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 protocols to drive the expression of Pax3 and Zic genes at the zebrafish neural plate border. This specification involves signals from the Wnt, BMP and FGF pathways. Wnt and FGF ligands are secreted by 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; Garcia-Castro et al., 2002), while the neural plate and 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 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.

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; Garcia-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 level of BMP signaling. Neural crest cells secrete 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 level 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).

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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 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 pathways.
Neural plate border enhancers

F1 fish containing reporter constructs were crossed to fish heterozygous for mCherry under control of the ubiquitously active EF1α promoter. Putative enhancer fragments were amplified from embryos using a Gateway-based technique similar to a previously described method (Fisher et al., 2006). A dual fluorescence vector was created by cloning sequences downstream of GFP. Supplementary material Table S1 shows primer sequences.

**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
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.
Two evolutionarily conserved enhancers drive \textit{pax3a} expression in the NPB

We wanted to elucidate the relationship between the BMP, Wnt and FGF pathways in \textit{pax3a} regulation by determining how they act on individual \textit{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 \textit{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. \textit{pax3a} IR1 contains two regions of high sequence identity between \textit{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). \textit{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 \textit{pax3a} NPB expression (Fig. 3C,E versus Fig. 1A,S), suggesting other elements are needed to refine \textit{pax3a} expression. A double in situ hybridization for GFP and \textit{pax3a} confirms that \textit{pax3a} expression and IR1 activity overlap (Fig. 3F,G). \textit{pax3a} IR2 contains a 142 bp region with 65% sequence identity between \textit{Fugu} and human. IR2 drives gene expression along the NPB during early segmentation (Fig. 3I-N) in a pattern more similar to endogenous \textit{pax3a} expression than IR1 (Fig. 3L versus Fig. 1A,S). A double in situ hybridization for GFP and \textit{pax3a} confirms that \textit{pax3a} expression and IR2 activity overlap (Fig. 3M). Both \textit{pax3a} enhancers drive gene expression in the dorsal neural tube at 24 hpf (Fig. 3H,O). We generated five independent zebrafish lines containing the \textit{pax3a} IR1:GFP transgene and three independent lines containing the \textit{pax3a} IR2:GFP transgene. The GFP expression patterns observed were consistent between lines.

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

\textit{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, \textit{pax3a} IR2 is FGF dependent, but Wnt independent. Assuming that IR2 regulates \textit{pax3a}, this demonstrates that at least some \textit{pax3a} regulation by FGF signaling is not mediated by Wnts. This is in contrast to work in \textit{Xenopus} suggesting FGFs regulate NPB genes mainly by inducing Wnt ligand expression (Hong et al., 2008).

\textit{pax3a} IR2 activity decreases upon \textit{bmp2b} overexpression and the activity shifts medially into a teardrop shape (Fig. 4K,L versus 4J). This is similar to the \textit{bmp2b} response observed for endogenous \textit{pax3a}. \textit{pax3a} IR1 activity also shifts medially in the trunk upon \textit{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 \textit{pax3a} IR2 is. The expression of neural markers also shifts medially upon \textit{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 \textit{pax3a} IR1 activity upon \textit{bmp2b} overexpression could result from \textit{wnt8a} upregulation with increased BMP levels (supplementary material Fig. S3).

The combined activity of two \textit{pax3a} enhancers is less susceptible to perturbation than either enhancer alone

Our results demonstrate that endogenous \textit{pax3a} expression is less susceptible to Wnt and BMP perturbations than the activity of \textit{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 \textit{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 \textit{pax3a} IR2 alone and endogenous \textit{pax3a} (Fig. 5A). When \textit{dkk1} was overexpressed by a shield stage heat shock we observed a slight decrease in GFP expression (Fig. 5B versus 5A). This decrease was
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 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 demonstrates that E1 activity overlaps with zic3 expression (Fig. 6E,F).
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 *dorsal neural tube* and the posterior paraxial mesoderm (Fig. 6M). and NPB (Fig. 6H-L).

Later, this sequence is active in the anterior in the posterior paraxial mesoderm and in the anterior 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 with 75% epiboly heat shock (100%, n=33) (K versus J) and further decreases and shifts medially (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.

*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 18 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. 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
possibly facilitating more robust control of BMP-responsive NPB gene expression. The fact that both heat shock (Fig. 7K versus 7J), but decreased dramatically with SU5402 treatment (Fig. 7P versus 7O). The sensitivity of zic3 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.

**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. 8L 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 (LaBonńe 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 soxE and foxd3 (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
that an intermediate level of BMP signaling is required, starting at mid-gastrulation to position and activate \textit{zic3} and \textit{pax3a} in the NPB (Fig. 1). We further find that Wnt and FGF act additively during this period to achieve maximum \textit{pax3a} expression (Fig. 2). Wnts are necessary for maximum \textit{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 neural crest specifiers \textit{foxd3} and \textit{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 \textit{snail} through Tcf/Lef-binding sites in its promoter (Vallin et al., 2001).

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

Our data suggest that \textit{zic3} and \textit{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 \textit{zic3} and \textit{pax3a} expression at the NPB, and tested their individual responsiveness to BMP, Wnt and FGF perturbations (Figs 3-7). We found that \textit{zic3} has two NPB enhancers: one responding to Wnt, FGF and BMP signals, and one responding to Wnt and BMP only (Fig. 9C). \textit{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 \textit{zic3} and \textit{pax3a} have BMP responsiveness encoded in both enhancers and each enhancer is responsive to FGF and/or Wnt signaling.

Although each \textit{pax3a} and \textit{zic3} enhancer recapitulates most aspects of endogenous NPB gene expression (except \textit{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 \textit{pax3a} expression is poorly buffered against FGF fluctuations. These results are consistent with recent \textit{Drosophila} studies indicating that multiple ‘shadow’ enhancers can buffer gene expression against environmental fluctuations (Perry et al., 2010). Our experiments with the \textit{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).
The expression of zic3 is buffered against variability by cryptic responsiveness to FGF hardwired into zic3. 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). 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. bmp2b overexpression shifts IR2 activity medially and weakens it, consistent with IR2 being active at intermediate BMP signaling levels.

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...
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.

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=7x10−7, n=16); zic3 E1, U,W versus T,V (57%, P=0.007, n=14); zic3 E2, Y,AA versus X,Z (73%, P=5x10−9, 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,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.

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).
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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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.

References
Neural plate border enhancers


