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Jagged-Notch signaling ensures dorsal skeletal identity in the vertebrate face

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SUMMARY

The development of the vertebrate face relies on the regionalization of neural crest-derived skeletal precursors along the dorsoventral (DV) axis. Here we show that Jagged-Notch signaling ensures dorsal identity within the hyoid and mandibular components of the facial skeleton by repressing ventral fates. In a genetic screen in zebrafish, we identified a loss-of-function mutation in *jagged 1b* (*jag1b*) that results in dorsal expansion of ventral gene expression and partial transformation of the dorsal hyoid skeleton to a ventral morphology. Conversely, misexpression of human jagged 1 (JAG1) represses ventral gene expression and dorsalizes the ventral hyoid and mandibular skeletons. We further show that *jag1b* is expressed specifically in dorsal skeletal precursors, where it acts through the Notch2 receptor to activate *hey1* expression. Whereas Jagged-Notch positive feedback propagates *jag1b* expression throughout the dorsal domain, Endothelin 1 (Edn1) inhibits *jag1b* and *hey1* expression in the ventral domain. Strikingly, reduction of Jag1b or Notch2 function partially rescues the ventral defects of *edn1* mutants, indicating that Edn1 promotes facial skeleton development in part by inhibiting Jagged-Notch signaling in ventral skeletal precursors. Together, these results indicate a novel function of Jagged-Notch signaling in ensuring dorsal identity within broad fields of facial skeletal precursors.

KEY WORDS: Jagged, Notch, Craniofacial, Skeleton, Zebrafish, Dorsoventral patterning

INTRODUCTION

The facial skeleton arises from cranial neural crest cells (CNCCs) that populate a series of pharyngeal arches in all vertebrate embryos. CNCCs of the mandibular and hyoid arches are further divided into dorsal and ventral domains that generate distinctly shaped cartilages and bones. In the larval zebrafish, ventral mandibular CNCCs generate the lower jaw Meckel's (M) cartilage, and dorsal mandibular CNCCs give rise to part of the palatoquadrate (Pq) cartilage (Crump et al., 2006). However, the pterygoid process (Ptp) of Pq, which functions as the larval upper jaw, arises from maxillary CNCCs (Eberhart et al., 2006). In the hyoid arch, ventral CNCCs give rise to the ceratohyal (Ch) and symplectic (Sy) cartilages and the branchiostegal ray (Br) bone, and dorsal CNCCs generate the hyomandibular (Hm) cartilage and opercle (Op) bone that support the gill covering (Fig. 1A,D). In general, dorsal mandibular and hyoid cartilages have plate-like morphologies, whereas their ventral cognates have rod-shaped morphologies. Moreover, the dorsal Op bone has a fan-shaped morphology that is distinct from the finger-shaped ventral Br bone.

Within the mandibular and hyoid arches, the secreted ligand Edn1 plays a central role in specifying ventral identity. In zebrafish and mice lacking Edn1 or the Endothelin type-A receptors (Ednras), the ventral facial skeleton either fails to develop or is transformed to a dorsal morphology (Kurihara et al., 1994; Clouthier et al., 1998; Miller et al., 2000; Ozeki et al., 2004; Nair et al., 2007). By contrast, Edn1 misexpression transforms the dorsal facial skeleton to a ventral morphology (Kimmel et al., 2007; Sato et al., 2008). Edn1 is thought to promote ventral skeletal development in part by activating the earlier expression of a network of ventral-specific genes in the mandibular and hyoid arches. Edn1 targets include *Dlx3/dlx3b*, *Dlx5/dlx5a*, *Dlx6/dlx6a*, *Msx1/msxe* and *epha4b* (*rtk2*) in ventral CNCCs of each arch, *bapx1* (*nkx3.2*) in dorsoventral (DV)-intermediate CNCCs of the mandibular arch, and *Hand2* (*dHand*)*/hand2* in the most ventral CNCCs of each arch (Thomas et al., 1998; Clouthier et al., 2000; Miller et al., 2000; Miller et al., 2003; Walker et al., 2006). Moreover, several of these Edn1 targets have been shown to be required for development of the ventral face. Compound $Dlx5^{-/-}$; $Dlx6^{-/-}$ mutant mice display transformations of the ventral mandibular skeleton (Beverdam et al., 2002; Depew et al., 2002), zebrafish lacking *bapx1* fail to form the jaw joint (Miller et al., 2003), and mutations in *Hand2/hand2* result in ventral skeletal loss in mice and zebrafish (Miller et al., 2003; Yanagisawa et al., 2003). However, whether patterning of the dorsal facial skeleton occurs simply by default (i.e. in the absence of ventral signaling) has remained unclear. Here, we show that dorsal skeletal identity requires active repression of ventral fates by Jagged-Notch signaling.

The Notch pathway is widely used during animal development to determine cell fates. Notch signaling occurs when transmembrane ligands of the Delta and Jagged/Serrate families engage Notch receptors on adjacent cells. Ligand binding then triggers cleavage and release of a Notch intracellular domain that translocates to the nucleus and activates the transcription of genes such as those of the Hey/Her/Hes class. In a process termed lateral inhibition, differential Notch signaling causes neighboring cells to adopt distinct fates. In other contexts, such as the fly wing, Notch signaling patterns fields of cells in organ primordia (Diaz-Benjumea and Cohen, 1995). In vertebrates, Jagged-Notch signaling has been implicated in the development of diverse organs,

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including the ear (Brooker et al., 2006; Kiernan et al., 2006), liver (Geisler et al., 2008; Lozier et al., 2008), pancreas (Golson et al., 2009) and cardiovascular system (High et al., 2008).

The role of Jagged-Notch signaling in craniofacial development is less clear. Several components of Jagged-Notch signaling are expressed in facial skeletal precursors, including zebrafish *jag1b* (Zecchin et al., 2005), mouse and human *Jag1*/*JAG1* (Mitsiadis et al., 1997; Kamath et al., 2002b), zebrafish and mouse *jag2*/*Jag2* (Jiang et al., 1998; Zecchin et al., 2005), and mouse *Notch2* (Higuchi et al., 1995; Mitsiadis et al., 1997). Heterozygous mutations in human *JAG1* or *NOTCH2* are linked to Alagille syndrome, which is characterized by defects in multiple visceral organs, an abnormal facial appearance and occasional craniosynostosis and deafness (Li et al., 1997; Oda et al., 1997; Kamath et al., 2002b; Kamath et al., 2002a; Le Caignec et al., 2002; McDaniell et al., 2006). Whereas $Jag1^{-/-}$ mice are embryonic lethal (Xue et al., 1999), $Jag2^{-/-}$ mice die at birth from cleft palate (Jiang et al., 1998). In zebrafish, combined reduction of *jag1b* and *jag2* function with morpholino oligonucleotides (MOs) has been reported to result in general reductions of facial cartilage (Lorent et al., 2004). However, a potential function of Jagged-Notch signaling in regional patterning of the facial skeleton has not been previously investigated.

Here, we employ mutant and transgenic analyses in zebrafish to demonstrate a novel role for Jagged-Notch signaling in patterning the dorsal face. In particular, we find that Jagged-Notch signaling limits the dorsal extent of ventral gene expression and helps determine dorsal skeletal morphology in the mandibular and hyoid arches. We further show that Jagged-Notch positive feedback and Edn1 inhibition are integrated through *jag1b* expression to restrict Notch activity to dorsal skeletal precursors. Moreover, compound mutant analysis reveals that a major function of Edn1 in ventral skeletal development is the repression of Jagged-Notch signaling. Together, our work defines a crucial role for Jagged-Notch signaling in DV facial patterning that might help to explain some of the craniofacial anomalies seen in Alagille syndrome.

MATERIALS AND METHODS

Zebrafish lines

Zebrafish (*Danio rerio*) embryos were raised at 28.5°C and staged as described (Kimmel et al., 1995). The $jag1b^{b1105}$ allele was identified in an ENU mutagenesis screen in which parthenogenic diploid progeny were analyzed for skeletal defects, and the *sucker*/$edn1^{tf216b}$ mutant is as described (Miller et al., 2000). $Tg(hsp70I:Gal4)^{kca4/+}$ (Scheer and Campos-Ortega, 1999) and *fli1a*:GFP (Lawson and Weinstein, 2002) zebrafish are as described. To create *UAS*:$JAG1^{el108}$ transgenic zebrafish, we used the Gateway (Invitrogen) Tol2kit (Kwan et al., 2007). Full-length human *JAG1* cDNA (Open Biosystems, clone 30528888) was inserted into pDONR221 by PCR using primers hJAG1-L2 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGAATTCCGCGGCGCAGCGATGCGTT-3′) and hJAG1-R2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTAGTCCCGCGGTCTGCTATACG-3′). The resultant pME-JAG1 vector was combined with p5E-UAS, p3E-polyA and pDestTol2CG2 to create *UAS*:JAG1, which also contains a *cmlc2*:EGFP transgene that expresses GFP in the heart (*cmlc2* is also known as *myl7* – Zebrafish Information Network). *UAS*:JAG1 was injected with Tol2 transposase RNA into one-cell stage embryos and stable line *el108* was isolated. For heat-shock induction, embryos were placed in a 40°C incubator at 20 hours post-fertilization (hpf) and then transferred to 28.5°C at 28 hpf.

Morpholino injections

One-cell stage embryos were injected with 3 nl of *jag1b*-MO (400 μM), *notch2*-MO (800 μM) or *edn1*-MO (27 μM) (Gene Tools, Philomath, OR, USA). *jag1b*-MO (previously known as *jag3*-MO) and *notch2*-MO have been demonstrated to block translation and mRNA splicing, respectively, and we confirmed inhibition of *notch2* splicing as described (Lorent et al., 2004). The concentration of *edn1*-MO used causes partial loss of Edn1 function (Miller and Kimmel, 2001).

In situ hybridization and skeletal analysis

Skeletal staining with Alcian Blue and Alizarin Red (Walker and Kimmel, 2007), live bone staining with Calcein Green (Kimmel et al., 2003), and colorimetric in situ hybridization experiments (Crump et al., 2004) were performed as described. For fluorescence in situ experiments, two modifications were made to the published protocol (Welten et al., 2006): hybridizations were conducted at 68°C and antibody concentrations were 1:500 anti-DIG-POD and 1:200 anti-DNP-peroxidase.

jag1a, *jag1b*, *jag2*, *notch2*, *hey1* and *ednra2* probes were synthesized with T7 RNA polymerase from PCR products using the following primers (shown 5′ to 3′): Jag1a-L, CCGCGTATGTTTGAAGGAGT; Jag1a-RT7, GCTAATACGACTCACTATAGGGCAGTTCTGTCCGGAGTAGC; Jag1b-3L, CACGTGACGAGTTCTTTGGA; Jag1b-4RT7, GCTAATACGACTCACTATAGGGACACCGGTATCCATTCACC; Jag2-L, TGGGACTGGGATAACTCCAC; Jag2-RT7, GCTAATACGACTCACTATAGGTCAAAGCCATTTTCCAGGTC; Notch2-L, ACCCTGTCATCATGGCAAAT; Notch2-RT7, GCTAATACGACTCACTATAGGACAGGTTCCCTGATTCATGC; Hey1-L, TCATTTAAAGATGCTTCATGCTG; Hey1-RT7, GCTAATACGACTCACTATAGGGTCTGTTTCTGTGCATCTGTTCA; Ednra2-L, CAATCATTTCCTGCATCGTG; and Ednra2-RT7, GCTAATACGACTCACTATAGGCAAGAGTTCACAGTCGCCAA. Published probes include *dlx2a* and *dlx3b* (Akimenko et al., 1994), *bapx1* (Miller et al., 2003), *epha4b* [referred to as EphA3 by Xu et al. (Xu et al., 1995)], *msxe* (Akimenko et al., 1995), *dlx5a* and *dlx6a* (Walker et al., 2006), *hand2* (Angelo et al., 2000) and *edn1* (Miller et al., 2000).

In all experiments, genotyping of embryos confirmed the observed phenotypes. For $jag1b^{b1105}$ genotyping, we amplified product using primers Jag1b-IDL (5′-GTACCAAATCCGGGTGACCT-3′) and Jag1b-IDR (5′-GTGGCTTTTTGGGTCATTATCA-3′) and digested with *Bts*CI to generate a 206 bp fragment in mutants and 134/72 bp fragments in wild types. $edn1^{tf216b}$ genotyping was performed using primers Edn1-IDL (5′-AGCGCGACAAATTCAATCAT-3′) and Edn1-IDR (5′-CAAAAGTAGACGCACTCGTTA-3′), followed by digestion with *Hpa*I to produce 178/20 bp fragments in mutants and a 198 bp fragment in wild types. The presence of *hsp70I*:Gal4 was detected by PCR using primers Gal4-IDL (5′-CTCCCAAAACCAAAAGGTCTCC-3′) and Gal4-IDR (5′-TGAAGCCAATCTATCTGTGACGG-3′). *UAS*:JAG1 embryos were selected by heart GFP, and *hsp70I*:Gal4-negative *UAS*:JAG1 siblings were used as controls.

Transplantations

Unilateral tissue transplantations were performed as previously described, with the non-recipient side acting as an internal control (Crump et al., 2004). Briefly, donor tissue from *fli1a*:GFP embryos injected with Alexa 568-dextran (Molecular Probes) was transplanted into different fate-map regions of $jag1b^{b1105}$; *fli1a*:GFP or *notch2*-MO; *fli1a*:GFP hosts at 6 hpf. Targeting of Alexa 568-positive donor tissue was assessed at 36 hpf by localization relative to *fli1a*:GFP. For endoderm transplants, donor embryos were also injected with Tar* RNA to promote endoderm targeting.

Imaging

Skeletons and in situ hybridization embryos were photographed on a Zeiss Axioimager.Z1 microscope using Axiovision software. Fluorescence images were captured on a Zeiss LSM5 confocal microscope and, except where indicated otherwise, *z*-stacks of ~40 μm were flattened into single projections. Levels were adjusted in Adobe Photoshop CS2, with care taken to apply identical adjustments to images from the same data set and to avoid removing information from the image. Dissected skeletons were cropped to remove surrounding soft tissue.

Statistical analysis

JMP 7.0 software (SAS) was used for one-way analysis of variance. A Tukey-Kramer honestly significant difference (HSD) test (α=0.05) showed significance for all *dlx3b* and *dlx5a* expression differences and for the

following comparisons in the skeletal analysis of Edn1 and Jagged-Notch interactions: M and Ch (*edn1* versus all others), Hm (*edn1*; *jag1b* versus all others) and Op (*edn1* versus *jag1b* and *edn1*; *notch2*-MO).

RESULTS

Identification of a zebrafish *jag1b* mutant with dorsal-specific facial skeletal defects

As part of an ENU mutagenesis screen conducted at the University of Oregon, we isolated a mutation, *b1105*, that displays defects in the facial skeleton (Fig. 1B). Linkage analysis, phenocopy with a *jag1b*-MO and gene sequencing revealed that the *b1105* lesion is a G-to-A transition in the zebrafish *jag1b* gene (see Fig. S1 in the supplementary material). The *jag1b*^{b1105} mutation converts tryptophan 223 to a premature stop codon, truncating the Jag1b protein within the extracellular DSL domain required for Notch binding (Fig. 1C) (Cordle et al., 2008). At 5 days post-fertilization (dpf), *jag1b*^{b1105} mutants have variable facial defects that ranged from mild reductions to more striking shape changes of dorsal hyoid and mandibular skeletal elements (Fig. 1I). In the hyoid arch of the most severely affected *jag1b*^{b1105} larvae, the dorsal Hm cartilage became more rod-shaped, partially resembling the ventral Ch, and the normally fan-shaped Op bone adopted the finger-shaped morphology of the ventral Br bone to which it occasionally fused (Fig. 1E,H). In the *jag1b*^{b1105} mandibular arch, the dorsal portion of Pq was truncated rather than transformed. Contrary to previous *jag1b*-MO studies (Lorent et al., 2004), we observed no defects in the ventral Ch, Sy and M cartilages or in the Br bone in *jag1b*^{b1105} larvae. The maxillary-derived Ptp and the neurocranium, which is derived from maxillary and frontonasal CNCCs (Wada et al., 2005), were likewise unaffected. *jag1b*^{b1105} larvae died by 7 dpf, precluding an examination of the DV morphology of later-forming facial bones. Nonetheless, our loss-of-function data suggest that, particularly in the hyoid arch, Jag1b is required for dorsal skeletal morphology.

Notch2 is required for development of the dorsal facial skeleton

We next investigated which Notch receptor mediates Jag1b signaling in the face. In humans, heterozygosity of either *NOTCH2* or *JAG1* can result in Alagille syndrome, and *Notch2* genetically interacts with *Jag1* in mouse (McCright et al., 2002). Moreover, of the four zebrafish Notch genes (*notch1a*, *notch1b*, *notch2* and *notch3*), we found that only *notch2* is expressed in the pharyngeal arches at DV patterning stages, 28-36 hpf (data not shown). We thus tested the requirement for Notch2 in facial skeleton patterning. Using a *notch2*-MO to block *notch2* mRNA splicing (Lorent et al., 2004), we found that Notch2 reduction results in partial transformation of dorsal Hm to a ventral rod-like morphology, the transformation of dorsal Op to a ventral finger-like morphology, and the truncation of dorsal Pq (Fig. 1F,H). *notch2*-MO larvae also infrequently developed ectopic cartilage near the DV boundaries within the mandibular and hyoid arches, suggesting an additional role for Notch2 in suppressing skeletal development at the DV interface (see Fig. S1 in the supplementary material). Overall, the

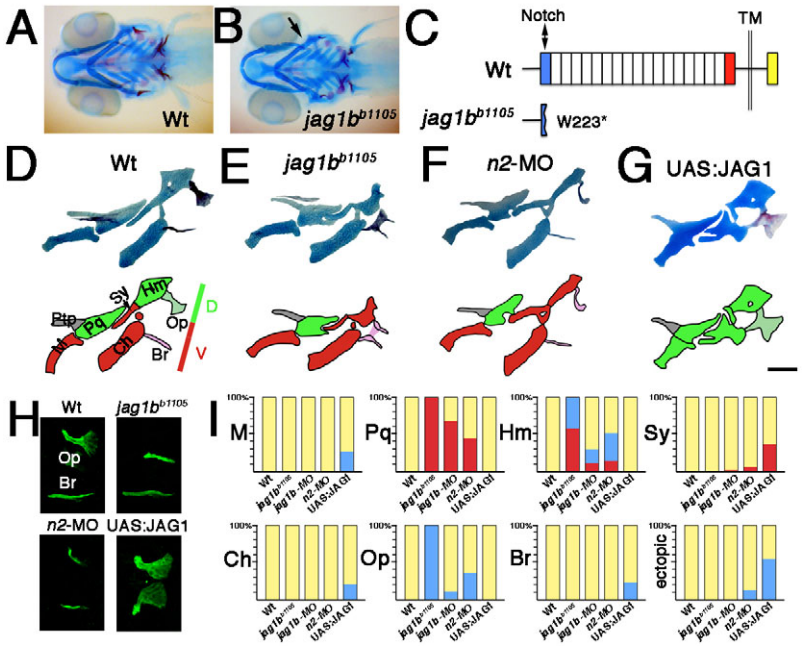

Fig. 1. Jag1b-Notch2 signaling regulates DV patterning of the zebrafish facial skeleton. (**A**,**B**) Skeletal staining at 5 dpf showing cartilage (blue) and bone (red). *jag1b*^{b1105} mutants display a characteristic kink (arrow) behind the eye, which is not seen in the wild type (Wt). (**C**) Schematic of Jag1b protein showing DSL (blue), EGF-like (white), cysteine-rich (red), transmembrane (TM) and intracellular (yellow) domains. The *jag1b*^{b1105} lesion is a nonsense mutation (W223*) that truncates Jag1b in the DSL domain required for Notch binding. (**D-G**) Dissected facial skeletons from wild-type (D), *jag1b*^{b1105} (E), *notch2*-MO (F) and 20-28 hpf heat shock-treated *hsp70l*:Gal4; *UAS*:JAG1 (G) larvae. Schematics (below) show ventral (red) and dorsal (green) elements derived from the mandibular and hyoid arches, with bones more lightly shaded. The maxillary-derived pterygoid process (Ptp) is in gray. Scale bar: 100 μm. (**H**) Calcein Green bone staining at 5 dpf shows Op-to-Br transformations in *jag1b*^{b1105} and *notch2*-MO larvae and Br-to-Op transformations in 20-28 hpf heat shock-treated *hsp70l*:Gal4; *UAS*:JAG1 larvae. (**I**) The proportion of wild-type, *jag1b*^{b1105}, *jag1b*-MO, *notch2*-MO and 20-28 hpf heat shock-treated *hsp70l*:Gal4; *UAS*:JAG1 larvae showing normal (yellow), reduced (red) or transformed (blue) skeletal elements. M, Meckel's; Pq, palatoquadrate; Hm, hyomandibular; Sy, symplectic; Ch, ceratohyal; Op, opercle bone; Br, branchiostegal ray bone. The proportion of larvae exhibiting ectopic cartilage (blue) is also shown.

skeletal phenotypes of *notch2*-MO larvae are comparable to those of *jag1b*-MO larvae, but weaker than those of *jag1b*^{b1105} mutants, which might be due to incomplete reduction of Notch2 function by the MO or partial compensation by other, weakly expressed Notch receptors (Fig. 1I). Nonetheless, as Notch2 reduction results in dorsal-specific skeletal defects that are similar to, although less severe than, those seen in *jag1b*^{b1105} mutants, we conclude that Notch2 at least partially mediates Jag1b signaling during DV facial patterning.

JAG1 misexpression transforms the ventral facial skeleton

We next tested whether Jagged-Notch signaling is also sufficient to promote a dorsal skeletal morphology. Using a heat shock-inducible Gal4/UAS system (Scheer and Campos-Ortega, 1999) to induce human JAG1 expression throughout zebrafish embryos at early facial patterning stages (20-28 hpf), we found that JAG1 misexpression results in ventral-specific defects of the mandibular and hyoid skeletons (Fig. 1G). In the most severely affected larvae, ventral M and Ch cartilages adopted plate-like morphologies similar to those of dorsal Pq and Hm cartilages. In addition, the ventral Sy cartilage and mandibular jaw joint were lost, and the ventral Br bone fused to the dorsal Op bone. By contrast, dorsal Hm and Pq cartilages and the maxillary-derived Ptp were unaffected. In less severe examples, the ventral Br bone was strikingly transformed to a mirror-image duplicate of the fan-shaped dorsal Op, the jaw joint was lost, and ectopic cartilage formed near Pq and the midline (Fig. 1H,I). As loss and gain of Jag1b function result in reciprocal DV skeletal transformations, we conclude that Jag1b is both necessary and sufficient for dorsal skeletal morphology in the hyoid and, to a lesser extent, the mandibular arches.

Jagged-Notch signaling inhibits ventral gene expression in the dorsal face

We next examined whether Jag1b-Notch2 signaling might control dorsal skeletal character by regulating earlier patterns of DV gene expression in CNCC-derived skeletal precursors. Whereas *dlx2a* was expressed throughout mandibular and hyoid CNCCs, double-fluorescence in situ hybridizations showed that *dlx3b* and *dlx5a* expression is restricted to more ventral CNCCs of 36 hpf wild-type embryos (Fig. 2A,E). By contrast, we observed a moderate dorsal expansion of *dlx3b* and *dlx5a* expression in *jag1b*^{b1105} and *notch2*-MO embryos (Fig. 2B,C,F,G). In particular, the dorsal expansion of *dlx3b* and *dlx5a* was more prominent in the hyoid arch, correlating with the stronger transformations seen in the dorsal hyoid skeleton. In order to rule out the possibility that *dlx3b* and *dlx5a* expansion is simply due to changes in arch size, we measured the areas of *dlx2a*, *dlx3b* and *dlx5a* expression in the hyoid arches of wild-type, *jag1b*^{b1105} and *notch2*-MO embryos. Normalization of hyoid arch size by measuring the ratio of *dlx3b* to *dlx2a* (wild type, 43%; *jag1b*^{b1105}, 61%; *notch2*-MO, 55%) and of *dlx5a* to *dlx2a* (wild type, 41%; *jag1b*^{b1105}, 55%; *notch2*-MO, 54%) confirmed that the percentage of the hyoid arch expressing *dlx3b* and *dlx5a* increases in both *jag1b*^{b1105} and *notch2*-MO embryos. By contrast, *dlx3b* and *dlx5a* expression was severely reduced in JAG1-misexpression embryos (Fig. 2D,H). Thus, Jagged-Notch signaling is also sufficient to inhibit *dlx3b* and *dlx5a* expression in ventral CNCCs.

We next examined whether Jag1b might regulate the expression of a broader cohort of ventral-specific genes. *dlx6a*, *epha4b* and *msxe* were expressed in a ventral-specific pattern, similar to that of *dlx3b* and *dlx5a* at 36 hpf. Compared with wild types, we found that the expression of *dlx6a*, *epha4b* and *msxe* also extended more dorsally in the hyoid and mandibular arches of *jag1b*^{b1105} mutants and was severely reduced in JAG1-misexpression embryos (Fig. 3A-I). The expression of the mandibular joint marker *bapx1* was also expanded dorsally in *jag1b*^{b1105} mutants and lost in JAG1-misexpression embryos (Fig. 3J-L). By contrast, the expression of *hand2*, one of the most ventrally restricted genes in the arches, was unaffected in *jag1b*^{b1105} embryos, although it was reduced in JAG1-misexpression embryos (Fig. 3M-O). Furthermore, the arch expression of *edn1* and its receptor, *ednra2*, were unaffected in *jag1b*^{b1105}, *notch2*-MO and JAG1-misexpression embryos (Fig. 3P-U and data not shown). We therefore conclude that the role of Jag1b and Notch2 in dorsal skeletal patterning correlates with an earlier requirement in limiting the dorsal extent of most, but not all, ventral gene expression in facial skeletal precursors.

jag1b and the Notch target *hey1* are selectively expressed in dorsal skeletal precursors

In order to understand where Jagged-Notch signaling functions to repress ventral gene expression, we analyzed the expression of *jag1b*, *notch2* and the Notch target gene *hey1* during arch patterning stages. At 28 hpf, double-fluorescence in situ hybridizations of *jag1b* with *notch2*, and of *hey1* with the CNCC-specific *dlx2a* probe, showed that *jag1b* and *hey1* are expressed in the dorsal-most CNCCs of the mandibular and hyoid arches and in

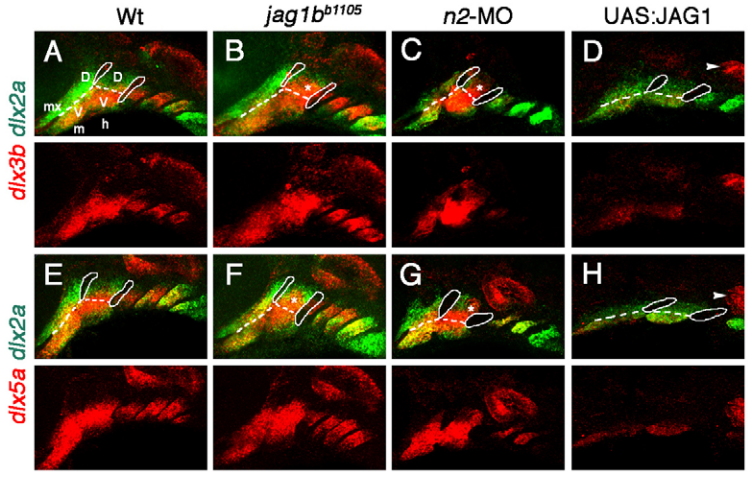

Fig. 2. Jag1b-Notch2 signaling inhibits *dlx3b* and *dlx5a* expression in the pharyngeal arches. (**A-H**) Double-fluorescence in situ hybridizations showing the expression of *dlx3b* or *dlx5a* (red) and *dlx2a* (green) at 36 hpf. Compared with wild types (A,E), *dlx3b* and *dlx5a* expression is expanded into the dorsal hyoid arches (asterisks) of *jag1b*^{b1105} (B,F) and *notch2*-MO (C,G) zebrafish embryos and is reduced in the ventral arches of 20-28 hpf heat shock-treated *hsp70l*:Gal4; *UAS*:JAG1 (D,H) embryos. The expression of *dlx3b* and *dlx5a* in otic placodes (arrowheads) is unaffected in *UAS*:JAG1 embryos. Endodermal pouches (solid lines) and DV arch boundaries (dashed lines) are indicated in the merged images. The maxillary domain (mx) and the dorsal (D) and ventral (V) domains of the mandibular (m) and hyoid (h) arches are indicated for wild type.

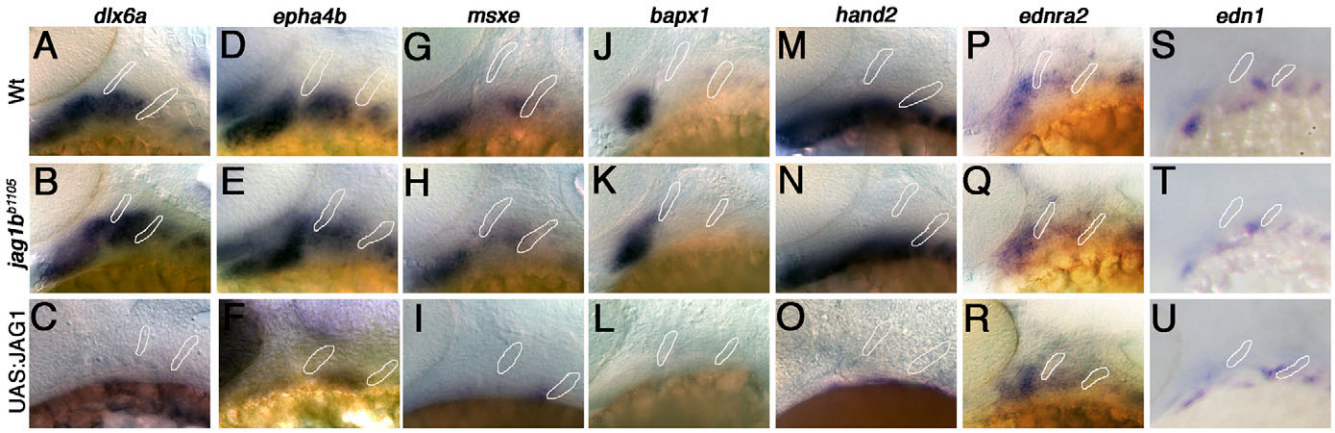

Fig. 3. Jag1b generally represses ventral gene expression in the pharyngeal arches. (**A-U**) In situ hybridizations showing the mandibular and hyoid arch expression of *dlx6a* (A-C), *epha4b* (D-F), *msxe* (G-I), *hand2* (M-O), *ednra2* (P-R) and *edn1* (S-U) at 36 hpf and of *bapx1* (J-L) at 40 hpf. The expression of *dlx6a*, *epha4b*, *msxe* and *bapx1* is dorsally expanded in *jag1b*^{b1105} zebrafish embryos (B,E,H,K) and is greatly reduced in 20-28 hpf heat shock-treated *hsp70l*:Gal4; *UAS*:JAG1 embryos (C,F,I,L). *hand2* expression is reduced in *UAS*:JAG1 embryos, whereas *ednra2* and *edn1* expression is unaffected in *jag1b*^{b1105} and *UAS*:JAG1 embryos. Endodermal pouches are outlined.

pouch endoderm (Fig. 4A,H). From 32 to 36 hpf, *jag1b* and *hey1* expression continued to be dorsally restricted, yet extended more ventrally to abut *dlx3b* and *dlx5a* expression; concomitantly, expression became more prominent in posterior CNCCs in each arch and endoderm expression disappeared (Fig. 4B,C,I,J and see Fig. S2 in the supplementary material). By 36 hpf, *hey1* also began to be expressed in ventral arch mesoderm. Double-fluorescence in situ hybridization revealed that *hey1* expression extended just ventral to that of *jag1b*, as predicted if Jag1b is activating Notch2 in adjacent cells, and extensive colocalization of *jag1b* with *dlx2a* confirmed that within the dorsal arches, *jag1b* is expressed primarily in CNCCs (see Fig. S2 in the supplementary material). In addition, *jag2*, but not *jag1a*, was co-expressed at low levels with *jag1b* in dorsal CNCCs at 36 hpf (see Fig. S3 in the supplementary material).

We also confirmed that *hey1* is a bona fide target of Jag1b-Notch2 signaling in CNCCs, as *hey1* CNCC expression was greatly reduced in *jag1b*^{b1105} and *notch2*-MO embryos and upregulated in JAG1-misexpression embryos (Fig. 4K-M). Of note, the expression of *hey1* in ventral arch mesoderm was lost in *notch2*-MO, but not in *jag1b*^{b1105}, embryos, suggesting a specific function of Jag1b in regulating *hey1* expression within CNCCs. Furthermore, in contrast to the dorsal-specific expression of *jag1b*, we found that *notch2* is more widely expressed throughout the pharyngeal arches from 28 to 36 hpf. Whereas higher expression of *notch2* was observed in ventral CNCCs that also express *dlx3b* and *dlx5a*, *notch2* was co-expressed at weaker levels with *jag1b*, *hey1* and *dlx2a* in dorsal CNCCs (Fig. 4A-C and see Fig. S2 in the supplementary material). This widespread expression of *notch2* was confirmed with two independent probes (data not shown). Thus, despite the stronger ventral expression of *notch2*, *hey1* expression indicates that Jag1b activates Notch2 specifically in dorsal CNCCs, consistent with the observed function of Notch2 in repressing ventral gene expression in dorsal skeletal precursors.

Jagged-Notch signaling functions within CNCCs for DV skeletal patterning

As *jag1b* and *notch2* are expressed in multiple arch tissues, we used mosaic rescue experiments to test in which tissues Jag1b and Notch2 are sufficient for facial skeletal patterning. In order to create tissue mosaics, we transplanted wild-type *fli1a*:GFP precursors at early gastrulation stages (6 hpf) into different fate-map domains of *jag1b*^{b1105}; *fli1a*:GFP or *notch2*-MO; *fli1a*:GFP hosts. *fli1a*:GFP specifically labels the CNCC component of the pharyngeal arches (Lawson and Weinstein, 2002), allowing us to assess the correct targeting of donor tissue to CNCCs, endoderm or ectoderm of the arches. Whereas transplantation of wild-type CNCC precursors rescued facial skeletal patterning in 30/39 *jag1b*^{b1105} embryos, transplantations of wild-type endodermal (0/5) or ectodermal (1/8) precursors did not reliably rescue (Fig. 5B-D). Similarly, wild-type CNCC precursor transplants rescued skeletal defects in 12/15 *notch2*-MO embryos (Fig. 5E). We therefore conclude that Jag1b and Notch2 function predominantly in CNCCs, and not in the surrounding endoderm or ectoderm, to pattern the dorsal facial skeleton.

Jagged-Notch signaling positively regulates *jag1b* expression in dorsal CNCCs

We next investigated how Notch activity is established throughout dorsal skeletal precursors. As Notch positively regulates the expression of Jagged/Serrate in other contexts (de Celis and Bray, 1997; Daudet et al., 2007), we examined whether Notch signaling also regulates *jag1b* expression in CNCCs. Indeed, we found that *jag1b* expression is severely reduced in dorsal CNCCs of *jag1b*^{b1105} and *notch2*-MO embryos and is expanded into ventral CNCCs of JAG1-misexpression embryos at 36 hpf (Fig. 4D-F). Conversely, the stronger ventral expression domain of *notch2* was expanded in *jag1b*^{b1105} and *notch2*-MO embryos and reduced in JAG1-misexpression embryos. We therefore conclude that Jagged-Notch signaling activates *jag1b* expression and represses strong *notch2* expression in dorsal CNCCs of the mandibular and hyoid arches.

Edn1 signaling restricts *jag1b* and *hey1* expression to dorsal CNCCs

As Edn1 is known to promote ventral gene expression, we examined whether Edn1 also inhibits the ventral expression of Jagged ligands. Similar to what we observe in JAG1-misexpression embryos, we found that *jag1b* expression expands into ventral arch CNCCs in *edn1* mutants (Fig. 4G). *jag2* expression also expanded ventrally in *edn1* mutants (see Fig. S3 in the supplementary

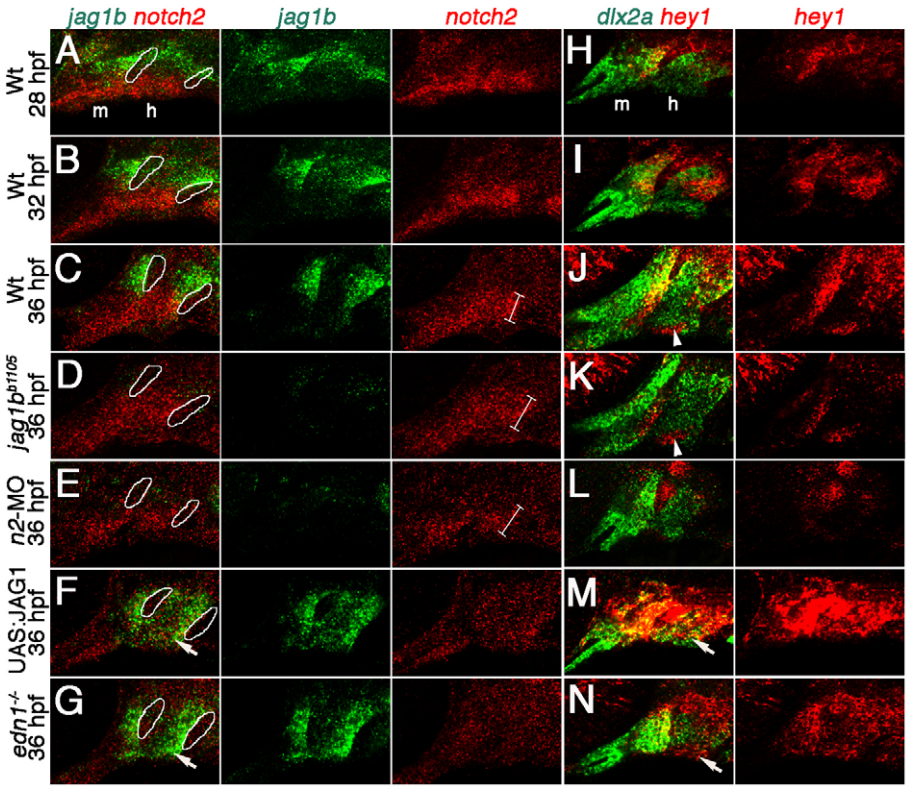

Fig. 4. Expression of *jag1b*, *notch2* and *hey1* is regulated by Jagged-Notch and Edn1 signaling. (**A-N**) Confocal sections of double-fluorescence in situ hybridizations showing mandibular (m) and hyoid (h) arch expression of *jag1b* (green) and *notch2* (red) (A-G) and of *dlx2a* (green) and *hey1* (red) (H-N). In wild types, *jag1b* and *hey1* CNCC expression expands ventrally from 28 hpf (A,H) to 32 hpf (B,I) and 36 hpf (C,J). In *jag1b*^{b1105} (D,K) and *notch2*-MO (E,L) zebrafish embryos, *jag1b* and *hey1* expression is reduced and strong ventral *notch2* expression (brackets) is expanded at 36 hpf. Arrowheads in J,K indicate ventral mesoderm expression of *hey1*, which is unaffected in *jag1b*^{b1105} mutants. In 20-28 hpf heat shock-treated *hsp70l*:Gal4; *UAS*:JAG1 (F,M) and *edn1*$^{-/-}$ (G,N) embryos, *jag1b* and *hey1* expression is expanded into ventral CNCCs (arrows) and *notch2* expression is reduced at 36 hpf. Endodermal pouches are outlined in A-G.

material), whereas ventral *notch2* expression was reduced. Concomitantly, *hey1* expression was upregulated in ventral CNCCs (Fig. 4N), suggesting that ectopic *jag1b* and/or *jag2* expression results in increased Notch activity in ventral CNCCs of *edn1* mutants. Thus, Edn1 acts oppositely to Jagged-Notch signaling to inhibit Jagged ligand expression and hence Notch activity in ventral skeletal precursors.

Reduction of Jag1b-Notch2 signaling partially rescues the ventral facial defects of *edn1* mutants

As we found that Edn1 represses Jag1b-Notch2 signaling, we reasoned that inappropriate Notch activity in the ventral domain might contribute to the ventral skeletal defects of *edn1* mutants. *edn1* mutants exhibit nearly complete loss of ventral M, Ch and Sy cartilages, ventral Br bone and joints, and the dorsal Op bone is variably reduced or expanded (Fig. 6C) (Miller et al., 2000; Kimmel et al., 2003). Remarkably, reduction of Jag1b or Notch2 function in *edn1*; *jag1b*^{b1105} and *edn1*; *notch2*-MO larvae substantially rescued ventral cartilage (Fig. 6D-G). Whereas the 'rescued' ventral mandibular M cartilage was morphologically abnormal, the ventral hyoid Ch and Sy cartilages were restored to a nearly normal morphology in some *edn1*; *jag1b*^{b1105} and *edn1*; *notch2*-MO larvae. Interestingly, heterozygosity of *jag1b* also partially rescued the ventral cartilage defects of *edn1* mutants at a low frequency, underscoring the critical balance of Jagged-Notch and Edn1 signaling required for DV skeletal patterning (Fig. 6E,G). Consistent with the rescue of ventral skeletal defects, we found that the earlier ventral expression of *dlx3b*, *dlx5a* and *dlx6a* is partially restored in *edn1*; *jag1b*^{b1105} mutants (Fig. 6H). Of note, *hey1* expression was reduced, but not completely absent, in ventral CNCCs of *edn1*; *jag1b*^{b1105} embryos, potentially reflecting the presence of residual low-level Notch signaling mediated by Jag2 (see Fig. S4 in the supplementary material).

In contrast to the rescue of *edn1*$^{-/-}$ phenotypes by loss of *jag1b*, partial reduction of Edn1 function with a low dose of *edn1*-MO did not rescue the dorsal Hm, Op and Pq cartilage defects of *jag1b*^{b1105} mutants (see Fig. S5 in the supplementary material). However, upon nearly complete loss of Edn1 function in *edn1*; *jag1b*^{b1105} mutants, there was a slight rescue of Hm shape and an increase in the frequency of expanded Op compared with that seen in *jag1b*^{b1105} single mutants. Thus, although the dorsal skeletal defects of *jag1b*^{b1105} mutants are not simply the consequence of increased Edn1, the presence of Edn1 appears to influence the penetrance of dorsal transformations in *jag1b*^{b1105} mutants. Nonetheless, our genetic analysis shows that for ventral skeletal patterning, Edn1 functions primarily as an upstream inhibitor of Jagged-Notch signaling.

DISCUSSION

Jagged-Notch signaling inhibits ventral identity in the dorsal face

Here, we demonstrate a novel function of Jagged-Notch signaling in ensuring dorsal identity in the mandibular and hyoid arches (Fig. 7). Several lines of evidence indicate that Jag1b-Notch2 signaling acts oppositely to Edn1 and ventral Dlx genes in DV facial patterning. In particular, reduction of Jag1b-Notch2 signaling in *jag1b*^{b1105} and *notch2*-MO larvae results in dorsal-specific defects that are similar to those seen upon Edn1 overexpression, including dorsal-to-ventral transformations of hyoid cartilage (Hm to Ch-like) and dorsal reductions of mandibular cartilage (Pq truncation) (Kimmel et al., 2007). Conversely, JAG1 misexpression results in ventral-specific skeletal defects similar to those seen upon reduction of Edn1 or combined Dlx3b/Dlx5a function, including loss of ventral Sy cartilage and joints and striking homeotic transformation of the ventral Br bone into a dorsal Op-like morphology (Miller and Kimmel, 2001; Walker et al., 2006).

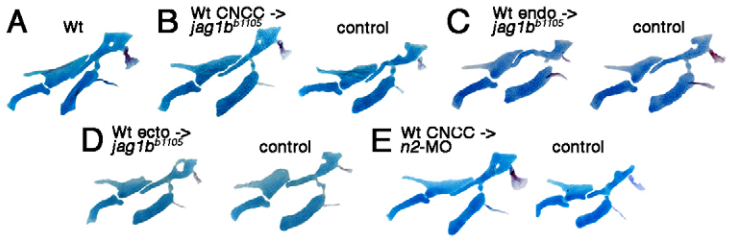

Fig. 5. Jag1b-Notch2 signaling functions within CNCCs for DV facial patterning. (**A-E**) Dissected zebrafish facial skeletons at 5 dpf with wild type (A) shown for reference. Transplantations of wild-type CNCC precursors (B), but not wild-type endoderm (C) or surface ectoderm (D) precursors, rescue the *jag1b*^{b1105} skeleton, as compared with the control non-recipient sides. Wild-type CNCC precursor transplants also rescue *notch2*-MO skeletal defects (E).

Moreover, JAG1 misexpression results in partial transformations of ventral M and Ch cartilages to dorsal plate-like morphologies, phenotypes that are not observed in zebrafish *edn1* mutants but which are reminiscent of the ventral mandibular transformations of *Edn1*$^{-/-}$, *Ednra*$^{-/-}$ and *Dlx5*$^{-/-}$; *Dlx6*$^{-/-}$ mice (Beverdam et al., 2002; Depew et al., 2002; Ozeki et al., 2004; Ruest et al., 2004). Thus, Jag1b-Notch2 signaling is specifically required for dorsal skeletal morphology and is also sufficient to alter the morphology of the ventral hyoid and mandibular skeleton.

The opposite effect of Jag1b-Notch2 and Edn1 signaling on skeletal morphology is also reflected at the level of DV gene expression. Edn1 has been shown to positively regulate a broad cohort of ventral genes (*dlx3b*, *dlx5a*, *dlx6a*, *epha4b*, *msxe* and *bapx1*) (Miller et al., 2000; Miller et al., 2003; Walker et al., 2006), and here we show that Edn1 also negatively regulates two newly characterized dorsal-specific genes, *jag1b* and *hey1*. By contrast, our gain- and loss-of-function studies demonstrate that Jag1b-Notch2 signaling negatively regulates these same ventral genes and positively regulates dorsal *jag1b* and *hey1*. Although we find that Jag1b and Notch2 function tissue autonomously within CNCCs for DV patterning, we do not know whether Jag1b-Notch2 signaling inhibits ventral gene expression directly through transcriptional repressors such as *hey1*, or indirectly via other downstream targets. Moreover, whereas changes in DV skeletal morphology correlate with earlier changes in DV gene expression in *jag1b*^{b1105} mutants and JAG1-misexpression animals, we cannot rule out the possibility that Jag1b and/or Notch2 have additional roles in the proliferation, survival and/or differentiation of skeletal precursors (Crowe et al., 1999; Nakanishi et al., 2007). Indeed, the stronger expression of *notch2* in ventral CNCCs suggests additional roles of Notch2 in arch development that are independent from its function in mediating Jag1b-dependent repression of ventral gene expression in dorsal skeletal precursors. Nonetheless, the mirror-image homeotic changes of hyoid bone seen in *jag1b*^{b1105} and JAG1-misexpression larvae, combined with the opposite effects on dorsal versus ventral gene expression and cartilage morphology, indicate a clear role for Jag1b-Notch2 signaling in promoting dorsal identity in the mandibular and hyoid arches.

Whereas Jag1b-Notch2 signaling plays a crucial role in ensuring dorsal identity in the face, except for the previously discussed hyoid bones, loss and gain of Jag1b-Notch2 signaling results in only partial transformations of DV skeletal character. There are

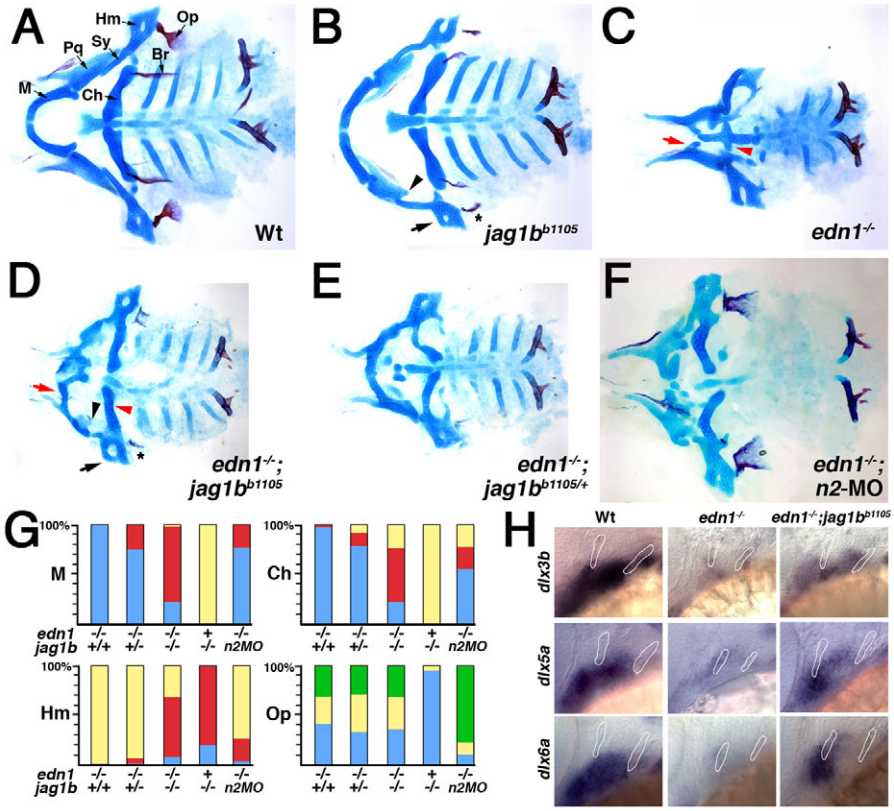

Fig. 6. Reduction of Jag1b-Notch2 signaling rescues the ventral defects of *edn1* mutants. (**A-F**) Ventral views of dissected zebrafish facial skeletons at 5 dpf, with elements labeled in wild type (A). *jag1b*^{b1105} mutants (B) have Pq reductions (arrowhead) and variable transformations of Hm (arrow) and Op (asterisk). In *edn1*$^{-/-}$ mutants (C), M (red arrow) and Ch (red arrowhead) are nearly absent. In *edn1*$^{-/-}$; *jag1b*^{b1105} larvae (D), development of ventral M and Ch is variably restored yet Pq and Hm defects are still evident. M and Ch development is also partially restored in some *edn1*$^{-/-}$; *jag1b*$^{b1105/+}$ (E) and *edn1*$^{-/-}$; *notch2*-MO (F) larvae. (**G**) Quantification of skeletal rescue, showing wild-type (yellow), weakly defective (red), severely defective (blue), and expanded (green) cartilage and bone. (**H**) In situ hybridizations showing *dlx3b*, *dlx5a* and *dlx6a* expression in arch CNCCs at 36 hpf. Compared with *edn1*$^{-/-}$ embryos, *edn1*$^{-/-}$; *jag1b*^{b1105} embryos show partial rescue of expression. Endodermal pouches are outlined. See Fig. 1 for abbreviations.

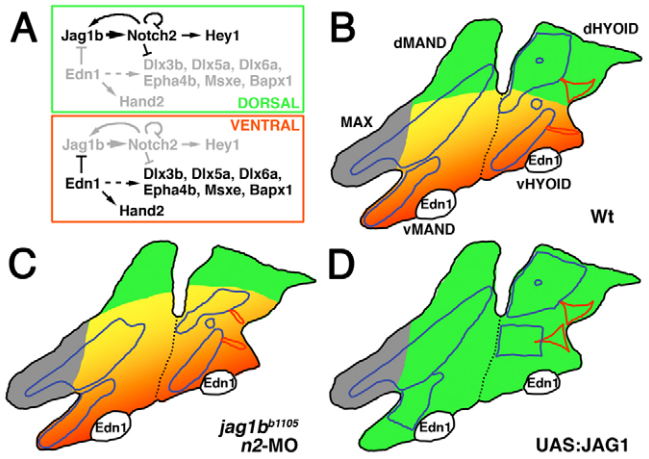

Fig. 7. Model of DV facial skeletal patterning in zebrafish. (**A**) In the wild-type dorsal domain, Jag1b activates the Notch2 receptor, promoting the expression of *jag1b* and *hey1* and repressing the expression of *dlx3b*, *dlx5a*, *dlx6a*, *epha4b*, *msxe*, *bapx1* and the otherwise strong expression of *notch2*. In the ventral domain, Edn1 represses *jag1b* expression and Notch signaling, permitting the expression of *dlx3b*, *dlx5a*, *dlx6a*, *epha4b*, *msxe* and *bapx1*. Edn1 promotes *hand2* expression independently of Jag1b in more ventral CNCCs. Separate from relieving Jagged-Notch inhibition, Edn1 might also directly promote *dlx3b*, *dlx5a*, *dlx6a*, *epha4b*, *msxe* and *bapx1* expression. (**B**) Schematic of the pharyngeal arches showing the dorsal (d) and ventral (v) portions of the mandibular (MAND) and hyoid domains, and the Jagged-Notch-independent maxillary (MAX) domain. The ectodermal Edn1 source, ventral fates (red-to-yellow gradient) and dorsal fates (green) are shown. Outlines indicate the fate-map origin of cartilage (blue) and bone (red) based on published data (Crump et al., 2006; Eberhart et al., 2006). (**C**) Ventral fates are moderately expanded in *jag1b*^{b1105} and *notch2*-MO larvae, resulting in reduction of dMAND cartilage and variable transformations of dHYOID cartilage and bone to ventral morphologies. (**D**) Dorsal fates are expanded in JAG1-misexpression larvae, resulting in partial transformations of vMAND and vHYOID cartilages and full transformation of vHYOID bone to a dorsal morphology.

several reasons why skeletal elements might adopt morphologies similar, but not identical, to their DV cognates upon Jag1b-Notch2 manipulation. First, the lack of full transformations in *jag1b*^{b1105} larvae could be due to residual Jagged-Notch signaling mediated by Jag2. Similarly, the partial transformations of *notch2*-MO larvae could be attributed to incomplete efficacy of the MO. However, our analysis of *jag1b*^{b1105}; *jag2* double mutants has not revealed any striking enhancement of facial defects over *jag1b*^{b1105} single mutants (data not shown), suggesting that the partial nature of the transformations is not due to residual Jagged-Notch activity. Second, cell-intrinsic changes in identity often do not lead to homeotic transformations of an identical nature, as a field of cells that adopts the identity of another may encounter different types of extrinsic signals or spatial constraints. For example, the loss of Hox paralog 2 genes, which function as homeotic selectors in anterior-posterior patterning, results in only partial duplications of the mandibular facial skeleton in the more posterior hyoid arch (Gendron-Maguire et al., 1993; Rijli et al., 1993; Miller et al., 2004). Analogously, Edn1 overexpression results in only partial transformations of dorsal skeletal elements, similar to what we observe in *jag1b*^{b1105} mutants (Kimmel et al., 2007; Sato et al., 2008). Third, the partial transformations of the dorsal skeleton in *jag1b*^{b1105} and *notch2*-MO embryos correlate with the only moderate expansion of *dlx3b*, *dlx5a*, *dlx6a*, *epha4b*, *msxe* and *bapx1* expression into the dorsal arches, with the expression of the most ventrally restricted gene, *hand2*, being unaffected. Hence, rather than inhibiting ventral identity throughout the dorsal domain, the function of Jagged-Notch signaling might be to refine the dorsal limit of a subset of ventral genes that are expressed up to the DV border. Therefore, as discussed below, it might be that other signaling pathways act in parallel with Jagged-Notch to repress ventral gene expression in dorsal skeletal precursors.

Jagged-Notch signaling patterns a distinct axis within the mandibular and hyoid arches

Our analysis also reveals that Jagged-Notch signaling has a more restricted role in patterning a DV axis of the mandibular and hyoid arches that is distinct from the maxillary-mandibular axis (Fig. 7B). Whereas *jag1b* and *hey1* are expressed in 'dorsal mandibular' CNCCs anterior to the first pouch and in dorsal hyoid CNCCs between the first and second pouches, they are not expressed in maxillary CNCCs anterior to the oral ectoderm. Concomitantly, ventral gene expression expands into the dorsal mandibular and hyoid domains, but not the maxillary domain, of *jag1b*^{b1105} and *notch2*-MO embryos. Our previous fate maps of wild-type arches (Crump et al., 2006; Eberhart et al., 2006) also help to explain why Hm and Op are strikingly transformed in shape, yet only a portion of Pq is lost, in *jag1b*^{b1105} mutants. Whereas Hm and Op derive entirely from dorsal hyoid CNCCs, only a portion of Pq derives from dorsal mandibular CNCCs, with the Ptp process of Pq deriving from maxillary CNCCs that are unaffected in *jag1b*^{b1105} mutants.

Species-specific differences might also account for the relative importance of Jagged-Notch signaling in facial patterning. Whereas the majority of the craniofacial skeleton of the larval zebrafish derives from mandibular and hyoid CNCCs, in mammals most of the facial skeleton derives from frontonasal, maxillary and ventral mandibular CNCCs, with dorsal mandibular and hyoid CNCCs contributing prominently to the ossicles of the middle ear. Recently, *Pofut1*$^{flox/-}$; *Wnt1-Cre* mice have been generated that lack Notch signaling throughout CNCCs owing to the tissue-specific deletion of *O*-fucosyltransferase 1, an essential component of Notch signaling (Okamura and Saga, 2008). The lack of severe craniofacial defects in *Pofut1*$^{flox/-}$; *Wnt1-Cre* mice is consistent with our findings in zebrafish that Jag1b and Notch2 are not required for the patterning of maxillary and frontonasal CNCCs. As we find that Jag1b and Notch2 inhibit ventral skeletal identity only in the dorsal mandibular and hyoid domains, it will be interesting to examine whether a role of JAG1 in development of the ossicles of the middle ear contributes to the conductive hearing loss seen in some Alagille syndrome patients (Le Caignec et al., 2002).

Jagged-Notch signaling propagates throughout dorsal skeletal precursors

Our analysis of *jag1b* transcriptional regulation also indicates a potential mechanism by which Notch patterns a broad field of dorsal skeletal precursors. In contrast to secreted morphogens, Jagged and Notch are transmembrane proteins and do not generally act at a distance. In the *Drosophila* wing, Notch signaling induces the expression of morphogens, in particular Wingless, which act at a distance to pattern the dorsal domain (Diaz-Benjumea and Cohen, 1995). However, our observation that the Notch2 target *hey1* is expressed throughout dorsal skeletal precursors suggests a more

direct role for Notch in dorsal facial patterning. Similar to what is observed during early inner ear development (Daudet et al., 2007), we find that Jagged-Notch transcriptional feedback serves to extend *jag1b* expression more ventrally over time. Such a mechanism would propagate Notch activity throughout the dorsal facial domain, ensuring that each cell within the dorsal field experiences Notch activity at some point during development.

Edn1 represses Jagged-Notch signaling in the ventral face

Whereas Jagged-Notch feedback extends Notch activity, we find that Edn1 signaling prevents Notch activity from spreading into the ventral domain by repressing *jag1b* expression. Previous studies have demonstrated a nearly complete loss of the ventral facial skeleton in zebrafish *edn1* mutants, suggesting that Edn1 is essential for development of the ventral facial skeleton (Miller et al., 2000). By contrast, we find that in the absence of Jag1b-Notch2 signaling, Edn1 is partially dispensable for development of the ventral face. Thus, the ventral gene expression and skeletal defects of *edn1* mutants can at least partially be attributed to aberrant *jag1b* expression and hence Notch activity in the ventral domain. However, the partial nature of the rescue in *edn1*; *jag1b*^{b1105} mutants suggests that Edn1 might also have Notch-independent roles in promoting ventral gene expression and skeletal development. Moreover, we find that partial reduction of Edn1 signaling fails to rescue *jag1b*^{b1105} defects, and the expression of *edn1* and its receptor *ednra2* are not regulated by Jag1b-Notch2 signaling. Thus, the dorsal-to-ventral transformations of *jag1b*^{b1105} mutants are not due to increased Edn1 signaling. What, then, might account for the striking ability to form a fairly normally patterned ventral hyoid skeleton even in the complete absence of Edn1? Studies in mouse suggest that unknown redundant signals function in parallel with Edn1 to promote ventral skeletal fates (Ruest et al., 2004). In the absence of Edn1, these redundant signals might be unable to sustain ventral skeletal development owing to ectopic repressive Notch activity in the ventral domain. However, in the absence of both Edn1 and Notch signaling, redundant ventralizing signals might now be able to partially support ventral skeletal development. Further investigation of ventral patterning in the absence of both Edn1 and Jagged-Notch signaling should help to reveal the extent of functional redundancy in specifying the DV axis of the facial skeleton.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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