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BMP, Wnt and FGF signals are integrated through evolutionarily conserved enhancers to achieve robust expression of Pax3 and Zic genes at the zebrafish neural plate border

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SUMMARY

Neural crest cells generate a range of cells and tissues in the vertebrate head and trunk, including peripheral neurons, pigment cells, and cartilage. Neural crest cells arise from the edges of the nascent central nervous system, a domain called the neural plate border (NPB). NPB induction is known to involve the BMP, Wnt and FGF signaling pathways. However, little is known about how these signals are integrated to achieve temporally and spatially specific expression of genes in NPB cells. Furthermore, the timing and relative importance of these signals in NPB formation appears to differ between vertebrate species. Here, we use heat-shock overexpression and chemical inhibitors to determine whether, and when, BMP, Wnt and FGF signaling are needed for expression of the NPB specifiers *pax3a* and *zic3* in zebrafish. We then identify four evolutionarily conserved enhancers from the *pax3a* and *zic3* loci and test their response to BMP, Wnt and FGF perturbations. We find that all three signaling pathways are required during gastrulation for the proper expression of *pax3a* and *zic3* in the zebrafish NPB. We also find that, although the expression patterns driven by the *pax3a* and *zic3* enhancers largely overlap, they respond to different combinations of BMP, Wnt and FGF signals. Finally, we show that the combination of the two *pax3a* enhancers is less susceptible to signaling perturbations than either enhancer alone. Taken together, our results reveal how BMPs, FGFs and Wnts act cooperatively and redundantly through partially redundant enhancers to achieve robust, specific gene expression in the zebrafish NPB.

KEY WORDS: FGF, Neural crest, Wnt, Enhancer, Pax3, Zic3

INTRODUCTION

A major theme in developmental biology is the need for cells to integrate multiple signals to make decisions about their fate. To achieve this, regulatory DNA must be structured so developmental control genes are expressed only when a cell is in the correct signaling environment. Furthermore, this regulation must be robust so development can proceed normally even when signaling levels fluctuate with environmental variability.

Neural crest development is a classic example of how multiple signals are integrated to make cell fate decisions. Neural crest precursors are initially specified at the border between the neural plate and the epidermal ectoderm, a region called the neural plate border (NPB). This specification involves signals from the Wnt, FGF and BMP pathways. Wnt and FGF ligands are secreted by the neural plate and underlying mesoderm (Bang et al., 1999; Monsoro-Burq et al., 2003; García-Castro et al., 2002), while the epidermal ectoderm produces BMP ligands and the neural plate secretes BMP antagonists, resulting in a BMP signaling gradient along the dorsoventral axis (Marchant et al., 1998; Smith and Harland, 1992; Sasai et al., 1995; Endo et al., 2002). The NPB is specified in the region of the ectoderm that receives an intermediate dose of BMP signaling, as well as Wnts and/or FGFs (LaBonne and Bronner-Fraser, 1998; Streit and Stern, 1999; Villanueva et al., 2002). The combination of these signals leads to the expression of NPB specifier genes, including members the Pax3/7 and Zic families (Sato et al., 2005; Monsoro-Burq et al., 2003; Bang et al., 1999). Experiments in *Xenopus* indicate that the combination of Pax3/7 and Zic genes is sufficient to induce neural crest (Sato et al., 2005).

How Wnt, FGF and BMP signaling specify the NPB is an area of active research. Much of what we know about this process comes from experiments in *Xenopus*. It is generally agreed that an intermediate level of BMP signaling is crucial for establishing the NPB and recent studies have shed light on the time dependence of the BMP and Wnt requirement (Steventon et al., 2009; Steventon and Mayor, 2012; Patthey et al., 2009). The relative importance of Wnts and FGFs in this process has been controversial. Studies in the past decade have fuelled a debate about whether FGF and Wnt signaling act in parallel to induce NPB genes (Monsoro-Burq et al., 2005) or whether FGF acts indirectly by activating the expression of Wnt ligands (Hong et al., 2008). The interactions of these pathways in NPB specification have been less well studied in zebrafish. The ease of transgenic zebrafish generation (Kawakami, 2004), coupled with excellent methods for temporally controlled gene overexpression make zebrafish an attractive model for studying NPB gene regulation.

To better understand how and when BMP, Wnt and FGF signals specify the NPB in zebrafish, we knocked down Wnt and FGF signaling, and overexpressed BMP ligand at various developmental time points and monitored the expression of the NPB specifiers *pax3a* and *zic3*. We find that full *pax3a* NPB expression requires all three signals during mid-to-late gastrulation, whereas *zic3* NPB expression only requires Wnt signaling and attenuation of BMP

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signaling. We also show that modulating BMP signaling affects both the position and intensity of *pax3a* and *zic3* expression within the ectoderm, while perturbing Wnt and/or FGF expression mainly affects expression intensity. We then investigated how BMP, FGF and Wnt signals are integrated by the cis-regulatory machinery of *zic3* and *pax3a*. To do this, we isolated four enhancers from the *Takifugu rubripes zic3* and *pax3a* loci that drive reporter expression in the forming zebrafish NPB and then tested their responses to Wnt, FGF and BMP perturbations. We used the *Fugu* genome for these enhancer studies because it is more compact than the zebrafish genome, allowing enhancers to be located more easily. We found that Wnts, FGFs and BMPs act in different combinations through these enhancers to achieve NPB expression. Based on these findings, we propose that the parallel influences of FGFs and Wnts on partially redundant enhancers drive sharp, intense NPB specifier expression and buffer the regulation of NPB genes against variability in signaling levels. Furthermore, we propose that lineage-specific differences in the relative influence of these evolutionarily conserved enhancers could account for observed differences in the importance of FGF versus Wnt signaling during NPB induction in frog and chick.

MATERIALS AND METHODS

Zebrafish stocks and husbandry

Adult fish were kept at 28.5°C on a 14 hour light/10 hour dark cycle. Embryos were obtained by natural crosses and staged as previously described (Kimmel et al., 1995).

Generation of transgenic zebrafish

Putative enhancer fragments were amplified from *Takifugu rubripes* genomic DNA and cloned upstream of the mouse Fos promoter and GFP using a Gateway-based technique similar to a previously described method (Fisher et al., 2006). A dual fluorescence vector was created by cloning mCherry under control of the ubiquitously active *Xenopus* EF1α promoter downstream of GFP. Supplementary material Table S1 shows primer sequences.

dkk1 and *bmp2b* were amplified using RT-PCR and cloned into a Gateway vector. *hsp70l-dkk1-mCherry* and *hsp70l-bmp2b tol2* plasmids were constructed using vectors from the Tol2 Gateway kits (Villefranc et al., 2007; Kwan et al., 2007).

Transgenesis constructs were injected as previously described (Kawakami, 2004; Kwan et al., 2007). Transgenic founders were identified using pair-wise crosses to wild-type fish. F1 fish were raised and their offspring used in experiments.

Site-specific mutagenesis was performed using PCR-based methods. Wild-type and mutant enhancers were cloned into the dual fluorescence vector. Mutant and wild-type constructs were injected on the same morning so GFP intensity could be directly compared. At the appropriate stage, GFP levels were scored in embryos expressing mCherry in the NPB or dorsal neural tube.

Signaling pathway manipulations

F1 fish containing reporter constructs were crossed to fish heterozygous for the *hsp70l-dkk1-mCherry* or *hsp70l-bmp2b* transgene. Similar transgenic lines have been described previously (Stoick-Cooper et al., 2007; Chocron et al., 2007). *hsp70:bmp2b* fish were incubated at 37°C for 1 hour then 28.5°C until fixed. *hsp70:dkk1-mCherry* fish were incubated at 39°C for 30 minutes then 33°C until fixed. Double in situ hybridizations were performed with probes for the gene of interest and *bmp2b* or *mCherry*. Coloration was carried out using NBT-BCIP for the gene of interest and Fast Red for *bmp2b* or *mCherry*. Embryos were sorted into mock heat shock and *bmp2b*- or *dkk1*-overexpressing groups based on Fast Red staining.

The *wnt8a* morpholino was a kind gift from Kristin Artinger (University of Colorado, Aurora, USA). The MO sequence is the same as *wnt8a* MO1 (Lekven et al., 2001). We injected the MO as described by Lewis et al. (Lewis et al., 2004).

SU5402 treatments were performed by incubating dechorionated embryos in 90 μM SU5402 in 1% DMSO/system water. Control embryos were incubated in 1% DMSO/system water alone. At the appropriate stage, embryos were removed from the treatment solution and immediately fixed.

A GR-Lef1-βcat construct was kindly provided by Yevgenya Grinblat (University of Wisconsin, Madison, USA). GR-Lef1-βcat mRNA was synthesized and injected as previously described (Ramel and Lekven, 2004). Dechorionated embryos were treated with dexamethasone and cycloheximide as previously described (Martin and Kimelman, 2008).

In situ hybridization, photography and mounting

Whole-mount in situ hybridization was performed as described previously (Thisse et al., 1993) with modifications (Melby et al., 1997). Digoxygenin- or fluorescien-labeled RNA probes were synthesized from cDNA templates. Embryos were cleared and mounted as described previously (Griffin et al., 1998).

RESULTS

Proper BMP, FGF and Wnt signaling during late gastrulation are crucial for *pax3a* and *zic3* expression at the NPB

Previous studies have shown that Wnt and FGF signals from the mesoderm and epidermis along with an intermediate level of BMP signaling are important for NPB induction (LaBonne and Bronner-Fraser, 1998; García-Castro et al., 2002). To determine the temporal requirement for these signals in zebrafish we used a heat-shock strategy to overexpress the Wnt antagonist *dickkopf1* (*dkk1*) or the BMP ligand *bmp2b*. Previous studies indicate that events during mid- to late gastrulation are important in NPB specification. For this reason we heatshocked embryos containing a *hsp70l:dkk1-mcherry* or *hsp70l:bmp2b* transgene beginning between 30% epiboly (4.7 hpf) and tailbud stage (10 hpf) and monitored NPB gene expression at 6 somites (12 hpf) and phenotype at 24 hpf. We found that shield stage (6 hpf) heatshock had a similar effect to earlier heat shocks and that heat shocks later than 75% epiboly had minor effects on gene expression (data not shown). For this reason, we concentrated on embryos that were heat shocked at shield stage (6 hpf) or 75% epiboly (8 hpf) and fixed at 6 somites (12 hpf). We used the FGF signaling inhibitor SU5402 to test the requirement for FGF signaling in regulating these genes during late gastrulation. SU5402 treatments have been shown to knockdown expression of direct FGF signaling targets in zebrafish (Roehl and Nüsslein-Volhard, 2001). We started SU5402 treatments at stages between 30% epiboly and tailbud stages and found that treatments beginning at 65% epiboly (7 hpf) disrupted NPB gene expression to a degree similar to earlier treatments, without confounding effects on gastrulation or axis formation. To confirm the efficiency of these manipulations, we monitored gene expression of the hindbrain marker *krox20* and the neural plate markers *sox2* and *sox3*, and observed the phenotypes of treated embryos at 24 hpf agreed with previously described phenotypes (supplementary material Fig. S1). The observed changes in gene expression patterns and phenotypes agreed with previously described results. We monitored *pax3a* and *zic3* expression in these embryos by in situ hybridization. *pax3a* is expressed in the anterior paraxial mesoderm near 12 hpf, so we used optical sections to confirm that any changes observed in *pax3a* expression occurred in the NPB (Fig. 1S-T).

zic3 NPB expression decreases dramatically with *dkk1-mcherry* overexpression (Fig. 1H,I versus 1G) and *pax3a* mRNA exhibits a more modest decrease (Fig. 1B,C versus 1A). The effect is stronger with a shield stage heat shock, indicating that Wnt signaling is crucial at late gastrulation for proper levels of

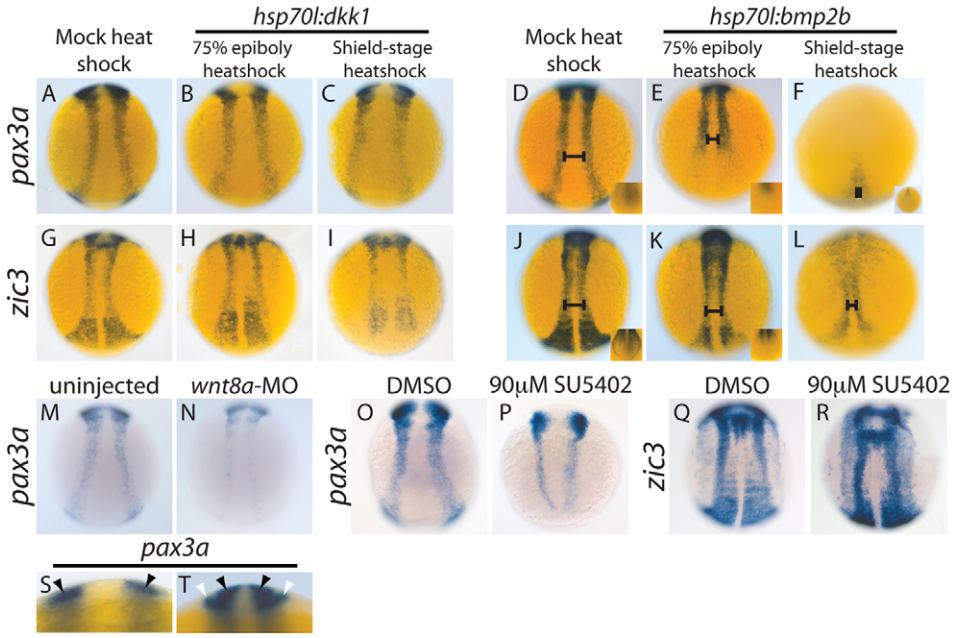

Fig. 1. BMP and Wnt signaling regulate *pax3a* and *zic3*, and FGF signaling regulates *pax3a* during late gastrulation. (**A-C**,**G-I**) *pax3a* and *zic3* expression decreases upon heat shock-mediated *dkk1* overexpression. *pax3a* and *zic3* expression decreases slightly in *hsp70l:dkk1* embryos heat shocked at 75% epiboly [*pax3a*, B versus A (81%, *n*=27); *zic3*, H versus G (93%, *n*=28)] and a shield stage heat shock leads to a greater decrease in expression [*pax3a*, C versus A (92%, *n*=49); *zic3*, I versus G (100%, *n*=33)]. (**D-F**,**J-L**) *pax3a* and *zic3* expression in the NPB decreases and shifts medially upon heat shock-mediated *bmp2b* overexpression. A 75% epiboly heat shock leads to a loss of posterior NPB expression (E versus D and K versus J, insets) and a slight medial shift of the anterior NPB domain (D,E,J,K, bars) (*pax3a*, 100%, *n*=22; *zic3*, 94%, *n*=18). A shield stage heat shock leads to a drastic decrease in *pax3a* and *zic3* expression throughout the anterior-posterior axis, and NPB expression shifts medially into a teardrop shape [*pax3a*, F versus D (100%, *n*=28); *zic3*, L versus J (100%, *n*=39)]. (**M**,**N**) Morpholino-mediated knockdown of *wnt8a* leads to a greater decrease in *pax3a* expression than does *dkk1* overexpression [N versus M (90%, *n*=20)]. (**O-R**) Inhibition of FGF signaling by SU5402 treatment beginning at 7 hpf leads to a decrease in *pax3a* expression by 12 hpf (84%, *n*=44) (P versus O). The same SU5402 treatment does not decrease NPB *zic3* expression (100%, *n*=27) (R versus Q). (**S**,**T**) Optical cross-sections allow the distinction between NPB and mesodermal *pax3a* expression. An optical cross-section of the trunk at ~12 hpf reveals *pax3a* expression in the ectoderm only (black arrowheads, S), whereas an optical cross-section slightly later reveals *pax3a* mRNA in the ectoderm (black arrowheads, T) and paraxial mesoderm (white arrowheads, T). The insets in D-F,J,K are posterior dorsal views, all other pictures are dorsal trunk views with anterior upwards.

NPB *pax3a* and *zic3* expression. Previous work suggests Wnt8a is the Wnt ligand involved in neural border/neural crest specification (Lewis et al., 2004). Interestingly, the effect of *dkk1* overexpression on *pax3a* expression was milder than the effect of *wnt8a* knockdown by morpholino (Lewis et al., 2004) (Fig. 1N versus 1M) (supplementary material Fig. S1). This difference suggests that the *wnt8a* MO may be disrupting *pax3a* NPB expression both directly and indirectly, potentially through its disruption of DV axis formation (Ramel and Lekven, 2004). Embryos treated with SU5402 beginning at 65% epiboly (7 hpf) exhibited decreased *pax3a* NPB expression (Fig. 1P versus 1O). The same SU5402 treatment did not decrease the level of *zic3* NPB expression, but led to a less organized *zic3* expression pattern (Fig. 1R versus 1Q).

hsp70l:bmp2b embryos heat shocked at 75% epiboly did not express *pax3a* or *zic3* in the posterior NPB (Fig. 1E versus 1D and 1K versus 1J, insets) and exhibited a slight medial shift in trunk NPB expression (Fig. 1E versus 1D and 1K versus 1J, bars). *pax3a* and *zic3* expression decreased dramatically when *hsp70l:bmp2b* embryos were heat shocked at shield stage and both expression patterns shifted to the midline (Fig. 1F versus 1D and 1L versus 1J). *pax3a* expression and the trunk and tail aspects of *zic3* expression are limited to a small tear-drop shape (Fig. 1F,L). This medial shift of *pax3a* and *zic3* expression is consistent with the idea that NPB gene expression requires a moderate level of BMP signaling. The decrease in expression accompanying the medial shift is consistent with the presence of a repressor of these genes in the neural plate. Our results indicate that BMP signaling during late gastrulation is important for the placement of the NPB. The temporal requirement for this signaling proceeds from anterior to posterior, as posterior gene expression is more severely affected by the 75% epiboly heat shock.

These results suggest BMP signaling positions *pax3a* and *zic3* expression in the ectoderm whereas Wnt signaling is required for the genes to be expressed at the proper level. FGF signaling is required for the proper level of *pax3a* expression, but not that of *zic3*.

Wnt and FGF signaling act redundantly to amplify NPB gene expression

The data described above demonstrate that a knockdown of Wnt or FGF signaling can decrease the expression level of *pax3a*, whereas FGF knockdown does not decrease the level of NPB *zic3* expression. We hypothesized that Wnt signaling may be sufficient to mask any effects of FGF knockdown on *zic3*. To test this, we treated *hsp70l:dkk1-mCherry* embryos with SU5402 while heat shocking them (Fig. 2). The combination of Wnt and FGF knockdown decreases *zic3* expression more than *dkk1*

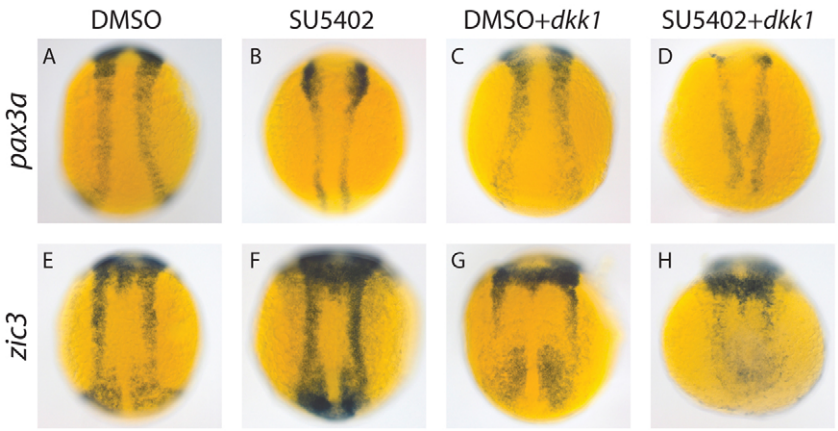

Fig. 2. Wnt and FGF signaling play partially redundant roles in regulating NPB specifiers. (**A-H**) Embryos treated with SU5402 starting at 7 hpf exhibited decreased *pax3a* expression (100%, *n*=28) (B versus A), but no decrease in *zic3* expression (100%, *n*=16) (F versus E). *dkk1* overexpression induced by 7 hpf heat shock decreases expression of *pax3a* (100%, *n*=24) (C versus A) and *zic3* (100%, *n*=16) (G versus E). When the Wnt and FGF signaling pathways are both attenuated with heat shock-induced *dkk1* overexpression and SU5402 treatment starting at 7 hpf, *pax3a* and *zic3* mRNA levels decrease more than when either pathway is knocked down alone [*pax3a*, D versus B,C (100%, *n*=24); *zic3*, H versus F,G (100%, *n*=17)]. All pictures are dorsal trunk views with anterior upwards.

overexpression alone (Fig. 2H versus 2G), demonstrating that these two pathways function redundantly to amplify *zic3* gene expression at the NPB. Similarly, *pax3a* expression decreases with Wnt or FGF knockdown (Fig. 2B,C versus 2A), and decreases further when both are inhibited (Fig. 2D).

Two evolutionarily conserved enhancers drive *pax3a* expression in the NPB

We wanted to elucidate the relationship between the BMP, Wnt and FGF pathways in *pax3a* regulation by determining how they act on individual *pax3a* enhancers. To identify these enhancers, we used comparative genomics to detect conserved non-coding sequences (Fig. 3A; supplementary material Fig. S2A). We identified two regions from the fourth intron of *Fugu pax3a* that drive gene expression in the NPB during early segmentation and later in the dorsal neural tube. We call these enhancers IR1 and IR2. *pax3a* IR1 contains two regions of high sequence identity between *Fugu* and human, a 5′ 286 bp region with 76% sequence identity and a 3′ 334 bp region with 65% sequence identity. The 3′ region is a likely homolog of a previously described mouse enhancer (Degenhardt et al., 2010). *pax3a* IR1 drives gene expression along the entire length of the NPB during early segmentation (Fig. 3B-G). This band of enhancer activity is wider than endogenous *pax3a* NPB expression (Fig. 3C,E versus Fig. 1A,S), suggesting other elements are needed to refine *pax3a* expression. A double in situ hybridization for GFP and *pax3a* confirms that *pax3a* expression and IR1 activity overlap (Fig. 3F,G). *pax3a* IR2 contains a 142 bp region with 65% sequence identity between *Fugu* and human. IR2 drives gene expression along the NPB during early segmentation (Fig. 3I-N) in a pattern more similar to endogenous *pax3a* expression than IR1 (Fig. 3J,L versus Fig. 1A,S). A double in situ hybridization for GFP and *pax3a* confirms that *pax3a* expression and IR2 activity overlap (Fig. 3M). Both *pax3a* enhancers drive gene expression in the dorsal neural tube at 24 hpf (Fig. 3H,O). We generated five independent zebrafish lines containing the *pax3a* IR1:GFP transgene and three independent lines containing the *pax3a* IR2:GFP transgene. The GFP expression patterns observed were consistent between lines.

The *pax3a* IR1 and IR2 enhancers have distinct responses to Wnt, BMP and FGF perturbations

To determine how the Wnt-, BMP- and FGF-regulated aspects of *pax3a* transcription are divided between the two enhancers, we crossed *pax3a* IR1 and IR2 transgenic fish into the *hsp70l:dkk1-mcherry* and *hsp70l:bmp2b* lines, and treated fish containing the transgenes with SU5402. *pax3a* IR1 activity dramatically decreases with *dkk1* overexpression (Fig. 4B,C versus 4A), whereas *pax3a* IR2 is unaffected by *dkk1* overexpression (Fig. 4E,F versus 4D). The decrease in *pax3a* IR1 activity is much greater than that of the endogenous gene (Fig. 4C versus Fig. 1C), indicating that additional enhancers buffer *pax3a* expression against variations in Wnt signaling levels.

pax3a IR1 and IR2 activities decrease dramatically upon SU5402 treatment (Fig. 4N versus 4M and 4P versus 4O), indicating FGF signaling is crucial for their function. Thus, *pax3a* IR2 is FGF dependent, but Wnt independent. Assuming that IR2 regulates *pax3a*, this demonstrates that at least some *pax3a* regulation by FGF signaling is not mediated by Wnts. This is in contrast to work in *Xenopus* suggesting FGFs regulate NPB genes mainly by inducing Wnt ligand expression (Hong et al., 2008).

pax3a IR2 activity decreases upon *bmp2b* overexpression and the activity shifts medially into a teardrop shape (Fig. 4K,L versus 4J). This is similar to the *bmp2b* response observed for endogenous *pax3a*. *pax3a* IR1 activity also shifts medially in the trunk upon *bmp2b* overexpression, but its activity intensifies and expands into the posterior epidermis (Fig. 4H,I versus 4G). The fact that the gene expression driven by IR1 intensifies and expands medially indicates that it is not repressed by factors in the neural plate, whereas *pax3a* IR2 is. The expression of neural markers also shifts medially upon *bmp2b* overexpression (supplementary material Fig. S1), but IR1 is able to drive expression in the most medial parts of the embryo, indicating that it is not highly repressed in these regions. The increase in *pax3a* IR1 activity upon *bmp2b* overexpression could result from *wnt8a* upregulation with increased BMP levels (supplementary material Fig. S3).

The combined activity of two *pax3a* enhancers is less susceptible to perturbation than either enhancer alone

Our results demonstrate that endogenous *pax3a* expression is less susceptible to Wnt and BMP perturbations than the activity of *pax3a* IR1 or IR2. We hypothesized that if we linked IR1 and IR2, the resulting transgene would be less sensitive to signaling perturbations. To test this, we made two independent stable transgenic lines in which *pax3a* IR2 and IR1 were placed upstream of the mouse Fos promoter driving GFP. Both lines expressed GFP in the NPB in a narrow band, similar to that driven by *pax3a* IR2 alone and endogenous *pax3a* (Fig. 5A). When *dkk1* was overexpressed by a shield stage heat shock we observed a slight decrease in GFP expression (Fig. 5B versus 5A). This decrease was

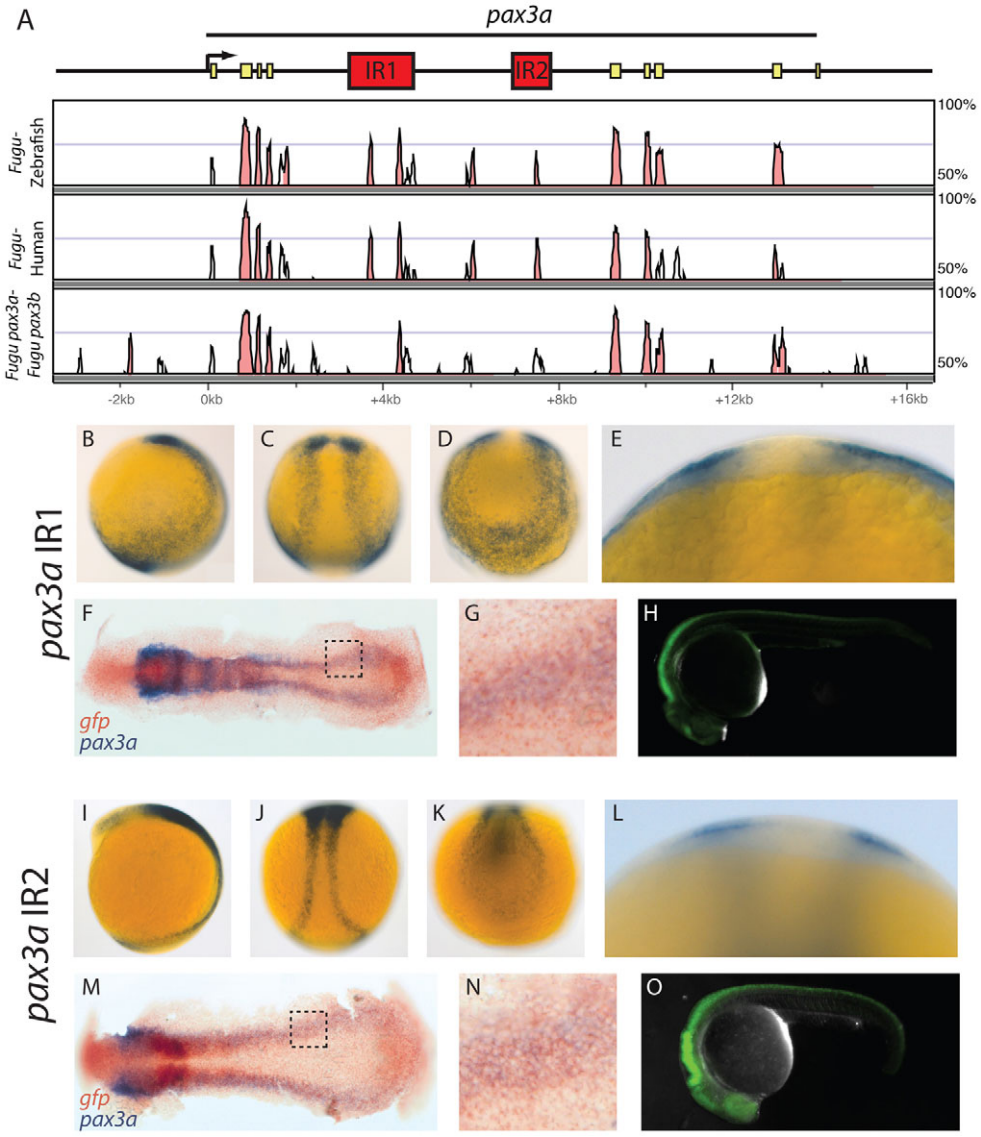

Fig. 3. Two enhancers in *pax3a* intron 4 drive gene expression in the NPB and dorsal neural tube. (**A**) The *Fugu pax3a* locus is shown along with a Vista plot (Frazer et al., 2004; Mayor et al., 2000) of sequence identity between *Fugu* and zebrafish, *Fugu* and human, and *Fugu pax3a* and its paralog *pax3b*. Enhancer regions are shown in red and *pax3a* exons are shown in yellow. (**B-O**) GFP expression driven by IR1 (B-H) and IR2 (I-O) is shown as detected by in situ hybridization for embryos at 12 hpf (B-G,I-N) and by fluorescence at 24 hpf (H,O). Optical cross-sections through the trunk region confirm that IR1 and IR2 drive gene expression in the ectoderm (E,L) and double in situ hybridization for GFP (brown) and *pax3a* (purple) demonstrate that *pax3a* expression overlaps with the activity of IR1 and IR2 (F,G,M,N). G and N are higher magnification views of the areas outlined in F,M, respectively. B,H,I,O are lateral views; C,J are dorsal trunk views; D,K are posterior dorsal views.

not as dramatic as when the same manipulation was performed for *pax3a* IR1 alone (Fig. 5B versus Fig. 4C), indicating the sensitivity of *pax3a* IR1 to changes in Wnt signaling levels can be masked by *pax3a* IR2. However, the activity of the compound enhancer is more significantly reduced upon *wnt8a* MO injection (Fig. 5G versus 5F), similar to the response observed for endogenous *pax3a* (Fig. 1N versus 1M). When *bmp2b* overexpression was induced in these fish, NPB GFP expression shifted medially in the trunk and expanded into the neural plate and epidermis in the posterior, while increasing in intensity (Fig. 5C versus 5A). This indicates that neural plate factors that repress *pax3a* IR2 cannot repress IR1 when the two enhancers are linked in this way. *pax3a* probably contains additional regulatory information that prevents IR1 from activating high levels of *pax3a* expression in the neural plate upon *bmp2b* overexpression. One likely candidate for this function is the *pax3a* promoter region, which has regulatory activity in mice (Milewski et al., 2004). SU5402 treatment beginning at 65% epiboly decreased IR2:IR1 activity similar to either enhancer alone (Fig. 5E versus 5D). Thus, the presence of these two enhancers cannot buffer against fluctuations in FGF levels. The drastic downregulation of endogenous *pax3a* upon SU5402 treatment demonstrates that endogenous *pax3a* is also not well buffered against decreases in FGF signaling (Fig. 1P versus 1O).

Two evolutionarily conserved enhancers drive *zic3* expression in overlapping regions of the NPB

We aligned the genomic regions containing *zic3* and *zic6* from *Fugu* and zebrafish (*zic3+6*) and the region containing *ZIC3* from human [*zic6* has been lost from the tetrapod lineage (Keller and Chitnis, 2007)]. Several non-coding sequences in this region are conserved between fish and human (Fig. 6A), and we placed some of these upstream of a minimal promoter driving GFP to test for enhancer activity. For a more detailed description of our enhancer search strategy see supplementary material Fig. S2B.

We found two regions that drive gene expression in the NPB and refer to these regions as E1 and E2. E1 lies 16 kb upstream of *zic3* and 11 kb downstream of *zic6* and contains a conserved region of 283 bp with 73% sequence identity between *Fugu* and human (Fig. 6A). This region drives gene expression along the NPB during early segmentation (Fig. 6B-F) and later in the dorsal neural tube (Fig. 6G). A double in situ hybridization for GFP and *zic3* indicates that E1 activity overlaps with *zic3* expression (Fig. 6E,F).

DEVELOPMENT

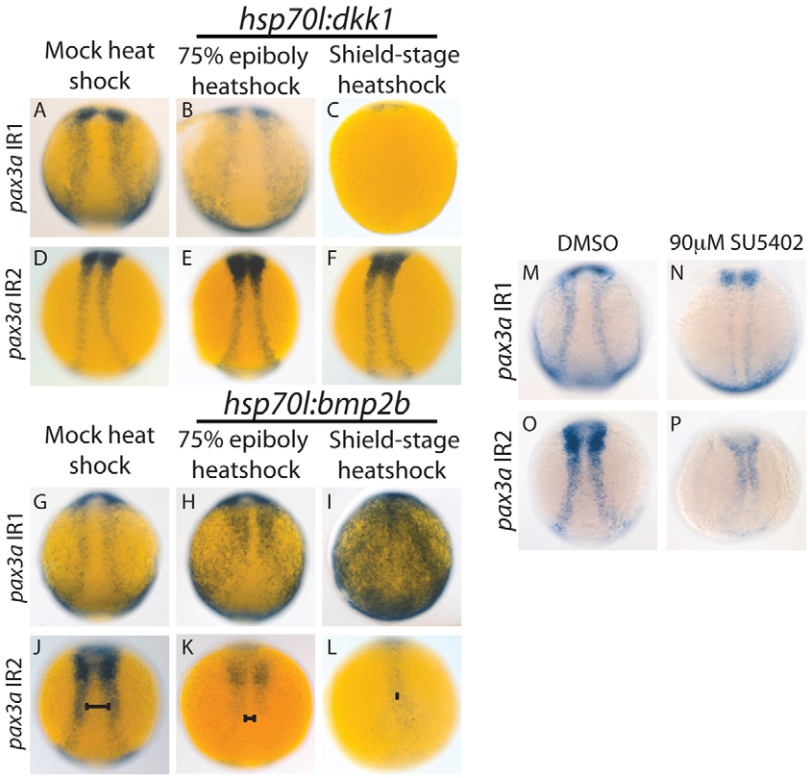

Fig. 4. *pax3a* IR1 requires Wnt and FGF signaling, and *pax3a* IR2 requires BMP and FGF signaling for full activity. (**A-F**) *dkk1* was overexpressed by heat shock in embryos with *pax3a* IR1:GFP or *pax3a* IR2:GFP. *pax3a* IR1 activity decreased with *dkk1* overexpression induced with a 75% epiboly heatshock (100%, *n*=25) (B versus A) and was almost completely lost with a shield stage heat shock (100%, *n*=12) (C versus A). *pax3a* IR2:GFP activity did not decrease when *dkk1-mCherry* overexpression was induced by heat shock at 75% epiboly (92%, *n*=12) (E versus D) or shield stage (89%, *n*=18) (F versus D). (**G-L**) *bmp2b* was overexpressed by heat shock. *pax3a* IR1activity intensifies and expands into the neural plate and epidermis when *bmp2b* overexpression is induced with a 75% epiboly heat shock (93%, *n*=41) (H versus G) and heat shock at shield stage leads to an even greater activity increase and causes expansion into the entire neural plate (84%, *n*=19) (I versus G). *pax3a* IR2 activity decreases and shifts medially (J-L, bars) upon *bmp2b* induction by 75% epiboly heat shock (100%, *n*=33) (K versus J) and further decreases and shifts medially into a tear-drop shape with a shield-stage heat shock (100%, *n*=14) (L versus J). (**M-P**) SU5402 treatment (90 μM) beginning at 7 hpf causes a decrease in the activity of *pax3a* IR1 (94%, *n*=17) (N versus M) and *pax3a* IR2 (100%, *n*=25) (P versus O). All pictures are dorsal trunk views with anterior upwards.

Interestingly, the sequence of E1 is similar to a previously identified *zic2a/zic5* NPB enhancer (Nyholm et al., 2007) and both of these are in roughly the same location relative to the genes they regulate. This suggests that these enhancers are paralogous and conservation of this enhancer sequence is partly responsible for the expression of vertebrate Zic genes in the developing neural crest.

zic3+6 E2 lies 53 kb upstream of *zic3* and 46.5 kb downstream of *zic6*. It contains a conserved 352 bp region with 70% sequence identity between *Fugu* and human (Fig. 6A). During early segmentation, *zic3+6* enhancer region E2 drives GFP expression in the posterior paraxial mesoderm and in the anterior neural plate and NPB (Fig. 6H-L). Later, this sequence is active in the anterior dorsal neural tube and the posterior paraxial mesoderm (Fig. 6M). A double in situ hybridization of GFP and *zic3* demonstrates that E2 activity and *zic3* expression overlap (Fig. 6K,L). The mesodermal expression is similar to endogenous *zic3* expression. The *zic3+6* E2-driven anterior dorsal neural tube expression is similar to that of *zic6* at 24 hpf (supplementary material Fig. S4). The ectodermal and mesodermal activities of this E2 are separable (supplementary material Fig. S5), but we performed analyses using full-length E2 to avoid losing important sequences not present in the trimmed-down NPB enhancer. *zic3* E2 is closer to *fgf13a* than is *zic3* or *zic6* (Fig. 6A), but *fgf13a* is not expressed before the 14-somite stage (Thisse et al., 2004), suggesting that it is not regulated by E2. Although these enhancers probably regulate both *zic3* and *zic6*, we refer to them as *zic3* enhancers as both have homologs in animals without a *zic6* gene. We generated two independent zebrafish lines with *zic3* E1:GFP and three independent zebrafish lines with *zic3* E2:GFP. The GFP expression patterns observed were consistent among lines.

zic3 E1 and E2 respond similarly to Wnt and BMP perturbations, but differently to FGF inhibition

We overexpressed *dkk1* in *zic3* E1 and E2 transgenic lines by heat shock at shield stage and 75% epiboly. We fixed *zic3* E1 embryos at six somites and *zic3* E2 embryos at four somites (11 hpf). The *zic3* E2 embryos were fixed earlier to observe GFP expression in the anterior NPB before neural closure in this region. *zic3* E1 activity decreased with *dkk1* overexpression induced by 75% epiboly heat shock (Fig. 7B versus 7A), and decreased more severely with shield stage heat shock (Fig. 7C versus 7A). *zic3* E2 activity decreased slightly upon 75% epiboly heat shock (Fig. 7E versus 7D), and its activity in the NPB was almost eliminated upon shield stage heat shock (Fig. 7F versus 7D).

We overexpressed *bmp2b* in *zic3* E1 and E2 transgenic lines by heat shock at shield stage and 75% epiboly. *zic3* E1 activity was

DEVELOPMENT

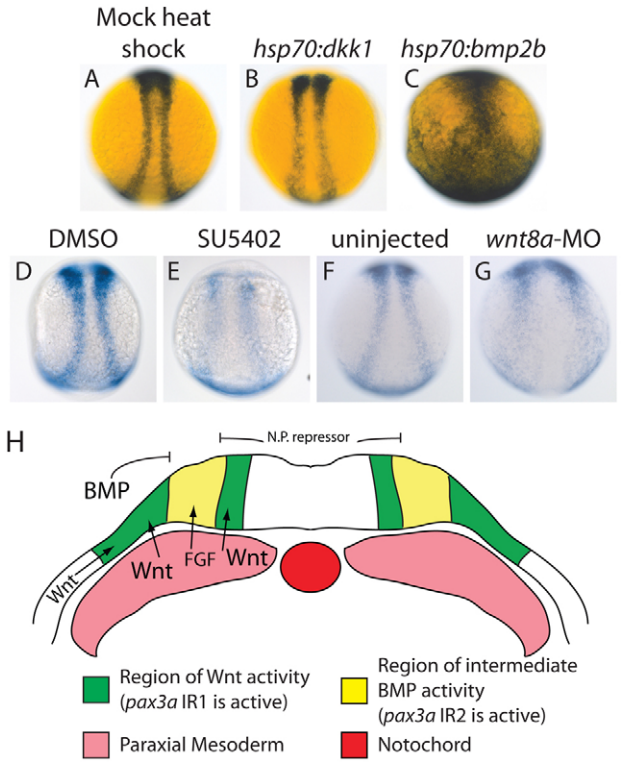

Fig. 5. The combination of *pax3a* IR1 and IR2 is less susceptible to signaling perturbations than either enhancer alone. (**A-G**) GFP expression driven by a *pax3a* IR2+IR1 composite enhancer is shown under various treatments. *dkk1-mCherry* overexpression beginning with a shield stage heat shock mildly decreases enhancer activity (60%, *n*=42) (B versus A). *bmp2b* overexpression beginning with a shield stage heat shock causes increased enhancer activity, a shift medially in the trunk region and expansion in the posterior (87%, *n*=31) (C versus A). SU5402 treatment starting at 7 hpf drastically decreases the activity of the composite enhancer (100%, *n*=14) (E versus D). *wnt8a*-MO injection causes a more severe decrease in activity than *dkk1* overexpression (80%, *n*=10) (G versus F). (**H**) A model for *pax3a* IR1 and IR2 activity. IR1 is activated by Wnt and FGF signaling in a wide band surrounding the NPB (green). IR2 activity is precisely positioned at the NPB by BMP signaling and a repressive neural plate factor and is amplified by FGF signaling (yellow). All pictures are dorsal trunk views with anterior upwards.

eliminated from the posterior NPB with the later heat shock (Fig. 7H versus 7G) and drastically decreased throughout the NPB upon shield stage heatshock (Fig. 7I versus 7G). E1-driven NPB GFP expression shifted medially with *bmp2b* overexpression similar to endogenous *zic3* (Fig. 7G-I, bars). *zic3* E2 activity did not noticeably decrease in *hsp70l:bmp2b* embryos upon 75% epiboly heat shock (Fig. 7K versus 7J), but decreased dramatically with shield stage treatment (Fig. 7L versus 7J). The fact that both enhancers drive BMP-responsive NPB gene expression demonstrates that they are partially functionally redundant, possibly facilitating more robust control of *zic3* expression in the anterior ectoderm.

To determine whether *zic3* E1 and E2 require FGF signaling, we treated transgenic embryos with SU5402 beginning at 65% epiboly. SU5402 treatment decreases *zic3* E1 activity (Fig. 7N versus 7M), while *zic3* E2 drives GFP expression at a similar level with SU5402 treatment (Fig. 7P versus 7O). The sensitivity of *zic3* E1 to SU5402 is surprising given the insensitivity of endogenous *zic3* expression to FGF inhibition. This suggests that other *zic3* enhancers can compensate for decreased E1 activity upon FGF signaling fluctuations, building additional robustness into *zic3* NPB expression. The Wnt/FGF knockdown experiments presented in Fig. 2 demonstrate Wnt signaling can mask the effect of FGF knockdown on *zic3* expression.

pax3a IR1, *zic3* E1 and *zic3* E2 are probably direct targets of canonical Wnt signaling

pax3a IR1 contains six putative high-affinity binding sites for Tcf/Lef transcription factors within its two regions of high sequence conservation (Fig. 8A; supplementary material Fig. S6A). Mutating these sites drastically decreased the activity of the enhancer (Fig. 8D,E versus 8B,C).

zic3 E1 contains three putative Tcf/Lef-binding sites in the most conserved region of the enhancer (Fig. 8F; supplementary material Fig. S6B). No decrease in enhancer activity was observed when we mutated these sites (Fig. 8I,J versus 8G,H).

zic3 E2 contains five putative Tcf/Lef-binding sites in the best conserved region of the enhancer (Fig. 8K; supplementary material Fig. S6C). Mutating these sites greatly decreases enhancer activity in the NPB and dorsal neural tube (Fig. 8N,O versus 8L,M).

To determine whether Tcf/Lef factors can induce *pax3a* IR1, *zic3* E1 or *zic3* E2 activity in the absence of translation, we injected embryos containing *pax3a* IR1:GFP, *zic3* E1:GFP or *zic3* E2:GFP transgenes with mRNA encoding the human Lef1 DNA-binding domain fused to the glucocorticoid receptor and β-catenin. This protein is a constitutively active Lef1 and is inducible by treatment with dexamethasone (Ramel and Lekven, 2004). Treatment of injected embryos with dexamethasone alone results in an increase in the activity of all three enhancers (supplementary material Fig. S7). We treated with cycloheximide beginning at 65% epiboly and added dexamethasone or ethanol 30 minutes later. We fixed the embryos at the two-somite stage and stained for GFP mRNA. In all three cases, GR-Lef1-βcat induction increases GFP expression in the absence of translation (Fig. 8P-BB), strongly suggesting the enhancers are direct targets of canonical Wnt signaling. This signaling is probably mediated through the putative Tcf/Lef-binding sites shown in Fig. 8A,K in the cases of *pax3a* IR1 and *zic3* E2.

DISCUSSION

zic3 and *pax3a* NPB expression is established by intermediate levels of BMP signal and amplified by Wnt and FGF signals during mid to late gastrulation

In frog and chick, neural crest induction requires intermediate levels of BMP signaling coupled with Wnt signals (LaBonne and Bronner-Fraser, 1998; García-Castro et al., 2002; Liem et al., 1995). In frog, an FGF requirement has also been shown (Mayor et al., 1997; Monsoro-Burq et al., 2003), though FGFs may act largely through Wnts to exert their influence (Hong et al., 2008). In both species these signals induce neural crest cells in part by activating NPB specifiers from the Zic and Pax3/7 families (Monsoro-Burq et al., 2003; Bang et al., 1999), which are necessary and sufficient for the expression of neural crest specifiers such as *foxd3* and *soxE* (Sato et al., 2005; Basch et al., 2006; Meulemans and Bronner-Fraser, 2004). BMPs and Wnts have also been shown to be important for neural crest development in zebrafish (Lewis et al., 2004; Dorsky et al., 1998; Nguyen et al., 1998). However, the earlier roles of these signals in zebrafish have not been intensively investigated. We find

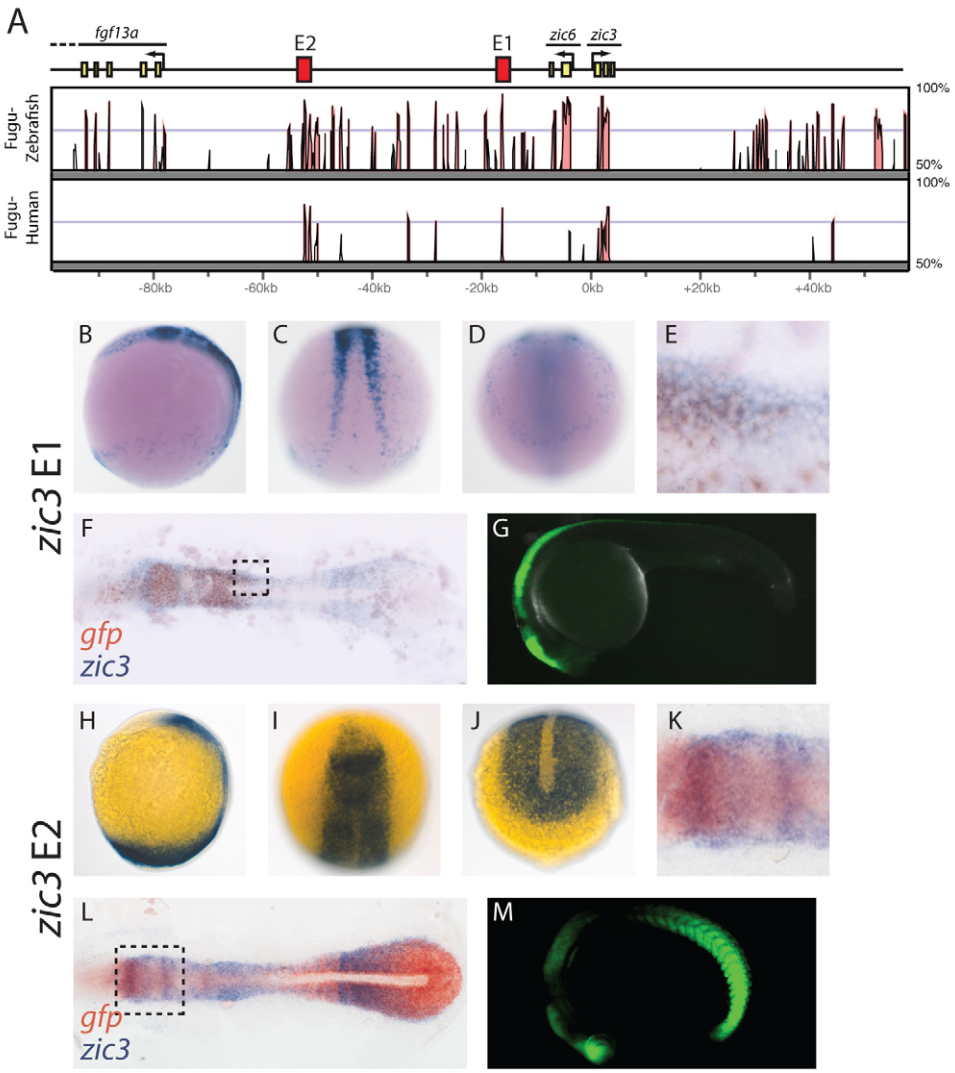

Fig. 6. Two *zic3* enhancers drive gene expression in the NPB and dorsal neural tube. (**A**) The *Fugu zic3/zic6* locus is shown along with a Vista plot of the level of sequence identity between *Fugu,* zebrafish and human *zic3* loci. Enhancers are shown in red and exons are shown in yellow. (**B-M**) GFP expression driven by E1 (B-G) and E2 (H-M) is shown as detected by in situ hybridization for embryos at 12 hpf (B-F,H-L) and by GFP fluorescence at 24 hpf (G,M). Double in situ hybridization for GFP (brown) and *zic3* (purple) demonstrate that *zic3* expression overlaps with the activity of E1 and E2 (E,F,K,L). E,K are higher magnification views of the areas outlined in F,L, respectively. B,H,G,M are lateral views; D,J are posterior dorsal views; C is a trunk dorsal view; I is an anterior dorsal view.

that an intermediate level of BMP signaling is required, starting at mid-gastrulation to position and activate *zic3* and *pax3a* in the NPB (Fig. 1). We further find that Wnt and FGF act additively during this period to achieve maximum *pax3a* expression (Fig. 2). Wnts are necessary for maximum *zic3* NPB expression, with the redundant role of FGFs becoming apparent only when Wnt signaling is attenuated. Thus, in zebrafish, intermediate levels of BMP during gastrulation establish the NPB, with Wnts and FGFs acting in parallel to amplify NPB specifier expression. Interestingly, Wnt inhibition at tailbud stage causes a dramatic reduction in the expression of the neural crest specifiers *foxd3* and *sox10* (Lewis et al., 2004). This probably reflects a later role for Wnts in inducing neural crest specifier expression distinct from its earlier role in establishing the NPB during gastrulation (Monsoro-Burq et al., 2005; Sato et al., 2005). Consistent with this, Wnts directly regulate the neural crest specifier *snail* through Tcf/Lef-binding sites in its promoter (Vallin et al., 2001).

zic3 and *pax3a* use similar cis-regulatory strategies to integrate Wnt, FGF and BMP signals

Our data suggest that *zic3* and *pax3a* use the same signals during the same time window to achieve similar NPB expression. We asked whether these signals are integrated by similar cis-regulatory systems. We isolated enhancers driving *zic3* and *pax3a* expression at the NPB, and tested their individual responsiveness to BMP, Wnt and FGF perturbations (Figs 3-7). We found that *zic3* has two NPB enhancers: one responding to Wnt, FGF and BMP signals, and one responding to Wnt and BMP only (Fig. 9C). *pax3a* also has two NPB enhancers, one that requires both Wnt and FGF signaling and is upregulated by BMPs, and one that requires FGF signaling and is downregulated by BMPs (Fig. 9A). Thus, both *zic3* and *pax3a* have BMP responsiveness encoded in both enhancers and each enhancer is responsive to FGF and/or Wnt signaling.

Although each *pax3a* and *zic3* enhancer recapitulates most aspects of endogenous NPB gene expression (except *zic3* E2, which is not active in the posterior), no single enhancer fully mimics the response of its cognate gene to signaling perturbations. In both cases, endogenous gene expression is less susceptible to perturbations than are the individual enhancers, indicating that gene expression driven by a combination of multiple enhancers imparts robustness. However, our experiments demonstrate that *pax3a* expression is poorly buffered against FGF fluctuations. These results are consistent with recent *Drosophila* studies indicating that multiple 'shadow' enhancers can buffer gene expression against environmental fluctuations (Perry et al., 2010). Our experiments with the *pax3a* IR1-IR2 combination indicate that this compound enhancer increases robustness and specificity over either single enhancer alone even in the absence of additional regulatory DNA (Fig. 5).

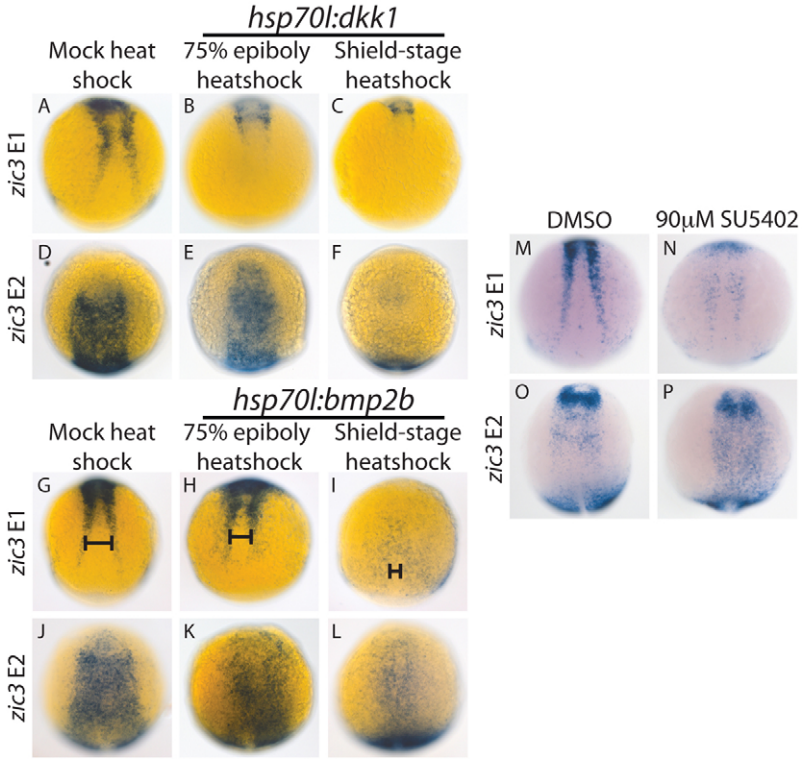

Fig. 7. *zic3* E1 is regulated by Wnt, BMP and FGF signaling, and *zic3* E2 is regulated by Wnt and BMP signaling. (**A-F**) *dkk1* was overexpressed by heat shock in embryos with *zic3* E1:GFP or *zic3* E2:GFP. *dkk1* overexpression beginning with a 75% epiboly heat shock decreases *zic3* E1 activity (100%, *n*=23) (B versus A) and slightly decreases *zic3* E2 activity (88%, *n*=50) (E versus D). The NPB activity of both enhancers is almost completely lost, with *dkk1* overexpression induced with a shield stage heat shock [E1, C versus A (100%, *n*=21); E2, F versus D (100%, *n*=55)]. (**G-L**) *bmp2b* was overexpressed by heat shock. *zic3* E1 loses activity in the posterior NPB and shifts medially in the anterior (H, bar) upon *bmp2b* induction with 75% epiboly heat shock (93%, *n*=29) (H versus G). Shield-stage heat shock causes a dramatic reduction of E1 activity throughout and causes a drastic medial shift (I, bar) (96%, *n*=23) (I versus G). *zic3* E2 activity does not change when *bmp2b* overexpression is induced with a 75% epiboly heat shock (79% with wild-type GFP level, *n*=38) (K versus J), but decreases with a shield-stage heat shock (100%, *n*=24) (L versus J). (**M-P**) SU5402 treatment beginning at 65% epiboly dramatically decreases *zic3* E1 activity (100%, *n*=27) (N versus M), but not *zic3* E2 activity (81% with wild-type GFP level, *n*=22) (P versus O). All E1 pictures are dorsal views of the trunk with anterior upwards; all E2 pictures are anterior dorsal views with anterior upwards.

zic3 expression is buffered against variability by cryptic responsiveness to FGF hardwired into *zic3* E1

zic3 uses FGF and Wnt signals in a partially redundant manner to further buffer its expression (Fig. 2E-H). *zic3* E1 is very sensitive to FGF signaling disruption, whereas SU5402 treatment does not decrease endogenous *zic3* NPB expression levels (Fig. 7). This suggests additional enhancers buffer *zic3* expression against FGF fluctuations. We find that *zic3* expression in the NPB decreases upon SU5402 treatment when Wnt signaling is also attenuated. So if the Wnt pathway is intact, it can compensate for decreased FGF signaling in *zic3* regulation. Thus, *zic3* expression is buffered both by the presence of redundant enhancers and by partially redundant regulation by Wnts and FGFs. NPB induction is a process that is probably susceptible to noise in the form of random signal variation, so it follows that *zic3* expression is well buffered against it.

pax3a achieves sharp, intense neural border expression by synergistic interactions between two enhancers with differential responsiveness to BMP and Wnts

Separate enhancers mediate the Wnt and BMP components of *pax3a* regulation at the NPB (Fig. 4), providing an opportunity to study these inputs independently. *pax3a* IR1, the Wnt-regulated enhancer, is active in a wider band at the NPB than endogenous *pax3a*, suggesting there is a broad zone in the ectoderm where Wnt signaling levels are permissive for *pax3a* expression (Fig. 5H, green region). This zone may correspond to the region of the ectoderm close enough to receive Wnt signals from the paraxial mesoderm. *pax3a* IR2 is active in a narrower band at the NPB than IR1 in a pattern closer to that of the endogenous gene, suggesting IR2 contains precise spatial information for *pax3a* expression (Fig. 5H, yellow zone). *bmp2b* overexpression shifts IR2 activity medially and weakens it, consistent with IR2 being active at intermediate BMP signaling levels. We propose that the decreased IR2 activity is the result of a repressor being present in the neural plate. Interestingly, *pax3a* IR1 activity increases when it expands into the neural plate, suggesting that it is not repressed by neural factors. The increase in IR1 activity may result from increased Wnt ligand expression upon *bmp2b* overexpression (supplementary material Fig. S3).

Our *pax3a* enhancer results indicate that BMP signaling positions NPB gene expression into a precise band within a larger area of the ectoderm that is receiving the proper Wnt signaling level (Fig. 5H). The combination of the *pax3a* IR1 and IR2 enhancers is able to read out this combination of signaling

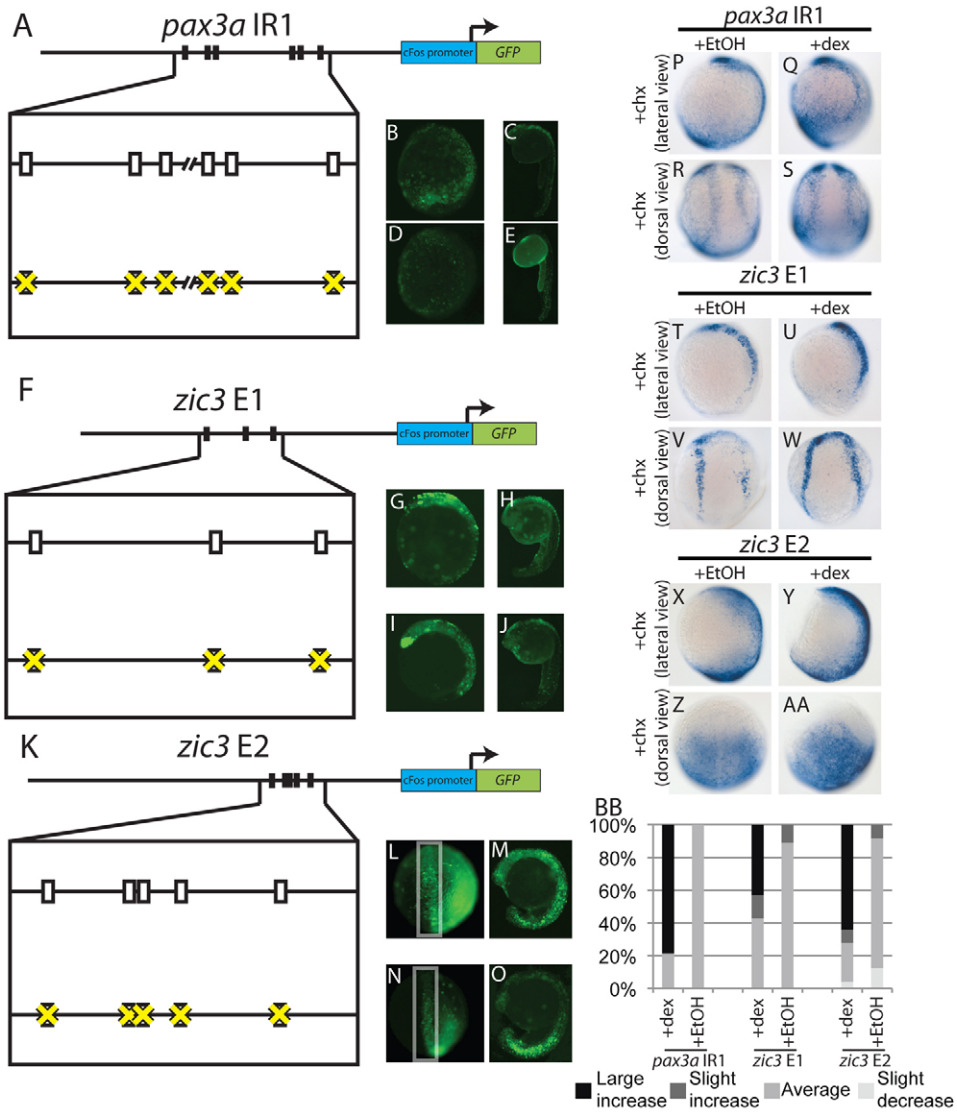

Fig. 8. *pax3a* IR1 and *zic3* E1 and E2 are probably direct targets of canonical Wnt signaling. (**A-E**) Mutating six putative Tcf/Lef-binding sites from *pax3a* IR1 (rectangles in A) decreases enhancer activity at 12 hpf (92%, *n*=36) (D versus B) and 24 hpf (100%, *n*=32) (E versus C). (**F-J**) Mutating three putative Tcf/Lef-binding sites (rectangles, F) does not affect *zic3* E1 activity at 12 hpf (89%, 16 out of 18 with wild-type GFP levels) (I versus G) and 24 hpf (61%, 11 of 18 with wild-type GFP levels) (J versus H). (**K-O**) Mutating five putative Tcf/Lef-binding sites (rectangles, K) reduces *zic3* E2 activity in the NPB at 12 hpf (87%, *n*=20) (N versus L) and the dorsal neural tube at 24 hpf (100%, *n*=40) (O versus M). (**P-AA**) Embryos containing *pax3a* IR1:GFP, *zic3* E1:GFP or *zic3* E2:GFP were injected with GR-Lef1-βcat mRNA. The activity of all three enhancers significantly increases with dexamethasone and cycloheximide treatment relative to ethanol and cycloheximide treatment [*pax3a* IR1, Q,S versus P,R (69%, $P=7\times10^{-7}$, *n*=16); *zic3* E1, U,W versus T,V (57%, *P*=0.007, *n*=14); *zic3* E2, Y,AA versus X,Z (73%, $P=5\times10^{-9}$, *n*=37)]. (**BB**) The distribution of GFP staining levels in embryos injected with GR-Lef1-βcat mRNA and treated with cycloheximide and dexamethasone or ethanol is shown for each enhancer. B-E,G-J,M,O,P,Q,T,U,X,Y are lateral views with dorsal towards the right; L,N,Z,AA are anterior-dorsal views with anterior upwards; R,S,V,W are dorsal trunk views with anterior upwards.

molecules and drive a strong, narrow band of *pax3a* expression in the NPB, although this enhancer combination does not work perfectly outside of its natural genomic context (Fig. 5). This type of enhancer synergy was recently described in *Drosophila* gap gene regulation and those experiments also indicate that genomic context is important for enhancer synergy to work properly (Perry et al., 2011). Gap gene and NPB gene regulation have much in common. Both gene types need to be activated in sharply defined regions within areas of broadly distributed regulatory molecules. Enhancer synergy may be a common strategy used to interpret such broad signal gradients.

Differences in the dominance of evolutionarily conserved enhancers could result in species-specific responses to experimental perturbations

Experiments in *Xenopus* and chick provide conflicting results as to whether FGF signaling specifies the NPB indirectly by acting through Wnt signaling (Liem et al., 1995; Hong et al., 2008; LaBonne and Bronner-Fraser, 1998) or through a Wnt-independent mechanism (Monsoro-Burq et al., 2003; Monsoro-Burq et al., 2005). The fact that *pax3a* IR2 is downstream of FGF signaling, but is insensitive to *dkk1* overexpression, clearly demonstrates that FGF signaling has a Wnt-independent role in zebrafish NPB induction (Fig. 4D-F,O,P).

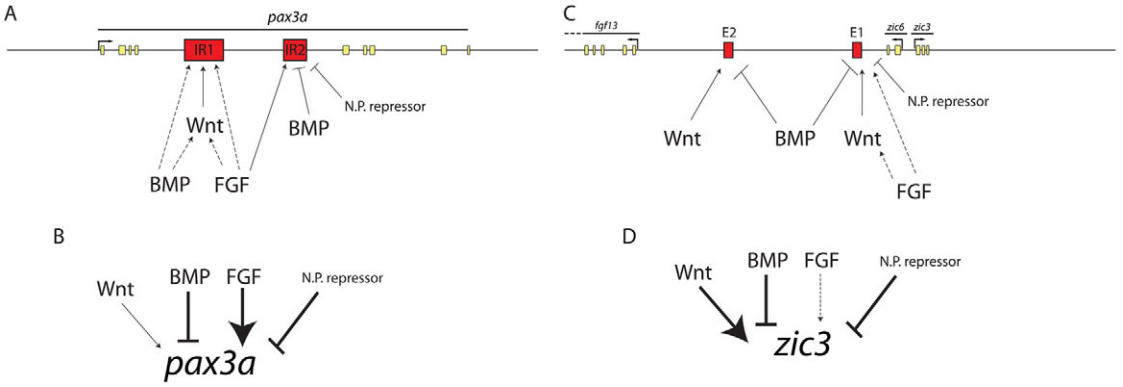

Fig. 9. *pax3a* and *zic3* are regulated by integrating inputs from multiple enhancers and signaling pathways. (**A**) *pax3a* IR1 requires Wnt and FGF signaling for its full activity and is activated by BMP signaling. The effect of Wnt signaling is probably direct. FGF and BMP signaling could be acting directly on IR1 or indirectly through Wnts (broken lines) as Wnt ligands are upregulated upon *bmp2b* overexpression (supplementary material Fig. S3) and Wnts are upregulated upon FGF overexpression in *Xenopus* (Hong et al., 2008). *pax3a* IR2 is repressed by BMP overexpression, requires FGF signaling for full activity and is probably repressed by a neural plate factor (N.P. repressor). (**B**) NPB *pax3a* expression has a strong requirement for FGF signaling and a weaker requirement for Wnt signaling. *pax3a* expression is repressed by BMP overexpression and probably by a repressor in the neural plate. (**C**) *zic3* E1 requires FGF and Wnt signaling for full activity and is repressed by BMPs. FGF could be acting indirectly by inducing expression of Wnt ligands. *zic3* E2 requires Wnt signaling for NPB activity and is repressed by BMP overexpression. (**D**) *zic3* NPB expression has a strong requirement for Wnt signaling and a cryptic requirement for FGF signals that only becomes apparent when Wnt signaling is attenuated. *zic3* in the NPB is repressed by BMP overexpression and probably also repressed by a factor in the neural plate.

Although our findings reveal a Wnt-independent function for FGFs in zebrafish neural border induction, they also suggest how NPB induction could evolve to rely on different combinations BMP, Wnt and FGF signals in divergent vertebrates. Our results reveal that *zic3* and *pax3a* are regulated by evolutionarily conserved enhancers responsive to different combinations of BMP, FGF and Wnt signals. Changes in the relative influence of these enhancers could thus cause *zic3* and *pax3/7* expression to respond differently to Wnt and FGF perturbations in different species. For example, if *pax3a* IR1 was lost, or *pax3a* IR2 somehow became dominant, *pax3a* would appear to be Wnt independent, whereas if *pax3a* IR2 was lost, or pax3a IR1 evolved to be dominant, *pax3a* regulation would be strongly Wnt dependent. Aside from the simple loss of partially redundant enhancers, changes in enhancer dominance could be driven by changes in the strength of transcription factor-mediated enhancer/promoter interactions. For example, one enhancer could potentially 'out-compete' the other by evolving to bind the basal transcriptional apparatus more stably. This mechanism would be similar to how long-range repressor elements 'squelch' transcription by blocking enhancer/promoter associations (Barolo and Levine, 1997; Gray et al., 1994).

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References

Bang, A. G., Papalopulu, N., Goulding, M. D. and Kintner, C. (1999). Expression of Pax-3 in the lateral neural plate is dependent on a Wnt-mediated signal from posterior nonaxial mesoderm. *Dev. Biol.* **212**, 366-380.
Barolo, S. and Levine, M. (1997). hairy mediates dominant repression in the Drosophila embryo. *EMBO J.* **16**, 2883-2891.
Basch, M. L., Bronner-Fraser, M. and García-Castro, M. I. (2006). Specification of the neural crest occurs during gastrulation and requires Pax7. *Nature* **441**, 218-222.
Brand, M., Heisenberg, C. P., Jiang, Y. J., Beuchle, D., Lun, K., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N. et al. (1996). Mutations in zebrafish genes affecting the formation of the boundary between the midbrain and hindbrain. *Development* **123**, 179-190.
Chocron, S., Verhoeven, M. C., Rentzsch, F., Hammerschmidt, M. and Bakkers, J. (2007). Zebrafish Bmp4 regulates left-right asymmetry at two distinct developmental time points. *Dev. Biol.* **305**, 577-588.
Degenhardt, K. R., Milewski, R. C., Padmanabhan, A., Miller, M., Singh, M. K., Lang, D., Engleka, K. A., Wu, M., Li, J., Zhou, D. et al. (2010). Distinct enhancers at the Pax3 locus can function redundantly to regulate neural tube and neural crest expressions. *Dev. Biol.* **339**, 519-527.
Dorsky, R. I., Moon, R. T. and Raible, D. W. (1998). Control of neural crest cell fate by the Wnt signalling pathway. *Nature* **396**, 370-373.
Endo, Y., Osumi, N. and Wakamatsu, Y. (2002). Bimodal functions of Notch-mediated signaling are involved in neural crest formation during avian ectoderm development. *Development* **129**, 863-873.
Fisher, S., Grice, E. A., Vinton, R. M., Bessling, S. L., Urasaki, A., Kawakami, K. and McCallion, A. S. (2006). Evaluating the biological relevance of putative enhancers using Tol2 transposon-mediated transgenesis in zebrafish. *Nat. Protoc.* **1**, 1297-1305.
Frazer, K. A., Pachter, L., Poliakov, A., Rubin, E. M. and Dubchak, I. (2004). VISTA: computational tools for comparative genomics. *Nucleic Acids Res.* **32**, W273-W279.
García-Castro, M. I., Marcelle, C. and Bronner-Fraser, M. (2002). Ectodermal Wnt function as a neural crest inducer. *Science* **297**, 848-851.
Gray, S., Szymanski, P. and Levine, M. (1994). Short-range repression permits multiple enhancers to function autonomously within a complex promoter. *Genes Dev.* **8**, 1829-1838.
Griffin, K. J., Amacher, S. L., Kimmel, C. B. and Kimelman, D. (1998). Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development* **125**, 3379-3388.
Hong, C. S., Park, B. Y. and Saint-Jeannet, J. P. (2008). Fgf8a induces neural crest indirectly through the activation of Wnt8 in the paraxial mesoderm. *Development* **135**, 3903-3910.
Kawakami, K. (2004). Transgenesis and gene trap methods in zebrafish by using the Tol2 transposable element. *Methods Cell Biol.* **77**, 201-222.
Keller, M. J. and Chitnis, A. B. (2007). Insights into the evolutionary history of the vertebrate zic3 locus from a teleost-specific zic6 gene in the zebrafish, Danio rerio. *Dev. Genes Evol.* **217**, 541-547.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253-310.
Kwan, K. M., Fujimoto, E., Grabher, C., Mangum, B. D., Hardy, M. E., Campbell, D. S., Parant, J. M., Yost, H. J., Kanki, J. P. and Chien, C. B. (2007). The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev. Dyn.* **236**, 3088-3099.
LaBonne, C. and Bronner-Fraser, M. (1998). Neural crest induction in Xenopus: evidence for a two-signal model. *Development* **125**, 2403-2414.
Lekven, A. C., Thorpe, C. J., Waxman, J. S. and Moon, R. T. (2001). Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. *Dev. Cell* **1**, 103-114.
Lewis, J. L., Bonner, J., Modrell, M., Ragland, J. W., Moon, R. T., Dorsky, R. I. and Raible, D. W. (2004). Reiterated Wnt signaling during zebrafish neural crest development. *Development* **131**, 1299-1308.
Liem, K. F., Jr, Tremml, G., Roelink, H. and Jessell, T. M. (1995). Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. *Cell* **82**, 969-979.
Marchant, L., Linker, C., Ruiz, P., Guerrero, N. and Mayor, R. (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev. Biol.* **198**, 319-329.
Martin, B. L. and Kimelman, D. (2008). Regulation of canonical Wnt signaling by Brachyury is essential for posterior mesoderm formation. *Dev. Cell* **15**, 121-133.
Mayor, C., Brudno, M., Schwartz, J. R., Poliakov, A., Rubin, E. M., Frazer, K. A., Pachter, L. S. and Dubchak, I. (2000). VISTA: visualizing global DNA sequence alignments of arbitrary length. *Bioinformatics* **16**, 1046-1047.
Mayor, R., Guerrero, N. and Martínez, C. (1997). Role of FGF and noggin in neural crest induction. *Dev. Biol.* **189**, 1-12.
Melby, A. E., Kimelman, D. and Kimmel, C. B. (1997). Spatial regulation of floating head expression in the developing notochord. *Dev. Dyn.* **209**, 156-165.
Meulemans, D. and Bronner-Fraser, M. (2004). Gene-regulatory interactions in neural crest evolution and development. *Dev. Cell* **7**, 291-299.
Milewski, R. C., Chi, N. C., Li, J., Brown, C., Lu, M. M. and Epstein, J. A. (2004). Identification of minimal enhancer elements sufficient for Pax3 expression in neural crest and implication of Tead2 as a regulator of Pax3. *Development* **131**, 829-837.
Monsoro-Burq, A. H., Fletcher, R. B. and Harland, R. M. (2003). Neural crest induction by paraxial mesoderm in Xenopus embryos requires FGF signals. *Development* **130**, 3111-3124.
Monsoro-Burq, A. H., Wang, E. and Harland, R. (2005). Msx1 and Pax3 cooperate to mediate FGF8 and WNT signals during Xenopus neural crest induction. *Dev. Cell* **8**, 167-178.
Nguyen, V. H., Schmid, B., Trout, J., Connors, S. A., Ekker, M. and Mullins, M. C. (1998). Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. *Dev. Biol.* **199**, 93-110.
Nyholm, M. K., Wu, S. F., Dorsky, R. I. and Grinblat, Y. (2007). The zebrafish zic2a-zic5 gene pair acts downstream of canonical Wnt signaling to control cell proliferation in the developing tectum. *Development* **134**, 735-746.
Patthey, C., Edlund, T. and Gunhaga, L. (2009). Wnt-regulated temporal control of BMP exposure directs the choice between neural plate border and epidermal fate. *Development* **136**, 73-83.
Perry, M. W., Boettiger, A. N., Bothma, J. P. and Levine, M. (2010). Shadow enhancers foster robustness of Drosophila gastrulation. *Curr. Biol.* **20**, 1562-1567.
Perry, M. W., Boettiger, A. N. and Levine, M. (2011). Multiple enhancers ensure precision of gap gene-expression patterns in the Drosophila embryo. *Proc. Natl. Acad. Sci. USA* **108**, 13570-13575.
Ramel, M. C. and Lekven, A. C. (2004). Repression of the vertebrate organizer by Wnt8 is mediated by Vent and Vox. *Development* **131**, 3991-4000.
Roehl, H. and Nüsslein-Volhard, C. (2001). Zebrafish pea3 and erm are general targets of FGF8 signaling. *Curr. Biol.* **11**, 503-507.
Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. *Nature* **376**, 333-336.
Sato, T., Sasai, N. and Sasai, Y. (2005). Neural crest determination by co-activation of Pax3 and Zic1 genes in Xenopus ectoderm. *Development* **132**, 2355-2363.
Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-840.
Steventon, B. and Mayor, R. (2012). Early neural crest induction requires an initial inhibition of Wnt signals. *Dev. Biol.* **365**, 196-207.
Steventon, B., Araya, C., Linker, C., Kuriyama, S. and Mayor, R. (2009). Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction. *Development* **136**, 771-779.
Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N. and Moon, R. T. (2007). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development* **134**, 479-489.
Streit, A. and Stern, C. D. (1999). Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity. *Mech. Dev.* **82**, 51-66.
Thisse, B. and Thisse, C. (2004). Fast release clones: A high throughput expression anaylsis. *ZFIN Direct Submission*.
Thisse, C., Thisse, B., Schilling, T. F. and Postlethwait, J. H. (1993). Structure of the zebrafish snail1 gene and its expression in wild-type, spadetail and no tail mutant embryos. *Development* **119**, 1203-1215.
Vallin, J., Thuret, R., Giacomello, E., Faraldo, M. M., Thiery, J. P. and Broders, F. (2001). Cloning and characterization of three Xenopus slug promoters reveal direct regulation by Lef/beta-catenin signaling. *J. Biol. Chem.* **276**, 30350-30358.
Villanueva, S., Glavic, A., Ruiz, P. and Mayor, R. (2002). Posteriorization by FGF, Wnt, and retinoic acid is required for neural crest induction. *Dev. Biol.* **241**, 289-301.
Villefranc, J. A., Amigo, J. and Lawson, N. D. (2007). Gateway compatible vectors for analysis of gene function in the zebrafish. *Dev. Dyn.* **236**, 3077-3087.