Gremlin 2 regulates distinct roles of BMP and Endothelin 1 signaling in dorsoventral patterning of the facial skeleton

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SUMMARY
Patterning of the upper versus lower face involves generating distinct pre-skeletal identities along the dorsoventral (DV) axes of the pharyngeal arches. Whereas previous studies have shown roles for BMPs, Endothelin 1 (Edn1) and Jagged1b-Notch2 in DV patterning of the facial skeleton, how these pathways are integrated to generate different skeletal fates has remained unclear. Here, we show that BMP and Edn1 signaling have distinct roles in development of the ventral and intermediate skeletons, respectively, of the zebrafish face. Using transgenic gain-of-function approaches and cell-autonomy experiments, we find that BMPs strongly promote hand2 and msxe expression in ventral skeletal precursors, while Edn1 promotes the expression of nkx3.2 and three Dlx genes (dlx3b, dlx5a and dlx6a) in intermediate precursors. Furthermore, Edn1 and Jagged1b pattern the intermediate and dorsal facial skeletons in part by inducing the BMP antagonist Gremlin 2 (Grem2), which restricts BMP activity to the ventral-most face. We therefore propose a model in which later cross-inhibitory interactions between BMP and Edn1 signaling, in part mediated by Grem2, separate an initially homogenous ventral region into distinct ventral and intermediate skeletal precursor domains.

KEY WORDS: BMP, Edn1, Gremlin 2, Jagged1, Notch, Craniofacial, Skeleton, Zebrafish, Dorsoventral patterning

INTRODUCTION
The facial skeleton develops from cranial neural crest cells (CNCCs) that populate a series of segments called the pharyngeal arches (Platt, 1893). Subsequently, skeletal elements of varying morphology develop from distinct DV domains within the arches (Crump et al., 2006; Eberhart et al., 2006), although a one-to-one correspondence between specific elements and DV expression domains has not been established. Initially, ventral CNCCs, unlike their dorsal counterparts, co-express Hand2 and the Dlx family members Dlx5 and Dlx6 (Charite et al., 2001). As development progresses, DV gene expression becomes further segregated within the arches, with ventral CNCCs of zebrafish expressing hand2, intermediate CNCCs expressing dlx3b, dlx5a, dlx6a and nkx3.2, and dorsal CNCCs expressing jag1b (Talbot et al., 2010; Zuniga et al., 2010). Mice also show a similar separation of ventral Hand2 and more intermediate Dlx5/6 expression (Barron et al., 2011). An important issue is how such distinct preskeletal domains are specified during development.

All three classes of genes (Hand2, Dlx and Jag1b) are required to form distinct DV structures of the facial skeleton. Loss of Hand2/hand2 function leads to reductions of the ventral skeletal expansion and expression of intermediate fates (Miller et al., 2003; Yanagisawa et al., 2003; Talbot et al., 2010), whereas Hand2 misexpression transforms the dorsal facial skeleton to a ventral morphology in mice (Sato et al., 2008). Dlx5–/–; Dlx6–/– compound mutants display loss of ventral Hand2 expression and transformation of the lower (ventral) jaw skeleton (Beverdam et al., 2002; Depew et al., 2002), and Dlx3b/4b/5a in zebrafish have important roles in development of the intermediate skeleton such as the jaw joint (Talbot et al., 2010). Similarly, reduction of Nkx3.2 in zebrafish causes joint fusions in the mandibular arch (Miller et al., 2003). Recent studies in zebrafish have also shown a prominent role for Jagged1b-Notch2 signaling in specifying the dorsal skeletal domain (Zuniga et al., 2010). Hence, at least in zebrafish, there is a clear functional separation between ventral, intermediate, and dorsal skeletons within the arches, and their disruption leads to specific craniofacial malformations.

Edn1 signaling specifies ventral and intermediate skeletal derivatives in the arches. Deficiencies in Edn1 or its receptors (Ednra in mouse and Ednra1/Ednra2 in zebrafish) result in reductions and/or dorsalization of the ventral and intermediate facial skeletons (Kurihara et al., 1994; Miller et al., 2000; Ozeki et al., 2004; Ruest et al., 2004; Nair et al., 2007). Cells lose expression of Dlx3-6/dlx3-6, Hand2/hand2, Nkx3.2/nkx3.2, Msx1/msxe and epha4b in the arches in Edn1–/– and Ednra–/– mouse mutants and edn1–/– zebrafish mutants (Miller et al., 2000; Ozeki et al., 2004; Ruest et al., 2004; Walker et al., 2006; Walker et al., 2007). Conversely, transgenic misexpression of Edn1 in mice or injection of human EDN1 protein in zebrafish transforms the dorsal skeleton (Kimmel et al., 2007; Sato et al., 2008). Edn1 also restricts Jagged1b-Notch2 activity to dorsal CNCCs in zebrafish, with loss of jag1b partially restoring ventral skeletal patterning in edn1 mutants (Zuniga et al., 2010). Notably, the facial skeleton forms largely normally in the absence of both Edn1 and Jagged1b-Notch2 signaling, suggesting the presence of additional signals that promote ventral skeletal identity.

BMP signaling is likely to be one such pathway that plays a role in development of the ventral facial skeleton (reviewed by Nie et al., 2006). Members of the Bmp2/4/7 subfamily are expressed in the arch epithelia of Nkx2.5CRE; Bmp4lacZ/flox mice reduces Hand2, Msx1 and Msx2 expression in ventral CNCCs and reduces/transforms the ventral mandibular skeleton (Liu et al., 2004; Liu et al., 2005). However, gain-of-function BMP experiments have given
conflicting results. In some cases, Bmp4-coated beads induce the formation of branched/duplicated Meckel’s cartilages (Mina et al., 2002; Mariani et al., 2008), but in other cases they cause CNCC death and skeletal loss (Shigetani et al., 2000; Mariani et al., 2008). BMPs also function in many other facets of CNCC development, such as induction (Lien et al., 1995; Nguyen et al., 1998; Steventon et al., 2009), apoptosis (Graham et al., 1994), migration (Kanzler et al., 2000) and skeletogenesis (Wozney et al., 1988), which complicates the interpretation of these studies. A further obstacle is genetic redundancy among BMPs. In zebrafish, four members of the Bmp2a/4 family – bmp2a, bmp2b, bmp4 and bmp7b – are expressed in the developing pharyngeal arches (Holzschuh et al., 2005; Wise and Stock, 2010). As such, loss-of-function studies have yielded little insights into DV patterning roles (Holzschuh et al., 2005; Wise and Stock, 2010). As such, loss-of-function analyses, we show that Grem2 promotes dorsal and intermediate skeletal fates by restricting BMP activity to the ventral arches. Edn1 and Jagged1b are also required for craniofacial development have not been previously investigated. With gain- and loss-of-function analyses, we show that Grem2 promotes dorsal and intermediate skeletal fates by restricting BMP activity to the ventral arches. Edn1 and Jagged1b are also required for BMP and Edn1 activity during craniofacial development. In so doing, we show that BMPs and Edn1 have distinct roles in establishing the dorsal and intermediate domains of the arches, respectively.

Several types of BMP antagonists regulate BMP activity, indicating that precise levels of BMP signaling are crucial for developmental patterning. Early arch primordia in the mouse express Noggin and Chordin, and mutations in either BMP antagonist disrupts development of the ventral mandibular skeleton (Stottmann et al., 2001). By contrast, members of the Gremlin family of BMP antagonists, including grem2 (prdc1) in zebrafish (Müller et al., 2006), are expressed in the arches at later stages (Hsu et al., 1998; Bardot et al., 2001). Functions for Gremlin proteins in craniofacial development have not been previously investigated. With gain- and loss-of-function analyses, we show that Grem2 promotes dorsal and intermediate skeletal fates by restricting BMP activity to the ventral arches. Edn1 and Jagged1b are also required for grem2 expression, suggesting that they promote intermediate and dorsal skeletal fates in part through Grem2-mediated repression of BMP activity.

MATERIALS AND METHODS
Zebrafish lines
Zebrafish were staged as described previously (Kimmel et al., 1995). We used the following mutant and transgenic strains: edn1(tf216b) (Zuniga et al., 2010) Tg(UAS:Bmp4;cmcl2:GFP)el49 and Tg(UAS:Bmp4;cmcl2:GFP)el47 (Sheer and Campos-Ortega, 1999), Tg(hsp70I:dnBmp1a-GFP)el40 (Pyati et al., 2005) and Tg(BRE:Bmp4;Grem2:GFP)el30 (Alexander et al., 2011). Tg(UAS:Bmp4;cmcl2:GFP)el40, Tg(UAS:Edn1;α-crystallin:Cerulean)el249 and Tg(UAS:Grem2;α-crystallin:Cerulean)el264 transgenic lines were generated using Gateway Cloning (Invitrogen) and the Tol2kit (Kwan et al., 2000).

β-galactosidase activity
Heat-shock treatments
For hsp70:Gal4; UAS:Bmp4 and hsp70:Gal4; UAS:Edn1 activations, embryos were placed in a programmable incubator at 40°C for 4-8 hours, as indicated, and then returned to 28.5°C. hsp70:dnBmp1a-GFP and hsp70:Gal4; UAS:Grem2 embryos were placed in a thermocycler at 39°C from 16-17 hours post-fertilization (hpf) for heat-shock induction. For shorter hsp70:Gal4; UAS:Bmp4 treatments, embryos were placed in 40°C pre-warmed embryo media at 21 hpf and transferred to 28.5°C embryo media after 1 or 3 minutes.

Morpholino injections
One-cell stage embryos were injected with 3 nl of hand2-morpho (MO) (600 μM) (Maves et al., 2009), grem2-MO #1 (300 or 600 μM), or grem2-MO #2 (400 μM) (GeneTools, Philomath, OR, USA). grem2-MO #1 (5’-GACAGAGCGCCACCATCTACTCATCT-3’) and grem2-MO #2 (5’-CTCGACACCTGTAAGGTTGATATG-3’) are translation blockers. Grem2:GFP was constructed by performing fusion PCR of the Grem2 cDNA template with primers Grem2-GFP-F (5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTTGTACAGCTC-3’) and Grem2-GFP-R (5’-GGGGACAAGTTTGTACCAAAAGAAGAGCTCGGCCACCATGAGCAGTAAGGTGGCGCTG-3’). For the in vitro translation, Grem2:GFP-1M (5’-AGTAAGGTGGCGCTGTGTCT-3’) and Grem2:GFP-2M (5’-GAGCGTGTCCGAGTCGGGGAAACAGCGCCACCTATCCGTCTACTGT-3’) were used with the GFP template with the following primers: Grem2:GFP-F (5’-GAGCGTGTCCGAGTCGGGGAAACAGCGCCACCTATCCGTCTACTGT-3’), Grem2:GFP-R (5’-GAGCGTGTCCGAGTCGGGGAAACAGCGCCACCTATCCGTCTACTGT-3’).

In situ hybridization and skeletal analysis
Skeletal staining and in situ hybridization analysis were as described previously (Zuniga et al., 2010). bmp4 and grem2 probes were synthesized with T7 RNA polymerase from PCR products amplified with the following primers: Bmp4-L (5’-GTGAGGCGCAACTCTTCTCTAG-3’), Bmp4-R (5’-GTAACTACGAGCTCATAATAGGTGTCTTATCCGACGACCCACCTCCT-3’), Edn1-L (5’-GGGGCAGCAGTTTGCTACAAAAGAAGACTCGTTTGTATCTATG-3’) and Edn1-R (5’-GGGGGACACATTTTACAAAGAAGCTGGGCCTGTTAGATGGTCTCTTGTTTCAAATCC-3’), Grem2-L (5’-GGGGGACACATTTTACAAAGAAGCTGGGCCTGTTAGATGGTCTCTTGTTTCAAATCC-3’) and Grem2-R (5’-GGGGGACACATTTTACAAAGAAGCTGGGCCTGTTAGATGGTCTCTTGTTTCAAATCC-3’). In all other probes are as described previously (Zuniga et al., 2010). Skeletal and colorimetric in situ hybridization images were acquired on a Leica D2500 upright microscope. Fluorescent images were captured on a Zeiss LSM 510 confocal microscope using ZEN software and presented as sections or flattened projections as indicated. Levels were adjusted in Adobe Photoshop CS4, with identical adjustments applied to images from the same dataset.

Cell transplantation
Tissue transplants were performed as described by Crampton et al. (2004). Briefly, donor cells from hsp70:dnBmp1a-GFP or flila:GFP embryos were transplanted into the CNCC precursor domain of wild-type 6 hpf hosts, and hosts were subjected to heat-shock induction from 16-17 hpf. Fluorescent in situ hybridization analysis was performed first with dtc3b, msxe or hand2 probes, followed by immunohistochemistry (Crampton et al., 2004) using 1:1000 rabbit polyclonal anti-GFP primary antibody (Torrey Pines Biolabs, East Orange, NJ, USA) and 1:300 AlexaFluor488 goat anti-rabbit secondary antibody (Invitrogen).
Using JMP 7.0 software, a Tukey-Kramer HSD test (α=0.05) was employed to show significance between multiple classes.

RESULTS

**Bmp4 and Edn1 are expressed in non-overlapping domains of ventral arch ectoderm**

Whereas previous studies have shown that bmp2a, bmp2b, bmp4 and bmp7b are expressed in or around the pharyngeal arches of zebrafish (Holzschuh et al., 2005; Wise and Stock, 2010), their expression relative to developing CNCCs had not been thoroughly characterized. Here, we found that bmp4 expression was restricted to ventral arch ectoderm at 24 hpf (Fig. 1A) and became localized to two domains of ventral ectoderm in the anterior mandibular and posterior hyoid arches at 36 hpf (Fig. 1D). Interestingly, edn1 expression also localized to ventral arch ectoderm at these stages (Fig. 1B,E), but did so in a slightly more dorsal domain that did not overlap with bmp4 expression (Fig. 1C,F). These distinct expression domains may indicate distinct roles in DV skeletal patterning.

**Distinct effects of Bmp4 and Edn1 misexpression on facial skeleton development**

To test the relative roles of Bmp4 and Edn1 in arch development, we took a gain-of-function approach. We created transgenic lines (UAS:Bmp4 and UAS:Edn1) in which zebrafish Bmp4 or Edn1 are expressed under the control of the Gal4-sensitive UAS promoter. In embryos doubly transgenic for these UAS lines and the heat-shock inducible hsp70I:Gal4 vector (Scheer and Campos-Ortega, 1999), the timing and dose of Bmp4/Edn1 is regulated by the stage and duration of heat-shock treatment. Strikingly, Tg(hsp70I:Gal4; UAS:Bmp4) embryos (referred to as UAS:Bmp4) subjected to heat-shock at postmigratory CNCC stages (20-24 hpf), had a range of defects in the dorsal and intermediate skeletons (Fig. 1H, Table 1;

<table>
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<tr>
<th>Table 1. Facial skeletal defects in UAS:Bmp4 and UAS:Edn1 zebrafish larvae</th>
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<td>Control</td>
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Percentage of animals showing defects. Arch-derived domains such as dorsal (D), intermediate (I), ventral (V) and maxillary (Mx) are listed next to each element. Intermediate-derived elements affected in UAS:Bmp4 but not UAS:Edn1 larvae are in bold. Animals displaying severe loss of the facial skeleton were not included.
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supplementary material Fig. S1), consistent with those induced by BMP4/7 beads (Alexander et al., 2011). Phenotypic variability was reflected in different levels of BMP activation as revealed by bmp4 expression and a BMP-response-element:GFP (BRE:GFP) transgenic line (Alexander et al., 2011) (supplementary material Fig. S1). Previous fate mapping and gene expression studies have shown that in the mandibular arch: (1) dorsal CNCCs generate the posterior portion of the palatoquadrate (Pq) cartilage; (2) intermediate CNCCs form the jaw joint and joint-proximal regions of Pq and Meckel’s (M) cartilage; and (3) ventral CNCCs form the majority of M. In the hyoid arch: (1) dorsal CNCCs form the hyomandibular (Hm) cartilage and opercle (Op) bone; (2) intermediate CNCCs form the symplectic (Sy), the joint, branchiostegal ray (Br) bones, and joint-proximal regions of ceratohyal (Ch) cartilage; and (3) ventral CNCCs form the majority of Ch (Fig. 1G). Defects in BMP-overexpressing embryos were most striking in the hyoid arch, where the dorsal Hm was typically transformed and fused in a mirror-image pattern to the ventral Ch, and intermediate Sy and joints were lost (Fig. 1H). In less severe classes, the dorsal Op bone was transformed to resemble the more ventral Br bone to which it fused (supplementary material Fig. S1F). In the mandibular arch, Pq and its Ptp process (a maxillary-derived element) became rod-shaped and resembled the ventral M, similar to effects of ectopic Bmps. However, in marked contrast to Bmp4 misexpression, Edn1 misexpression never altered the intermediate-domain-derived joints or Sy in the hyoid arch. Hence, whereas Bmp4 misexpression affects development of both the dorsal and intermediate regions of the facial skeleton, Edn1 misexpression defects are largely confined further dorsally.

**Distinct effects of Bmp4 and Edn1 misexpression on DV gene expression**

We next asked whether misexpression of Bmp4 and Edn1 has distinct effects on DV gene expression. Strikingly, Bmp4 misexpression strongly upregulated expression of hand2 throughout arch CNCCs at 36 hpf (Fig. 2B,G), whereas Edn1 misexpression slightly reduced hand2 expression (Fig. 2C,H). By contrast, expression of the intermediate genes dlx3b, dlx5a and dlx6a was expanded throughout arch CNCCs of all UAS:Edn1 embryos, yet was variably reduced or mosaically expanded in different UAS:Bmp4 arches (Figs 2, 3). Expression of the intermediate (joint) marker nks3.2 was also expanded in UAS:Edn1 embryos and absent in UAS:Bmp4 embryos (Fig. 3J-L). We next analyzed the expression of eph4b, as well as msxe, which marks a ventral subset of the broader dlx3b-expressing intermediate domain in wild types (Fig. 3S-U). Whereas msxe and eph4b were markedly expanded in UAS:Bmp4 embryos, they were only moderately so in UAS:Edn1 embryos (Fig. 3A-F). Furthermore, dorsal genes such as jag1b and hey1 were similarly reduced in UAS:Bmp4 and UAS:Edn1 embryos (Fig. 3M-R). In contrast to

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**Fig. 2. Distinct effects of Bmp4 and Edn1 misexpression on hand2 and dlx3b/5a expression.** (A-J) Confocal sections of in situ hybridization for hand2 (green) with dlx3b (red, A-E) or dlx5a (red, F-J) in the mandibular (1) and hyoid (2) arches of 36 hpf control hsp70I:Gal4 (A,F) and hsp70I:Gal4; UAS:Bmp4 embryos subjected to a 20-24 hpf heat-shock (B,G), as well as hsp70I:Gal4; UAS:Edn1 embryos subjected to a 20-28 hpf heat-shock (C,H). (A,F) In controls, hand2 (n=48/48) was restricted to the ventral domain, and dlx3b (n=30/30) and dlx5a (n=18/18) to intermediate domains. (B,G) In UAS:Bmp4 embryos, hand2 was upregulated (n=23/32), dlx3b was variably expanded (n=11/21) or reduced (n=9/21), and dlx5a was also variably expanded (n=4/20) or reduced (n=9/20). (C,H) In UAS:Edn1 embryos, hand2 was reduced (n=34/34) and dlx3b (n=20/20) and dlx5a (n=14/14) were expanded. (D,E,I,J) Un-injected hsp70I:Gal4; UAS:Bmp4 embryos subjected to a 3-minute heat-shock at 21 hpf (D,I) never showed co-localization of hand2 with dlx3b (n=0/29) or dlx5a (n=0/21), whereas hand2 colocalized with dlx3b (n=21/35) and dlx5a (n=16/16) in hand2-MO-injected embryos (E,J). Anterior is towards the left and dorsal is upwards. Scale bar: 50 μm.
Cell-autonomous requirements for BMP signaling in CNCCs

Whereas reduction of BMP signaling in Tg(hsp70I;dnBmpr1a-GFP) embryos results in loss of hand2, msxe and dlx3b expression, BMP signaling also regulates edn1 expression in the ventral ectoderm (Alexander et al., 2011). To discriminate cell-autonomous roles of BMP signaling from indirect roles such as edn1 regulation, we transplanted wild-type Tg(fli1a:GFP) or Tg(hsp70I;dnBmpr1a-GFP) CNCC precursors into wild types and examined gene expression in the GFP+ donor cells. Whereas wild-type fli1a:GFP donor CNCCs showed normal dlx3b (n=5), msxe (n=3) and hand2 (n=5) expression when compared with unlabeled host CNCCs, hsp70I:dnBmpr1a-GFP+ donor CNCCs showed a cell-autonomous lack of hand2 (n=5/5) and msxe (n=6/6) expression yet no change in dlx3b (n=0/6) expression (Fig. 4). Thus, our mosaic analyses indicate that BMP signaling acts cell-autonomously in CNCCs for ventral (hand2 and msxe) but not intermediate (dlx3b) gene expression.

Bmp4 can induce hand2 and msxe independently of Edn1

The cell-autonomous requirement for BMP responsiveness in hand2 and msxe expression suggests that BMPs promote expression directly, rather than through induction of ectodermal edn1 expression. To further investigate this model, we analyzed whether ectopic Bmp4 can induce DV gene expression in the genetic absence of Edn1. As previously reported (Miller et al., 2003; Talbot et al., 2010), we tested whether the strong induction of Hand2 by Bmp4 caused the loss of intermediate gene expression seen in our gain-of-function experiments. Indeed, reduction of Hand2 function with a hand2-MO (Maves et al., 2009) resulted in expansion of dlx3b and dlx5a expression through the arches of UAS:Bmp4 embryos (Fig. 2E,F). Hence, Bmp4 acts in a dose-dependent manner during DV patterning, with lower BMP promoting intermediate gene expression and higher BMP promoting Hand2, which subsequently inhibits expression of intermediate Dlx genes.

Edn1 and Jag1b promote Gremlin2 expression in dorsal and intermediate CNCCs

As BMPs strongly promote hand2 expression, yet normally only do so in the ventral-most regions of the arches, we investigated whether BMP antagonists restrict BMP signaling ventrally. Gremlin2 was a good candidate as it is expressed in the arches during these
early stages of DV patterning (Müller et al., 2006). Using double fluorescent in situ hybridization with the CNCC marker dlx2a, we found that grem2 was expressed in dorsal and intermediate CNCCs of the arches (Fig. 6A; supplementary material Fig. S4A). The grem2 expression domain partially overlaps with intermediate dlx3b and dorsal jag1b expression, and most strongly overlaps with expression of the Jag1b-Notch2 target gene hey1 (supplementary material Fig. S4). Consistently, we found that grem2 expression was substantially reduced in jag1b<sup>b1105</sup> mutants (Fig. 6B). grem2 expression was also reduced in edn1<sup>−/−</sup> mutants and expanded in UAS:Edn1 embryos (Fig. 6C,D). This induction by Edn1 is required to suppress BMP signaling in intermediate and dorsal domains, as arch expression from a BRE:GFP transgenic line (Alexander et al., 2011) expanded in edn1<sup>−/−</sup> mutants (Fig. 6G,H). By contrast, BMPs inhibit grem2 as expression was shifted ventrally in hsp70I:dnBmpr1a-GFP embryos and lost in UAS:Bmp4 embryos (Fig. 6E,F). Hence, a combination of Jag1b and Edn1 activation and BMP inhibition restricts grem2 expression to dorsal-intermediate CNCCs.

**Grem2 is required for dorsal and intermediate skeletal patterning**

To investigate whether Grem2 is required to restrict BMP activity to ventral CNCCs, we designed two independent translation-blocking MOs against grem2. Of the two MOs, grem2-MO #1 was used for further analysis as it most effectively blocked translation from a Grem2:GFP fusion construct containing the MO-recognition site (Fig. 7A). Injection of grem2-MO into BRE:GFP fish increased BMP activity in the dorsal and intermediate arches at 36 hpf (Fig. 7D,E). In addition, grem2-MO caused dorsal and intermediate skeletal defects similar to those seen with moderate increases in BMP signaling (Fig. 7J; supplementary material Fig. S5). Skeletal transformations were most apparent in the hyoid arch, with the dorsal Hm adopting a rod-shaped morphology and the Op bone acquiring a finger-like appearance (n=21/36; supplementary material Fig. S5B), and less frequently Hm and the intermediate Sy and joints were lost (n=14/36; supplementary material Fig. SSC). Consistent with these dorsal and intermediate skeletal defects, the expression of dlx3b, and to a lesser extent hand2, was moderately expanded in 36 hpf grem2-MO-injected embryos (Fig. 7G). As with moderate Bmp4 misexpression (Alexander et al., 2011), reducing Grem2 function rescued development of the ventral (M and Ch) but not the intermediate (Sy and joints) skeleton in 15/24 edn1<sup>−/−</sup> mutants (Fig. 7M). These effects were specific because: (1) co-injection of grem2-MO #1 and #2 at sub-threshold doses caused highly penetrant synergistic effects on dorsal skeletal development; (2) grem2-MO #2 also restored the ventral facial skeleton in 6/12 edn1<sup>−/−</sup> mutants; and (3) arch misexpression of Grem2 (see details below) partially rescued the dorsal skeletal defects of grem2-MO-injected embryos (supplementary material Fig. S5). These data strongly indicate that Grem2 is required for patterning of the dorsal and intermediate facial skeleton.

**Grem2 misexpression dorsalizes the ventral facial skeleton**

In order to test Grem2 sufficiency in dorsal skeletal patterning, we misexpressed it in the arches by subjecting Tg(hsp70I:Gal4; UAS:Grem2) embryos to a 16-17 hpf heat shock (referred to as UAS:Grem2). Similar to the skeletal defects of edn1<sup>−/−</sup> mutants (Fig. 7L) and hsp70I:dnBmpr1a-GFP embryos (Alexander et al., 2011), Grem2 misexpression caused specific defects in the ventral and intermediate skeletons. In particular, the ventral (M and Ch) and intermediate (Pq and Sy) cartilages were variably reduced and altered in shape, and the intermediate-domain-derived joints were lost in 56/72 UAS:Grem2 larvae (Fig. 7K; supplementary material Fig. SSG,H). Consistent with Grem2 inhibiting ventral and intermediate skeletal development, ventral hand2 and intermediate dlx3b expression were almost completely lost in UAS:Grem2 embryos (Fig. 7H), again resembling hsp70I:dnBmpr1a-GFP (Alexander et al., 2011) and edn1<sup>−/−</sup> (Fig. 5E) embryos. We
therefore conclude that the ventral exclusion of grem2 expression is crucial for development of the ventral and intermediate facial skeleton.

**DISCUSSION**

Here, we show that BMP and Edn1 signaling play distinct roles in specifying ventral and intermediate domains, respectively, of the pharyngeal arches. Whereas misexpression of Bmp4 or Edn1 can partially compensate for the loss of the other at early stages, we find that BMP activity later becomes restricted and plays a more prominent role in development of the ventral-most facial skeleton. This restriction of BMP activity to the ventral face is accomplished in part by Edn1- and Jag1b-mediated induction of the BMP antagonist Grem2 in the intermediate and dorsal face. Together, these results support a model of DV facial patterning in which cross-inhibitory interactions between initially redundant BMP and Edn1 signaling pathways result in the segregation of facial skeletal precursors into distinct ventral and intermediate domains.

BMPs and Edn1 have distinct roles in DV patterning of the face

Whereas Edn1 and BMP signaling are both required for ventral and intermediate facial skeletal development (Alexander et al., 2011), our gain-of-function studies reveal that misexpression of Bmp4 but not Edn1 disrupts the development of the intermediate skeleton, including the joints. Such a result is consistent with BMPs having a distinct role in promoting ventral at the expense of intermediate skeletal fates. These different roles of BMPs and Edn1 in ventral versus intermediate facial patterning are also reflected in the earlier regulation of DV gene expression. Whereas Bmp4 misexpression strongly induces the ventral genes *hand2* and *msxe*, Edn1 more prominently induces intermediate genes such as *dlx3b/5a/6a* and *nkx3.2*. Previous studies have shown that as arch development progresses, *hand2* becomes restricted to a distinct ventral domain from the more intermediate expression of *dlx3b/5a/6a* and *nkx3.2* (Miller et al., 2003; Talbot et al., 2010). Moreover, we show here that DV gene expression is further refined, with *msxe* expression...
marking a ventral-intermediate domain within the broader dlx3b-positive intermediate domain. Hence, BMPs may serve to segregate ventral hand2+/msxe+/dlx3b- and ventral-intermediate hand2+/msxe-/dlx3b+ skeletal precursors from more intermediate hand2-/msxe-/dlx3b- precursors (Fig. 8).

Our findings in zebrafish also agree with those in avians and mice showing that Msx1, Msx2 and Hand2 are regulated by BMP signaling (Tucker et al., 1998; Liu et al., 2004; Liu et al., 2005; Mariani et al., 2008), whereas Dlx3, Dlx5 and Dlx6 (but not Hand2) are strongly induced by Edn1 (Sato et al., 2008). Although BMPs promote edn1 expression in the ectoderm (Alexander et al., 2011), two lines of evidence argue that BMP signaling also regulates hand2 and msxe expression more directly: (1) Bmp4 can induce the expression of hand2 and msxe, but not dlx3b, in the genetic absence of Edn1; and (2) BMP responses are required cell-autonomously in CNCCs for the expression of hand2 and msxe but not dlx3b. We therefore conclude that BMP signaling probably functions directly to regulate Hand and Msx gene expression in the ventral arches, but may function indirectly through Edn1 to regulate Dlx family expression in intermediate domains. Conversely, the more prominent role of Edn1 in intermediate skeletal development would explain why the intermediate skeletal is particularly sensitive to partial reductions of Edn1 signaling (Miller and Kimmel, 2001; Walker et al., 2006).

An important consideration is that the roles of BMPs and Edn1 may change as arch development progresses. DV gene expression is highly dynamic within the developing pharyngeal arches, with Hand2 and Dlx family gene expression colocalizing in the early ventral arches and later becoming segregated into distinct ventral and intermediate domains (Talbot et al., 2010; Barron et al., 2011). Dlx5 and Dlx6 are required for the initial arch expression of Hand2 in mice (Depew et al., 2002; Ruest et al., 2004), and we find that BMPs and Edn1 have overlapping roles in early dlx5a/6a expression (Alexander et al., 2011). However, once Hand2 reaches a specific level it begins to inhibit Dlx family and Nkx3.2 gene expression (Alexander et al., 2011). Thus, as arch development progresses, arch elongation and the expression of Greml2 in dorsal-intermediate domains would progressively restrict BMP activity and hence Hand2 to the ventral-most arches, where it would inhibit Dlx family and Nkx3.2 expression. In this model, the lack of Hand2 in the intermediate domain presumably allows continued Dlx family and Nkx3.2 expression (Fig. 8B).

Edn1 and Jag1b function through Greml2 to restrict BMP activity to the ventral face

Our genetic data indicate that the later restriction of BMP activity to the ventral arches is crucial for proper development of intermediate and dorsal skeletal precursors. Whereas previous studies in mice have shown roles for the BMP antagonists Noggin and Chordin in restricting BMP activity during mandibular development, Noggin is expressed in ventral arch epithelium and Chordin weakly throughout the arches (Stottmann et al., 2001). By contrast, we show here that zebrafish greml2 is expressed in a dorsal-intermediate arch domain that opposes ventral bmp4 expression. Consistent with Greml2 restricting BMP signaling to the ventral domain, reduction of Greml2 results in upregulated BMP activity and altered skeletal development in the dorsal and intermediate face.

As Edn1 is a potent inducer of greml2, Edn1 may pattern the intermediate domain in part by keeping BMP activity below the threshold required for hand2 expression, thus preventing Hand2 repression of dlx3b/5a/6a and nkd3.2 expression. In the dorsal domain, Jag1b would further contribute to greml2 induction and
BMP inhibition. A role for Jag1b in Grem2 induction would explain why loss of Jag1b rescues ventral skeletal defects in edn1−/− mutants (Zuniga et al., 2010), similar to depleting Grem2 (this paper) or misexpressing Bmp4 (Alexander et al., 2011). In edn1−/− mutants, a reduction of grem2 expression correlates with increased BMP activity, yet this is not sufficient to support ventral development in the absence of Edn1. Significantly, some residual BMP activity, yet this is not sufficient to support ventral edn1−/− protein throughout the arches can largely restore normal DV robustness. For example, the underlying ventral bias of BMP threshold. Such a self-reinforcing BMP network would create levels depending on the initial activity relative to a specific domains, with BMP signaling self-reinforcing to either high or low DV patterning is that it creates a sharp boundary between two states. In one model, arch CNCCs are exposed to different ratios of Bmp4 and Edn1, with the former higher ventrally. Distinct diffusion coefficients or the unique expression domains of these ligands might explain such differences. Indeed, we observe that bmp4 is expressed in a more ventral domain of facial ectoderm than edn1. Alternatively, the added input of Jagged-Notch signaling on grem2 expression might increase total Grem2 levels beyond what can be inhibited by BMP, thus reducing BMP activity to below a threshold required to maintain itself in dorsal and intermediate CNCCs. Future modeling studies will be needed to understand how BMP, Edn1 and Jagged-Notch signaling are integrated to generate such highly reproducible pre-skeletal domains within the developing face.

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