larger band in *hdac4*^{aik3/aik3} mutants (indicated by white asterisk in F) was sequenced and determined to be identical to the lower band. G and H: Genomic DNA samples were genotyped by PCR and show differences in band sizes indicating mutant (841 bp *hdac4*^{aik2/aik2}, 858 bp *hdac4*^{aik3/aik3}), wild type (822bp), and heterozygous fish (mutant and wild type bands). I and J: At 7dpf, mutant *hdac4*^{aik3/aik3} fish (J) appear normal compared to wild type siblings (I). H₂O = negative control. 100 bp ladder.

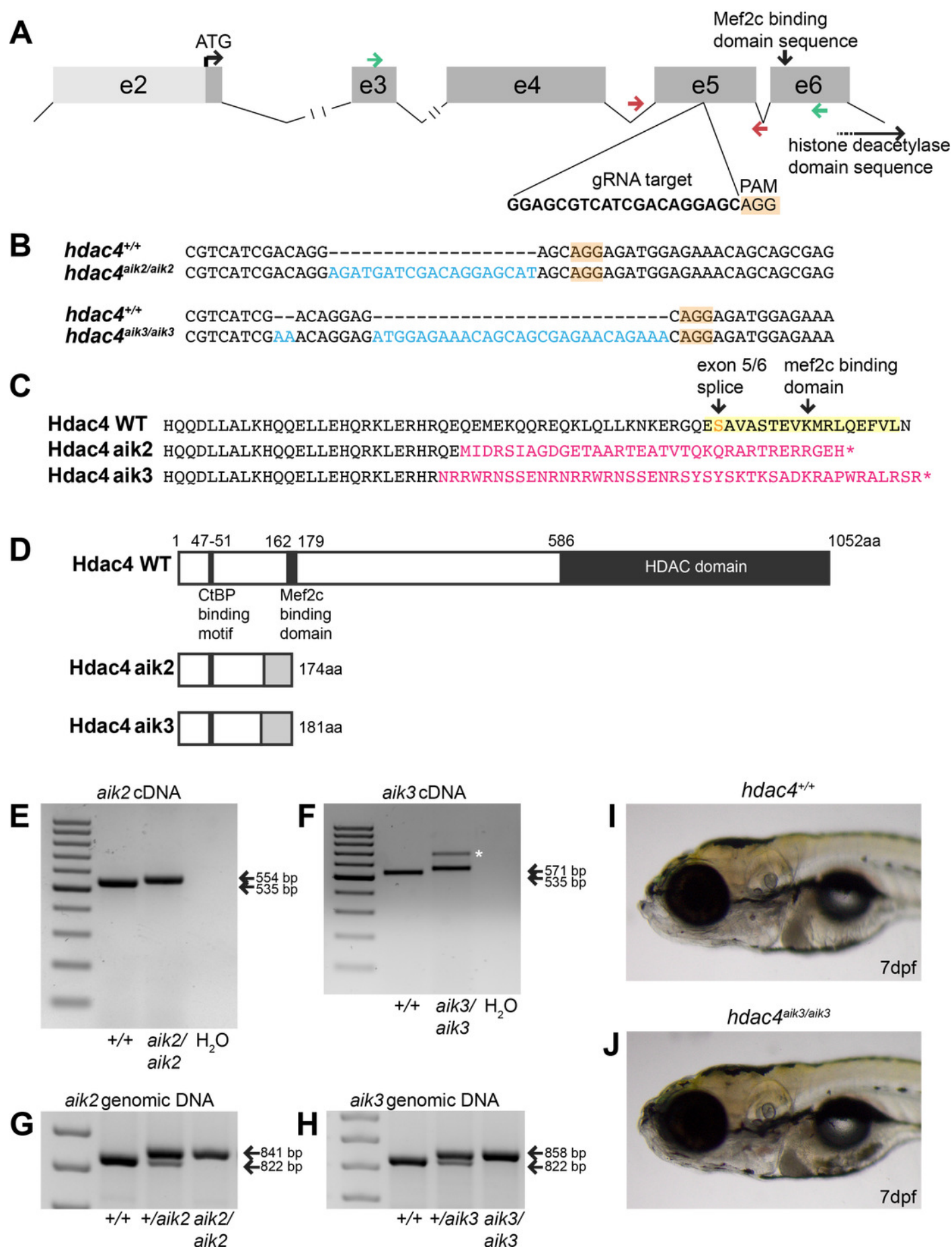


Figure 2

Analysis of ossification of the pharyngeal skeleton of *hdac4* zygotic mutants.

A: Schematic showing elements of the 7 dpf larvae pharyngeal skeleton. Cartilage is indicated in blue, bone is indicated in red. B and C: Total scores assigned to 7 dpf larvae scored for *aik2* and *aik3* lines, respectively. D and D': wild type larval pharyngeal skeleton and enlarged view of ceratohyal. E and E': *hdac4^{aik2/aik2}* mutant larvae showing "mild" increase of ossification of ceratohyal. Arrow indicates irregular border of ossification and asterisk indicates bone collar on dorsal surface of ceratohyal. Lower arrow indicates spread of ossification to ventral aspect of ceratohyal. F and F': *hdac4^{aik2/aik2}* mutant showing "moderate" increase of ossification of ceratohyal. Asterisks indicate bone collar on dorsal and ventral surfaces of the ceratohyal. G and H: Bar graphs comparing ratios of bone to total area of the ceratohyal for *aik2* and *aik3*. I and J: Bar graphs comparing ratios of bone to total area of the hyosymplectic for *aik2* and *aik3*. Abbreviations: ch = ceratohyal, bsr = branchiostegal ray, de = dentary, en = entopterygoid, hs = hyosymplectic, ih = interhyal, m = Meckel's cartilage, op = opercle, pq = palatoquadrate, q = quadrate. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Scale bar = 100 microns. Cartilage is stained blue (Alcian Blue), bone is stained red (Alizarin Red).

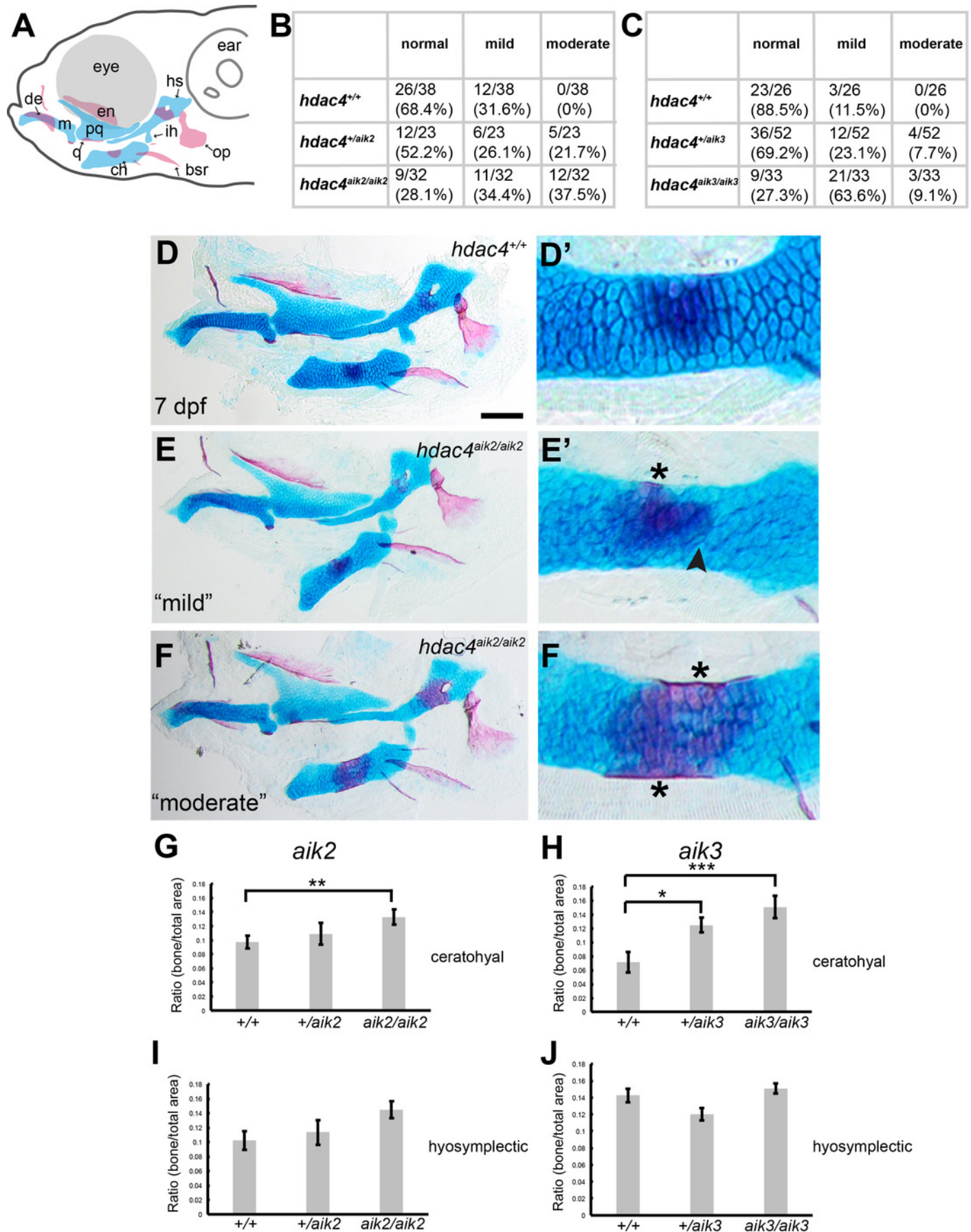


Figure 3

Expression of *hdac4* and *sox9a* mRNA in the pharyngeal skeleton of wild type embryos at 72 hpf.

A: Schematic of skeletal elements, lateral view for B-D, blue indicates cartilage, red indicates bone. B-D: expression of *hdac4* and *sox9a*, arrows indicate expression in hyosymplectic, ceratohyal, and palatoquadrate cartilages. E: Schematic of skeletal elements, lateral view for F-H. F-H: expression of *hdac4* and *sox9a*, arrows indicate expression in ceratohyal and hyosymplectic cartilages. I: Schematic of skeletal elements, ventral view for J-L. J-L: expression of *hdac4* and *sox9a*, arrows indicate expression in the posterior pharyngeal arches. Abbreviations: bb = basibranchial, ch = ceratohyal, hs = hyosymplectic, ih = interhyal, m = Meckel's cartilage, op = opercle, pa3-7 = posterior pharyngeal arches, pq = palatoquadrate. Scale bar = 50 microns.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

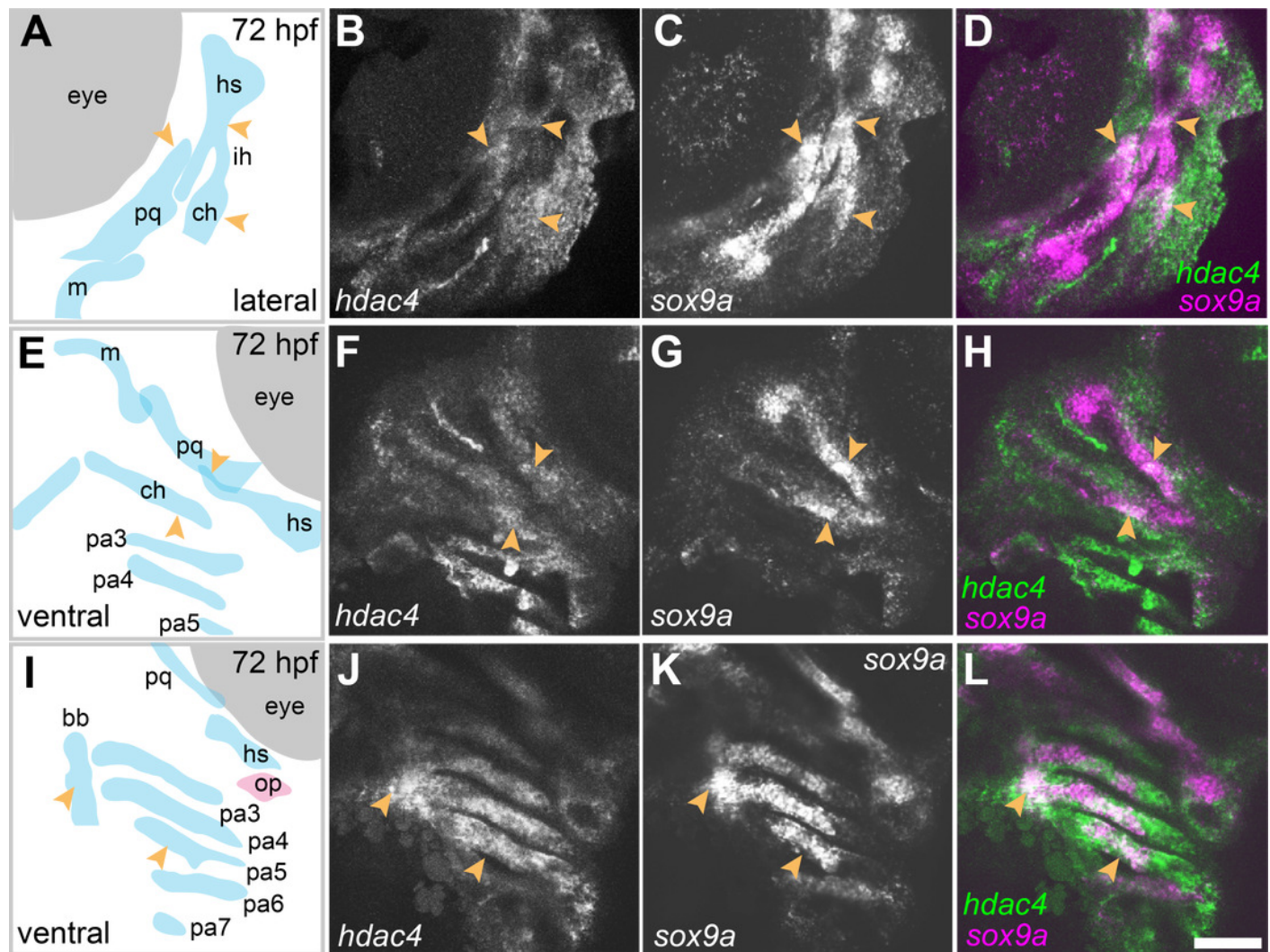


Figure 4

Expression of *runx2a*, *runx2b*, and *sp7* mRNA in the pharyngeal skeleton of wild-type and *hdac4^{aik3/aik3}* mutant larvae at 4 dpf.

A, C, E, G, I, K: Schematic view of skeletal elements, ventral view, blue indicates cartilage, red indicates bone. B and D: expression of *runx2a* in wild type (B) and mutant (D), arrows indicate expression in the ceratohyal. F and H: expression of *runx2b* in wild type (F) and mutant (H), arrows indicate expression in the ceratohyal. J and L: expression of *sp7* in wild type (J) and mutant (L), arrows indicate expression in the ceratohyal. M: schematic showing overlapping domains of expression of *runx2a*, *runx2b*, and *sp7* in wild type larvae. N: schematic showing expanded *runx2a* and *sp7* domains in mutant larvae. Abbreviations: ch = ceratohyal, bsr = branchiostegal ray, pa3 = pharyngeal arch 3, pq = palatoquadrate. Scale bar = 50 microns.

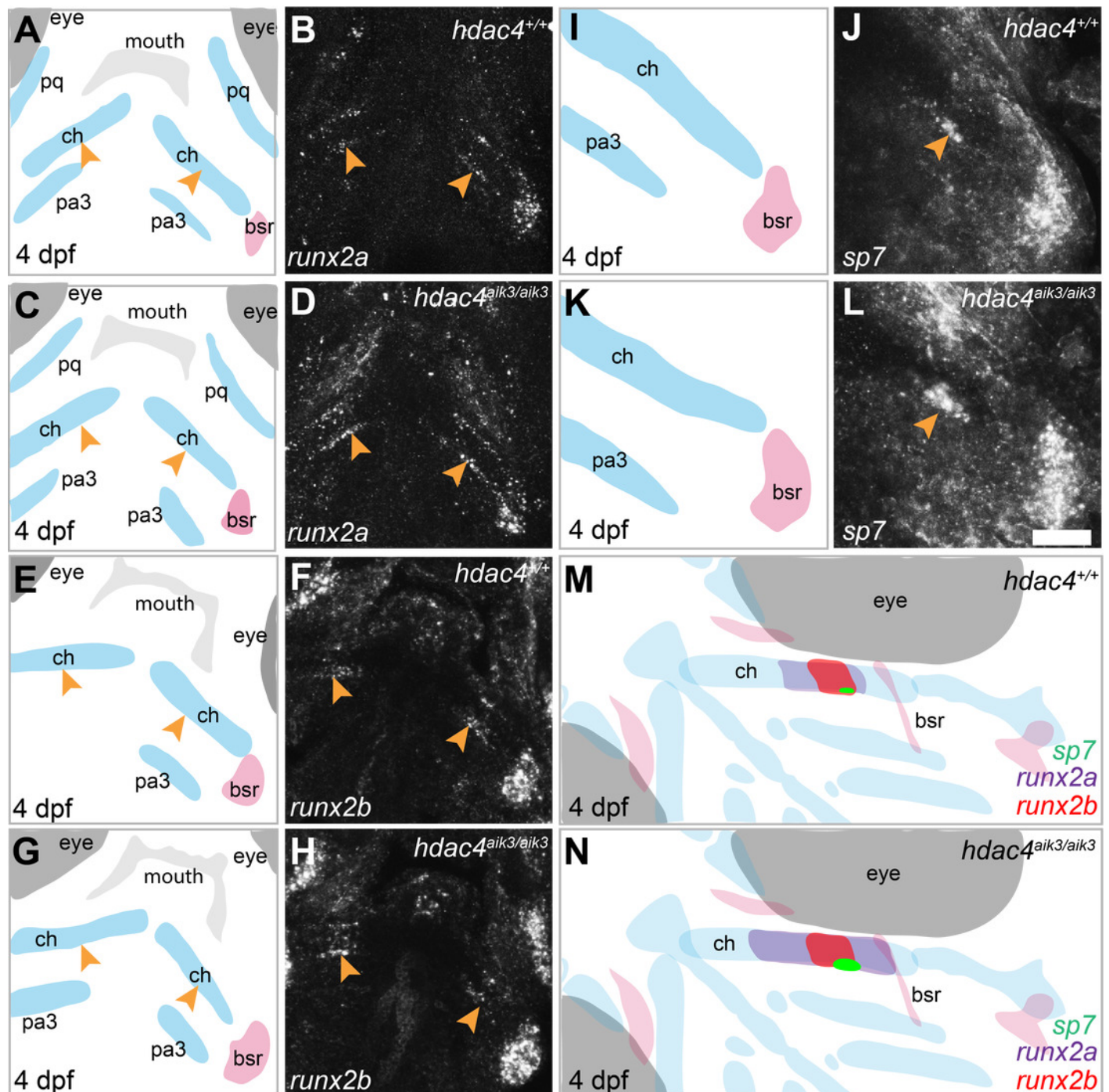


Figure 5

Analysis of maternal-zygote mutant and heterozygote skeletal patterning

A: Wild type (non-sibling) larval pharyngeal skeleton at 7 dpf. B-D: maternal-zygotic mutant (B and C) and heterozygote (D) pharyngeal skeletons at 7 dpf showing precocious angular articular bone (aa, indicated in B and C), dorsal hypohyal (hhd, indicated in B and D), ossification of the symplectic of the hyosymplectic (indicated by asterisk in B). E: Wild type posterior pharyngeal arches 3 and 4 showing first and second ceratobranchial cartilages (cb1, cb2), ventral view, 7 dpf. F: maternal-zygotic mutant showing ossification of the ceratobranchial cartilages, indicated by arrows (also in C). G: Total scores of maternal zygotic mutants and heterozygotes for first arch (M, Pq defect), neurocranium, and early ossification defects. H: Maternal-zygotic heterozygote at 72 hpf, lateral view. I: Maternal-zygotic mutant at 72 hpf, lateral view. J: Maternal-zygotic mutant showing loss of first arch cartilage, a small remnant entopterygoid bone (en) and quadrate (q) associated with the palatoquadrate are present (see Fig. 2A for reference). K and L: Maternal-zygotic heterozygote neurocrania, ventral view, 7 dpf. L: Maternal-zygotic mutant neurocranium, ventral view, 7 dpf. A-D, H-M: Scale bar = 200 microns, E and F: Scale bar = 50 microns. Cartilage is stained blue (Alcian Blue), bone is stained red (Alizarin Red).

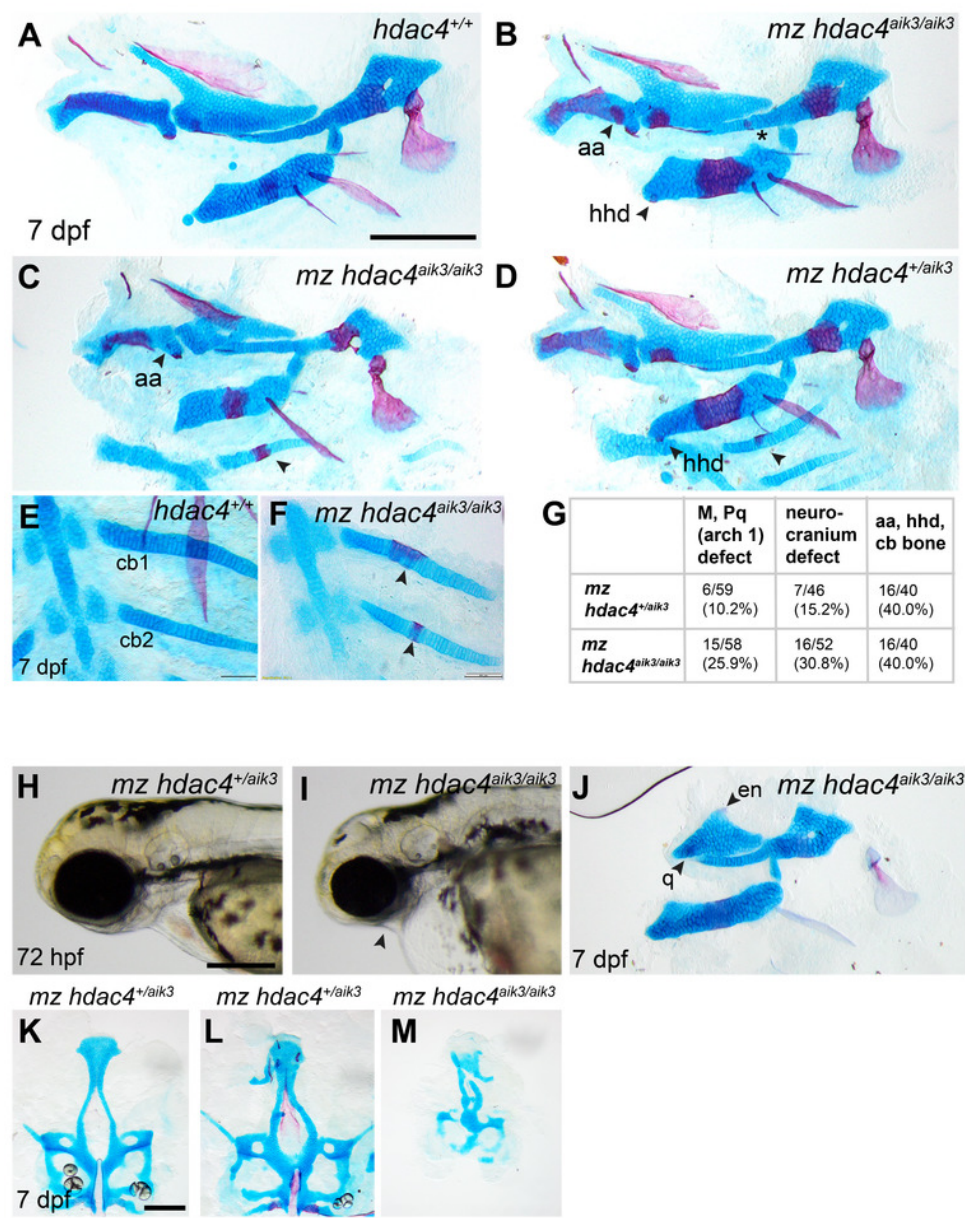


Figure 6

RNA-seq mRNA levels of *hdac4* transcripts from zygotic to larval day 5 stage.

Bars indicate numbers of *hdac4* transcripts per million transcripts read at each stage.

Between the 128 and 256-cell stage, maternal transcripts degrade and zygotic transcripts become predominant. See <http://www.ebi.ac.uk/gxa/experiments/E-ERAD-475> for Expression Atlas data.

