Wnt Signaling Interacts with Bmp and Edn1 to Regulate Dorsal-Ventral Patterning and Growth of the Craniofacial Skeleton

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Abstract
Craniofacial development requires signals from epithelia to pattern skeletogenic neural crest (NC) cells, such as the subdivision of each pharyngeal arch into distinct dorsal (D) and ventral (V) elements. Wnt signaling has been implicated in many aspects of NC and craniofacial development, but its roles in D-V arch patterning remain unclear. To address this we blocked Wnt signaling in zebrafish embryos in a temporally-controlled manner, using transgenics to overexpress a dominant negative Tcf3, (dntcf3), (Tg(hsp70ltcf3-GFP), or the canonical Wnt inhibitor dickkopf1 (dkk1), (Tg(hsp70ldkk1-GFP) after NC migration. In dntcf3 transgenics, NC cells in the ventral arches of heat-shocked embryos show reduced proliferation, expression of ventral patterning genes (hand2, dlx3b, dlx5a, msxe), and ventral cartilage differentiation (e.g. lower jaws). These D-V patterning defects resemble the phenotypes of zebrafish embryos lacking Bmp or Edn1 signaling, and overexpression of dntcf3 dramatically reduces expression of a subset of Bmp receptors in the arches. Addition of ectopic BMP (or EDN1) protein partially rescues ventral development and expression of dlx3b, dlx5a, and msxe in Wnt signaling-deficient embryos, but surprisingly does not rescue hand2 expression. Thus Wnt signaling provides ventralizing patterning cues to arch NC cells, in part through regulation of Bmp and Edn1 signaling, but independently regulates hand2. Similarly, heat-shocked dkk1+ embryos exhibit ventral arch reductions, but also have mandibular clefts at the ventral midline not seen in dntcf3+ embryos. Dkk1 is expressed in pharyngeal endoderm, and cell transplantation experiments reveal that dntcf3 must be overexpressed in pharyngeal endoderm to disrupt D-V arch patterning, suggesting that distinct endodermal roles for Wnts and Wnt antagonists pattern the developing skeleton.

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Introduction
A fundamental question in skeletal biology is how cartilages and bones with distinct shapes arise from skeletogenic precursor cells. Much of the craniofacial skeleton derives from neural crest (NC) cells that migrate in streams into the pharyngeal arches and contain anterior-posterior (A-P) patterning information obtained prior to migration [1–3]. However, these NC cells also become intimately associated with epithelia, including surface ectoderm and pharyngeal endoderm, which produce signals important for skeletal patterning. For example, FgB from the facial ectoderm regulates A-P polarity of the mandibular arch as well as NC proliferation/survival [4–6]. Surgical disruption of the pharyngeal endoderm in chick [7], or mutations that disrupt endoderm in zebrafish, lead to severe cartilage malformations [8–10]. Endoderm-derived Fg3 induces cartilage formation [9] and sphingosine phosphate-1 from endoderm modulates Shh signaling to promote mandibular growth and patterning [11–13]. Ectoderm-derived Shh induces upper jaw and neurocranial structures [14,15]. Thus, craniofacial skeletal shapes reflect interplay between epithelial signals and intrinsic properties of mesenchyme, but the mechanisms underlying these interactions remain unclear.

One well-studied example of such epithelial-mesenchymal interactions in the pharyngeal skeleton is the induction of ventral skeletal fates along the dorsal-ventral (D-V) axis of the mandibular arch by ectodermal Endothelin 1 (Edn1) and Bone morphogenetic proteins (Bmps) [16–22]. Conditional loss of Bmp4 in the facial ectoderm in mice inhibits ventral mandibular growth and patterning [23]. Loss of Edn1 and/or any of several components of its signal transduction pathway leads to severe jaw truncations, both in mice and in humans, and in some cases transformations of ventral tissues (the lower jaw) to a more dorsal identity [22,24–27]. Initially both Edn1 and Bmps induce similar subsets of ventral/intermediate genes as well as restricting Fg1 signaling to the dorsal domain [16,19,20,28]. But once arch primordia are established, effects of Bmps become more ventrally restricted to domains that no longer depend on Edn1, particularly the transcription factor Hand2. Uniform application of Bmp or Edn1 proteins can restore many aspects of D-V patterning in Bmp- and Edn1-deficient zebrafish embryos, suggesting that other ventralizing signals must interact to control D-V patterning [22].

Wnts are good candidates for additional signals involved in D-V patterning based on their localized expression and known requirements in craniofacial development. Several Wnt ligands

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Wnts are good candidates for additional signals involved in D-V patterning based on their localized expression and known requirements in craniofacial development. Several Wnt ligands
**Author Summary**

Craniofacial abnormalities are among the most common birth defects. Understanding the molecular mechanisms underlying craniofacial disorders is crucial for developing treatment strategies. Much of the craniofacial skeleton arises from specialized embryonic structures known as pharyngeal arches. Patterning of these arches requires precise spatial and temporal expression of multiple genes, which is coordinated between tissues by secreted signals. Wnts are secreted ligands expressed throughout the pharyngeal arches yet their role in craniofacial patterning remains unclear. In this study we examine the role of Wnts in craniofacial patterning using transgenic zebrafish to inhibit downstream Wnt signaling. We show that Wnt signaling deficient embryos have lower jaw specific defects, which strongly resembles loss-of-function phenotypes in both the Bmp and Edn1 signaling pathways. Through rescue experiments we find that Wnts are upstream regulators of both Bmp and Edn1 signaling. We thus have uncovered a crucial requirement for Wnt signaling in craniofacial patterning.

show restricted expression in facial epithelia (ectoderm, endoderm) in zebrafish, chick and mouse embryos [29–32] and Frizzled (Fzd) receptors are expressed throughout arch NC cells and endoderm [31,33,34]. In addition, expression of Lef1, Tcf1, β-catenin (βcat), [35], and transgenic Wnt signaling reporter lines (Lef/Tcf promoters driving β-gal or LacZ expression) in mice all are ventrally (distally) restricted in the mandibular as well as distal maxillary prominences [36,37]. Like Bmps, Wnt signaling is necessary for early NC cell induction [38,39] and also plays later roles in NC migration, fate specification, and proliferation [40]. In zebrafish, conditional overexpression of a dominant negative Tcf3 (dntcf3) during NC cell specification dramatically reduces NC cell numbers [41], similar to depletion of Fzd3 receptors in Xenopus [42]. Wnt1+/−/Wnt3+/− double mutant mice show reduced proliferation of pre-migratory NC cells [43]. Conditional loss of βcat in the pharyngeal ectoderm impairs growth of the facial prominences [44], while conditional loss of βcat in cranial NC tissue leads to apoptosis and a nearly complete loss of NC-derived craniofacial structures [45]. Finally, loss of Tcf4/Lef1 function or overexpression of the Wnt inhibitor Dickkopf-1 (Dkk1) results in smaller facial structures and clefting between the frontonasal and maxillary prominences [36,37]. Similarly, Wnt signaling is important for facial midline development in humans as incipient cleft lip and palate have been mapped to genetic variations in Wnt ligands [46].

In this study, we examine temporal requirements for Wnt signaling in zebrafish D-V craniofacial development. We utilize two transgenic lines, Tg(hsp701:dkk1-GFP)(dkk1+) and Tg(hsp701:tcf3-GFP)(dntcf3+), to interfere with Wnt signaling conditionally, in a stage-specific manner. Tg(hsp701:dkk1-GFP) embryos overexpress dkk1, a secreted negative regulator, while Tg(hsp701:tcf3-GFP) embryos overexpress a dominant negative form of the Tcf3 transcription factor. Both methods of inhibiting Wnt signaling after NC cell migration result in proliferation and ventral patterning defects in the mandibular and hyoid arches. Interestingly, dkk1+ embryos also show unique cleavage of the lower jaw. Defects in ventral-intermediate specific gene expression and expansion of the dorsal specific jag1b resemble loss of Bmp and Edn1 signaling [19,20]. We show that Wnt signaling promotes Bmp signaling through regulation of expression of two specific Bmp receptors, bmpr1ab and bmpr1ba, in the pharyngeal arches. Ectopic Bmp protein can rescue mycn, dlx3b, dlx5a but not hand2 in the absence of Wnt signaling, demonstrating that Wnts participate in a regulatory network with Bmp and Edn1 signaling, but separately in hand2 regulation, to control D-V pharyngeal patterning. Chimeric analyses reveal that dntcf3 acts cell autonomously in pharyngeal endoderm, which also expresses dkk1. This suggests that Wnts regulate patterning in the endoderm, which through some as yet unknown signal impacts D-V patterning upon neighboring skeletal progenitors in the NC.

**Results**

Wnt responses are highest in the ventral pharyngeal arches

Numerous Wnt ligands (Wnt2, Wnt4, Wnt5a/b, Wnt 6, and Wnt7a/b) and receptors (Fzd1, Fzd3, Fzd4, Fzd6, Fzd7, Fzd8, and Fzd10) are expressed broadly in the pharyngeal endoderm, endoderm, neural crest (NC), and mesoderm [29,32,34,35]. To determine which regions of the pharyngeal arches respond directly to Wnt signaling we used in situ hybridization (ISH) to examine expression of the direct downstream Wnt target mycn (Fig. 1A,B); an oncogene with roles in regulating Wnt-dependent morphogenesis and proliferation [47,48]. mycn mRNA was detected throughout the arches but at higher levels in the ventral domain, primarily within the NC mesenchyme (arrowheads in Fig. 1A,B). To further address which pharyngeal tissues respond directly, we examined expression of a transgenic Wnt reporter zebrafish Tg(7xTCF-Xla.Siam:GFP)red/7xTCF-GFP [49], which contains seven TCF response elements driving expression of GFP, thus acting as a live reporter in cells where stabilized β-catenin (βcat) interacts with Tcf transcription factors. ISH for GFP mRNA at 28 hours postfertilization (hpf) revealed regions of 7xTCF-GFP expression in the ventral first and second arches (Fig. 1C), which in transverse sections appeared localized both to arch NC cells and pharyngeal endoderm, but not pharyngeal ectoderm (Fig. 1D).

Blocking Wnt signaling after NC migration disrupts the ventral arch skeleton

To bypass earlier requirements for Wnts in embryogenesis we took a conditional loss-of-function approach using heat shock-inducible transgenic zebrafish lines to inhibit Wnt signaling in a temporally-controlled manner. Tg(hsp701:tcf3-GFP) (hs-dntcf3) embryos overexpress a truncated form of the transcription factor tcf3 with GFP replacing the βcat-interacting domain, under control of heat shock promoter 70 [41]. With a similar hs70 promoter, Tg(hsp701:dkk1-GFP) (hs-dkk1) embryos overexpress full length dkk1b tagged with a GFP [50], which prevents Fzd-Lrp co-receptor binding [51,52].

To verify that Wnt-βcat signaling was affected in hs-dkk1+ and hs-dntcf3+ embryos we used ISH to examine the expression of mycn and axin2, both direct Wnt targets, after heat shocking during stages of craniofacial patterning. At 4 hours post heat shock (hps) hs-dntcf3+ embryos heat shocked at 22 hpf showed severe reductions in expression of mycn in the arches, eyes, and brain and axin2 (which shows only very weak or no expression in pharyngeal arches), in the eyes and brain (Fig. 1E–H). Similarly, compared with controls (Fig. S1A, C) at 2 hps hs-dkk1+ embryos heat shocked at 24 hpf showed reduced mycn in the arches, eye, and brain and axin2 expression in the brain but to a lesser extent than in hs-dntcf3+ embryos (Fig. S1B, D).

To determine stage-specific defects caused by disrupting Wnt signaling in hs-dntcf3+ and hs-dkk1+ embryos we performed quantitative real time PCR (qPCR) analysis of direct Wnt targets. In hs-dntcf3+ embryos, axin2 and mycn expression was reduced.
significantly from 2–8 hphs (Fig. 1I), but recovered and slightly increased by 24 hphs (Fig. 1I). *Lef1*, a transcriptional cofactor in the Wnt pathway [53,54], was also significantly reduced in hs-*dntcf3*+ embryos from 2–8 hphs (Fig. 1I). In hs-*dkk1*+ embryos, *axin2* expression was severely reduced from 1–8 hphs, while *Lef1* downregulation started later, from 4–8 hphs (Fig. S1E). Thus both hs-*dntcf3* and hs-*dkk1* lines deplete Wnt signaling almost immediately after heat shock and repress Wnt signaling for up to 8 hphs.

Alcian blue staining of cartilage in larvae at 96 hpf revealed that, compared with controls, hs-*dkk1*+/+ heterozygotes heat shocked for 30 min at 16–28 hpf developed mandibular clefting and reduced Meckel’s cartilages (Mc), as well as mild reductions in other craniofacial cartilages (Fig. 2A, B, D). Homozygous hs-*dntcf3*+/+ larvae displayed dramatic shortening of Mc ventrally in the first arch, as well as the symplectic (Sy), a more intermediate/dorsal element of the second arch (Fig. 2C, E; [19,20]). Thus, hereafter “dntcf3+” refers to homozygous hs-*dntcf3*+ embryos/larvae heat shocked for 30 min between 20–22 hpf.

Heterozygous hs-*dntcf3*+ larvae heat shocked slightly later (22–24 hpf) also showed mild reductions in Mc, but in this case Mc was fused to the more dorsal palatoquadrate (Pq) in arch 1 (Fig. 2F, H). The ceratohyal (Ch) in ventral arch 2 was also variably reduced, but more posterior cartilages appeared largely unaffected. Cartilage defects in homozygous hs-*dntcf3* larvae similarly were much more severe (Fig. 2E, G) – 41% showed reduced Mc and Ch reduction/loss, while 66% showed joint fusion between Mc and Pq (Fig. 2H). Thus, hereafter “dntcf3+” refers to heterozygous hs-*dntcf3*+/− embryos/larvae heat shocked for 12 min between 22–24 hpf.

To determine tissue-specific requirements for Wnt signaling in the pharyngeal arches, we transplanted *dntcf3*+ cells at gastrula...
stages either into the fate map position that gives rise to NC or co-injected with Taram-A mRNA to drive them to an endodermal fate, into non-transgenic WT hosts [9]. While dntcf3+/NC cells in chimeras that virtually filled the entire mandibular arch caused no discernable cartilage defects (not shown), large grafts of dntcf3+ endodermal cells into the pharyngeal region induced D-V patterning defects that resembled dntcf+/embryos, including reduced Mc and fused Mc-Pq (Fig. S2A). These results suggest that the critical direct response to Wnt occurs in the endoderm (which expresses 7XTCF:GFP) and is indirectly relayed to surrounding NC cells.

To verify that cartilage defects in dkk1+ and dntcf3+ larvae reflect specific requirements for Wnt signaling we attempted to rescue them using the compound 6-bromoindirubin-3’-oxime (BIO), which stabilizes Wnt signaling by inhibiting GSK-3 [55]. BIO treatments of 7xTCF:GFP embryos at 24 hpf caused ectopic gfp expression and direct Wnt targets were upregulated in a dose-dependent manner as determined by ISH and qPCR analysis at 30 hpf (Fig. S3). Treatment of wild type embryos with BIO resulted in an overall reduction of cartilages in a dose-dependent manner, which correlated with reduced proliferation of cartilage precursors (Fig. S4). Despite their smaller sizes, dorsal cartilages acquired more rod-like morphologies similar to ventral Mc and Ch, suggesting partial ventralization (Fig. S4A–C). BIO treatments of both heat shocked dntcf3+ and dkk1+ transgenics partially rescued cartilage defects, including Mc-Pq joint fusions and Ch was consistently restored in dntcf3+/embryos heat shocked at different stages from 16–30 hpf. (E) Histogram quantifying the frequency of cartilage defects in dkk1+ embryos heat shocked at different stages from 16–30 hpf. Abbreviations: Ch, ceratohyal; Hm, hyomandibular; Mc, Meckels; Pq, palatoquadrate; Sy, symplectic. Scale bar: 100 μm.

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Figure 2. Requirements for Wnt signaling in craniofacial cartilage development. (A–C, F, G) Alcian blue stained cartilage at 96 hpf, dissected and flat mounted, ventral views, anterior to the left. (A) Wild-type control, (B) dkk1+/− heterozygote, (G) dntcf3−/+ homozygote, heat shocked as described (see Results). Arrowheads indicate clefting of Meckel’s cartilage (Mc). (D) Histogram quantifying the frequency of Mc defects in dkk1+/embryos heat shocked at different stages from 16–36 hpf. (E) Histogram quantifying average Mc length, p<0.01. (H) Histogram quantifying the frequency of cartilage defects in arches 1 and 2 in dntcf3+/embryos heat shocked at different stages from 16–30 hpf. Abbreviations: Ch, ceratohyal; Hm, hyomandibular; Mc, Meckel’s; Pq, palatoquadrate; Sy, symplectic. Scale bar: 100 μm.

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To confirm these apparent defects in proliferation we used an antibody that recognizes phosphoHistone3 (pH 3), a protein involved in chromosome condensation in mitotic cells [57], which
marks a subset of pcna+ dividing cells. In dkk1+ and dntcf3+ embryos pH 3 staining was reduced throughout the eye and brain (Fig. S6A–B, D–E). At 4 hphs, dkk1+ embryos had a 75% reduction in pH 3+ cells in the arches compared to controls (Fig. S6A, B). Similarly, at 3 hphs dntcf3+ embryos had approximately 50% fewer pH 3+ cells in the arches than controls.
Thus depleting Wnt signaling in embryos between 24–30 hpf severely impairs proliferation in the arches, which correlates with reductions in cartilage and in Wnt target gene expression (Figs. 1, 2).

Blocking Wnt signaling disrupts expression of ventral arch patterning genes

To investigate roles for Wnt signaling in D-V patterning within the arches, we examined expression of genes that mark distinct ventral, intermediate and dorsal regions of the arch primordia in dntcf3+ and dkk1+ embryos with ISH [19]. hand2 expression in the ventral-most domains of each arch was severely reduced in both dkk1+ (53%, n = 15) and dntcf3+ (59%, n = 17) embryos (Fig. 4A–C, Q), with a small domain of expression remaining at the arch 1–2 boundary in dntcf3+ embryos (Fig. 4C). Similarly, expression of dlx3b and dlx6a in the intermediate domains of each arch were mildly reduced in dkk1+ (44%, n = 18, 23%, n = 21) and severely reduced in dntcf3+ embryos (83%, n = 12; 90%, n = 21) (Fig. 4D–I, Q). Finally, expression of the Notch ligand, jag1b in the dorsal-most domains of each arch [28], was variably expanded ventrally in dkk1+ (10.5%, n = 57) embryos and consistently expanded in dntcf3+ embryos (55.5%, n = 9) as well as chimeras in which dntcf3+ cells were transplanted into the pharyngeal endoderm (Fig. 4J–L, Q; Fig. S2B–C). These gene expression changes were not simply due to an overall loss of arches or NC cells, since dlx2a expression (Fig. 4N, P) as well as sox10:lynTdtom expression throughout the D-V extent of the arch NC were unaffected in the arches of both dkk1+ and dntcf3+ embryos (Fig. S7).

Blocking Wnt signaling disrupts Bmp and Edn1 signaling in the arches

Because dntcf3+ embryos showed D-V defects in cartilage morphology and gene expression that more closely resembled Bmp- and Edn1-deficient embryos than dkk1+ we focused on dntcf3+. To examine interactions between Wnt and Bmp signaling in the arches we used an antibody that recognizes phosphorylated Smad1/5/8 (pSmad1/5/8) in dntcf3+ embryos. In controls pSmad1/5/8 localized to ventral arches 1 and 2 where levels of Bmp signaling have been shown to be highest at 24 hpf (Fig. 5A–C; [19]). Anti-pSmad1/5/8 staining was slightly reduced in the first arch at 2 hphs (24–26 hpf) in dntcf3+ embryos (Fig. 5D), in both arches by 4 hphs (Fig. 5E), and virtually lost altogether at 6 hphs (Fig. 5F). Western blots confirmed that pSmad1/5/8 levels were much lower than controls at 6 hphs (Fig. 5G, H). To examine potential interactions between Wnt and Edn1 signaling in the arches we performed qPCR for Edn1 in dntcf3+ embryos at 6 hphs.

Figure 4. Requirements for Wnt signaling in dorsal-ventral arch patterning. (A–P) Whole mount ISH for genes involved in dorsal-ventral (D-V) patterning in control (A, D, G, J), dkk1+ embryos heat shocked at 20–22 hpf (B, E, H, K), and dntcf3+ embryos heat shocked at 22–24 hpf (C, F, I, L), lateral views, anterior to the left. hand2 (B, C), dlx3b (E, F), and dlx6a (H, I) expression is reduced, while jag1b (K, L) expression expands ventrally (arrowheads) particularly in dntcf3+ embryos, compared with controls. (M–P) dlx2a expression in control (M, O), dntcf3+ (N), and dkk1+ (P) embryos. The dorsal boundary of dlx2a expression (arrowheads) appears to shift ventrally in some cases, (N, P) compared to the control (M, O). (Q) Histogram quantifying percentages of dkk1+ and dntcf3+ embryos with defects in D-V patterning. Scale bar: 100 μm.

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(Fig. S6D’, E’, F). Thus depleting Wnt signaling in embryos between 24–30 hpf severely impairs proliferation in the arches, which correlates with reductions in cartilage and in Wnt target gene expression (Figs. 1, 2).
expression was significantly reduced relative to control (Fig. 5I). These results reveal an indirect role for Wnts in D-V patterning through regulation of both Bmp and Edn1 signaling.

Exogenous Bmp or Edn1 proteins partially rescue ventral patterning in Wnt-deficient arches

Bmps act together with Edn1 to promote ventral-intermediate cell fates in the arches [16,17,21,22,58]. Therefore we examined the ability of Bmp and Edn1 to restore ventral-intermediate gene expression in Wnt signaling-deficient embryos. Beads coated in human recombinant BMP4/7 heterodimers effectively induce Bmp target genes in zebrafish pharyngeal arches [19]. Similarly, microinjection of a 25 ng/ul BMP4/7 solution extracellularly on one side of the head induced Bmp signaling, as measured by expression of the transgenic Bmp-response element reporter (Brc:Gfp; [19]) at 8 hours post injection (hpi) (Fig. 6A, B). Unilateral injections of BMP4/7 protein into dntcf3+ embryos at 4 hphs partially rescued cartilage defects on the injected side (Fig. 6C–J). Typically this restored Mc length and Ch, but not the Mc-Pq joint, and rescue was dose-dependent (Fig. 6C, D). These results suggest that Wnt signaling acts upstream of, or possibly in parallel to, Bmp signaling to promote ventral cartilage cell fates in the arches. EDN1 protein injections have previously been shown to rescue an Edn1 mutant phenotype and partially rescue a Bmp loss of function phenotype [16,19]. EDN1 injections into dntcf3+ embryos also partially rescued Mc length.
but notably were more proficient at rescuing Ch and joint development (Fig. 6C–D, L).

While both Bmp and Edn1 signaling induce many of the same genes that specify ventral-intermediate NC cell fates in the early arches, by later stages Bmps become much stronger inducers of hand2 (ventral) and msxe (ventral-intermediate) [19,20]. Strikingly, neither BMP4/7 nor EDN1 protein injections at 4 hphs were sufficient to rescue hand2 expression in dntcf3+ embryos (Fig. 7A–D, Q). BMP4/7 but not EDN1 restored msxe expression (Fig. 7E–H), particularly in the mandibular arch near the injection site (Fig. 7G). In contrast, both BMP4/7 and EDN1 injections restored dlx3b and dlx5a expression in the intermediate domain (Fig. 7I–P, Q). These results suggest that hand2 expression absolutely requires Wnt signaling to respond to Bmps, while other signals can partially substitute for Wnts in induction of more intermediate-dorsal NC cell fates.

**Wnt signaling regulates expression of Bmp receptors**

To further investigate how Wnts might regulate the ability of NC cells to respond to Bmp signaling, we examined whether or not dntcf3+ embryos show any changes in expression of Bmp receptors. Zebrafish have four type 1 receptors (Bmpr1aa, ab, ba, bb) and two type II receptors (Bmpr2a and b). Whole mount ISH for all six receptors revealed that only bmpr1ab, bmpr1ba, and bmpr1bb are expressed strongly in the arches at 24 hpf (Fig. S9C–F, I–J, M–R). bmpr1aa, bmpr2a, and bmpr2b were detected much more broadly throughout the embryo at this stage (Fig. S9A–B, G–H, K–L). Bmpr1ab expression extended throughout arches 1 and 2, while bmpr1ba and bmpr1bb expression was restricted to more intermediate and ventral domains (Fig. S9M–O). Transverse sections additionally showed that bmpr1ab and bmpr1ba expression is limited to arch NC cells and not surrounding epithelia (Fig. S9P–R). In dntcf3+ embryos bmpr1ab was severely reduced (57% n = 35).
Figure 7. Bmp and Edn1 restore ventral-intermediate gene expression, but not hand2, in dntcf3+ embryos. (A–P) Whole mount ISH for ventral-intermediate patterning genes in heat shocked controls (A, E, I, M), dntcf3+ embryos (B, F, J, N), and dntcf3+ embryos with either 25 ng BMP4/7 (C, G, K, O), or 5 ng EDN1 (D, H, L, P), lateral views, anterior to the left. Asterisks in G and K indicate cases in which rescue occurred in arch 1 but not arch 2. (Q) Histogram quantifying the number of embryos with reductions in arch expression for each gene. Quantification obtained by counting number of embryos with rescue of gene expression in three independent experiments (minimum of 10 embryos per protein injection experiment). Statistical method used is a paired student t-test. * P<.05, ** P<.001. Scale bar: 100 μm.
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Changes in Bmp receptor expression in dntcf3+ embryos were further quantified by qPCR analysis. At 6 hphs we compared the relative expression of arch specific Bmp receptors (bmpr1ab, bmpr1ba, bmpr1bb) with ubiquitously expressed bmpr2a and tgfbr1a, a TGF-B receptor expressed in the arches unrelated to Bmp signaling [59]. There was no detectable reduction in tgfbr1a or bmpr2a expression in dntcf3+ embryos (Fig. 8H). At 6 hphs, both bmpr1ab and bmpr1ba expression were reduced (Fig. 8I) but bmpr1bb expression showed no difference from controls (Fig. 8H).

A time series analysis revealed no change in bmpr1ab and bmpr1ba expression at 2 hphs, despite reduced Wnt signaling (see Fig. 1), but levels dropped dramatically by 4 hphs. bmpr1ba but not bmpr1ab expression recovered substantially by 8 hphs. This suggests differential requirements for Wnt signaling in induction of Bmp receptors.

Dkk1b functions in the pharyngeal endoderm

Dkk1+ embryos exhibit a unique clefting of the mandible not seen with dntcf3+. Although primarily known as a repressor of Wnt signaling, Dkk1 has also been reported to positively regulate the Wnt-PCP pathway [60]. To gain further insights into its
tissue-specific functions, we examined dkk1b expression in pharyngeal arch primordia. Of the five known dkk genes in zebrafish, only dkk1b is expressed in the embryonic arches [61]. We found that between 28–48 hpf dkk1b expression localized to the pharyngeal endoderm, particularly the pouches between arches (Fig. S10A–C). Consistent with this, expression was lost in runx ggo [ggo] mutants, which lack pouches [10] (Fig. S10D–E). dkk1b expression was also detected in the stomodeum (oral ectoderm) at 28 hpf (Fig. S10A) and later in the ectoderm of the mouth at 48 hpf (Fig. S10F).

Signals from the pharyngeal endoderm and oral ectoderm are necessary for craniofacial patterning, and chondrogenesis [9,10,13–15]. To determine if there were gross defects in these epithelial layers in dntcf embryos, we examined nkd2, 3, and found that its expression in the pharyngeal endoderm was disorganized in the first two pouches (Fig. S11B,F) and severely reduced in the more posterior pouches (Fig. S11B). In contrast, anterior pouches appeared unaffected in dkk1+ embryos, while the more posterior pouches were occasionally disorganized (Fig. S11D,H). Expression of pitx2ca in the oral ectoderm (Fig. S11I–R) was delayed in dkk1+ embryos until 26 hphs (Fig. S11O), by 30 hphs the mouth opening was abnormally elongated laterally and by 51 hphs showed a ventral midline fold (Fig.S11P–R). Thus pharyngeal pouch and mouth defects differ between dntcf and dkk1 embryos, which could account for some of the differences in their effects on growth and morphogenesis of the lower jaw.

Discussion

We show that Wnt signaling promotes proliferation and provides ventral-intermediate patterning cues to NC cells in the pharyngeal arches by participating in a regulatory network with Edn1 and Bmp (Fig. 9). By overexpressing Dkk1 or dnTcf3 to disrupt Wnt signaling, we show that Wnt promotes expression of ventral (hand2) and ventral-intermediate genes (dlx3b, dlx5a, msxe) and their corresponding skeletal derivatives, and acts upstream or in parallel to the ventralizing activities of Edn1 and Bmp. Unlike Edn1 and Bmp, however, our chimeric analyses suggest that direct responses to Wnt signaling occur in the pharyngeal endoderm, which also expresses dkk1. This endoderm must secondarily produce as yet unknown signals important for D-V patterning, which regulate the competence of NC cells to respond to Bmp signaling, in part by transcriptionally regulating Bmp receptors. Overexpression of dkk1 also causes a unique midline clefting of the mandible, which we suggest reflects a role in formation of the mouth.

Wnt-mediated patterning and growth in craniofacial development

Direct Wnt responses in the ventral first and second arches resemble the pattern of TOP:Gal expression in mice, including distal (ventral) arch 1 [36,37]. Mycn, a direct transcriptional Wnt target, is also expressed in both fish and mouse arch NC cells, where it is likely to inhibit Wnt-β-catenin signaling [62], and provide negative feedback. Murine Mycn is expressed in highly proliferative cells and mutants show hypoplasia of the mandibular arch [63,64]. Similarly, we find reduced proliferation in the pharyngeal arches in Wnt-deficient zebrafish and smaller craniofacial cartilages (Fig. 3; Fig. S5). Thus, Wnt signaling may promote growth of the ventral arches through induction of mycn expression.

We show a critical requirement for Wnt signaling in arch growth and patterning that is distinct from its earlier roles in NC induction and migration. Earlier heat shocks of dntcf3 or dkk1 zebrafish (10–20 hpf) disrupt premigratory NC formation [41,65] similar to Wnt1/Wnt3a mutant mice [43]. Unlike recent conditional loss- and gain-of-function studies of βcat in the pharyngeal ectoderm in mice [44], we find no changes in cell survival in the arches in dntcf3+ embryos.

D-V defects in gene expression in the arches caused by overexpressing dntcf3 or dkk1 at these later stages point to a problem with canonical Wnt signaling. Both reduce expression of canonical Wnt target genes up to 8 hphs (Fig. 1I; Fig. S1), and both lead to ventral cartilage and joint defects. However, dkk1 overexpression has more subtle effects (restricted primarily to Mc and the jaw joint) than dntcf3. Defects in dntcf3+ embryos are also stronger when heat shocked at slightly earlier stages (15–22 hpf), than dntcf3+ (22–24 hpf). These differences may reflect distinct functions for the two transgenes, or a delay due to the time required for Dkk1 to competitively bind with the Lrp5/6 co-receptor, whereas Tcf3 directly binds βcat and Wnt target genes. Heat shocking dntcf3 or dkk1 at earlier stages eliminates cartilage, consistent with requirements for canonical Wnt signaling in NC induction. Both mycn and axin2 expression recover by 24 hphs in heat shocked embryos indicating a transient requirement for canonical Wnt signaling prior to skeletal cell differentiation.

Wnt and the D-V signaling network in pharyngeal arch development

Bmps and Edn1 secreted by the pharyngeal ectoderm both promote ventral-intermediate skeletal cell fates in the arches [19,20] and our results implicate Wnt as an additional ventralizing factor. Overexpression of dntcf3 leads to reduced hand2, dlx3b, and dlx5a ventrally and expansion of dorsal jag1b expression (Fig. 4), and similar but less severe changes in gene expression result from dkk1 overexpression. Loss of the positive Wnt regulator, R-spondin, in mice also disrupts expression of Hand2, Dlx5, Dlx6, and Msx6 [66], suggesting a conserved requirement for Wnt signaling in promoting ventral-intermediate cell fates.

How are these different ventralizing signals integrated during D-V arch patterning? Wnts can either activate or inhibit Bmp signaling in different developmental contexts [67–71], pSmad1/5/8 expression is reduced in the pharyngeal arches of dntcf3+ embryos (Fig. 5), suggesting a novel role for Wnts upstream of Bmp signaling during arch development. Consistent with this model, microinjection of Bmp4/7 protein directly into the arch primordia at 4 hphs rescues ventral cartilages (Mc, Ch) in dntcf3+ embryos, but not joint fusions (Fig. 6C, F). Similar injections of Bmp4/7 protein into edn1−/− mutant zebrafish rescues ventral cartilages but not joint fusions, while ectopic Edn1 can rescue joint defects caused by a loss of Bmp [19]. We show that injection of EDN1 protein rescues joint fusions in Wnt deficient embryos (Fig. 6D, L). Wnts also induce Edn1 expression in the pharyngeal ectoderm [66]. Taken together, these results suggest that Wnt signaling influences ventral cell fates in the arches through both Bmps and Edn1, and joint patterning specifically through Edn1.

Another clue to specificity in the D-V arch patterning system comes from the fact that msxe expression (a direct BMP target and marker of more intermediate identities) is induced by Bmp4/7 in edn1−/− mutants [20], and by Edn1 protein in the absence of Bmp signaling [19]. Surprisingly, however, msxe expression in dntcf3+ embryos is only rescued by Bmp4/7, and not EDN1, while dlx3b and dlx5a expression is rescued in both. This suggests that Edn1 can only induce msxe expression in the arches in the presence of Wnt signaling. Thus Wnt controls the competence for arch cells to respond to Edn1 in addition to inducing expression of Edn1 itself. Mouse mutant in the essential Wnt receptor co-factor, Lrp6, also lack expression of Msx1 and Msx2, in the arches [72], possibly as a result of defects in Bmp signaling.
Neither BMP4/7 nor EDN1 overexpression rescues hand2 expression in dntcf3+ embryos, revealing a critical requirement for Wnt in induction of hand2 [17,23,73–75]. Both Bmp and Edn1 induce hand2 expression and specify the ventral arch domain initially, but later Bmps maintain hand2 in the ventral domain while Edn1 promotes expression of more ventral-intermediate genes. We pinpoint a critical period for Wnt signaling in D-V patterning between 24–30 hpf, when hand2 expression is unresponsive to Edn1. Our results suggest that Bmp signaling requires Wnt signaling to induce hand2 expression in the arches. Consistent with this model, Wnt signaling directly regulates Hand2 transcription in chondrocytes [76]. Failure of hand2 induction is not simply due to loss of cells, since ventral NC cells are still present in the arches of dntcf3+ embryos (Fig. 4N–O; Fig. S7). BMP protein can also induce hand2 throughout the D-V extent of the arch [20]. Our results suggest that Wnt signaling plays a critical role in regulating the competence of cells to respond to Bmps and to express hand2.

We propose that Wnt signaling activates a signal (factor X) from the pharyngeal endoderm that primes the ability of NC cells to respond to Bmp signaling, in part through the transcriptional regulation of Bmp receptors (Fig. 9). Similarly in D-V patterning of the mouse limb Wnt signaling is thought to act upstream of Bmpr1a [71]. Three type I Bmp receptors, bmp1ab, bmp1ba, bmp1bb, have arch-specific expression in zebrafish, similar to mice [77]. These are expressed in nested patterns within the arches: bmpr1ab throughout and bmpr1ba/bb only in intermediate-ventral domains. Thus Bmpr1 receptors may have distinct roles in different spatial domains (in addition to their cell-type specific roles [78,79]), but this has been difficult to test due to early lethality in traditional Bmpr knockout [80,81]. We show that overexpression of dntcf3+ inhibits bmpr1ab and bmpr1ba, but not bmpr1bb and bmpr2a, expression in the arches. This reduction in Bmpr expression occurs later than most direct Wnt targets (Fig. 8I; 4–8 hphs) suggesting that it is indirect, consistent with our model that Wnt activates an unknown signal from the endoderm important in this process. bmpr1ab expression also recovers by 8 hphs, before bmpr1ab expression and within the period during which Wnt signaling is significantly reduced (Fig. 1I) indicating that bmpr1ab is particularly sensitive. This could help explain the inability of Bmp protein to rescue hand2 expression in dntcf3+ embryos if bmpr1ab plays a specific role in hand2 induction. In contrast, intermediate-ventral genes such as msxe and dlx3b, may be rescued by Bmp protein because other Bmp receptors are sufficient for...
their induction. Such distinct transcriptional roles for Bmp receptors could help fine-tune D-V domains within an arch despite the relatively broad expression of Bmp ligands.

**Distinct roles for Wnt signaling in pharyngeal endodermal development**

Both dntcf3+ and dkk1+ embryos appear to function in the pharyngeal endoderm, which is an important signaling center in craniofacial development [7,9]. Our chimeric analyses demonstrate a cell autonomous requirement for dntcf3 in endoderm (Fig. S2) and dkk1 expression is restricted to this epithelium. Interestingly, we do not detect dkk1 expression in the first pharyngeal pouch, which lies between arches 1 and 2 (Fig. S10). This could help explain why Wnt signaling only appears to be required for D-V patterning in these arches; the more posterior ceratobranchials (arches 3–7) are largely unaffected in heterozygous dkk1+ and dntcf3+ embryos (Fig. 2B,F). These results suggest a previously unrecognized role for Wnts and Wnt antagonists in endoderm and the existence of an as yet unknown factor X produced by endoderm that is important in D-V patterning of the NC (see Fig. 9).

The distinct and highly penetrant clefting of the mandible observed in dkk1+ embryos is never observed in dntcf3+ embryos. Disruption of the canonical Wnt pathway can cause clefting of the palate in mice [36,46,67], but such midline clefts in the lower jaw are rare. Midline facial defects, particularly of the frontonasal palate in mice [36,46,67], but such midline clefts in the lower jaw were never observed in dkk1+ embryos [66,84]. Humans with Richieri-Costa-Pereira syndrome also exhibit clefting of the lower jaw similar to what we describe in dkk1+ embryos [68,84]. Dkk1 not only inhibits canonical Wnt signaling [52] but can also activate the non-canonical Wnt-PCP pathway during zebrafish gastrulation [60]. Non-canonical Wnt signaling has also been implicated in craniofacial development [5]. Wnts in Craniofacial Development

Overexpression of dkk1 also leads to elongation and ventral clefting of the mouth, which is not observed in dntcf3+ embryos. Both loss- and gain-of-function mutations in mammalian Dkk1 result in midline clefts in the frontonasal and maxillary prominences [36]. NC cells fated to form the lower jaw lie adjacent to the oral ectoderm (stomodeum), which secretes important signals such as Shh [14,15] and Bmps [19]. Dkk1 not only inhibits canonical Wnt signaling [52] but can also activate the non-canonical Wnt-PCP pathway during zebrafish gastrulation [60]. Non-canonical Wnt signaling has also been implicated in craniofacial development [5].

**Materials and Methods**

**Ethics statement**

All zebrafish work was performed using protocols approved by the University of California, Irvine Institutional Animal Care and Use Committee (Protocol # 2000-2149-4).

**Animals**

Adult Tg(hsp701:dkk1-GFP) (dkk1) [50] and Tg(hsp701:ctf3-GFP) (dntcf3) [41] fish were genotyped by performing PCR analysis for gfp (sense, GTGATGCAACATACGGAAAAC; antisense, GCCATGTGTAATCCACGACGC) using genomic DNA extracted from fin clips as template. Zygosity of fish was determined by outcrossing genotyped transgenic adults with wild type adults and scoring the Mendelian ratio of GFP positive to GFP negative after heat shocking as described below. To observe NC, we outcrossed adult homozygous Tg(hsp701:ctf3-GFP) fish with sox10:LynTdtomato fish to make a stable double transgenic line. Adult heterozygous Tg(7xTCF-Xla.Siam:GFP) (7xTCF/GFP) transgenics [49] were in-crossed and sorted by GFP expression for downstream phenotypic analysis. Tg(BRE:gfpr) [bre:gfpr] [19] heterozygous adults were in-crossed, selected for strong GFP expression, and used for protein injection (see below).

**Heat shock conditions**

Heat shocks were performed in a thermal cycler at 39°C for either 12 min (dntcf3) or 30 min (dkk1). Fluorescence was checked 1-hour post heat shock and GFP-negative embryos were separated and used as controls. Embryos were then raised in a 28.8°C incubator until they were fixed for in situ analysis, RNA extraction, or protein extraction at various time points after heat shock or 4 dpf for skeletal staining.

**Phenotypic analysis**

Alican blue staining of cartilage was performed on 96 hpf embryos as previously described [19]. In situ hybridization was performed on embryos fixed in 4% PFA for one hour at room temperature. Probes used include gfp [19], dlt2, dlt3b, dlt6a [85], mycn [86], hand2 [87], jag1b [28], mses, dkk1b, nka2 [17], ednA1, ednAR2 [Nair et al, 2007], and pitx2ca [88]. The axin2a probe was synthesized directly from a PCR product with a T7 promoter and used for in situ hybridization. Dkk1 not only inhibits canonical Wnt signaling [52] but can also activate the non-canonical Wnt-PCP pathway during zebrafish gastrulation [60]. Non-canonical Wnt signaling has also been implicated in craniofacial development [5]. Wnts in Craniofacial Development

**Protein injection**

Tg(hsp701:ctf3-GFP) embryos were heat shocked as described, anesthetized, and then embedded in 1% low melt agarose in embryo medium. Human EDN1 (Siga-Maldrich) was diluted to 10 μg/μl and human recombinant BMP4/7 (R&D Systems) was diluted to 10 μg/μl and human recombinant BMP4/7 (R&D Systems) was added as control. Animals were then heat shocked and/or injected at 1-cell stage with either 4 dpf for skeletal staining.
diluted to either 50 ng/μl or 10 ng/μl. A 0.5 nl droplet of protein solution was pressure injected into the arch region 4 hours post heat shock (~26 hpf) using a glass needle. Embryos were then carefully removed from the agarose using forceps and fixed for phenotypic analysis 4 hours later (~30 hpf) or 4 dpf for alcin stain using 4% PFA at room temperature for 1 hour.

6-bromoindirubin-3’-oxime (BIO) treatment

Dechorionated control, dkk1+ and dntcf3+ embryos were placed in dishes containing 50 μm or 100 μm of BIO (30 mm stock in DMSO) (Sigma) diluted in embryo medium (EM) at 2 hours post heat shock [55]. The dishes were placed in a 28.5°C incubator for 6 hours and then washed several times with EM. Embryos were fixed for in situ hybridization, harvested for RNA, or allowed to develop to 4 dpf and then fixed for alcin blue staining.

Western blot

Protein was extracted from dechorionated embryos by adding 2 μl/embryo of sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol blue) and homogenizing with a pestle. The sample was boiled in a 95°C heat block for 10 min and then immediately placed on ice. Before loading into a 10% SDS gel the sample was spun down at 1300 rpm for 5 min. The membrane was blocked in 3% BSA and 3% Donkey Serum in TBST (1XTBS 1300 rpm for 5 min. The membrane was blocked in 3% BSA and 3% Donkey Serum in TBST (1XTBS

Quantitative real time PCR analysis

Total RNA was extracted from control, dkk1+, and dntcf3+ embryos at various time points post heat shock using Trizol reagent (Ambion). cDNA synthesis was performed with 1 μg of RNA using Protoscript M-MuLV First Strand cDNA synthesis kit (New England Biosystems). qPCR was performed using Light QPCR was performed using Light Cycler 480 SYBR Green Master (Roche Applied Science) in a Cycler 480 SYBR Green Master (Roche Applied Science) in a

Supporting Information

Figure S1 Wnt signaling defects in dkk1+ embryos. (A–D) Whole mount in situ hybridization (ISH) for mycn (A, B) and axin2 (C, D) expression in dkk1+ embryos at 24 hpf (2 hplps), lateral views, anterior to the left. Arrowheads indicate expression at the mid-hindbrain boundary. (E) Quantitative, real-time PCR (qPCR) analysis for axin2 (red bars) and left1 (orange bars) in dkk1+ embryos, normalized to nontransgenic, heat-shocked controls, with e1alpha as an internal control. Abbreviations: e, eye.

Figure S2 Wnt signaling in the pharyngeal endoderm is necessary for D-V arch patterning. (A) Dissected, flat mounted alcin blue/alizarin stained cartilage/bone at 5 dpf. Anterior to the left. Transplantation of dntcf3+ endoderm results in D-V patterning defects including reduced Mc, fused Mc-Pq, and loss of Hs. A dotted line indicates the extent of the endoderm transplant, which was lost in dissection. Asterisk indicates joint fusion. (B–C) Lateral views of in situ hybridization for jag1b in a control (B) and a mosaic embryo in which dntcf3+ cells were transplanted into the endoderm in WT hosts. White arrowheads indicate expansion of jag1b expression into the ventral domain of arches 1 and 2. Asterisks indicate transplanted, dnx9/mib-1 stained donor cells. Abbreviations: Ch, ceratohyal; Hm, hyomandibular; Hs, hyosymphetic; Mc, Meckel’s; Pq, palatoquadrate.

Figure S3 BIO rescues expression of Wnt targets in dntcf3+ and dkk1+ embryos. (A–F) Whole mount ISH for gfp (A–C) and axin2 (D–F) at 30 hpf in embryos treated with DMSO, 50 μM BIO, and 100 μM BIO for 6 hpf at 24 hpf. With increasing concentrations of BIO, 7xTcf-GFP embryos show an increase in ectopic gfp expression, particularly throughout the brain (B,C). Similarly, axin2 expression expands in a dosage-dependent manner (E,F). (G–H) qPCR analysis for Wnt targets left1 (G) and axin2 (H) in control, dntcf3+, and dkk1+ embryos treated with DMSO, 50 μM BIO, and 100 μM BIO at 6 hpf. Red line indicates normalized expression levels in DMSO treated non-transgenic controls with e1alpha as an internal control. BIO induces expression of Wnt targets in non-transgenic controls and rescues expression in dntcf3+ and dkk1+ embryos (G,H).

Figure S4 BIO rescues expression of Wnt targets in dntcf3+ and dkk1+ embryos. (A–I) Dissected, flat mounted alcin stained cartilage at 4 dpf, ventral views, anterior to the left; non-transgenic control (top row), dntcf3+ (middle row) and dkk1+ (bottom row), untreated (A, D, G), or treated with 50 μM (B, E, H) or 100 μM (C, F, I) BIO for 6 hours. White arrowheads in G–H indicate Mc clefting, and black arrowheads indicate missing Ch in D. Asterisks in D and G indicate Mc-Pq joint fusions. High concentrations of BIO reduce overall cartilage size and cause specific loss of dorsal cartilages (C). BIO treatments rescue Mc-Pq joint fusions in both dntcf3+ and dkk1+ embryos (E, F, H, I, M) as well as Ch in dntcf3+ embryos (E, F, M). High BIO concentrations rescue Mc clefting in dkk1+ embryos (I). (J–L) Whole mount ISH for pcna in control embryos treated with DMSO, 50 μM BIO, and 100 μM BIO. (M) Histogram quantifying the percentage of dntcf3+ embryos showing Ch loss.
or Mc-Pq joint fusion in response to BIO treatments. Abbreviations: Ch, ceratohyal; Hm, hyomandibular; Mc, Meckel’s; Pq, palatoquadrate.

**Figure S5** Cell death is not increased in dkk1+ and dntcf3+ embryos. (A–H) Lateral views of live acridine orange staining at 6 hphs in control (A, B, E, F), dkk1+ (C, D), and dntcf3+ (G, H) embryos. White arrowheads indicate apoptotic cells. Abbreviations: c, eye.

**Figure S6** Arch proliferation defects in dkk1+ and dntcf3+ embryos. (A, B, D, E) Anti-phosphoHistone3 (pH 3) staining in controls (A, A’) and dkk1+ (B, B’) embryos stained 3 hphs and in controls (D, D’) and dntcf3+ (E, E’) embryos stained 4 hphs; lateral views, anterior to the left. (A’, B’, D’, E’). Enlargements of boxed areas encompassing presumptive first and second arches used for quantification. (C, F) Histograms quantifying numbers of pH 3 positive cells in the pharyngeal region of control and dkk1+ embryos, p < 0.05 (C) and dntcf3+ embryos, p = 0.06 (F). Scale bar: 100 μm.

**Figure S7** Neural crest migration in dntcf3+ embryos. (A–H) Live images of Tg(sox10:lyn-Tdtomato) fluorescence in cranial neural crest cells in the arches in control (A–D) and dntcf3+ (E–H) embryos at 2 hour intervals post heatshock, lateral views, anterior to the left.

**Figure S8** BIO disrupts dorsal-ventral patterning gene expression. (A–L) Lateral views of whole mount ISH for D-V patterning genes in embryos treated with DMSO (A, D, G, J), 50 μM BIO (B, E, H, K), and 100 μM BIO (C, F, I, L) for 6 hours at 24 hpf. With increasing concentrations of BIO, expression of the ventral-intermediate gene mnx expands dorsally (B, C) (black arrowheads). In response to BIO treatments, expression domains of hom1 (H–I) and dlx3b (E–F) only expand slightly. Expression of the dorsally restricted pgj1b is reduced (asterisk) in the first and second arches of BIO treated embryos (K–L).

**Figure S9** Bmp receptor expression during pharyngeal arch development. (A–O) Whole mount ISH for bmp11a (A, B), bmp11b (C, D, O), bmp11b (E, F, M), bmp2a (G, H), bmp1va (L, J, N), and bmp2b (K, L), lateral views, anterior to the left. (P–R) Transverse sections through 24 hpf embryos showing expression of bmp1lab (P), bmp1lba (Q), and bmp1lbb (R) in neural crest cells of the pharyngeal arches. Arrowheads indicate ventral restriction of bmp1lab (P) and bmp1lbb (Q) expression compared with bmp1lbb (R). Abbreviations: e, eye; hb, hindbrain; mh, mid-hindbrain boundary; nc, notochord; nt, neural tube; pa, pharyngeal arches; s, somites.

**Figure S10** Dkk1 expression in pharyngeal endoderm. (A–F) Whole mount ISH for dkk1b in wild type (A–D, F) and van gogh (vg) (E) embryos, (A–E) lateral and (F) ventral views, anterior to the left. Arrowhead in A indicates expression in the first arch. Asterisks in A and B indicate the first pharyngeal pouch. Arrowheads in C-E indicate the first and second pouches. (F) Ventral view at 48 hph showing dkk1b expression in the oral ectoderm (arrowhead) surrounding the mouth opening (dotted line). Scale bar: 100 μm.

**Figure S11** Defects in endoderm and oral ectoderm in dkk1+ embryos [A–L, N–Q]. Whole mount ISH, anterior to the left. (A–H) Dorsal and lateral views showing nka.2 expression in pharyngeal pouch endoderm at 50 hphs in controls (A, C, E, G), dntcf3+ (B, F), and dkk1+ (D, H) embryos. Normal pouches are numbered. Asterisks and black lines indicate defective pouches. (I–L, N–Q) Ventral views showing pitx2ca expression in control (I–L) and dkk1+ (N–Q) embryos. (M, R) Transverse sections of the mouth showing pitx2ca expression in control (M) and dkk1+ (R) embryos at 51 hpf. Arrowheads indicate expression in oral ectoderm. Dotted lines in K, L, P and Q indicate the mouth opening. White arrowhead indicates a ventral midline fold in the mouth. Scale bars: 100 μm.

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**Author Contributions** Conceived and designed the experiments: CA SP TFS. Performed the experiments: CA SP PLP TFS. Analyzed the data: CA SP PLP TFS. Contributed reagents/materials/analysis tools: CA SP PLP TFS. Wrote the paper: CA PLP TFS.

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