Zebrafish furin mutants reveal intricacies in regulating Endothelin1
signaling in craniofacial patterning☆

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Abstract

Endothelin1 (Edn1) signaling promotes ventral character to the facial skeleton. In zebrafish edn1 mutants, the ventral jaw structures are severely reduced and fused to their dorsal counterparts, with a loss of joints that normally form at an intermediate dorsal–ventral position. Loss of function at another locus, sturgeon, also yields joint losses, but only mild reductions in the ventral jaw structures. We show that sturgeon encodes one of two orthologs of Furin present in zebrafish, and that both furin genes may function partially redundantly to activate Edn1 signaling. Supporting this hypothesis, early expression of edn1-dependent genes is downregulated in sturgeon (furinA) mutants. Later in development, expression of most of these genes recovers to near wild-type levels in furinA mutants but not in edn1 mutants. The recovery explains the less severe furinA mutant skeletal phenotype and suggests that late gene expression is dependent on a critical level of Edn1 signaling not present in the more severe edn1 mutants. However, expression defects in the intermediate joint-forming domains in both mutants persist, explaining the joint losses observed later in both mutants. We further show that in both mutants the arches fail to correctly undergo ventral elongation before skeletogenesis begins and propose a model in which this failure is largely responsible for the loss of an Edn1-dependent compartmentation of the arch into the intermediate and ventral domains.

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Introduction

How facial skeletons are patterned to obtain specialized characteristics across diverse vertebrates is an intriguing question. That some of the principles of facial patterning are shared, yet the outcome so different, speaks of the intricacies involved in the regulation. Genetic and pharmacological studies demonstrate that Endothelin1 (Edn1) signaling patterns the ventral facial skeleton in mice, chicks, and fish. Mice homozygous for targeted mutations in the known Edn1 pathway genes, including the ligand Edn1, the Edn1 receptor Ednra, the small G proteins Gαq and Gα11, and an Edn1-specific cleaving enzyme Ece-1, have jaw defects that include reductions in ventral jaw structures, joint fusions, and malformation and loss of some dorsal structures (Clouthier et al., 2000; Ivey et al., 2003; Kurihara et al., 1994; Yanagisawa et al., 1998). Closer analysis of ventral jaw structures in Edn1 and Ednra mutant mice suggest a homeotic-like transformation of the lower jaw to an upper jaw identity (Ozeki et al., 2004; Ruest et al., 2004). Mice doubly homozygous for deletions in the Edn1 targets Dlx5 and Dlx6 also have lower jaws whose size and shape resemble upper jaw structures, suggesting that an Edn1-dependent Dlx5 and Dlx6 code determines lower jaw identity in mice (Beverdam et al., 2002; Depew et al., 2002). In the chick, pharmacological inactivation of Ednra also results in lower jaw defects (Kemp et al., 1998). Similarly, zebrafish edn1 mutants have greatly reduced ventral jaw cartilages and
an absence of joints between dorsal and ventral jaw elements (Miller et al., 2000). In addition, partial reductions in Edn1 function result in homeotic-like size and shape changes to dermal bones of the anterior arches, supporting a conserved role for Edn1 in controlling facial patterning and helping to explain facial diversity among vertebrates (Kimmel et al., 2003).

In this paper, we show that zebrafish have two co-orthologs of the single mammalian furin gene. We name the zebrafish furin co-orthologs, furinA and furinB, and present evidence that one of these, furinA, is the gene mutated in sturgeon. sturgeon (furinA) mutants, which we shorten to furinA for clarity, have mild jaw phenotypes in which the jaw joints are variably lost and the ventral pharyngeal cartilages are typically only mildly reduced in length. As a partial reduction in Edn1 function can phenocopy the furinA mutant jaw defects, it is possible that furinA and edn1 function in the same genetic pathway, controlling jaw morphogenesis (Miller and Kimmel, 2001).

Furin is an endoprotease belonging to a family of proprotein convertases with seven mammalian members and related to bacterial subtilin (Molloy et al., 1999; Thomas, 2002). A null mutation of furin in mice yields embryos that die between embryonic days 10.5 and 11.5 with severe ventral closure defects, including failure of the heart tube to fuse and undergo looping morphogenesis, and failure of the embryo to undergo axial rotation (Roebroek et al., 1998). These severe early defects in the mutants are likely due to failure of Furin processing of multiple proproteins, such as BMP4 and TGFβ1, which have important functions in embryogenesis (Molloy et al., 1999). The early embryonic lethality of furin mutant mice has precluded later phenotypic analysis of Furin’s role in craniofacial patterning.

Edn1 is synthesized as a large, inactive proprotein, proEdn1, which is initially cleaved at two Furin-like cleavage sites to produce an intermediate form, termed bigEdn1 (Denault et al., 1995). bigEdn1 is then cleaved by an endothelin-converting enzyme (Ece) to yield the mature, active, 21-amino acid Edn1 peptide (Xu et al., 1994). Our analysis of furinA mutants and furinB morpholino (MO)-injected embryos supports in vitro results of Denault et al. (1995) that Furin cleaves Edn1, and further suggests that other proprotein convertases play only a minor role in activating Edn1 in vivo. The furinA mutants have also allowed us to dissect specific requirements of Edn1 signaling in craniofacial development. A critical level of Edn1 signaling, that is reached in furinA mutants but not in edn1 mutants, is required to maintain late ventral expression of dlx3b, dlx5a, dlx6a, and hand2. In contrast, we show that bapx1 expression in the intermediate domain of the first arch does not recover in furinA mutants, and that the intermediate domains of both the first and second arches have ectopic expression of homeodomain-containing transcription factors and skeletal regulators. These results demonstrate that the intermediate arch domains are exquisitely sensitive to reductions in Edn1 signaling. Furthermore, we find defects in the early elongation of the ventral arch in both furinA and edn1 mutants. We propose that Edn1

Fig. 1. furinA mutants have jaw and fin defects. (A and B) Live views at 5 dpf. furinA mutants have an open-mouth phenotype and fail to form a swim bladder (white asterisk in panel A). (C and D) Flat mounts of Alcian green-stained cartilages at 5 dpf. Wild-type DV joint regions are indicated with arrows in panel C. Fusions at joint regions in furinA mutants are indicated with asterisks in panel D. Cartilages are labeled as followed: pq (palatoquadrate), mc (Meckel’s cartilage), hm (hyomandibula), ch (ceratohyal), sy (symplectic), and ih (interhyal). Two bones of the hyoid arch are also lightly stained with Alcian green: op (opercle), bsrp (branchiostegal ray posterior). (E and F) Live views of the tail fin at 2 dpf. furinA mutant larvae have mildly ruffled fins. Scale bars: 50 μm.
signaling is required for both the early elongation of the ventral arch and in establishing a DV identity to this Edn1-dependent arch domain.

Materials and methods

Fish stocks and maintenance

Fish were raised under standard conditions at 28.5°C and staged as described (Kimmel et al., 1995; Westerfield, 1993). furinB<sup>ed204e</sup> and furinA<sup>td204e</sup> alleles were generously provided by Drs. Tatjana Piotrowski and Christiane Nüsslein-Volhard. The zucker<sup>ed204e</sup> (endothelin1) mutants have been previously described (Miller et al., 2000). We obtained homozygous mutant embryos from natural matings of heterozygous carriers maintained on an inbred background. The limb phenotype confirmed by PCR genotyping 480 mutant diploid embryos. (Miller et al., 2000); bapx1 (Miller et al., 2003); runx2b (Flores et al., 2004); and sox9a (Yan et al., 2002).

Cloning of a zebrafish barx1 ortholog

We used RT-PCR with the degenerate primers 5’GCCGGAGTCCACNGGTCATATGGG3’ and 5’GCCGGATCTCTTCTGTRACANG-TYTTAC3’ to amplify Barx1-related homeobox fragments from 5 dpf zebrafish larvae. 3’ RACE PCR (Chenchik et al., 1996) produced a 577-bp fragment (exclusive of the polyA tail) of the putative zebrafish barx1. This sequence, which was used as a probe for in situ hybridization, differs by only two nucleotides from positions 521 to 1097 of the predicted zebrafish transcript ENSDART00000023889, identified as the ortholog of mouse Barx1 by reciprocal BLAST analysis (http://wwwensembl.org/Danio_rerio).

Identification, mapping, and phylogenetic analysis of sturgeon (furinA) and furinB

sturgeon<sup>ed419</sup> and sturgeon<sup>ed404e</sup> were mapped on a hybrid genetic background, TLAB and WIK/AB, respectively, by bulked segregant analysis between the microsatellite markers Z1206 and Z20963 on LG 7 (Knapik et al., 1996). We mapped a zebrafish EST (f23d03, Incyte Genomics, Inc.) encoding a protein highly similar to human FURIN to within the sturgeon interval. We call this zebrafish gene furinA. A zebrafish fragment of a potential furinA duplicate, which we call furinB, was obtained by RT-PCR of 24 hpf zebrafish total RNA with the degenerate primers 5’CAYGGNACNMGNTGYGCGNGGAG3’ and 5’ATNSWRTNTGTNRANCCRTCRCAG3’. Full-length furinB sequence was obtained by standard methods and mapped to LG 25. Phylogenetic relatedness of zebrafish/furinA and furinB with mammalian proprotein convertase converge sequences were determined using the ClustalX and Njplot programs.

Sequence and co-segregation analysis of furinA alleles

The furinA cDNAs were prepared from pools of mutant and wild-type sibling embryos by RT-PCR and sequenced directly using an ABI automated sequencer. The primers 5’GGACAGCTGCAATTGCGACGGTTA3’ and 5’ATNSWRTNTGTNRANCCRTCRCAG3’ were used to turn the <sup>tg419</sup> lesion into a co-dominant polymorphism following <sup>Mse</sup> restriction enzyme digestion and segregation with mutant phenotype confirmed by PCR genotyping 480 mutant diploid embryos.

Morpholino antisense oligonucleotide injections

Translation blocking and splice site blocking morpholinos (MOs) were purchased from Gene Tools, Inc. furinB exon 9 splice acceptor MO: 5’

Table 1

<table>
<thead>
<tr>
<th>Arch</th>
<th>% Severe ventral reductions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2 (4/228)</td>
</tr>
<tr>
<td>2</td>
<td>3 (7/228)</td>
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<tr>
<td>3</td>
<td>16 (37/228)</td>
</tr>
<tr>
<td>4</td>
<td>11 (24/228)</td>
</tr>
<tr>
<td>5</td>
<td>3 (6/228)</td>
</tr>
<tr>
<td>6</td>
<td>1 (2/228)</td>
</tr>
<tr>
<td>7</td>
<td>1 (2/228)</td>
</tr>
</tbody>
</table>

At 5 dpf, larvae were fixed and cartilage stained with Alcian green. Phenotypes were scored on both sides of the larvae. Severe ventral reductions refer to cartilages that are less than half the size of its wild-type counterpart.

Fig. 2. The sturgeon gene is furinA. (A) sturgeon (furinA) maps to Danio rerio chromosome 7. (B) PCR genotyping illustrating co-segregation of nonsense lesion with furinA<sup>ed419</sup> mutant phenotype following MseI digestion. (C and D) Sequence chromatograms of furinA<sup>+</sup> (wt), furinA<sup>ed419</sup>, and furinB<sup>ed404e</sup> alleles around lesion sites in furinA. Arrows indicate lesions. (E) Schematic of protein domains in furinA<sup>+</sup> (wt), furinA<sup>ed419</sup>, and furinB<sup>ed404e</sup> alleles. Abbreviations: sp (signal peptide), pro (propeptide), cys (cysteine rich), tm (transmembrane), cyt (cytosolic).
AGCCAGATCGACCCTAGAAACACAT3′; furinA exon 9 splice donor MO: 5′GAGGGACTCACAATCTGTTTCTCAT3′; dlx5a MO: 5′CGAATACTCAGTCAATGTTTGGAT3′ (underlined sequence is complementary to start codon); edn1 MO (Miller and Kimmel, 2001); and dlx3b MO (Liu et al., 2003). Roughly 2 nl of MOs diluted to 0.5–10 mg/ml in 0.2 M KCl and 0.2% phenol red were pressure injected into 1–4 cell zebrafish embryos.

Measurement of ventral arch tissue

Nomarski differential interference contrast (DIC) optical views of whole-mount Dlx-stained embryos were obtained using a Zeiss Axiophot microscope. A pixel measurement from pouch 1 (p1) to the yolk was taken directly from the screen image and converted to μm.

GenBank Accession numbers are as follows:

DQ499476 furinA;
DQ499477 furinB; and
DQ499478 barx1.

Results

furinA mutants have defects in intermediate domain elements of the pharyngeal skeleton and mildly ruffled fins

In contrast to the severe loss of ventral facial cartilage in edn1 mutants, furinA mutants typically have wild-type looking anterior arch ventral cartilages that are abnormally fused to dorsal cartilages (Fig. 1; Table 1). Cartilages of the posterior arches are also typically wild type, with a low penetrance of cartilage reduction observed in the third and fourth arches (Table 1). In addition, we observe shape changes and bony fusions between the opercle and posterior branchiostegal ray, and altered muscle insertion points near joint domains (Supplemental Fig. 1) (Kimmel et al., 2003). These phenotypes are seen in mutants bearing any of the three furinA alleles and are consistent with altered development at the interface of dorsal and ventral skeletal elements, which we previously defined as a distinct intermediate domain (Miller et al., 2003). The furinA mutants also have a ruffled fin phenotype, in which the edges of all fins, including pectoral fins, are uneven (Figs. 1E and F). Because we observe a disorganization of the collagen fibrils of the mutant fins, it is possible the fin phenotype arises due to a defect in the processing of fibrillar procollagen precursors (data not shown).

The sturgeon gene is furinA

We mapped sturgeon*tg419 and sturgeon*td204e to zebrafish chromosome 7 and identified furinA as a candidate for sturgeon (Fig. 2A; see Materials and methods). Sequencing of furinAtg419 mutants revealed a C-to-A transversion predicted to result in an early stop codon at amino acid 307, thus truncating FurinA within the catalytic domain (Figs. 2C and E). We confirmed segregation of this lesion with mutant phenotypes in 480 diploid mutants (Fig. 2B). In furinAtd204e, a T-to-A splice site mutation transforms a conserved GT donor site to GA. We confirmed by

![Fig. 3. Both FurinA and FurinB function are required to activate Edn1 signaling. Flat mounts of Alcian-stained cartilages and bones at 5–6 dpf. (A) Wild-type, (B) furinA mutant, (C) furinA MO-injected larva, (D) furinB MO-injected larva, (E) furinB MO injected into furinA mutant, (F) low edn1 MO-injected larva, (G) edn1 mutant, and (H) dlx3b MO + dlx5a MO-injected larva. Wild-type DV joint regions are indicated with arrows in panel A. Fusions at joint regions are indicated with asterisks in panels B–H. Scale bar: 50 μm.](image-url)
RT-PCR that furinA<sup>ad204e</sup> creates a skipped exon 9 within the catalytic domain (data not shown). In furinA<sup>b963</sup>, a T-to-A transversion results in a transformation of the start codon to lysine, with no potential alternate start methionines nearby in the sequenced cDNA (Figs. 2D and E). We predict that all three alleles produce null or strong loss of FurinA function.

To further confirm that a loss of FurinA function accounts for the phenotypes we observe in sturgeon mutants, we designed a splice-blocking furinA MO against the same splice site mutated in sturgeon<sup>ad204e</sup>. Injection of this MO resulted in a dose-dependent phenocopy of the sturgeon mutant cartilage phenotype (Figs. 3A–C; Table 2).

furinA expression overlaps with edn1 expression

If FurinA indeed activates Edn1 signaling, furinA expression should overlap with edn1 expression. We have shown previously that from 18 to 36 hpf, edn1 expression is ventrally restricted in the pharyngeal arches (Fig. 4E) (Miller et al., 2000). We find furinA expression to be ubiquitous before and throughout the time period that edn1 is expressed in the pharyngeal arches (Figs. 4A and C; data not shown). Thus, furinA is expressed at the right time and place for activation of Edn1.

Loss of maternal FurinA function does not enhance jaw defects in furinA mutants

Because we suppose that Furin cleavage is absolutely required for Edn1 function, how do we explain why the phenotypes of furinA and edn1 mutants do not match (Figs. 3B and G)? One hypothesis is that a maternal source of FurinA functions to partially activate Edn1 signaling. By RT-PCR analysis, we show that furinA is maternally expressed (Supplemental Fig. 2A). As a small percent (less than 2%) of furinA<sup>tg419</sup> mutants can be raised to be homozygous viable breeding adults, we bred homozygous furinA mutant females to determine the contribution of maternal furinA to jaw patterning. We did not observe a significant difference in joint losses between embryos with a loss of both maternal and zygotic furinA compared to embryos with a loss of zygotic furinA alone (Supplemental Fig. 2B). Furthermore, losses of both maternal and zygotic furinA did not result in ventral pharyngeal cartilage losses.

### Table 2

<table>
<thead>
<tr>
<th>Class</th>
<th>Number scored</th>
<th>Joint loss</th>
<th>Ventral reductions</th>
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<tr>
<td></td>
<td></td>
<td>Arch 1</td>
<td>Arch 2</td>
</tr>
<tr>
<td>furinA MO into wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninjected</td>
<td>50</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>8 ng</td>
<td>118</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>10 ng</td>
<td>146</td>
<td>1%</td>
<td>5%</td>
</tr>
<tr>
<td>12 ng</td>
<td>116</td>
<td>7%</td>
<td>23%</td>
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<tr>
<td>20 ng</td>
<td>140</td>
<td>30%</td>
<td>50%</td>
</tr>
<tr>
<td>furinB MO into wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uninjected</td>
<td>62</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>4 ng</td>
<td>82</td>
<td>0%</td>
<td>0%</td>
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<td>6 ng</td>
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<td>4%</td>
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<td>8 ng</td>
<td>90</td>
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<td>6%</td>
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<td>10 ng</td>
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</tr>
<tr>
<td>furinB MO into tg419</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Uninjected</td>
<td>20</td>
<td>30%</td>
<td>65%</td>
</tr>
<tr>
<td>1 ng</td>
<td>32</td>
<td>72%</td>
<td>100%</td>
</tr>
<tr>
<td>2 ng</td>
<td>22</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>diox&lt;sup&gt;3b&lt;/sup&gt; MO into wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 ng (SY strongly reduced = 9%)</td>
<td>66</td>
<td>6%</td>
<td>0%</td>
</tr>
<tr>
<td>diox&lt;sup&gt;5a&lt;/sup&gt; MO into wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 ng</td>
<td>72</td>
<td>0%</td>
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<tr>
<td>(SY strongly reduced = 11%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>diox&lt;sup&gt;3b&lt;/sup&gt; MO + diox&lt;sup&gt;5a&lt;/sup&gt; MO into wild type</td>
<td></td>
<td></td>
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<tr>
<td>6 ng each</td>
<td>138</td>
<td>85%</td>
<td>33%</td>
</tr>
<tr>
<td>(SY strongly reduced = 90%)</td>
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Morpholinos were injected at 1–4 cell stage, larvae fixed at 4–5 dpf, and cartilage stained with Alcian green. The joint loss phenotype refers to larvae in which the joint is lost due to fusion between dorsal and ventral cartilages at the joint region. Ventral and symplectic (SY) reduction refers to larvae in which the cartilage is less than half the size of its wild-type counterpart. Both sides of the larvae were scored, and number scored refers to sides scored. Injections of 6–10 ng of furinB MO into furinA mutants resulted in embryos with edema, shortened axis, enhanced fin defects, and strong reductions of all pharyngeal cartilages.


Fig. 4. furinA expression overlaps with edn1 expression. furinA antisense and sense in situ hybridizations at 3 somite stage (11 hpf) (A and B) and 25 hpf (C and D). (E) edn1 in situ hybridization at 30 hpf. a1 and a2 label pharyngeal arch 1 and 2. Dotted lines label pharyngeal pouches 1 and 2. Scale bar: 50 μm.
furinB and furinA were frequently less severe and also of lower penetrance than MO to arches (data not shown).

FurinA and FurinB are members of the FURIN subfamily and of zebrafish FurinA and FurinB and the 7 known mammalian redundant. At very low

do determine whether the two furin genes function partially

An alternative hypothesis to explain why a complete loss of FurinA function causes only a partial loss of Edn1 function is that furin genes are duplicated in zebrafish and have partially redundant functions. We isolated a potential furin duplicate, which we call furinB, based on sequence analysis. A phylogenetic analysis of the full-length amino acid sequences of zebrafish FurinA and FurinB and the 7 known mammalian proprotein convertase family members confirmed that zebrafish FurinA and FurinB are members of the FURIN subfamily and are co-orthologs to the single FURIN gene in mammals (Fig. 5).

Like furinA, furinB is maternally and ubiquitously expressed, and its expression also overlaps with edn1 in the pharyngeal arches (data not shown).

Both FurinA and FurinB activate Edn1 signaling

To understand the function of specific Dlx genes, we injected MOs to dlx3b, dlx5a, and dlx6a singly and in combination into wild-type embryos (Table 2, and data not shown). Single Dlx MO injections yielded a low penetrance of skeletal phenotypes, whereas combined injection of dlx3b and dlx5a MOs resulted in a high penetrance of specific skeletal defects, resembling those in furinA mutants and low edn1 MO injections (Table 2; Figs. 3B, F, and H; and data not shown). Interestingly, we did not observe significant alterations to the ventral cartilages in single or combined dlx3b, dlx5a, and dlx6a MO injections, suggesting a difference in the requirement for Dlx gene function between intermediate and ventral arch domains.

We have previously shown that hand2 is an edn1-dependent bHLH transcription factor required for ventral pharyngeal cartilages

increase in the penetrance of joint losses and a small percent of injected mutants with ventral cartilages losses in both the first and second arches (Table 2). Slightly higher doses of furinB MO into furinA mutants increases the penetrance of joint losses in the anterior arches, and ventral cartilage reductions in arches 1–4, but not in arches 5–7 (Table 2; Fig. 3E, data not shown). The similarity of this enhanced phenotype to the edn1 mutant phenotype (Fig. 3G) is striking (Miller et al., 2000).

We do not provide direct evidence for an Edn1 cleavage defect in furinA mutants, as unfortunately Edn1 is expressed at low levels and currently available antibodies fail to detect Edn1 in the pharyngeal arches. To more directly support a role for FurinA within the Edn1 pathway, we generated double mutants in furinA and edn1. We observed a strong genetic interaction between furinA and edn1, with loss of a single copy of edn1 enhancing the furinA mutant jaw phenotype. Furthermore, furinA;edn1 double mutants resemble edn1 single mutants, suggesting that Edn1 is the principal target of FurinA in controlling jaw morphogenesis (Supplemental Fig. 4).

furinA is required for early but not late edn1-dependent expression of Dlx genes and hand2

We next determined whether the expression of genes downstream of Edn1 signaling is reduced in furinA mutants. In mice, a nested code of Distalless homeodomain (Dlx) transcription factor expression patterns the DV axis of the pharyngeal arches (Depew et al., 2002). In zebrafish, dlx2a expression is at near wild-type levels in edn1 mutants at 30 hpf (Fig. 6C). The expression of dlx5a, dlx6a, and dlx3b is strongly reduced in edn1 mutants at 24 and 30 hpf (Figs. 6F, I, and L; and data not shown). At 36 hpf, these genes are still strongly downregulated, with the exception that dlx5a is expressed at near wild-type levels dorsally but not ventrally in the arches (Figs. 6O, R, and U).

In furinA mutant embryos at 24 and 30 hpf, dlx2a is expressed at near wild-type levels, whereas the expressions of dlx5a, dlx6a, and dlx3b are moderately reduced (Figs. 6B, E, H, and K; and data not shown). At 36 hpf, expressions of dlx5a, dlx6a, and dlx3b have recovered to near wild-type levels in furinA mutants with the exception that dlx3b remains reduced in the posterior region of the second arch (Figs. 6N, Q, and T).

To understand the function of specific Dlx genes, we injected MOs to dlx3b, dlx5a, and dlx6a singly and in combination into wild-type embryos (Table 2, and data not shown). Single Dlx MO injections yielded a low penetrance of skeletal phenotypes, whereas combined injection of dlx3b and dlx5a MOs resulted in a high penetrance of specific skeletal defects, resembling those in furinA mutants and low edn1 MO injections (Table 2; Figs. 3B, F, and H; and data not shown). Interestingly, we did not observe significant alterations to the ventral cartilages in single or combined dlx3b, dlx5a, and dlx6a MO injections, suggesting a difference in the requirement for Dlx gene function between intermediate and ventral arch domains.

We have previously shown that hand2 is an edn1-dependent bHLH transcription factor required for ventral pharyngeal cartilages.
(Miller et al., 2000; Miller et al., 2003). In edn1 mutants at 30 and 55 hpf, hand2 expression is strongly reduced in both the first and second arch (Figs. 7C and F). In furinA mutants, hand2 expression is strongly reduced in the first arch and moderately reduced in the second arch at 30 hpf but is expressed at near wild-type levels in both arches at 55 hpf (Figs. 7B and E).

We conclude that furinA is required for early, but not late, edn1-dependent ventral expression of Dlx genes and hand2. We also observed ectopic hand2 expression in the second arch in furinA mutants at 55 hpf (Fig. 7E, asterisks). This expression lies just dorsal to the ventral hand2 expression domain in wild-type embryos and appears to correspond to the place where we later see ectopic cartilage within the joint region of the second arch in furinA mutants. Furthermore, our analysis of dlx and hand2 expression in furinA/furinB MO embryos strongly supports the hypothesis that furinA and furinB redundantly activate edn1-dependent signaling (Supplemental Fig. 5).

Intermediate arch domain defects in furinA and edn1 mutants

We have previously hypothesized that the jaw and support joints are part of an intermediate domain of the first and second arches, respectively, specified early in development (Miller et al., 2003). For example, barx1 is expressed in the first arch intermediate domain at 32 hpf and later, at 54 hpf, the barx1 domain clearly includes the jaw joint and associated cartilages around the joint. barx1 expression is strongly reduced in both edn1 and furinA mutants (Fig. 8B) (Miller et al., 2003). To understand the intermediate domain defects in furinA mutants more fully, including the ectopic expression of hand2 in the second arch, we looked at the expression of genes regulated by Edn1 and whose expression is restricted to distinct dorsal and ventral arch domains.

At 38 hpf, goosecoid (gsc) is expressed in dorsal and ventral domains in the second arch (Miller et al., 2000). In edn1 mutants, the ventral second arch gsc expressing domain is strongly reduced, but the dorsal second arch domain is present and often slightly expanded in size (Miller et al., 2000). In furinA mutants at 45 hpf, both dorsal and ventral expression domains are present but some cells in the second arch intermediate domain ectopically express gsc (Fig. 8D, asterisk).

We analyzed the expression of barx1, a homeobox gene found to be dependent on Ednra signaling in mice (Clouthier et al., 2000). In wild-type embryos, barx1 is expressed in dorsal and ventral domains of both the first and second arches at 30 and 35 hpf, and by 48 hpf these domains are clearly separated by a nonexpressing intermediate domain (Figs. 9A–C). In edn1 mutants, we observe strong ectopic expression of barx1 within the intermediate arch domains at 48 hpf, and a reduction of barx1 expression in the ventral second arch (Fig. 9D). In furinA mutants, barx1 is ectopically expressed within the intermediate domain of the second arch at 48 hpf, with only a small percent of mutants having ectopic barx1 expression in the first arch, suggesting a difference in arch sensitivities to reductions to Edn1 signaling (Fig. 8E).
As furinA mutants have ectopic cartilage and bone within the intermediate domain of the pharyngeal arches, we looked at expression of sox9a and runx2b, early markers of chondrogenic and osteogenic potential (Eames et al., 2004; Flores et al., 2004; Yan et al., 2002, 2005). In 48 hpf wild-type embryos, the second arch intermediate domain contains sox9a positive symplectic and interhyal cartilage precursors (Fig. 8G). sox9a is expressed more weakly in these precursors than in the hyomandibular and ceratohyal precursors, located in the dorsal and ventral domains, respectively. The identification of these cartilage precursors is supported by time-lapse and fate-mapping analyses (Crump et al., 2004). In furinA and edn1 mutants, sox9a is ectopically expressed at 48 hpf within the intermediate domain of both the first and second arches, reflecting the joint fusions we observe in both mutants (Fig. 8H, and data not shown). In 55 hpf wild-type embryos, runx2b is strongly expressed in dorsal and ventral domains in the second arch separated by a nonexpressing intermediate domain, and less strongly expressed in similar domains in the first arch (Fig. 8I, and data not shown). In furinA mutants at 55 hpf, runx2b is ectopically expressed in the intermediate second arch domain, whereas in edn1 mutants this ectopic expression is variably present, consistent with the gain/loss bone phenotypes observed in both mutants (Fig. 8J) (Kimmel et al., 2003).

furinA and edn1 mutants have early defects in elongation of the ventral arch

We have observed an early defect in the DV length of the anterior pharyngeal arches in both furinA and edn1 based on the distance from the ventral aspect of pharyngeal pouch 1 to the yolk. To analyze this more carefully, we measured the distance from the ventral aspect of pharyngeal pouch 1 to the yolk in wild-type and furinA and edn1 mutant embryos at 24, 30, and 36 hpf. Between 24 and 30 hpf, wild-type embryos have a ventral arch elongation of 17.8 ± 0.8 μm (mean ± SE, n = 5) compared to 7.3 ± 1.4 μm for furinA mutants and 1.6 ± 0.6 μm for edn1 mutants (Fig. 10). Between 30 and 36 hpf, wild-type embryos have a ventral arch elongation of 17.7 ± 1.6 μm compared to 15.6 ± 1.7 μm for furinA mutants and 14.0 ± 0.9 μm for edn1 mutants. These data show that edn1 mutants have a strong defect in early elongation of the ventral arch, whereas the defect in furinA mutants is much milder, and that later elongation of the ventral arch is largely Edn1 independent. In furinA;furinB MO embryos, ventral arch length is reduced similar to edn1 mutants (Supplemental Fig. 5).

Fig. 8. Intermediate arch domains are misspecified in furinA mutants. (A, B) bapx1 expression is strongly reduced in furinA mutants at 48 hpf. (C, D) At 45 hpf, gsc is ectopically expressed in a second arch intermediate domain in furinA mutants. (E, F) barx1 is ectopically expressed in a second arch intermediate domain in furinA mutants at 48 hpf. (G, H) sox9a is ectopically expressed in a second arch intermediate domain in furinA mutants at 48 hpf. (I, J) runx2b is ectopically expressed in a second arch intermediate domain in furinA mutants at 55 hpf. Arrows indicate a second arch intermediate domain in wild-type embryos. Asterisks indicate ectopic expression in a second arch intermediate domain in furinA mutant embryos. a1 and a2 label pharyngeal arch 1 and 2. Scale bars: 50 μm.
These results raise the possibility that Edn1 signaling temporally regulates the position of the ventral Edn1 source through control of early ventral arch elongation.

Discussion

Piotrowski et al. (1996) and Kimmel et al. (1998) proposed that sturgeon and sucker (edn1) act in the same pathway controlling DV patterning in the pharyngeal arches, and here we provide molecular support for this hypothesis. We demonstrate that sturgeon encodes FurinA, providing genetic evidence that a major Furin function during craniofacial development is to activate Edn1 signaling.

Partial redundancy of duplicate furin genes in early embryonic development

The duplicated furin co-orthologs, furinA and furinB, both function in craniofacial development. Partially reducing furinB function in furinA mutants copies the severe phenotypes of edn1 mutants, demonstrating that both furin genes are critical Edn1 activators. Stronger knockdown results in severe early embryonic defects, reminiscent of knocking out the single furin gene in mice (Roebroek et al., 1998). Hence, redundancy in furin function probably extends to activation of other signaling pathways. At the same time, all of our evidence suggests that Edn1 is the major Furin target promoting jaw development. The duplication of furin genes in zebrafish has enabled us to genetically dissect out this specific role of furin in craniofacial development.
development from its many roles in early embryonic development.

Critical levels of Edn1 signaling and DV patterning

We observe DV-nested expression of Dlx genes, under control of Edn1, and reminiscent of the pattern in mouse that functional analyses reveal control DV skeletal fates (Depew et al., 2005). At 24 hpf, dlx2a is broadly expressed and dlx3b, dlx5a, and dlx6a are more ventrally restricted to regions that develop both intermediate and ventral arch skeletal fates (Crump et al., 2004; Crump, personal communication), and which are most sensitive to Edn1 loss. furinA and edn1 mutants have reduced early expression of dlx3b, dlx5a, and dlx6a, suggesting that Edn1 signaling initially specifies a domain containing both intermediate and ventral arch fates through the regulation of Dlx genes. The pattern is dynamic: At 30 and 36 hpf, the expression of dlx5a extends more dorsally than that of dlx3b and dlx6a, but is still nested relative to dlx2a, and includes the intermediate domain. The ventral expression of the Edn1-dependent Dlx genes recovers at 36 hpf in furinA mutants but not in edn1 mutants, suggesting that a critical level of early Edn1 signaling, achieved in furinA mutants but not in edn1 mutants, is required for the late ventral Dlx gene expression. That this recovery is functionally significant is suggested by the matching skeletal phenotypes of furinA mutants, low edn1 MO-injected embryos, and combined dlx3b and dlx5a MO-injected embryos, in which the prominent defects are in the intermediate domain, not the ventral domain. Interestingly, in edn1 mutants we do observe late dlx5a expression in a dorsal but not ventral arch domain. It is possible that this late dorsal dlx5a expression is largely edn1 independent as mice mutant in Edn1 and Ednra also have late dorsal but not ventral dlx5a expression (Ozeki et al., 2004; Ruest et al., 2004).

Edn1 also regulates hand2 expression, present in the ventral arches, and essential for ventral skeletal development (Miller et al., 2000, 2003; Yelon et al., 2000). Whereas edn1 mutants have a strong reduction of arch hand2 expression, an early reduction in furinA mutants then recovers to near wild-type levels, explaining their nearly normal ventral pharyngeal cartilages. These results also suggest that a critical level of Edn1 signaling, which is reached in furinA mutants but not in edn1 mutants, is required to maintain late hand2 expression.

How might a critical level of Edn1 signaling function to promote late Dlx and hand2 expression? Late Hand2 expression in mouse mandibular mesenchyme is dependent on early Edn1/Ednra signaling but later becomes independent (Fukuhara et al., 2004): epithelial-derived signals, possibly Fgfs, function as signal relays to promote late Hand2 expression only if the mandibular mesenchyme had been “primed” by early Edn1/Ednra signaling. In support of this conclusion, late Ednra conditional knockout resulted in normal late Hand2 and Dlx5 expression and normal mandible patterning (Ruest et al., 2005). Our results with recovery of Dlx and hand2 expression in furinA mutants fit nicely with these findings and further suggest that a critical level of Edn1 signaling, that is achieved in furinA mutants, is required to prime cranial neural crest cells to respond to later Edn1-independent relay(s).

Temporal model for Edn1 regulation of intermediate/ventral arch domain patterning

In furinA mutants and low edn1 MO knock downs, we see specific defects in intermediate arch fates. Why is this domain so exquisitely sensitive to reductions in Edn1 signaling? We propose that a Dlx code (dlx3b, dlx5a, and dlx6a) specifies a domain that contains both intermediate and ventral arch fates. That hand2 expression, whose transcription in mice has been shown to be regulated through a Dlx6-dependent enhancer element, is more ventrally restricted than dlx6a expression, suggests other factors in addition to dlx6a must be responsible for restricting hand2 expression to a smaller domain (Fig. 11A) (Charite et al., 2001).

How then are intermediate and ventral arch fates specified to be different? We had previously proposed compartmentalization into three arch domains based in part on gsc expression at 38 hpf (Miller et al., 2000). Here we show that like gsc, barx1 is patterned in three compartments in the second arch-dorsal and ventral expression separated by a nonexpressing intermediate domain. Both gsc and barx1 are ectopically expressed within the intermediate domain in furinA and edn1 mutants, indicating Edn1 normally represses their expression within this region. Further, we observe expansion of sox9a and runx2b into the intermediate domain of furinA mutants, where we later observe ectopic cartilage and bone. These altered gene expression patterns all suggest misspecification of the intermediate domain.

One possibility is that Edn1-dependent elongation of the ventral arch is critical to properly segregate intermediate and ventral fates, and the segregation never occurs in furinA and edn1 mutants, both of which have early defects in ventral arch elongation. Early reduction of arch length would have the effect of placing a ventral Edn1 source closer to where the intermediate domain should form and result in its loss (Fig. 11). In furinA mutants, a sufficient level of Edn1 signal is achieved to specify intermediate and ventral arch fates through Dlx gene expression, but not to properly segregate these fates due to the early elongation defects. In edn1 mutants, an insufficient level of Edn1 signal results in both a failure to specify and to segregate intermediate and ventral arch fates. Furthermore, elongation of the ventral arch may promote repeated subdivisions of the ventral arch into sequentially smaller domains, allowing for fine control of facial patterning. It is of great interest to identify the relay(s), currently unknown in mice as well as fish, promoting Edn1-dependent arch elongation. Ongoing studies are in progress to identify these molecules. Further analysis of Edn1 pathway mutants, such as furinA, will also enable us to more fully dissect the requirement for Edn1 signaling in patterning specific skeletal elements within pharyngeal arches.

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Appendix A. Supplementary data

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References


