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DYNAMIC EXPRESSION OF NEURAL PLATE BORDER SPECIFERS ESTABLISHES AND MAINTAINS ECTODERMAL DOMAINS IN THE DEVELOPING *XENOPUS* EMBRYO

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ABSTRACT

As gastrulation ends, the newly formed ectoderm of the developing vertebrate embryo is divided into broad domains of neurogenic and non-neurogenic tissues via the well-studied process of neural induction. Interactions between these two domains, along with the long-range inductive signals that establish the main body axes, result in the formation of a lateral neurogenic zone (LNZ) with a mixed developmental fate. The medial region of this domain will give rise to the neural crest, while the lateral and anterior regions will become preplacodal ectoderm. Considerable work has been done to clarify the transcriptional program that specifies these domains, yet the mechanism by which genes expressed in adjacent ectodermal domains pattern the lateral neurogenic zone and its derivatives remains to be elucidated. In this investigation, we use gain-of-function experiments in whole *Xenopus* embryos to examine the influence of five neural plate border genes upon the specification and maintenance of preplacodal ectoderm and neural crest fates. We demonstrate that overexpression of any of a suite of neural plate border specifiers in the lateral neurogenic zone is sufficient to inhibit expression of the preplacodal ectoderm marker *Six1*, and that this activity is attenuated as the embryo progresses through neurulation. We also show that within the lateral neurogenic zone, neural plate border genes of the *Zic* family promote neural crest fate at the expense of preplacodal ectoderm. Together, these results suggest that the dynamic redistribution of neural plate border genes within the lateral neurogenic zone is a critical event in the specification of its two major derivatives. We propose a model in which a small set of 'lateral neurogenic zone specifiers' lies upstream of the neural plate border specifiers, and after induction of this latter gene suite, the former withdraw from the LNZ into the epidermis, permitting specification of the preplacodal ectoderm.

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INTRODUCTION

The dynamic *Xenopus* ectoderm

As gastrulation ends, the newly formed ectoderm of the developing vertebrate embryo is divided into broad domains of neurogenic and non-neurogenic tissues via the well-studied process of neural induction (reviewed in De Robertis and Kuroda, 2004; Wilson and Edlund, 2001). The neurogenic domain, termed the neural plate, will develop into the structures of the central nervous system, while the non-neurogenic domain will give rise to the epidermis (Figure 1A). In the vertebrate model *Xenopus laevis,* interactions between these two ectodermal regions, along with dorsoventral and anteroposterior signaling, will subsequently induce an intermediate domain that has a mixed developmental fate (reviewed in Baker and Bronner-Fraser, 2001; Meulemans and Bronner-Fraser, 2004). This intermediate domain has previously been referred to as the 'neural plate border,' but recent evidence suggests that the neural plate border is in fact a derivative of a broader 'lateral neurogenic zone' (Figure 1B) that is competent to give rise to both neurogenic and non-neurogenic cell populations (Moody, 2007). The medial region of the lateral neurogenic zone will give rise to the aforementioned neural plate border, which subsequently induces neural crest, a precursor of the peripheral nervous system and much of the cranial skeleton. The more lateral region, as well as the ectoderm immediately anterior to the neural plate, will give rise to the characteristically "horseshoe-shaped" preplacodal ectoderm (Figure 1C), which will then coalesce into discrete cranial sensory placodes (Figure 1D). These cranial placodes later differentiate into both structural and neural elements of the cranial sensory organs, including the olfactory epithelium, lens, auditory/vestibular organs, lateral line, and cranial sensory ganglia (reviewed in Schlosser and Northcutt, 2000; Schlosser 2006).

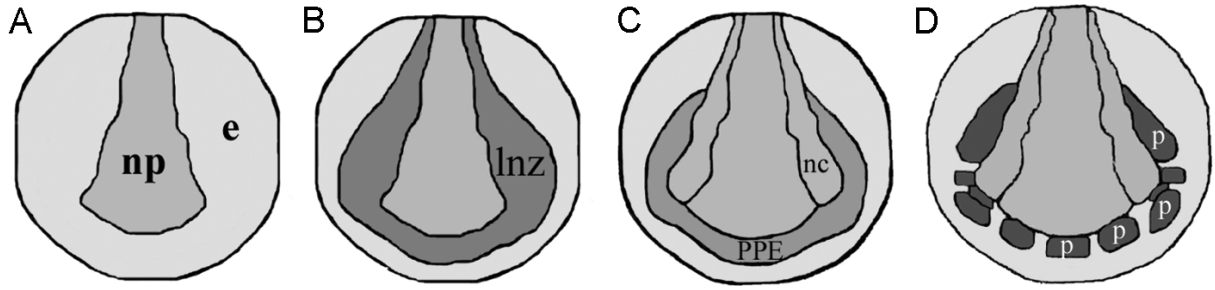

Figure 1. Early differentiation of the *Xenopus* ectoderm; embryos are viewed from the anterior, with dorsal above and ventral below. (A) The neural plate (NP) is induced in a strip of dorsal ectoderm along the midline, while the ventral ectoderm takes on an epidermal (E) fate. (B) The lateral neurogenic zone (LNZ) arises between the NP and E. (C) The LNZ is subdivided into medial neural crest (NC) and more lateral preplacodal ectoderm (PPE). (D) The PPE breaks up into individual placodes (p) with distinct developmental fates (from Moody, 2007).

In the early stages of *Xenopus* neurulation (see Figures 1A through 1C), the transient domains of the ectoderm are anatomically indistinguishable from one another; however, the dynamic expression of distinctive sets transcription factors in each domain specifies their developmental fates before cell and tissue morphologies have changed. The collective activities of such molecular markers constitute a coordinated transcriptional program that acts to both establish and maintain the borders between the ectodermal domains of the neurulating embryo. The bulk of the research in this area has focused on the events that shape the neural plate and neural crest, but recent identification of markers specific to the preplacodal ectoderm (Brugmann *et al.*, 2004; David *et al.,* 2001) has allowed more comprehensive investigations into the mechanisms that control the specification of ectodermal domains. Though this investigation primarily

examines events within the lateral neurogenic zone, our results support and expand a broad model of ectodermal patterning that has been emerging for decades.

Molecular specification of ectodermal domains

Primary neural induction

The complex molecular program that patterns the vertebrate ectoderm is largely initiated by dorsally localized antagonism of BMP signal transduction. In the ventral ectoderm, where BMP signaling remains active, binding of the ligand to a BMP receptor initiates a signal cascade that promotes expression of epidermal genes (Suzuki *et al*., 1997; Feledy *et al*., 1999; Beanan and Sargent, 2000; Luo *et al*., 2001a; Tribulo *et al*., 2003). Conversely, in the dorsal ectoderm this cascade is blocked by secreted BMP antagonists (namely Noggin, Chordin, Cerberus, and Follistatin) in cooperation various FGF and Wnt signals (reviewed in De Robertis and Kuroda, 2004). The absence of BMP signaling permits the dorsal ectoderm to follow a default neural fate, inducing expression of neural plate-associated markers like *Sox2* and *Zic* family genes that subsequently initiate the neurogenic program (Mizuseki *et al.,* 1998; Nakata *et al*., 1997).

The neural plate border

Preliminary models of neural induction interpreted this primary event as a binary developmental decision between neural and non-neural fate. However, there is now considerable evidence for the simultaneous specification of an intermediate region or regions via the same inductive signals, in combination with planar intraectodermal interactions between presumptive neural plate and epidermis. Early fate mapping analyses in *Xenopus* observed a domain between the neural plate and epidermis wherein neural and non-neural fates overlap (Keller, 1975; 1976), a phenomenon that was later confirmed in preliminary gene expression experiments. These investigations determined that, while some neural markers become confined to the neural plate at the exclusion of epidermal genes, others co-express with non-neural markers to a broad band of intermediate ectoderm that was at the time termed the 'neural plate border' (reviewed in Baker and Bronner-Fraser, 2001). This domain is characterized by the expression of 'neural plate border specifying' genes that code for several of the Dlx, Msx, Pax, and Zic family transcription factors. Though the exact expression patterns of these genes do differ from one another, especially later in neurulation, they appear to have a common developmental function: the induction of neural crest fate via mediation of BMP and Wnt signals (reviewed in Meulemans and Bronner-Fraser, 2004).

This activity of the neural plate border has been well documented, especially in the frog model (Figure 2). *Pax3* and *Msx1* have been placed downstream of Wnt signaling (Bang *et al*., 1997), and *Zic1, Zic3, Dlx5*, and *Msx1* become upregulated in response to an attenuated BMP signal (Aruga *et al*., 2002; Luo *et al*., 2001b; Nakata *et al*., 1997; Tribulo *et al.,* 2003). In *Xenopus* embryos and ectodermal explants, *Zic* family genes are sufficient to induce expression of the neural crest markers *Snail, Slug*, *FoxD3*, and *Twist* (Brewster, *et al*., 1998; Mizuseki et al., 1998; Nakata et al., 2000, 1997; Sasai et al., 2001). Msx1 is both necessary and sufficient for the expression of *Snail, Slug*, and *FoxD3* (Tribulo, *et al.,* 2003), and while Dlx5 behaves much like Msx1 in establishing the border zone, misexpression of the *Dlx5* gene has been observed to repress, rather than induce, a neural crest fate (Luo *et al*., 2001b; McLarren *et al*., 2003; Woda *et al*., 2003). Additionally, the expression pattern of *Dlx5* diverges from the other neural plate border specifiers as neurulation progresses (Matsuo-Takasaki *et al*., 2005). Thus, although *Dlx5*

has been placed upstream of *Msx1* as an early neural plate border specifier (Meulemans and Bronner-Fraser, 2004; Woda *et al.*, 2003), its distinct behavior may require the division of this gene suite into two distinct subgroups, or perhaps a revision of the definition of a neural plate border specifying gene itself.

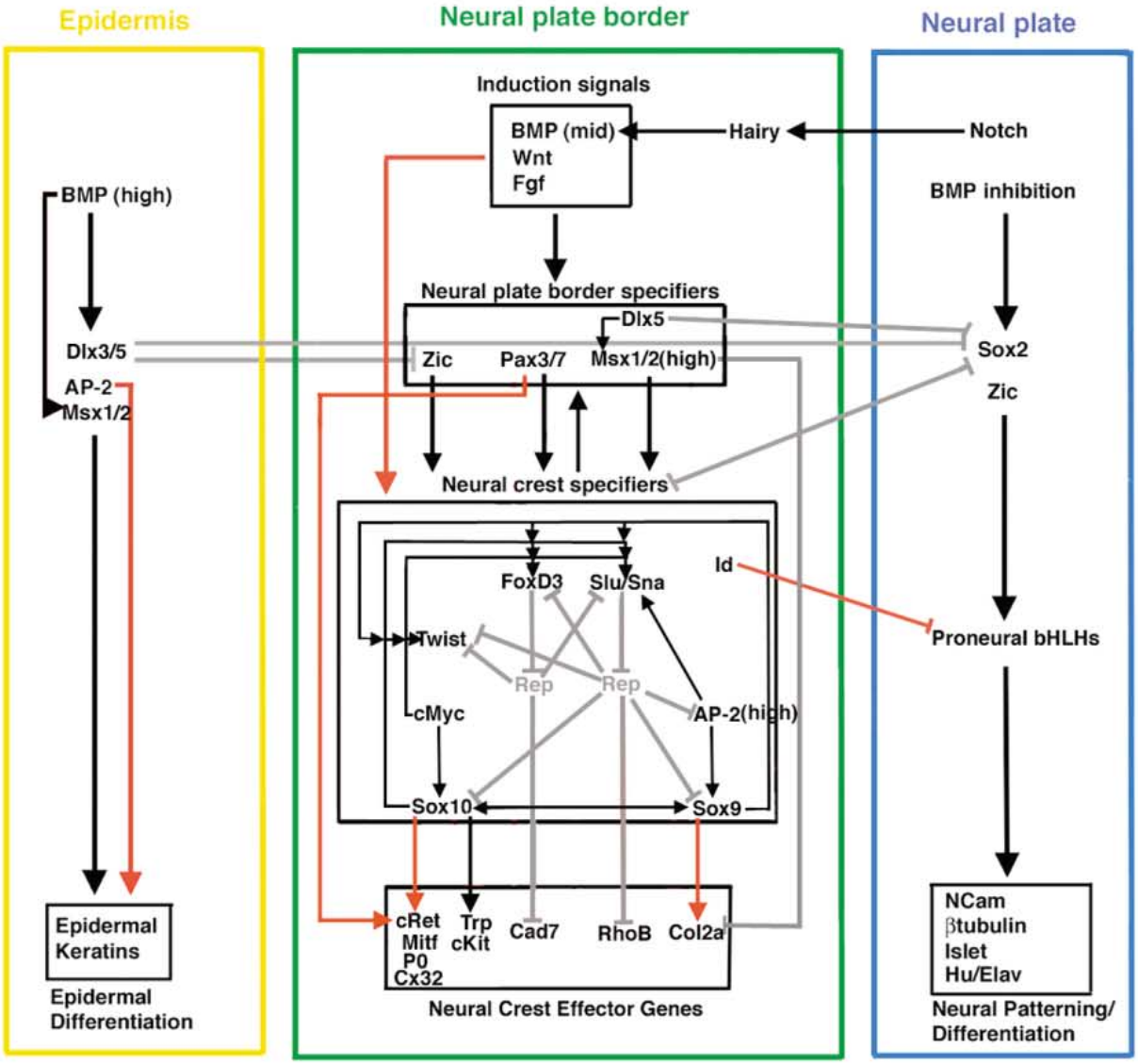

Figure 2. A putative vertebrate neural plate border gene network with a focus on neural crest specification. **Red** arrows indicate proven direct regulatory interactions. **Black** arrows are genetic interactions suggested by gain- and loss-of-function analyses primarily in *Xenopus*. **Gray** lines indicate repression (from Meulemans and Bronner-Fraser, 2004).

Evidence for a lateral neurogenic zone

Like the neural crest, the preplacodal ectoderm is patterned in part by locally moderated inductive signaling of BMP, Wnt, and FGF ligands. Nevertheless, while attenuation of the BMP signal is required to induce expression of preplacodal marker genes, misexpression of BMP antagonists is not sufficient to specify ectopic preplacodal ectoderm *in vivo* (Brugmann *et al.*, 2004; Glavic *et al.*, 2004; Ahrens and Schlosser, 2005). A considerable body of literature has

shown that neural plate-derived signals are required for the induction of individual cranial placodes (reviewed in Baker and Bronner-Fraser, 2001), yet relatively little work has been done to elucidate the role of local intraectodermal signals in the specification of the panplacodal fate. A few recent studies, however, have begun to delve into this matter, and demonstrate that neural plate tissue grafted into non-neural ectoderm induces both neural crest and preplacodal ectoderm at the graft boundary (Woda *et al*., 2003; Glavic *et al*., 2004; Ahrens and Schlosser, 2005). This result suggests the existence of an even broader intervening precursor zone, designated here as the 'lateral neurogenic zone' that spans both the neural plate border (from which the neural crest is derived) and the flanking non-neural ectoderm destined to become the preplacodal ectoderm.

Though there is some debate as to whether this local neural/non-neural signaling produces a true lateral neurogenic zone that is initially competent to give rise to both neural crest and preplacodal ectoderm, several recent genetic studies support this model. The forkhead-family transcription factor Foxi1 is of particular interest; it is first expressed in the anterior ventral ectoderm of the gastrula, and later in the putative lateral neurogenic zone surrounding the anterior neural plate before withdrawing into the epidermis proper (Matsuo-Takasaki *et al*., 2005). Like other epidermal genes, *Foxi1* is induced by BMP signaling, and antisense morpholino (MO) knockdown in whole embryos expands the neural plate. However, these MO knockdown experiments of *Foxi1* expression had another, more interesting effect: the loss of neural crest and preplacodal fates, suggesting that expression of *Foxi1* across the neural/non-neural boundary is required for the specification of both these domains. As mentioned previously, the *Dlx* family genes, homologs of *Drosophila* distal-less, appear to have a related function. *Dlx3*, *Dlx5*, and *Dlx6*, like *Foxi1*, are initially expressed throughout the non-neural ectoderm that abuts the neural plate, and are induced by BMP signaling (Luo *et al*, 2001a; 2001b; Woda *et al*., 2003). Early expression of *Dlx* genes represses neural fate and is required for the specification of both derivatives of the putative lateral neurogenic zone (Feledy *et al*., 1999; Beanan and Sargent, 2000; Luo *et al.,* 2001a; Woda *et al*., 2003). These results strongly suggest the transient presence of a lateral neurogenic zone that, in addition to its early function in placing the neural/non-neural border, is a shared precursor of the neural crest and preplacodal ectoderm.

Maintenance of domain boundaries

The existence of a lateral neurogenic zone that is competent to give rise to two distinct derivatives, however, begs an important question: once established, how is this domain subdivided into neural crest and preplacodal ectoderm? The dynamic expression patterns of the neural plate border genes suggest an answer. Much like *Foxi1*, *Dlx5* gradually withdraws from the lateral neurogenic zone and becomes localized to the flanking non-neural ectoderm just outside of this domain (Luo *et al*., 2001b; Matsuo-Takasaki *et al*., 2005). At the same time, the other neural plate border specifiers (*Msx, Pax, Zic*) become restricted to the medial portion of the lateral neurogenic zone, forming the definitive neural plate border (Aruga *et al*., 2002; Bang *et al.,* 1997; Mizuseki *et al*, 1998; Nakata *et al*., 2007; Suzuki *et al*., 1997). As mentioned previously, these neural plate border specifiers have been implicated in the placement of the neural/non-neural border and the induction of neural crest (Meulemans and Bronner-Fraser, 2004). It has been proposed, however, that these neural plate border specifiers also act to maintain and refine the boundaries between the various ectodermal domains via local, planar transcriptional interactions (Brugmann *et al*., 2004; Moody, 2007).

As of yet, only a few disparate studies have examined this broader maintenance function, and many preliminary results appear to contradict one another. Experiments in support of the lateral neurogenic zone model, as previously discussed, show that genes of the *Dlx* family are required for expression of both neural crest and preplacodal ectoderm markers. Somewhat counterintuitively, *in vivo* overexpression of *Dlx* genes in the presumptive lateral neurogenic zone, either singly (Brugmann *et al*., 2004) or together (Woda *et al*., 2003), reduced the size of the preplacodal ectoderm, suggesting that the activities of this gene family differ based on the temporal and/or spatial environment. Overexpression of *Zic2* had a similar inhibitory effect on the expression of preplacodal ectoderm genes, and was itself (along with *Dlx5* and *Dlx6*) repressed by Six1, the primary preplacodal ectoderm marker (Brugmann *et al*., 2004). This type of mutually repressive interaction between neighboring ectodermal regions is observed for a few genes in epidermis, neural plate, and neural crest as well (Brugmann *et al*., 2004; Matsuo-Takasaki *et al*., 2005), and may constitute a fundamental mechanism whereby broad, overlapping bands of gene expression resolve into discrete functional domains.

Nevertheless, the mechanism by which genes expressed in adjacent ectodermal domains pattern the lateral neurogenic zone and its derivatives remains to be elucidated. In this investigation, we use gain-of-function experiments in whole *Xenopus* embryos to examine the influence of five neural plate border genes upon the specification and maintenance of preplacodal ectoderm and neural crest fates. We demonstrate that overexpression of any of a suite of neural plate border specifiers in the lateral neurogenic zone is sufficient to inhibit expression of the preplacodal ectoderm marker *Six1*, and that this activity is attenuated as the embryo progresses through neurulation. We also show that within the lateral neurogenic zone, neural plate border genes of the *Zic* family promote neural crest fate at the expense of preplacodal ectoderm. Together, these results suggest that the bidirectional withdrawal of neural plate border genes from the lateral neurogenic zone is a critical event in the specification of its two major derivatives. We propose a model in which a small set of 'lateral neurogenic zone specifiers' lies upstream of the neural plate border specifiers, and after induction of this latter gene suite, the former withdraw from the LNZ into the epidermis, permitting specification of the preplacodal ectoderm.

MATERIALS AND METHODS

Expression transcripts

Capped and polyadenylated mRNAs were synthesized by in vitro transcription (Ambion mMessage mMachine Kit) from the following linearized plasmids: pCTSDlx5 (Luo et al., 2001), pCS2^{+}Msx1 (Suzuki et al., 1997), pCS2^{+}ZicR1 (Nakata et al., 1998), pCS2^{+}Myc-Zic2 (Nakata et al., 1998), and pCS2^{+}Zic3 (Nakata et al., 1997). The *Dlx5* and *Msx1* mRNAs were transcribed with Ambion T7 polymerase; Ambion SP6 polymerases were used for all *Zic* family transcripts.

RNA microinjection

Fertilized *Xenopus laevis* eggs were obtained by gonadotropin-induced natural matings of adult frogs and prepared for injection as described (Moody, 2000). The *Dlx5* (200 pg; Luo *et al.*, 2001b), *Msx1* (120 pg; Suzuki *et al*., 1997), *Zic1* (200 pg; Nakata et al., 1998), *Zic2* (100 pg; Nakata et al., 1998), and *Zic3* (100 pg; Nakata et al., 1997) transcripts were each mixed with a nucleus-localized β-galactosidase (nβgal) lineage tracer mRNA (100 pg) and microinjected into the V1.2 blastomere at the 16-cell stage (Moody, 1987). Injected embryos were then allowed to develop until early, mid-, or late neurula stages.

Whole-mount *in situ* hybridization

Embryos were staged according to Nieuwkoop and Faber (1967), and fixed and processed for whole mount *in situ* hybridization according to standard protocols (Sive et al., 2000) using a digoxigenin-labeled *Six1* mRNA probe (Pandur et al., 2000) or *FoxD3* mRNA probe (Sasai et al., 2001). Embryos were analyzed for whether the *Six1* or *FoxD3* expression domains were expanded or decreased in size compared to the uninjected side of the same embryo. For each transcript/probe combination at each stage (here referred to as a 'bin'), the "percent change" was calculated as the number of embryos displaying expansion or repression of the probed expression domain, divided by the total number (n) of embryos in the bin.

RESULTS

Neural plate border specifiers inhibit *Six1* expression

Since relatively little work has been done upon the interaction between the neural plate border (NPB) and the preplacodal ectoderm (PPE) following the initial studies identifying the genetic markers of these domains, we first sought to determine whether the NPB specifiers function to establish and/or maintain the boundaries of the PPE. We individually overexpressed five of these NPB specifiers in the lateral neurogenic zone (LNZ), thereby expanding the NPB zone into the normally non-neural tissues of the presumptive PPE. Each embryo was injected with an expression cocktail of NPB specifier mRNA and nβgal mRNA as a lineage tracer; to target overexpression to the LNZ on one side of the embryo, the cocktail was injected into a single LNZ precursor blastomere at the 16-cell stage. Then, using *Six1* expression as a marker for the PPE, we examined the effect of the expanded NPB zone upon the PPE domain at three stages of Xenopus development: late gastrula/early neurula, mid-neurula, and late neurula. All five NPB specifiers (*Dlx5, Msx1, Zic1, Zic2, Zic3*) significantly reduced *Six1* expression on the injected side of the embryo (Figure 3A); *Six1* expression on the uninjected (control) side remained unchanged. Embryos injected with nβgal alone served as a secondary control, and showed no change in *Six1* expression on either side (Figure 3B). The robust repression of *Six1* expression by all five NPB specifiers suggests that the withdrawal of these genes from the lateral half of the LNZ is required for the specification of PPE fate.

With the exception of *Zic3*, each injected mRNA transcript had a similarly repressive activity at each developmental stage bracket, exhibiting a generally downward trend in regulatory potency over time (Figure 3C). At the late gastrula/early neurula stage (St. 13-14), repression of *Six1* expression was at its highest for *Dlx5* (93.6%, n = 27), *Msx1* (93.8%, n = 32), *Zic1* (90.9%, n = 44), and *Zic2* (90.0%, n = 10) transcripts. By mid-neurula (St. 15-17), repression of *Six1* had decreased for each of these NPB mRNAs: *Dlx5* (80.5%, n = 41), *Msx1* (76.1%, n = 109), *Zic1* (76.4%, n = 55), and *Zic2* (79.6%, n = 49). At the late neurula stage (St. 18-19), repression of *Six1* expression was at its lowest for *Dlx5* (70.5%, n = 44), *Zic1* (69.7%, n = 33), and *Zic2* (69.5%, n = 59). Repression by *Msx1* (76.3%, n = 38) remained essentially the same as in mid-neurula stage. As mentioned, the behavior of the *Zic3* mRNA transcript did not follow this general trend; at early neurula, repression of *Six1* was at its lowest (85.2%, n = 27), increasing at mid-neurula to its highest (93.2%, n = 74), and finally, by late neurula, decreasing only slightly (91.3%, n = 23). Nevertheless, this general attenuation of repressive activity reveals the temporal sensitivity of transcriptional regulation within the developing *Xenopus* ectoderm, and may suggest molecularly distinct mechanisms for the induction and subsequent maintenance of the PPE domain.

Zic family transcription factors expand *FoxD3* expression

As already mentioned, previous studies in *Xenopus* suggest that certain neural plate border specifiers are required for neural crest specification (Brugmann *et al.*, 2004; Meulemans and Bronner-Fraser, 2004; Monsoro-Burq *et al.*, 2005). Since the lateral neurogenic zone is competent to give rise to both neural crest and PPE (Moody, 2007), overexpression of NPB specifiers should expand the neural crest at the expense of the PPE. We tested this premise with the same suite of five NPB specifiers as in the previous experiment, individually overexpressing each in the lateral neurogenic zone via an identical mRNA microinjection

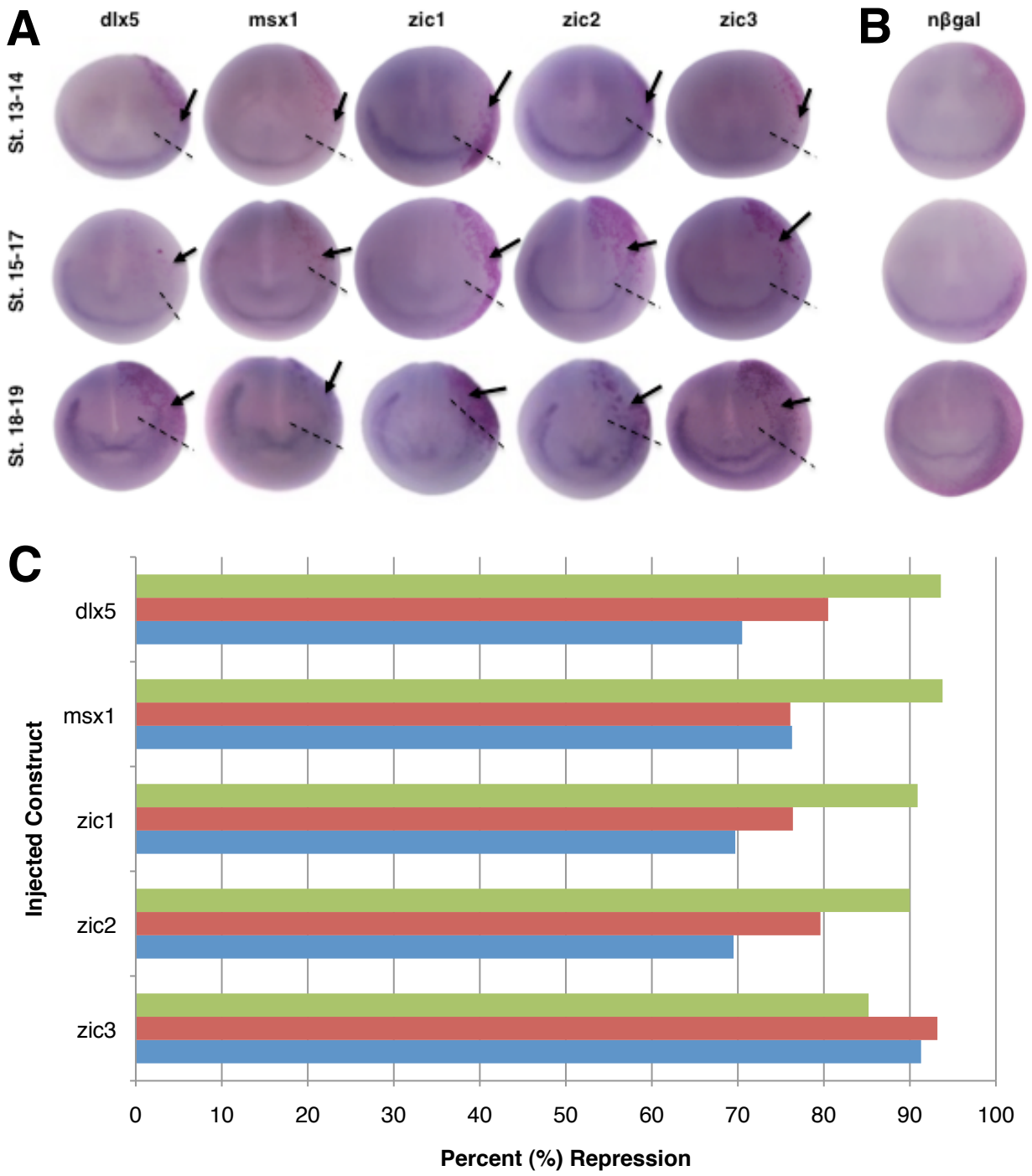

Figure 3. *Six1* is inhibited by overexpression of neural plate border (NPB) genes. (A) Embryos injected with NPB specifier mRNA (*Dlx5, Msx1, Zic1, Zic2, Zic3*) exhibit strongly repressed *Six1* expression on the injected (right) side. Dashed lines mark the furthest limit of the repressed *Six1* expression domain, and arrows indicate the position of the domain of the control side. (B) Control embryos injected with nβ-galactosidase (magenta stain) alone show symmetrical *Six1* expression. (C) Repression of *Six1* by most NPB specifiers is strongest at early neurula stages, and decreases in potency as neurulation proceeds; repression by *Zic3* is the only significant exception to this trend. **Green** bars represent data from early neurula (St. 13-14) bin, **red** from mid-neurula (St. 15-17), and **blue** from late neurula (St. 18-19).

method. Using *FoxD3* expression as a marker for the neural crest, we examined the effect of the expanded NPB zone upon the neural crest domain at mid-neurula (stages 15-17), when *FoxD3* expression is most robust. Interestingly, though expansion of the *FoxD3* expression domain was observed for each injected mRNA (Figure 4A), repression of this domain was also observed in a significant proportion of injected embryos, resulting in a nearly binary distribution of contradictory responses (Figure 4B).

This incongruous behavior was most pronounced for the *Dlx5* and *Msx1* transcripts (Figure 4B); repression of the *FoxD3* domain by *Dlx5* (58.8%, n = 34) and *Msx1* (59.6%, n = 52) far outweighed the incidence of domain expansion (29.4% and 40.4%, respectively). It is important to note that this result is expected for *Dlx5*, which has been previously observed to inhibit, rather than promote, neural crest fate (Luo *et al.*, 2001b; McLarren *et al.*, 2003; Woda *et al.*, 2003). Thus, we confirm that *Dlx5* has a unique regulatory activity within the neural crest, consistent with a model in which *Dlx5*, possibly along with *Foxi1*, comprises a set of genes distinct from the other established NPB specifiers. Repression of the *FoxD3* domain by *Msx1* still requires explanation, since previous work has established this gene as an inducer of neural crest (Tribulo, *et al.*, 2003), and it appears to localize to the presumptive neural crest along with the other NPB specifiers, as opposed to the flanking non-neural ectoderm.

Alternatively, all transcripts of the *Zic* gene family exhibited considerable bias towards expansion, despite the occurrence of some repression (Figure 4B). Additionally, *FoxD3* domain expansion by *Zic1* (69.1%, n = 55), *Zic2* (78.8%, n = 33), and *Zic3* (68.8%, n = 32) was more robust, with larger expanded domains than those by *Dlx5* or *Msx1* (data not shown). These findings confirm previous work implicating *Zic2* in the induction of neural crest, and constitute the first evidence that the other *Zic* family genes *Zic1* and *Zic3* also act as canonical neural plate border specifiers within the LNZ.

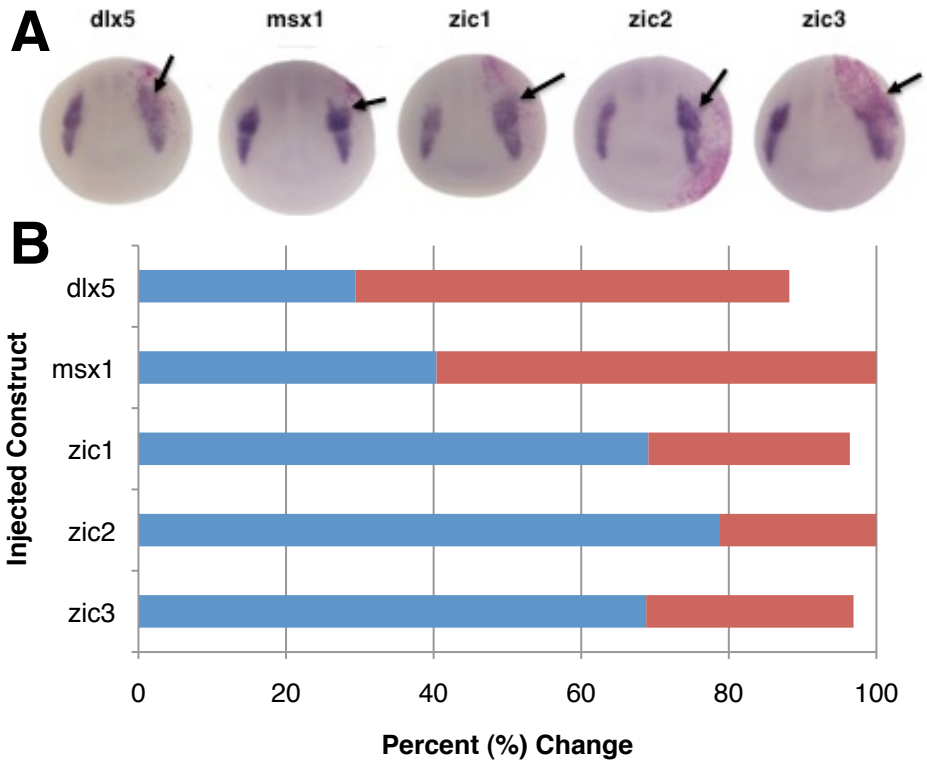

Figure 4. *FoxD3* is expanded by overexpression of *Zic* family NPB genes. (A) Embryos injected with NPB specifier mRNA (*Dlx5, Msx1, Zic1, Zic2, Zic3*) exhibited expansion of FoxD3 expression on the injected (right) side. Arrows indicate expanded domains. (B) All injected mRNA transcripts produced mixed expansion (**blue**) and repression (**red**) of the *FoxD3* domain. Only *Zic* family genes expanded *FoxD3* expression for the majority of embryos; overexpression of *Dlx5* and *Msx1* tended to repress *FoxD3*, though this was expected for *Dlx5*.

DISCUSSION

PPE specification requires withdrawal of NPB specifiers from the LNZ

Though the activities of long-range inductive signals such as BMP, FGF, and Wnt are required for the induction of intermediate ectodermal domains (namely, the neural crest and the preplacodal ectoderm), these molecules, either alone or in combination, are not sufficient to pattern the living embryo. A growing body of research suggests that a complex program of local transcriptional interactions between neighboring regions is essential for the specification and maintenance of ectodermal borders, yet the mechanisms that drive this program remain largely unknown.

NPB specifiers withdraw bidirectionally to specify PPE borders

Here we demonstrate that overexpression of neural plate border (NPB) specifiers in the lateral neurogenic zone (LNZ) is sufficient to inhibit expression of the preplacodal ectoderm (PPE) marker *Six1*, and that this activity is attenuated as the embryo progresses through neurulation. Though seemingly in conflict with previous studies showing that *Dlx* family genes are in fact required for expression of preplacodal markers (Feledy *et al.*, 1999; Beanan and Sargent, 2000; Luo *et al.*, 2001a; Woda *et al.*, 2003), our findings instead illuminate the importance of temporospatial sensitivity of gene function in the *Xenopus* ectoderm. The aforementioned studies of the *Dlx* family were concerned with gene expression during gastrulation stages, and often relied upon animal cap explant experiments that reduce the complex signaling environment of the living embryo into a molecularly homogenous tissue. Thus, the observed repression of preplacodal fate by *Dlx5* during neurulation suggests that, while early, broad expression of *Dlx5* is indeed required to establish the lateral neurogenic zone, withdrawal of this same gene away from the presumptive PPE into the epidermis permits expression of *Six1* and the initiation of a preplacodal fate. This behavior is nearly identical to that of *Foxi1*: early expression of *Foxi1* is required for specification of PPE, but overexpression of *Foxi1* reduces the size of the *Six1* domain *in vivo* (Matsuo-Takasaki *et al.*, 2005). As such, we suggest that *Dlx5* and *Foxi1* be classified as separate from the canonical neural plate border specifiers of the *Msx* and *Zic* families.

The later expression patterns of the these canonical NPB specifiers (*Msx1, Zic1, Zic2, Zic3*) are quite distinct from that of *Dlx5*; after establishing the neural/non-neural boundary, these genes become restricted to the medial half of the LNZ, rather than the flanking non-neural ectoderm (Aruga *et al.*, 2002; Bang *et al.,* 1997; Mizuseki *et al*, 1998; Nakata *et al.*, 2007; Suzuki *et al.*, 1997). Nevertheless, in this investigation we observe that any gene of the tested NPB suite, including *Dlx5*, acts to inhibit PPE fate. This shared transcriptional regulatory activity suggests an interesting mechanism for the specification of PPE: *Six1* expression, which initiates the preplacodal program, requires the bidirectional withdrawal of NPB specifiers away from the lateral part of the LNZ. The canonical NPB specifiers move medially to establish the true neural plate border region, and in the process delimit the medial border of the PPE. Conversely, the non-canonical NPB specifiers (*Dlx5* and *Foxi1*) move laterally into the presumptive epidermis, defining the lateral border of the PPE. Thus, only between these two borders is the ectoderm competent to express *Six1* and take on a preplacodal fate. It should be noted, however, that this cannot be the sole mechanism at work: the PPE is constrained to the anterior cranial ectoderm, indicating the importance of anteroposterior signaling in positioning this domain.

The border maintenance function of NPB specifiers remains unclear

Robust repression of *Six1* expression by *Dlx5, Msx1, Zic1*, and *Zic2* at the early neurula stage, followed by a shared downward trend in regulative potency, supports a model in which most neural plate border specifiers play a major role in the specification, rather than maintenance, of preplacodal ectoderm borders. However, *Six1* has been suggested to positively regulate its own expression (Brugmann *et al*., 2004), so this decrease in repressive capacity may simply be due to the increase in levels of *Six1* expression that occur as neurulation progresses. Distinguishing between these two possibilities would demand modification of the endogenous activating function of Six1. It has been established that Six1 requires a cofactor Eya1 to act as an effective activator of transcription (Silver *et al*., 2003; Brugmann *et al*., 2004); knockdown of *Eya1* in combination with the gain-of-function experiments featured in this investigation should shed light on this question.

Canonical NPB specifiers promote neural crest over preplacodal fate

Our examination of *FoxD3* expression in response to overexpression of NPB specifiers has, for the most part, confirmed a large body of previous work that places these genes upstream of neural crest markers (Meulemans and Bronner-Fraser, 2004). We show that Zic family transcription factors promote neural crest fate while simultaneously repressing PPE fate, suggesting a mechanism whereby dynamic expression of canonical NPB genes patterns the LNZ. Based on previous models, we expected Msx1 to behave much like the transcription factors of the Zic family, promoting neural crest at the expense of PPE. However, we observe that overexpression of *Msx1* in the LNZ results in an entirely binary distribution of *FoxD3* domain repression and expansion, with a slight bias toward repression. This type of response suggests the dose-dependent mediation by another regulatory molecule, and indeed, a recent investigation has identified several factors that influence the inductive capacity of *Msx1* (Monsoro-Burq *et al*., 2005). In animal cap explant studies, *Msx1* cannot induce *FoxD3* expression without the presence of Noggin, a neural inducer secreted from the dorsal midline. Furthermore, only injection of a mid-level dose (250 pg) of *Msx1* mRNA could induce neural crest fate in a neuralized *in vitro* environment. Based on these findings, we propose two possible reasons for our inconclusive results: 1) the low dose of *Msx1* mRNA (120 pg) used in our investigation was not sufficient to induce *FoxD3* expression, and 2) overexpression of *Msx1* expanded the neural plate border laterally into non-neural (Noggin-absent) ectoderm that was not competent to express *FoxD3*. Repeating our gain-of-function experiments with a gradient of doses of *Msx1* mRNA should help to clarify the role of this gene as a neural plate border specifier.

Similarly, recent experiments in whole *Xenopus* embryos and animal cap explants show that *Zic1* is necessary and sufficient to promote a preplacodal fate, rather than repress it (Hong and Saint-Jeannet, 2007). However, this inductive activity becomes repressive in the presence of another neural plate border specifier Pax3, whose expression domain abuts, but does not include, the neural plate. Consequently, overexpression of *Zic1* in Pax3-positive LNZ should result in repression of PPE fate, as is observed. As with *Dlx5*, it is clear that the regulatory activities of *Zic1* are highly dependent upon space- and time-specific information. Again, this demonstrates the need for a more dynamic model of ectodermal patterning that integrates the sensitivity of many transcription factors to the spatiotemporal changes in the molecular environment of the developing *Xenopus* embryo.

Two distinct classes of NPB specifiers pattern the *Xenopus* ectoderm

Taken as a whole, our results strongly support the existence of a lateral neurogenic zone (LNZ) that is subdivided into discrete neural crest and preplacodal fates via the bidirectional withdrawal of neural plate border genes away from the presumptive PPE. We confirm that Dlx5 behaves in a manner distinct from the Zic family transcription factors (and very likely Msx1 as well), and for this reason, we propose a model in which *Dlx5*, along with *Foxi1*, acts upstream of the canonical NPB specifiers as a functionally and spatiotemporally separate class of genes. Though these genes do act to specify the neural/non-neural boundary during early neurulation, we suggest that they be reclassified as 'lateral neurogenic zone' specifiers to properly distinguish between the two. Thus, the LNZ specifying genes act first to place the neural/non-neural border by mediating long-range inductive signals (BMP, FGF, Wnt). After the neural plate is established, the LNZ specifiers, along with the aforementioned ligands, induce expression of the canonical NPB specifiers in the same broad domain. At this point, the expression patterns of the two gene classes diverge: the LNZ specifiers withdraw into the presumptive epidermis to delimit the lateral PPE border, and the canonical NPB specifiers become localized to the presumptive neural crest to form the medial PPE border. This event, then, functions as the determining developmental decision that subdivides the LNZ into neural crest and PPE. What remains to be determined is whether these classes of genes continue to maintain the borders of the domains that they specify, or if other factors and mechanism are required.

Though our findings are suggestive of several important regulatory events in the developing *Xenopus* ectoderm, the gain-of-function studies featured in this investigation cannot confirm a direct transcriptional interaction between two genes. There may be as-yet-unidentified intermediates between any of our proposed interactions within the model, and some of these may even explain some observed inconsistencies (i.e. the binary expansion/repression of *FoxD3* by *Msx1*). Nevertheless, recent work in mouse has identified an enhancer of the rostral preplacodal ectoderm, and report direct binding activity with the Dlx5 and Msx1 transcription factors (Sato *et al*., 2010). This promising finding is powerful evidence of a direct molecular interaction between the neural plate border specifiers and the genes of the lateral neurogenic zone, which further supports our general model of vertebrate ectodermal patterning via highly dynamic local transcriptional interactions.

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Using both genetic and biochemical approaches, examined the function of the *ask1* gene in ubiquitylation pathways of *Arabidopsis thaliana*; specifically, investigated putative downstream targets of ask1 involved in floral organ development, and carried out RNA silencing of two ask gene family members to create a novel tissue-specific double mutant.

Undergraduate Researcher • Department of Anatomy, George Washington University • 2009 to 2010

Investigated the transcriptional interactions that establish and maintain the boundaries between the ectodermal domains of the developing *Xenopus* nervous system. Performed gain-of-function assays testing the role of several neural plate border-specifying genes in the differentiation of the lateral neurogenic zone into the neural crest and preplacodal ectoderm via mRNA microinjection followed by *in situ* hybridization of whole embryos.

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Investigated gene flow among tubeworm populations of the East Pacific Rise cold seeps using experimental and theoretical approaches to population genetics. Through the collaboration of marine ecology and population genetics laboratories, constructed phylogenies based on sequencing data of select mitochondrial gene markers.

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Assisted a postdoctoral fellow in the use of molecular, cellular, genetic, and transgenic techniques to study developmental angiogenesis in the zebrafish. Specifically, investigated the function of rap1 genes in the regulation of endothelial junction integrity using gene cloning, morpholino knockdown, *in situ* hybridization, RT-PCR, siRNA knockdown, and development of transgenic lines.

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