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dlx3b/4b are required for the formation of the preplacodal region and otic placode through local modulation of BMP activity

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ABSTRACT

The vertebrate inner ear arises from the otic placode, a transient thickening of ectodermal epithelium adjacent to neural crest domains in the presumptive head. During late gastrulation, cells fated to comprise the inner ear are part of a domain in cranial ectoderm that contain precursors of all sensory placodes, termed the preplacodal region (PPR). The combination of low levels of BMP activity coupled with high levels of FGF signaling are required to establish the PPR through induction of members of the *six/eya/dach*, *iro*, and *dlx* families of transcription factors. The zebrafish *dlx3b/4b* transcription factors are expressed at the neural plate border where they play partially redundant roles in the specification of the PPR, otic and olfactory placodes. We demonstrate that *dlx3b/4b* assist in establishing the PPR through the transcriptional regulation of the BMP antagonist *cv2*. Morpholino-mediated knockdown of Dlx3b/4b results in loss of *cv2* expression in the PPR and a transient increase in Bmp4 activity that lasts throughout early somitogenesis. Through the *cv2*-mediated inhibition of BMP activity, *dlx3b/4b* create an environment where FGF activity is favorable for PPR and otic marker expression. Our results provide insight into the mechanisms of PPR specification as well as the role of *dlx3b/4b* function in PPR and otic placode induction.

Introduction

The interface between neural and non-neural ectoderm gives rise to several cell types, including neural crest, paired placodes and, in anamniotes, Rohon–Beard sensory neurons (Baker and Bronner-Fraser, 1997; Gans and Northcutt, 1983; Holland and Holland, 2001; Meulemans and Bronner-Fraser, 2004; Northcutt and Gans, 1983; Schlosser, 2006). The paired placodes, transient thickenings of ectodermal epithelium, arise lateral and adjacent to neural crest and Rohon–Beard domains in the presumptive head. During late gastrulation and early segmentation stages, placodal cells comprise a domain of cranial ectoderm that contains precursors of all sensory placodes (termed the preplacodal region, or PPR) (reviewed in Bailey and Streit, 2006; Ohyama et al., 2007; Riley, 2003; Schlosser, 2006). Evidence from studies in *Xenopus*, zebrafish, and chick suggest that the convergence of multiple activities of signaling molecules is required for the establishment of the PPR. A balance of FGF activity and antagonism of both BMP and WNT signaling are required to induce expression of members of the *Eyes absent* (*Eya*)/*Sine oculis* (*Six*)/*Dachshund* (*Dach*), *Iroquois* (*Iro*), and *Distalless* (*Dlx*) families of transcription factors during late gastrulation, which are the earliest markers of PPR fate (Ahrens and Schlosser, 2005; Brugmann et al., 2004; Glavic et al., 2004; Litsiou et al., 2005; Nguyen et al., 1998). In particular, modulation of BMP activity at the neural plate border is instrumental in the establishment of the PPR and also patterns adjacent Rohon–Beard and neural crest domains (Nguyen et al., 1998; Nguyen et al., 2000; Rossi et al., 2008; Tribulo et al., 2003).

In *Xenopus* and zebrafish, a BMP gradient model has been proposed in which BMP activity is high in ventral/lateral regions and progressively lower in more dorsal/medial regions during gastrulation. High levels of BMP activity are required to induce epidermis, low levels are required to specify neural plate, and intermediate levels are required to specify neural crest and Rohon–Beard domains (Aybar and Mayor, 2002; Nguyen et al., 1998; Nguyen et al., 2000; Tribulo et al., 2003). Although the PPR lies lateral to the domain of neural crest, evidence from *Xenopus*, zebrafish and chick suggests that BMP activity must be lower in the PPR than in adjacent neural crest and epidermal territories (Ahrens and Schlosser, 2005; Glavic et al., 2004; Litsiou et al., 2005). For example, implantation of Bmp4-containing beads near the PPR is sufficient to inhibit expression of the PPR marker *Six1* (Ahrens and Schlosser, 2005). Thus, it appears that establishment of the PPR requires lower levels of BMP activity than that required for neural crest and Rohon–Beard formation, contradictory to a simple gradient model.

While it is apparent that attenuation of BMP activity is critical in establishing the PPR, it is not yet clear how this attenuation is achieved. Tissue grafting experiments have revealed that potential BMP antagonists originate from tissues other than the PPR. Grafting of chicken head mesoderm onto extraembryonic ectoderm yields host tissue with PPR characteristics (Litsiou et al., 2005). Likewise, transplantation of neural ectoderm into domains of ventral ectoderm yields similar results in *Xenopus*, demonstrating the role these tissues

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have in creating an environment suitable for the formation of the PPR (Ahrens and Schlosser, 2005). However, the BMP antagonists involved in this process remain unidentified.

Members of the *Dlx* family of transcription factors are thought to play intrinsic roles in the formation of the PPR, although the mechanisms by which they do so are unclear. *Dlx* genes are required but not always sufficient for the expression of PPR markers from the *Eya*/*Six*/*Dach* families. For example, ectopic expression of *Six1* in *Xenopus* and chick can only be achieved in the presence of functional Dlx3 and Dlx5, respectively (Woda et al., 2003). In zebrafish, *dlx3b/4b* are initially expressed along the entire neural plate border, which includes the PPR, at the end of gastrulation. Expression becomes restricted to the otic and olfactory placodes during somitogenesis (Ekker et al., 1992; Feledy et al., 1999; Pera et al., 1999). Only rudimentary otic and olfactory placodes form when *dlx3b/4b* function is lost, and the resulting size of these sensory organs is significantly reduced (reviewed in Ohyama et al., 2007; reviewed in Riley, 2003). Induction of early otic and olfactory markers, such as *pax2a* and *eya1*, is severely compromised, suggesting that *dlx3b/4b* function early in the process of otic and olfactory induction. Thus, it has been suggested that *Dlx* genes may act as competence factors for placode induction (Hans et al., 2007; Hans et al., 2004).

In amniotes, *Dlx5* and *Dlx6* are expressed in a similar pattern to *dlx3b/4b* in zebrafish (Acampora et al., 1999; Yang et al., 1998). However, inactivation of *Dlx5/6* in mouse does not appear to affect induction of the otic or olfactory placodes, but rather their subsequent development (Merlo et al., 2002; Robledo and Lufkin, 2006; Robledo et al., 2002). The reason for the discrepancy in phenotypes between zebrafish and mouse embryos lacking these *Dlx* paralogues is currently unclear.

To better understand the role of *dlx3b/4b* during the establishment of the PPR and otic placodes, we examined signaling activities involved in PPR and otic placode induction. We have identified that a BMP signaling modulator, Cv2, is critical for the formation of the PPR. The predominant function of this protein is as a BMP antagonist, although its proteolytic cleavage may allow Cv2 to act as an agonist of BMP activity (Rentzsch et al., 2006; Zhang et al., 2007; Zhang et al., 2008). We show that *cv2* lies transcriptionally downstream of *dlx3b/4b*, and that the full-length protein is required to modulate BMP activity to levels conducive for PPR formation. Morpholino-mediated knockdown of Dlx3b/4b results in loss of *cv2* expression in the PPR and a transient increase in Bmp4 activity that is first observed at the end of gastrulation. This is followed by a transient decrease in FGF activity that can be rescued when *cv2* or *fgf-receptor 1* (*fgfr1*) mRNA is supplied in Dlx3b/4b morphants. Ectopic expression of either *dlx3b* or *cv2* is sufficient to drive PPR marker expression. Conversely, loss of *cv2* has similar effects on PPR development as loss of *dlx3b/4b*, indicating that a significant aspect of *dlx3b/4b* function at the end of gastrulation is mediated through *cv2*. Our results suggest a model in which *dlx3b/4b*-mediated modulation of BMP signaling through *cv2* lies upstream of *Six*/*Eya*/*Dach* genes and FGF responsiveness in the specification of the PPR and induction of the otic placode. Furthermore, our findings provide a possible explanation for the difference in function of the *Dlx* genes between mouse and zebrafish.

Materials and methods

Animals

Wild-type (AB) mutant zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR). Embryos were maintained at 28.5 °C and staged using standard criteria (Kimmel et al., 1995). *Tg (hsp70l:dnBmpr-GFP)^{w30}* (*tBR*) transgenic zebrafish were obtained from the Kimelman lab (University of Washington, Seattle). This transgenic line contains a truncated Type I Bmp receptor containing GFP in place of the kinase domain under the control of a heat-shock promoter (Pyati et al., 2005). *tBR* embryos were heat shocked at 37 °C for 1 h at bud stage according to Pyati et al. (2005). They were then sorted according to GFP expression and raised at 28.5 °C until fixation. Where appropriate, wild-type or control morpholino-injected embryos were heat-shocked at the same stage as controls.

In situ hybridization

The following probes were used: *bmp4* (Nikaido et al., 1997), *chordin* (Miller-Bertoglio et al., 1997), *cv2* (Rentzsch et al., 2006), *dlx3b* (Ekker et al., 1992), *erm* (Raible and Brand, 2001), *eya1* (Sahly et al., 1999), *fgfr1* (Scholpp et al., 2004), *fgfr2* (Poss et al., 2000), *fgfr3* (Sleptsova-Friedrich et al., 2001), *fgfr4* (Thisse et al., 1995), *pax2a* (Krauss et al., 1991), *six4.1* (Kobayashi et al., 2000), *spry4* (Fürthauer et al., 2001), and *tbx2b* (Dheen et al., 1999).

Real-time quantitative RT-PCR

RNA was isolated from three sets of 20 embryos of each experimental sample using the RNeasy kit (Qiagen). The SYBR Green I (Roche Applied Science) RNA amplification kit was used on the LightCycler according to the manufacturer's instructions and published protocols (Rajeevan et al., 2001). The primers used for

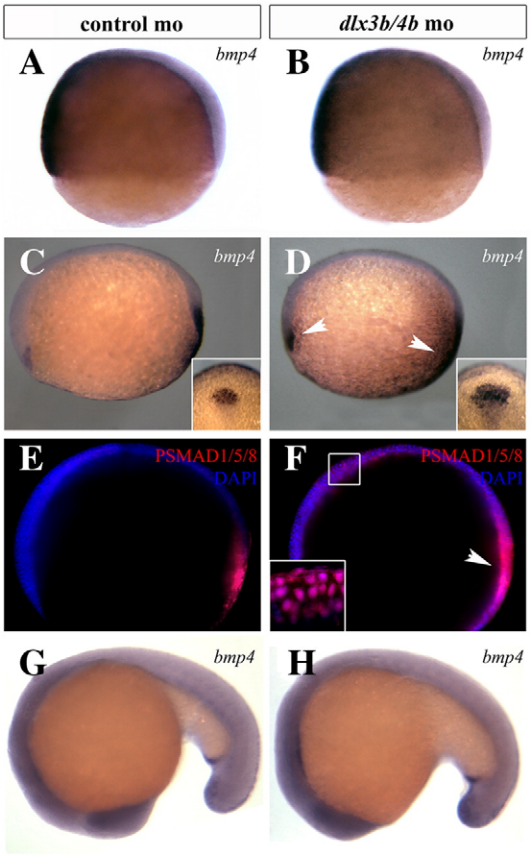

Fig. 1. BMP activity is transiently increased during early somitogenesis in Dlx3b/4b morphants. (A,B) *bmp4* expression is unaffected in Dlx3b/4b morphants prior to the onset of *dlx3b/4b* expression in 70% epiboly embryos. (C,D) At bud stage, the domain of *bmp4* expression is expanded in the prechordal plate and tailbud of Dlx3b/4b morphants (D). Insets depict the increase of the prechordal domain. (E,F) Antibodies against PSMAD1/5/8 (red) reveal an increase in cells responding to BMP activity of Dlx3b/4b morphants (F). DAPI-stained nuclei are blue. Inset in (F) is a high magnification image depicting PSMAD1/5/8 co-localization with DAPI-stained nuclei. (G,H) *bmp4* expression in Dlx3b/4b morphants is comparable to controls at 18-somites. All views are lateral views, with ventral to the left in (A,B), anterior to the left in (C–F), and anterior to the bottom in (G,H). Insets in (C,D) are dorsal views, with anterior to the top.