Bmps and Id2a Act Upstream of Twist1 To Restrict Ectomesenchyme Potential of the Cranial Neural Crest

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Introduction

The neural crest is a transient, migratory cell population that arises at the boundary between the neural and non-neural ectoderm [1]. Although both cranial and trunk neural crest cells differentiate into non-ectomesenchyme derivatives, such as neurons, glia and pigment cells, CNCCs also generate ectomesenchyme derivatives, in particular many of the cartilage-, bone-, and teeth-forming cells of the head [2]. Whereas much is known about how individual non-ectomesenchyme lineages are specified, how the ectomesenchyme lineage is specified remains actively debated [3]. When the ectomesenchyme versus non-ectomesenchyme lineage decision is made during CNCC development also remains unclear. Whereas cultured avian CNCCs can clonally generate both lineages [2], lineage tracing experiments in zebrafish embryos have failed to identify a common precursor [4,5].

In zebrafish, CNCCs are first apparent within the anterior neural plate border at 10.5 hours-post-fertilization (hpf), when they begin to express sox10, foxD3, sox9b, and fli1a. Within the next few hours, three streams of CNCCs can be seen migrating away from the neural tube to more ventral positions. Starting around 15.5 hpf, ectomesenchyme precursors begin to downregulate early CNCC genes such as sox10, foxD3, sox9b, and fli1a [6,7] and up-regulate ectomesenchyme-specific genes such as dlx2a [8] and fli1a [9]. These ectomesenchyme cells then go on to populate a series of pharyngeal arches from which develops the support skeleton of the jaw and gills in zebrafish, and the jaw, middle ear, and larynx in mammals [10]. Whereas dlx2a and fli1a are uniquely expressed in the ectomesenchyme lineage, Dlx2a appears to be dispensable for ectomesenchyme formation [8] and the function of Fli1a in ectomesenchyme development remains unknown.

One factor critical for ectomesenchyme specification in mouse is the basic-helix-loop-helix (bHLH) transcription factor Twist1. Both the conventional Twist1 knockout and a conditional Twist1 neural-crest-specific (Wnt1-CRE) knockout display defective ectomesenchyme development, including abnormal perdurance of Sox10 and loss of expression of many arch ectomesenchyme genes [11,12]. Furthermore, the neural-crest-specific knockout showed severe reductions of the CNCC-derived craniofacial skeleton, although the lower jaw was less affected. Zebrafish have two Twist1 orthologs, with twist1b being expressed in early CNCCs and twist1a restricted to ectomesenchyme precursors from 16 hpf onwards [13]. Here, we show that these two Twist1 genes function redundantly for zebrafish ectomesenchyme development, with Twist1 depletion resulting in both perdurance of sox10 and loss of fli1a expression.

As Twist1 genes are expressed throughout the early CNCC domain, an important yet unanswered question is how Twist1 function is specifically regulated in ectomesenchyme precursors. Twist1 function can be regulated by post-translational modification (e.g. phosphorylation), as well as choice of dimerization partners. In particular, Inhibitor of differentiation (Id) proteins, which share HLH but not basic DNA-binding domains with bHLH factors, influence Twist1 homodimer versus heterodimer formation by sequestering Twist1 binding partners such as E2A [14,15]. Id genes are widely expressed in the early neural crest, and Id2 has been shown to promote neural crest at the expense of epidermis in avians [16]. In zebrafish, Id2a has been shown to...
regulate neuron and glia formation in the retina, albeit non-cell-autonomously, yet its role in CNCC development has not been explored [17]. In this study we find a novel role of Id2a in CNCC lineage decisions, with down-regulation of id2a in migrating CNCCs being essential for ectomesenchyme specification.

Upstream signals that specify ectomesenchyme could originate from the ectoderm where CNCCs are born, from the mesoderm along which CNCCs migrate, or from the endoderm/ectoderm upon which CNCCs condense within the pharyngeal arches. Previous studies have suggested roles for Fgf signaling, in particular Fgf20b and Fgfr1, in ectomesenchyme specification in avians and zebrafish [18,19]. It was further proposed that CNCCs might acquire ectomesenchyme identity upon arrival in the pharyngeal arches, potentially as a result of endoderm-secreted Fgfs [18]. In contrast, lineage-tracing experiments have revealed that CNCCs in zebrafish are largely restricted to single lineages before migration [4,5], suggesting that ectomesenchyme fates may be specified at their birthplace within the neural plate border ectoderm.

Bmps such as bmp2b in zebrafish are prominently expressed in the non-neural ectoderm, where they influence neural crest induction, migration, survival, and differentiation [20,21,22]. However, a role for Bmp signaling in ectomesenchyme lineage decisions has not been previously described, likely because of the earlier essential roles of Bmps in neural crest induction [21]. By manipulating Bmp signaling specifically in migratory CNCCs, we have uncovered an additional function of Bmp signaling in restricting ectomesenchyme potential, in part by maintaining high levels of Id2a. We therefore propose a model in which the early migration of CNCCs away from an ectodermal Bmp source results in down-regulation of Bmp activity and id2a expression, which in turn promotes Twist1-dependent establishment of ectomesenchyme fates.

Results

Twist1a and Twist1b are redundantly required for ectomesenchyme specification in zebrafish

In order to test whether Twist1 genes are required for ectomesenchyme development in zebrafish, we designed translation-blocking MOs to deplete both zebrafish Twist1 orthologs - Twist1a and Twist1b. Injection of twist1a-MO or twist1b-MO alone resulted in only very subtle changes in sox10 and no changes in fli1eGFP transgene expression [Figure S1]. In contrast, embryos injected with both MOs (twist1a/1b-MO) displayed abnormal persistence of sox10 and reduced expression of fli1a in arch CNCCs at 18 and 24 hpf, respectively [Figure 1A, 1B, 1E, 1F]. Analysis of doubly transgenic sox10;dsRed; fli1a:GFP embryos injected with twist1a/1b-MO confirmed that CNCCs still formed arches of largely normal size at 28 hpf in the absence of Twist1 function yet failed to initiate fli1a:GFP expression [Figure S1]. Furthermore, arch expression of dlc2a was relatively unaffected in 18 hpf twist1a/1b-MO embryos [Figure 1I, 1J]. Consistent with the earlier ectomesenchyme gene expression defects, we also found that depletion of both Twist1 proteins resulted in severe reductions of the ectomesenchyme-derived skeleton of the face and anterior neurocranium at 5 days-post-fertilization (dpf) [Figure 1M, 1N]. Single depletion of either Twist1a or Twist1b resulted in milder craniofacial defects, particularly in the ventral mandibular and hyoid cartilages [Figure S1]. Importantly, injection of a twist1b mRNA not targeted by the MOs, but not a control krt5 mRNA, partially rescued skeletal defects in twist1a/1b-MO embryos, thus showing specificity of MO-generated defects for Twist1 [Figure S1]. Hence, despite differences in timing of their expression, Twist1a and Twist1b function redundantly for ectomesenchyme development.

Twist1 function is required in migratory CNCCs for ectomesenchyme specification

We next investigated whether Twist1 functions in CNCCs for ectomesenchyme specification. To do so, we developed a transgenic strategy to misexpress a dominant-negative Twist1b protein specifically in migratory CNCCs. Caenorhabditis elegans has a single Twist gene, hlh8, which is required for mesodermal patterning and vulval muscle development [23]. Mutation of Glutamine 29 to Lysine in the DNA-binding domain of Hlh8 has been shown to dominantly interfere with its function [24]. As this residue is conserved in zebrafish Twist1 genes (Glutamine 84 in Twist1b), we reasoned that the equivalent mutation might also result in a dominant-negative protein. We therefore constructed a transgenic line in which Twist1b-E84K (referred to as dnTwist1b) was expressed under the control of the sox10 neural crest promoter [25]. Although the endogenous sox10 gene is first expressed in pre-migratory CNCCs at 10.5 hpf, in situ hybridizations for krt5 mRNA in sox10;Gal4VP16; UAS; hlh8-Gal4VP16; UAS; krt5GR embryos revealed transgene expression within migratory CNCCs at 13 hpf, but not earlier within premigratory CNCCs at 11 hpf [Figure S2]. Hence, this sox10;Gal4VP16 line allows us to alter genetic pathways specifically in migratory CNCCs. Consistent with a requirement for Twist1 in migratory CNCCs, we observed persistent sox10 and reduced fli1a expression in the arches of sox10;Gal4VP16; UAS; dnTwist1b embryos, similar to what we observed in twist1a/1b-MO embryos [Figure 1]. We also observed a partial reduction of arch dlc2a expression upon dnTwist1b misexpression, as well as reductions of the facial skeleton, particularly in the jaw region [Figure 1K, 1L, 1Q, 1P]. Hence, the similar phenotypes of twist1a/1b-MO embryos and transgenic embryos with CNCC-specific disruption of Twist1 function indicate that zebrafish Twist1 genes function largely in migratory CNCCs for ectomesenchyme development.

Twist1 genes globally activate ectomesenchyme and inhibit non-ectomesenchyme gene expression

Twist1 could regulate the transition from early multipotent CNCCs to ectomesenchyme precursors, or alternatively fate
Figure 1. Twist1 genes are required for ectomesenchyme specification in zebrafish. (A–D) Whole mount in situ hybridizations of sox10 expression at 18 hpf show ectopic expression in the arches (numbered and second arch indicated by black arrow) of twist1a/1b-MO (n = 16/16) and sox10:Gal4VP16; UAS:dnTwist1b (n = 4/4) embryos compared to un-injected (n = 0/14) and sox10:Gal4VP16 only (n = 0/9) controls. White arrowheads indicate otic expression. (E–H) Whole mount in situ at 24 hpf show reduction of fli1a expression in the arch ectomesenchyme (numbered) of twist1a/1b-MO (n = 12/12) and sox10:Gal4VP16; UAS:dnTwist1b (n = 5/5) embryos compared to un-injected (n = 0/13) and sox10:Gal4VP16 only (n = 0/8) controls. Insets in E and F highlight arch ectomesenchyme which is reduced in twist1a/1b-MO embryos. Vascular expression of fli1a (red arrowheads) is unaffected. (I–L) Whole mount in situ at 18 hpf show a slight reduction of dlx2a in sox10:Gal4VP16; UAS:dnTwist1b embryos (n = 4/4) but not un-injected (n = 0/6), twist1a/1b-MO (n = 0/8), and sox10:Gal4VP16 only (n = 0/6) embryos. (M–P) Skeletal staining at 5 dpf shows severe loss of CNCC-derived head skeleton in twist1a/1b-MO embryos (n = 21/21) and primarily jaw reductions in sox10:Gal4VP16; UAS:dnTwist1b embryos (n = 9/9) compared to no defects in un-injected (n = 0/24) and sox10:Gal4VP16 only (n = 0/16) controls. Whereas only small remnants remain of the CNCC-derived skeleton (arrows), the mesoderm-derived otic capsule cartilage (arrowheads) and posterior neurocranium are less affected in twist1a/1b-MO embryos. (Q–T) In situ for dct expression at 28 hpf show normal melanophore precursors in un-injected (n = 14), twist1a/1b-MO (n = 12), sox10:Gal4VP16 only (n = 8), and sox10:Gal4VP16; UAS:dnTwist1b (n = 8) embryos. (U–X) In situ for xdh expression at 28 hpf show normal xanthophore precursors in un-injected (n = 7), twist1a/1b-MO (n = 5), sox10:Gal4VP16 only (n = 9), and sox10:Gal4VP16; UAS:dnTwist1b (n = 8) embryos. (Y–AB) In situ for foxd3 expression at 48 hpf reveal largely normal patterns of glia in un-injected (n = 9), twist1a/1b-MO (n = 8), sox10:Gal4VP16 only (n = 9), and sox10:Gal4VP16; UAS:dnTwist1b (n = 8) embryos. Scale bar = 50 μm.

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choices between ectomesenchyme and non-ectomesenchyme lineages. In order to examine these possibilities without bias, we performed microarray-based gene expression profiling of purified sox10:GFP-positive CNCCs from 18 hpf un-injected or twist1a/1b-MO-injected embryos (Figure 2A, 2B). We next compared the relative enrichment of transcripts in GFP-positive versus GFP-negative CNCCs of un-injected and twist1a/1b-MO embryos to identify CNCC-specific genes up or down regulated by Twist1 depletion (Figure 2C). By comparing the GFP+/GFP– ratio between un-injected and twist1a/1b-MO embryos (as opposed to simply comparing expression levels between GFP+ fractions), we controlled for possible non-specific effects of MO toxicity on gene expression (i.e. non-specific toxicity is predicted to equally affect GFP+ and GFP– fractions and thus toxicity effects would cancel out in the ratio). By analyzing data sets with respect to published gene expression patterns (Table S1), we found that the downregulated set included many known ectomesenchyme genes (e.g. lamina, grem2, tox2a, podh10a, and fli1a) and the up-regulated set included genes expressed in non-ectomesenchyme lineages such as neurons (e.g. mra2b, atpa1a1, and aac2), glia (e.g. rab3ip), and pigment (e.g. gch2 and ednrb1) (Figure 2C). In order to validate several of the most differentially expressed genes, we next performed in situ hybridization. Whereas the ectomesenchyme expression of fli1a was severely reduced (Figure 1F), the non-ectomesenchyme expression domains of nr4a2b and gch2 were increased in intensity and expanded in size in twist1a/1b-MO embryos at 18 hpf, ectopic arch expression (arrows) of sox10 and foxd3 at 24 hpf, but no change in sox9b and tfap2a expression at 24 hpf in twist1a/1b-MO versus un-injected controls. Scale bar = 50 μm.

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Figure 2. Gene expression profiling in twist1a/1b-MO embryos. (A,B) sox10:GFP-positive and -negative cells were isolated from un-injected or twist1a/1b-MO embryos at 18 hpf by FACS. (C) Fold changes of the GFP+/GFP– ratios between twist1a/1b-MO and un-injected controls show the top 25 up-regulated (blue) and down-regulated (red) genes after Twist1 depletion. Color codes indicate where genes are expressed based on the published literature (see Table S1 for references). (D–O) Confocal projections of fluorescent in situ hybridizations show expanded expression of gch2 and nr4a2b at 18 hpf, ectopic arch expression (arrows) of sox10 and foxd3 at 24 hpf, but no change in sox9b and tfap2a expression at 24 hpf in twist1a/1b-MO versus un-injected controls. Scale bar = 50 μm.

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observed ectopic arch expression of foxd3 but not sox9b and tfap2a (Figure 2J–2O). Whereas several studies have established roles for Sox10 and Foxd3 in maintaining neural crest multipotency [26], these transcription factors also have later roles in promoting non-ectomesenchyme lineages such as melanocytes, neurons, and glia [27,28]. The lack of sox9b and tfap2a upregulation in twist1a/1b-MO embryos argues against a general perdurance of early CNCC expression, with the ectopic expression of sox10 and foxd3 likely reflecting the upregulation of non-ectomesenchyme gene expression observed in our microarray analysis. However, despite the ectopic expression of sox10 and foxd3 in 24 hpf twist1a/1b-MO arches, we failed to observe ectopic foxd3-positive glia, HuC/D-positive cranial ganglionic neurons, kdr-positive melanophores, or sdr-positive xanthophores in the arches of either twist1a/1b-MO or sox10;Gal4VP16; UAS:dnFgfr1a larvae at later stages (Figure 1Q–1AB and Figure S3). Instead, the sox10:GFP-positive arches of twist1a/1b-MO embryos were reduced at 36 hpf, and even more so at 48 hpf, with LysoTracker staining revealing increased cell death in arch CNCCs and putative CNCCs in the dorsal neural tube (Figure S4 and data not shown). In addition, expression of the early chondrocyte marker sox9a was very reduced in arch CNCCs of 48 hpf twist1a/1b-MO embryos (Figure S4G) yet was less affected in the mesoderm-derived precursors of the otic capsule cartilage which is less sensitive to loss of Twist1a/1b function (Figure 1N). Hence, arch CNCCs undergo cell death and fail to initiate skeletal gene expression in the absence of Twist1 function yet do not form ectopic non-ectomesenchyme derivatives.

Twist1 is required for Fgf signaling in ectomesenchyme precursors

As Fgf signaling has also been shown to promote ectomesenchyme fates [18,19], we next examined whether Twist1 might be required for Fgf signaling during ectomesenchyme development. In zebrafish embryos, pea3 expression is entirely dependent on Fgf signaling [29], and we observed decreased pea3 expression in the arches of twist1a/1b-MO embryos at 10 hpf (Figure 3B), consistent with Twist1 being required for Fgf signaling in migrating CNCCs. We next investigated to what extent loss of Fgf signaling accounts for the ectomesenchyme gene expression defects seen upon Twist1 depletion. To do so, we inhibited Fgf signaling specifically in migratory CNCCs through transgenic misexpression of a dominant-negative version of Fgfr1a (dnFgfr1a). In sox10;Gal4VP16; UAS:dnFgfr1a embryos, we observed persistent sox10 and reduced dlx2a expression in the arches, yet, unlike Twist1-deficient embryos, fli1a expression was largely unaffected (Figure 3C–3H). This effect of Fgf inhibition on ectomesenchyme formation was not due to transcriptional regulation of Twist1 genes as both twist1a and twist1b were expressed normally in 24 hpf sox10;Gal4VP16; UAS:dnFgfr1a embryos (Figure 3I–3L). We therefore conclude that Fgf signaling likely functions downstream of Twist1 to repress sox10 and activate dlx2a expression yet plays less of a role in fli1a expression.

Twist1 regulates fli1a expression through an ectomesenchyme-specific enhancer

As we determined that Fgf signaling is not required for fli1a ectomesenchyme expression, we next examined whether Twist1 might more directly activate fli1a expression. We first used a comparative genomics and transient transgenic approach to identify ectomesenchyme-specific regulatory regions of the fli1a gene. A ~15 kb region centered around the first exon of the fli1a gene had previously been shown to drive expression in both the ectomesenchyme and vasculature [30], and we used the mVISTA program [31] to identify seven short (<500 bp) sub-regions (A–H) that were conserved between zebrafish, puffer fishes (Takifugu rubripes and Tetraodon nigroviridis), stickleback (Gasterosteus aculeatus), and medaka (Oryzias latipes). By testing the ability of these sub-regions to drive GFP expression in conjunction with the hsp70l core promoter, we identified two vasculature-specific enhancers (G and H) and one ectomesenchyme-specific enhancer (F) (Figure 4A). Element F, located ~1.5 kb upstream of the fli1a promoter, was sufficient to drive GFP expression in the ectomesenchyme from 19 hpf onwards, as confirmed by co-localization with a sox10:dsRedc

Figure 3. Fgf signaling depends on Twist1 and regulates a subset of ectomesenchyme gene expression. (A,B) In situ at 18 hpf show that expression of the Fgf target gene pea3 is reduced in the arches (numbered) of twist1a/1b-MO embryos (n = 8/8) compared to uninjected controls (n = 0/12). (C–L) sox10;Gal4VP16; UAS:dnFgfr1a embryos show ectopic arch expression of sox10 at 18 hpf (n = 8/8) versus controls (n = 0/8), reduction of dlx2a at 24 hpf (n = 3/3) versus controls (n = 0/3), but no change in fli1a at 24 hpf (n = 0/7) versus controls (n = 0/5). twist1a expression was unchanged at 24 hpf in dnFgfr1a embryos (n = 10) compared to controls (n = 7), as was twist1b expression in dnFgfr1a embryos (n = 8) compared to controls (n = 8). Black arrows indicate the second arch, white arrowheads the ear, and red arrowheads the vasculature. Scale bar = 50 μm. doi:10.1371/journal.pgen.1002710.g003
transgene (Figure 4C). We also identified homologous sequence to enhancer F, 1.7 kb upstream of the mouse Fli1 gene, with both the mouse and fish Fli1 genes containing a perfectly conserved element, CAGATG, that matches the bHLH binding site, CANNTG (though Twist1 most strongly prefers CATATG) [32] (Figure 4B). This putative Twist1 binding site was required for enhancer function, as mutation to GTATAC abolished the ability of the fli1a element F enhancer to direct ectomesenchyme expression in zebrafish embryos and to potentiate Twist1-dependent transgene expression in mammalian 293T cells transfected with wild-type or mutant fli1a-F enhancer reporter constructs, with or without a Twist1 expression plasmid. Asterisks indicate significant comparisons using a student’s t-test (p<0.05). Arbitrary units and standard error of the mean are shown.

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Figure 4. Twist1 regulates fli1a expression through an ectomesenchyme-specific enhancer. (A) Schematic shows the fli1a genomic locus with hatch marks at 1 kb intervals. Predicted enhancers (grey boxes, “A–H”) and the first exon (red box) are shown. Below are the various enhancer constructs analyzed, with the hsp70I core promoter in blue and GFP in green. Wild-type (black) and mutant (red) versions of the putative Twist1 binding site are also shown, as is the percentage of transient transgenic embryos showing GFP expression in the pharyngeal arches or vasculature. (B) Alignment of the central portion of the F enhancer between five fish species and mouse. The putative bHLH/Twist1 binding site is boxed in red. (C,D) Confocal projections of 32 hpf sox10:dsRed embryos injected at the one-cell-stage with fli1a-F enhancer constructs. The wild-type (C) but not mutant (D) enhancer drives arch expression. (E and F) Confocal sections of merged GFP and DIC channels show that a stable Tg(fli1a-F-hsp70I:GFP) line displays arch GFP expression in 32 hpf wild-type (E) but not twist1a/1b-MO (F) embryos. (G) Luciferase activity relative to renilla firefly activity in 293T cells transfected with wild-type or mutant fli1a-F enhancer reporter constructs, with or without a Twist1 expression plasmid. Asterisks indicate significant comparisons using a student’s t-test (p<0.05). Arbitrary units and standard error of the mean are shown.

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Exclusion of id2a expression is necessary for ectomesenchyme specification

As Twist1 genes are expressed throughout the early CNCC domain (e.g. twist1b in zebrafish), we next examined whether dimerization partners might regulate the ectomesenchyme-specific function of Twist1. The Id class of proteins modulates Twist1 function by preventing the formation of Twist1-class B heterodimers [14,15], and several Id family members are expressed in early CNCCs [33,34,35]. Intriguingly, we found that zebrafish id2a was broadly co-expressed with sox10 in early CNCCs (12 and 14 hpf) yet by 16.5 hpf was restricted to sox10-positive non-ectomesenchyme and excluded from ectomesenchyme expressing dlx2a and twist1a (Figure 5). As the down-regulation of id2a expression tightly correlates with ectomesenchyme specification, we next investigated whether the exclusion of Id2a from ectomesenchyme precursors was required for their differentiation. Indeed, when Id2a was misexpressed throughout migratory CNCCs in sox10:Gal4VP16; UAS:Id2a embryos, we observed persistent expression of sox10 and reduced expression of fli1a in arch CNCCs, as well as mild reductions of dlx2a expression (Figure 6A–6F). The effect of Id2a misexpression was specific to the ectomesenchyme, as we observed severe reductions of the craniofacial skeleton but no defects in the non-ectomesenchyme-derived melanophore and xanthophore precursors (Figure 6G–6L) and HuC/D-positive cranial ganglionic neurons (Figure S3). However, we note that the sox10 promoter also drives later expression in chondrocytes [36], and thus we cannot rule out that the skeletal defects of sox10:Gal4VP16; UAS:Id2a animals are additionally or alternatively due to this later phase of Id2a misexpression. In any event, the ectomesenchyme
specificity of gene expression and differentiation defects suggests that Id2a does not generally inhibit CNCC differentiation but instead specifically restricts ectomesenchyme fates. Furthermore, the highly similar defects seen upon Id2a misexpression and depletion of Twist1 are consistent with Id2a functioning to inhibit Twist1 in CNCCs. However, this inhibition of Twist1 function by Id2a is likely at the protein and not transcriptional level as twist1a and twist1b expression were unaffected by Id2a misexpression (Figure 6M–6P).

**Bmp signaling regulates id2a expression in non-ectomesenchyme CNCCs**

We next investigated the mechanism by which id2a expression becomes restricted to non-ectomesenchyme precursors. Bmps are
well known regulators of Id gene expression [37], and we found that CNCC-specific misexpression of Bmp4 in sox10:Gal4VP16;UAS:Id2a embryos resulted in ectopic expression of id2a in arch ectomesenchyme (Figure 5I). To analyze whether down-regulation of Bmp activity also coincides with loss of id2a expression in wild-type ectomesenchyme precursors, we performed immunostaining for the phosphorylated form of Smad1/5/8 (pSmad1/5/8), which is thought to broadly indicate canonical Bmp activity [38]. Consistent with the known role of Bmps in neural crest induction [39], all CNCCs displayed high levels of pSmad1/5/8 at pre-migratory stages (12 hpf) (Figure 7A). In contrast, ectomesenchyme precursors down-regulated pSmad1/5/8 by 16.5 hpf, while more dorsal non-ectomesenchyme precursors continued to display high pSmad1/5/8 (Figure 7B,7C). Cross-sectional views demonstrated that ventral CNCCs with low pSmad1/5/8 were located deep to the ectoderm whereas dorsal CNCCs with high pSmad1/5/8 had yet to delaminate and remained in the neural plate ectoderm (Figure 7D).

Bmp4 misexpression inhibits ectomesenchyme specification

We next examined whether the observed down-regulation of Bmp activity in ectomesenchyme precursors was necessary for their formation. Strikingly, Bmp4 misexpression in migratory CNCCs of sox10:Gal4VP16;UAS:Bmp4 embryos resulted in profound defects in ectomesenchyme specification, including persistent sox10 expression and reduced expression of fli1a and dlx2a (Figure 8A–8H). Importantly, the reduction of fli1a and dlx2a arch expression was not simply due to a lack of CNCC formation, migration, or survival. The examination of sox10-positive CNCCs in 15 hpf Bmp4-misexpression embryos revealed no major defects in CNCC induction or migration (Figure S5), and Lysotracker staining also revealed no major increases in CNCC apoptosis (Figure S6). Moreover, although fewer CNCCs were found in the arches of Bmp4-misexpression embryos at later stages (as shown by lineage tracing using the mKR red fluorescent protein at 24 hpf), those CNCCs found in the arches also displayed reduced fli1a and dlx2a expression (Figure 8E–8H). As with Twist1 depletion or Id2a misexpression, Bmp4 misexpression also resulted in specific reductions of the ectomesenchyme-derived craniofacial skeleton without affecting the non-ectomesenchyme-derived foxd3-positive glia, dct-positive melanophores, and xdh-positive xanthophores (Figure 8I–8P).

The ectoderm is a likely source of Bmps for ectomesenchyme specification

We also investigated which tissue is the likely source of Bmps for the ectomesenchyme lineage decision. A previous study had...
reported that ablation of mesoderm and endoderm, by injection of mRNA encoding the Nodal antagonist Antivin, resulted in severe reductions of \(dlx2a\)-positive ectomesenchyme at 24 hpf [40], yet it was unclear whether the loss of \(dlx2a\) reflected defects in the specification or later endoderm-mediated survival of ectomesenchyme precursors [41,42,43,44]. By performing similar Antivin mRNA injections, we found normal \(dlx2a\) and \(fibla\) induction at 15.5 hpf and \(sox10\) down-regulation by 19 hpf in the presumptive ectomesenchyme of embryos lacking endoderm and mesoderm (Figure 9). Hence, rather than signals deriving from the arch endoderm and/or mesoderm [18], our data are more consistent with signals from the non-neural ectoderm, such as Bmps, influencing ectomesenchyme fates.

**Id2a is required for Bmps to inhibit ectomesenchyme formation**

As Id2a and \(Bmp4\) misexpression resulted in similar ectomesenchyme defects, we next examined whether Id2a might mediate the negative effects of Bmps on ectomesenchyme development. To do so, we injected a previously validated translation-blocking \(id2a\)-MO [17] into one-cell-stage embryos to deplete Id2a protein. Whereas injection of \(id2a\)-MO caused no ectomesenchyme defects on its own, injection into \(sox10\);\(Gal4\VP16\); \(UAS\);\(Bmp4\) embryos fully rescued persistent \(sox10\) and reduced \(dlx2a\) arch expression, as well as partially rescuing \(fibla\) arch expression (Figure 10). In contrast, injection of \(twist1b\) mRNA failed to rescue \(sox10\) persistence in \(sox10\);\(Gal4\VP16\); \(UAS\);\(Bmp4\) embryos (Figure S7). Together, these results support a model in which, rather than inhibiting \(twist1a\) or \(twist1b\) at the transcriptional level, Bmps negatively regulate Twist1 ectomesenchyme function through induction of the Id2a negative regulator.

**Discussion**

Here we present evidence for a new model of ectomesenchyme formation. Upon delamination from the neural plate border, early migrating CNCCs downregulate Bmp activity and \(id2a\) expression, thus allowing activation of Twist1. Twist1 then promotes ectomesenchyme and inhibits non-ectomesenchyme gene expression through both potentiation of Fgf signaling and activation of genes such as \(fli1a\) (Figure 11). A salient feature of our model is that the integration of Bmp inhibition at their origin and Fgf activation along their migratory route would confer temporal and spatial specificity to the generation of ectomesenchyme fates from CNCCs.

**Twist1 promotes ectomesenchyme at the expense of non-ectomesenchyme gene expression**

The CNCC-derived craniofacial skeleton is nearly completely lost in zebrafish with reduced \(Twist1a\) and \(Twist1b\) function. This loss of skeleton does not appear to be due to defects in the generation or migration of CNCCs as shown by the presence of largely normal numbers of migrating \(dlx2a\)-positive CNCCs at 18 hpf and \(sox10\);\(dsRed\)-positive arch CNCCs at 28 hpf in \(twist1a/1b\)-MO embryos. Instead, we found that \(Twist1a\) and \(Twist1b\) are redundantly required for a subset of ectomesenchyme gene expression, in particular the early induction of \(fibla\) and inhibition of \(sox10\), with the later severe loss of facial skeleton precisely correlating with a loss of pre-chondrogenic \(sox9a\) expression, as well as increased cell death in the arches. However, \(dlx2a\) expression was much less affected in \(twist1a/1b\)-MO embryos, as well as in mice with CNCC-specific deletion of Twist1, suggesting that some aspects of ectomesenchyme formation may be Twist1-independent [11]. Our data in zebrafish are generally in agreement with previous studies of Twist1 function in mouse. Conventional \(Twist1^{−/−}\) mutant mice also displayed arch persistence of \(Sox10\) and loss of later \(Sox9\) expression, although \(Fibl\) and early \(Ddx2\) expression were not examined and lethality at E10.5–E11.5 precluded an analysis of the extent of craniofacial skeletal loss [12]. In addition, Twist1 function was required largely in CNCCs for \(Sox10\) repression and craniofacial skeleton development in both zebrafish (this study) and mouse [11], although in both species the facial skeleton was not as affected as in \(twist1a/1b\)-MO zebrafish. These phenotypic differences might reflect incomplete efficacy of the \(dn\)Twist1b transgene at reducing endogenous Twist1 function, or alternatively additional non-

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**Figure 7. Bmp activity is selectively down-regulated in ectomesenchyme precursors.** (A) Confocal projection of \(pSMAD1/5/8\) immunostaining at 12 hpf shows that all \(sox10\);\(GFP\)-positive CNCCs display high \(pSMAD1/5/8\). (B–D) At 16.5 hpf, a projection (B) and a high-magnification section (C, boxed area in B) show that \(pSMAD1/5/8\) remains high in the dorsal non-ectomesenchyme but is lower in the more ventral ectomesenchyme. An orthogonal section (D, taken at the level of the line in C) shows that high \(pSMAD1/5/8\) CNCCs remain in the neural epithelium whereas low \(pSMAD1/5/8\) CNCCs are positioned more medial and ventral. The eye also displays high \(pSMAD1/5/8\). Abbreviations: D, dorsal; V, ventral; A, anterior; P, posterior; M, medial; L, lateral; m, mandibular arch ectomesenchyme; h, hyoid arch ectomesenchyme; n, non-ectomesenchyme; e, ectomesenchyme; ve, ventral epithelium. Scale bar = 50 μm. doi:10.1371/journal.pgen.1002710.g007
autonomous roles of mesodermal Twist1 expression (which is also lost in twist1a/1b-MO larvae) in the migration, survival, and/or differentiation of arch CNCCs. Indeed, a role for mesodermal Twist1 in the migration of CNCCs has been inferred from studies in mouse, although we did not observe major defects in CNCC migration in twist1a/1b-MO zebrafish.

In theory, Twist1 could promote the transition from a multipotent CNCC precursor to a more lineage-restricted ectomesenchyme cell, or alternatively influence fate choices between ectomesenchyme and non-ectomesenchyme lineages. However, the observation that only a subset of early CNCC genes persisted in twist1a/1b-MO arches, combined with the normal development of CNCC-derived cranial pigment and glial cells, suggest that CNCCs do not remain as multipotent precursors in the absence of Twist1 function. Instead, the ectopic arch expression of sox10 and foxd3 in Twist1-depleted embryos, at a stage when these genes are normally co-expressed in the non-ectomesenchyme, suggests that presumptive ectomesenchyme precursors partially adopt a non-ectomesenchyme expression profile in the absence of Twist1. This conclusion was confirmed by our microarray analysis in which non-ectomesenchyme but not early CNCC genes were the most upregulated in Twist1-deficient embryos. Unexpectedly, some non-ectomesenchyme genes, such as nr4a2b and gch2, were upregulated in the non-ectomesenchyme domain but not ectopically expressed in Twist1-deficient arches, suggesting that Twist1 may have additional roles in restricting non-ectomesenchyme gene expression within the early non-ectomesenchyme domain. Consistent with only a subset of non-

Figure 8. Misexpression of Bmp4 in migrating CNCCs inhibits ectomesenchyme formation. (A–D) Whole mount in situ at 18 hpf show ectopic expression of sox10 in the arches (numbered) of sox10:Gal4VP16; UAS:Bmp4; UAS:mKR embryos (n = 8/8) compared to sox10:Gal4VP16; UAS:mKR controls (n = 0/8) and reductions of dlx2a in sox10:Gal4VP16; UAS:Bmp4; UAS:mKR embryos (n = 4/4) compared to sox10:Gal4VP16; UAS:mKR controls (n = 0/4). Arrows indicate the second arch and white arrowheads the developing ear. (E–H) Double fluorescent in situ for mKR (red) and fli1a or dlx2a (green) at 24 hpf show reduction of fli1a arch expression in sox10:Gal4VP16; UAS:Bmp4; UAS:mKR embryos (n = 5/5) compared to sox10:Gal4VP16; UAS:mKR controls (n = 0/9) and reduction of dlx2a arch expression in sox10:Gal4VP16; UAS:Bmp4; UAS:mKR embryos (n = 4/4) compared to sox10:Gal4VP16; UAS:mKR controls (n = 0/3). The mKR transgene was included as a lineage tracer for CNCC-derived cells that migrated into the arches. (I,J) Skeletal staining at 5 dpf shows severe loss of craniofacial skeleton in sox10:Gal4VP16; UAS:Bmp4; UAS:mKR embryos (n = 7/7) compared to sox10:Gal4VP16; UAS:mKR controls (n = 0/9). (K,L) In situ for foxd3 at 48 hpf reveal largely normal patterns of glia in sox10:Gal4VP16; UAS:Bmp4; UAS:mKR embryos (n = 11) and normal xdh-positive xanthophore precursors in sox10:Gal4VP16; UAS:Bmp4; UAS:mKR embryos (n = 15) and sox10:Gal4VP16; UAS:mKR controls (n = 10). Scale bars = 50 μm.

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ectomesenchyme genes showing ectopic arch expression, we also found no evidence for pigment cells, functional glia, or neurons forming ectopically in Twist1-deficient arches. Hence, loss of Twist1 function is not sufficient for arch CNCCs to generate non-ectomesenchyme derivatives.

Twist1 promotes ectomesenchyme through both Fgf-dependent and -independent gene activation

Our data also indicate that Twist1 promotes ectomesenchyme fates through two largely parallel pathways. The finding that Twist1 was required for expression of the Fgf target gene *poe* in arch ectomesenchyme suggests a role for Twist1 in potentiating Fgf signaling. Twist1 might do so by regulating expression of Fgf ligands and/or receptors, as Twist1 has been shown to regulate the CNCC expression of both *Fgf10* and *Fgfr1* in mice [12]. Hence, Twist1 could function upstream of autocrine/paracrine Fgf signaling within CNCCs that promotes ectomesenchyme development. However, although Fgf signaling was required to repress *sox10* and activate *dlx2a* expression, we found that *fli1a* ectomesenchyme expression was Fgf-independent. Instead, *fli1a* appears to be a potentially direct target of Twist1, and we have identified a conserved ectomesenchyme enhancer element of *fli1a* that is Twist1-dependent. However, chromatin immunoprecipitation experiments (currently not feasible due to the lack of a high quality Twist1 antibody) will eventually be needed to show enhancer occupancy by Twist1. Moreover, the function of Fli1a in ectomesenchyme formation also remains to be investigated. Targeted disruption of the *Fli* gene in mouse results in embryonic lethality by E12.5 due to severe hemor-

**Figure 9. Mesoderm and endoderm are not essential for ectomesenchyme formation.** (A–D) In situ at 12 hpf show loss of axial *ntl* and mesodermal *myod* expression in Antivin-mRNA-injected embryos compared to un-injected controls. (E,F) Confocal projections of *her5:GFP* expression at 19 hpf show loss of pouch endoderm (arrows) but not brain (arrowheads) in Antivin-mRNA-injected embryos compared to un-injected controls. (G,H) In situ at 15.5 hpf show normal ectomesenchyme induction of *dlx2a* in Antivin-mRNA-injected embryos (n = 12) and un-injected controls (n = 13). Arches are numbered. (I,J) Double fluorescent in situ at 15.5 hpf show normal induction of *dlx2a* (green) in *sox10*-positive CNCCs (red, yellow indicates co-localization) in Antivin-mRNA-injected embryos (n = 15) and un-injected controls (n = 9). (K,L) Double fluorescent in situ at 19 hpf show complementary expression of *fli1a* (green) in ectomesenchyme and *sox10* (red) in non-ectomesenchyme in Antivin-mRNA-injected embryos (n = 5) and un-injected controls (n = 8). Scale bar = 50 μm.

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Id2a restricts Twist1 activity to the ectomesenchyme

The expression of Twist1 genes throughout early CNCCs in both zebrafish [13] and mice [46,47] suggests that Twist1 function is regulated at the post-translational level during ectomesenchyme formation, either through enzymatic modifications or changes in dimerization partners. Here we find evidence for the latter, with the Bmp target gene Id2a functioning oppositely to Twist1 for ectomesenchyme specification. Previous work suggests that Id2a does not inhibit Twist1 function per se, but instead would modulate what types of dimers Twist1 forms [14,15]. In the future, the identification of binding partners for Twist1 in CNCCs will allow us to test what roles Twist1 heterodimers versus homodimers play in ectomesenchyme specification. Furthermore, we note that Twist1 genes continue to be expressed in arch ectomesenchyme and likely have additional roles in facial skeletal patterning, for example by interacting with other bHLH factors such as Hand2 [48]. Hence, different Twist1 heterodimers may have distinct roles during craniofacial development (i.e. ectomesenchyme formation versus arch patterning), with the later disruption of these heterodimers in Twist1-deficient and Id2a-misexpression embryos also contributing to some aspects of the observed facial skeletal defects.
A Bmp-dependent switch for ectomesenchyme formation

Previous studies have shown both temporal and spatial components to the specification of the large diversity of neural crest derivatives. In the trunk, neural crest cell fate is influenced both by migration pathways [49,50] and signals at their destinations [51]. In contrast, CNCCs appear to be largely fate-restricted before migration in zebrafish, with Wnts from the dorsal ectoderm promoting pigment at the expense of neural and glial fates but having little effect on ectomesenchyme derivatives [4,5]. Instead, we find that Bmps, also from the ectoderm, actively inhibit ectomesenchyme fates in more dorsal CNCCs. Furthermore, this observed role of Bmps in regulating ectomesenchyme specification is not simply an indirect effect of earlier roles of Bmps.
in neural crest development, as prolonging Bmp activity did not result in decreased amounts of CNCC being generated or defects in non-ectomesenchyme derivatives.

The down-regulation of Bmp activity in ectomesenchyme precursors could reflect CNCCs distancing themselves from a Bmp source in the non-neural ectoderm (e.g., Bmp2b) and/or active inhibition of Bmp signaling during migration. Whereas the Bmp antagonist Noggin1 is expressed in the paraxial mesoderm surrounding migratory CNCCs in zebrafish [52], genetic ablation of endoderm and mesoderm did not disrupt ectomesenchyme gene expression, although there are likely other sources of Bmp inhibitors [53]. Alternatively, the down-regulation of Bmp activity could result from the delamination of CNCCs from the neural plate ectoderm, with the basement membrane of the ectodermal epithelium, under which CNCCs migrate, serving as a barrier to apical Bmp secretion and signaling [34].

Although we found that Bmp down-regulation was essential for ectomesenchyme formation, transgenic inhibition of Bmp (or Id2a depletion) did not result in ectopic ectomesenchyme (data not shown). Therefore, low Bmp activity is not sufficient to specify ectomesenchyme fates. Instead, Bmp down-regulation might allow CNCCs to respond to ectomesenchyme-promoting signals present only in the early CNCC milieu. In particular, our data suggest that activation of Twist1 at low Bmp levels allows migrating CNCCs to respond to Fgfs. Although the exact source of Fgfs remains to be determined, we propose that a combination of low Bmp and high Fgf signaling uniquely present in early migrating CNCCs generates ectomesenchyme fates.

### Materials and Methods

#### Ethics statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the University of Southern California Institutional Animal Care and Use Committee.

#### Zebrafish lines and transgenic constructs

Zebrafish were staged as described [55]. Previously reported lines include Tg(hsp70I:Gal4)404 [56], Tg(~/~.Hors2;EGFP)7011 [57], and Tg(UAS:Bmp4; cmlc2:GFP)el49. Tg(UAS:kikGR; cmlc2:GFP)el15, Tg(UAS:mKR; cmlc2:GFP)el151, Tg(UAS:dnTwist1b; UAS:mcherryCAAX; cmlc2:GFP)el179, and Tg(UAS:dnTwist1b; UAS:ID2a; cmlc2:GFP)el411. mutagenesis was then performed with primers twdnEK1L: 5'-GCACTCGGCCGCAACATGAGAGCACTTTGTAACAAGAGTCGCTGTCATTGTTGGC-3', and twdnEK1R: 5'-GTCGCCACCATGCTGTG-3', respectively. UASSmK was made by combining p5E-UAS, pME-mKR, p3E-polyA, and pDestTol2pA2. The dnTwist1b clone was made by first amplifying the full-length zebrafish Twist1b gene from Xenopus laevis [60] and cloning the PCR product into pDONR221; primers used were 5'-CAGGCTCGGCCACCATGCCCGAAGAGCCCGCGGAGA-3', and 5'-CTACCGAGTATACTGGGGCCCTCGAGG-3'.

PCR based mutagenesis was then performed with primers twdnEK1L: 5'-GCCCTTTCCGACGACACTTA-3', and twdnEK1R: 5'-CTGTCGCCCTGGCAAGCCTTT-3' as described [59] to change nucleotide G250 of the twist1b cDNA to A, which results in conversion of amino acid 84E to K, with the PCR product cloned into pDONR221 to create pME-dnTwist1b. To construct pME-dnFgfr1a, we amplified a truncated form of zebrafish fgfr1a based on the previously reported dominant-negative Fgfr receptor in Xenopus laevis [60] and cloned the PCR product into pDONR221; primers used were 5'-GGGACAACTTTTGTAACAAGAGTCGCTGTCATTGTTGGC-3', and 5'-GGGGACCACTTTGTAACAAGAGTCGCTGTCATTGTTGGC-3'.

Full-length zebrafish sox10 was amplified with primers sox10F: 5'-TACGACTCACTATAGGTACCGGCAGTCCAATTTC-3', and sox10R: 5'-ATGAAGGCAATAAGATTTTACACATAAACCGGCGGAGAATGTCGCCACCATGCTGTG-3'.

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Full-length zebrafish sox10 was amplified with primers sox10F: 5'-TACGACTCACTATAGGTACCGGCAGTCCAATTTC-3', and sox10R: 5'-ATGAAGGCAATAAGATTTTACACATAAACCGGCGGAGAATGTCGCCACCATGCTGTG-3'.
In situ hybridization, immunohistochemistry, and skeletal analysis

Aliased Blue staining of cartilage and Alizarin Red staining of bone at 5 dpf, as well as in situ hybridizations, were performed as described [61]. Reported probes included sox9a and sox9b [62], ftop2a [63], and fil1a [9], id2a, sox10, fil1a, twist1a, nla, det, xdh, pea3, kikGR, mkr, gh2, d4a2b, and foxd3 probes were synthesized with T7 RNA polymerase from PCR products amplified from multistage zebrasfish cDNA, with the exception of id2a which was amplified from an id2a cDNA plasmid (Zebrafish International Resource Center). We used the following PCR primers: id2a F:id2aF and id2aR, twist1a: 5'-CACAGTGTCGGGTAAGTAGT-3' and 5'-GCTAAATGGCGTCAGTATGTTGAG-3', det: 5'-CTGACTGGACTTGGCAG-3', pea3: 5'-CCCATATGGAGAGGACA-3', kikGR: 5'-GAAGCTCCAGCAACAC-3', xdh: 5'-TGAAACACTCTTGAGCACCTC-3' and 5'-GCTATACAGCTACTATAGGGTCTTTTCCT-3'.

Assays with Lysotracker Red were performed as described [53].

For MO validation, we performed rescue experiments with in vitro injected mRNA never rescued (n = 0/11). Scale bars = 50 μm.

Luciferase assay

The Twist1 expression vector was made by cloning the zebrasfish twist1b cDNA into pCDNA3. Luciferase reporter constructs were generated by cloning wild-type and mutant versions of the fil1a-F enhancer into the Pg3 basic vector (Progema #E1715). We then transfected 29T7 cells with the Twist1b expression and luciferase reporter plasmids and used the Dual Luciferase Assay kit (Promega #E1910) per manufacturer instructions to measure firefly luciferase activity relative to renilla luciferase activity 48 hours after transfection. All experiments were performed in triplicate.

Statistical analysis

Statistics were performed with JMP7 software. One-way analysis of variance was calculated with a Tukey-Kramer HSD test (alpha = 0.05) and standard errors of the mean were plotted.

Supporting Information

Figure S1 Twist1a and Twist1b function redundantly to specify ectomesenchyme. (A-C) In situ at 18 hpf show sox10 expression in un-injected, twist1a-MO, and twist1b-MO embryos. A few ectopic sox10-positive cells are seen in the second arches (arrows) of twist1a-MO and twist1b-MO embryos. White arrowheads denote the developing ear. (D-G) Confocal projections of fil1a-F-GFP; twist1a-F-red doubly transgenic embryos at 28 hpf show normal fil1a-F expression in un-injected control, twist1a-MO, and twist1b-MO embryos and loss of fil1a-F expression in twist1a/1b-MO embryos. Arrows indicate fil1a-F-GFP vascular expression which is unaffected in twist1a/1b-MO embryos. (H-L) Skeletal staining shows malformed mandibular and hyoid skeletons in twist1a-MO and twist1b-MO embryos compared to un-injected controls. In addition, co-injection of a Twist1b mRNA not targeted by the MOs partially rescued the head skeleton of twist1a/1b-MO embryos (n = 24/24), whereas co-injection of a control kikGR mRNA never rescued (n = 0/11). Scale bars = 50 μm.

Figure S2 Time-course of sox10-Gal4VP16-dependent transgene expression. (A-D) In situ for kikGR mRNA in sox10-Gal4VP16; UAS:kikGR embryos show transgene expression in migratory CNCs at 13 hpf but not in pre-migratory CNCs at 11 hpf. sox10:Gal4VP16 control embryos show no expression.

(TIF)
Figure S3  Cranial ganglionic neurons are unaffected in twist1a/1b-MO and Id2a misexpression embryos. (A–D) Confocal projections of anti-Hu/C/D immunofluorescence show neurons of the trigeminal (tg), anterior lateral line (all), otic (ot), and posterior lateral line (pll) ganglia at 36 hpf. No major differences in the pattern of anti-Hu/C/D staining was observed between uninjected control (n = 6), twist1a/1b-MO (n = 6), sox10:Gal4VP16 only control (n = 9), and sox10:Gal4VP16: UAS:Id2a (n = 8) embryos. Scale bar = 50 μm. (TIF)

Figure S4  Cell death and sox9a expression in twist1a/1b-MO embryos. (A–D) Confluent projections of Lysotracker Red staining in 36 hpf sox10:GFP transgenic embryos show increased cell death in the pharyngeal arches (shown in high-magnification views in C and D from the boxes in A and B) in twist1a/1b-MO-injected embryos (n = 6) compared to uninjected controls (n = 6). Arrowhead shows increased cell death in more dorsal CNCCs as well. Scale bar = 50 μm. (E) Quantification of Lysotracker-positive cells per arch area. Mandibular and hyoid arches were used for the analysis. Asterisk indicates statistical significance using a Tukey-Kramer HSD test (α = 0.05). (F,G) In situ hybridizations for sox9a at 48 hpf show very reduced expression in the pharyngeal arches of twist1a/1b-MO embryos (n = 5/5) compared to uninjected controls (n = 0/12). Dorsal (D) and ventral (V) pre-chondrogenic domains of the mandibular (1) and hyoid (2) arches are shown for the control. sox9a expression in the pre-chondrogenic domain of the mesoderm-derived otic capsule cartilage (arrows) was less affected in twist1a/1b-MO embryos. (TIF)

Figure S5  Induction and early migration of CNCCs is unaffected by Bmp4 misexpression. In situ for sox10 at 15 hpf show no difference in early migrating CNCCs between sox10:Gal4VP16; UAS:Bmp4 embryos (n = 5) and sox10:Gal4VP16 only controls (n = 12). Scale bar = 50 μm. (TIF)

Figure S6  Cell death analysis in sox10:Gal4VP16; UAS:Bmp4 embryos. Compared to sox10:Gal4VP16 only controls (n = 8), Lysotracker staining reveals no major increase in cell death at 24 hpf in sox10:Gal4VP16; UAS:Bmp4 embryos (n = 8). Scale bar = 50 μm. (TIF)

Figure S7  Injection of twist1b mRNA fails to rescue sox10 ectomesenchyme persistence in Bmp4 misexpression embryos. (A–C) sox10 expression at 18 hpf was similarly upregulated in the arches of uninjected sox10:Gal4VP16; UAS:Bmp4 embryos (n = 11/11) and twist1b-mRNA-injected sox10:Gal4VP16; UAS:Bmp4 embryos (n = 5/5) compared to sox10:Gal4VP16 only controls (n = 0/10). Scale bar = 50 μm.

Table S1  Summary of CNCC genes affected by Twist1 depletion in the microarray analysis. (XLS)

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Author Contributions
Conceived and designed the experiments: AD JGC. Performed the experiments: AD. Analyzed the data: AD JGC. Contributed reagents/materials/analysis tools: AD JGC. Wrote the paper: AD JGC.

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