A BMP regulatory network controls ectodermal cell fate decisions at the neural plate border

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SUMMARY
During ectodermal patterning the neural crest and preplacodal ectoderm are specified in adjacent domains at the neural plate border. BMP signalling is required for specification of both tissues, but how it is spatially and temporally regulated to achieve this is not understood. Here, using a transgenic zebrafish BMP reporter line in conjunction with double-fluorescent in situ hybridisation, we show that, at the beginning of neurulation, the ventral-to-dorsal gradient of BMP activity evolves into two distinct domains at the neural plate border: one coinciding with the neural crest and the other abutting the epidermis. In between is a region devoid of BMP activity, which is specified as the preplacodal ectoderm. We identify the ligands required for these domains of BMP activity. We show that the BMP-interacting protein Crossveinless 2 is expressed in the BMP activity domains and is under the control of BMP signalling. We establish that Crossveinless 2 functions at this time in a positive-feedback loop to locally enhance BMP activity, and show that it is required for neural crest fate. We further demonstrate that the Distal-less transcription factors Dlx3b and Dlx4b, which are expressed in the preplacodal ectoderm, are required for the expression of a cell-autonomous BMP inhibitor, Bambi-b, which can explain the specific absence of BMP activity in the preplacodal ectoderm. Taken together, our data define a BMP regulatory network that controls cell fate decisions at the neural plate border.

KEY WORDS: Zebrafish, BMP signalling, Neural plate border, Neural crest, Preplacodal ectoderm, Bmper, Bambib

INTRODUCTION
The construction of tissues from pluripotent stem cells is providing a promising approach for the treatment of diseases, but the development of such methods necessitates a detailed understanding of how different tissues are normally specified during embryonic development. Tissue specification and patterning in developing embryos are known to require networks of tightly regulated signalling pathways, such as those activated by the transforming growth factor β (TGFβ) superfamily, Wnt, FGF and Notch ligands (Tzahor, 2007; Stuhlmueller and García-Castro, 2012). Yet how these pathways are spatially and temporally regulated to orchestrate growth and differentiation during embryogenesis is not well understood. Furthermore, the importance of their precise regulation is underlined by the observation that some of the pathways are frequently dysregulated in diseases in adult organisms (Kho et al., 2004; Dreesen and Brivanlou, 2007).

One of the pathways that is crucial for embryonic development and whose dysregulation is also implicated in human disease is the bone morphogenetic protein (BMP) signalling pathway (Blanco Calvo et al., 2009; Wu and Hill, 2009; Lowery and de Caestecker, 2010). BMPs are members of the TGFβ superfamily of ligands. For activation of the canonical BMP signalling pathway, BMP ligands bind as homo- or heterodimers to type I-type II serine/threonine kinase receptor complexes. Upon ligand binding, the constitutively active type II receptor phosphorylates the type I receptor. This activates the type I receptor and promotes the binding and subsequent phosphorylation of the main signalling effectors – the receptor-regulated Smads (R-Smads). In particular, Smad1 and Smad5 are activated in response to BMP and growth differentiation factor (GDF) signalling. Phosphorylated Smad1/5 then forms complexes with Smad4, which accumulate in the nucleus, where they regulate gene expression (Ramel and Hill, 2012). Monitoring nuclear phosphorylated R-Smads can thus be used as a readout for pathway activation (von der Hardt et al., 2007; Tucker et al., 2008; French et al., 2009).

During zebrafish and Xenopus gastrulation, a gradient of BMP signalling is required for the establishment of the dorsoventral (DV) axis and for ectodermal patterning (reviewed by Ramel and Hill, 2012). This gradient, which is high ventrally and low dorsally, is initially established between 30% and 40% epiboly in zebrafish as a result of an interplay between BMP ligands, dorsally expressed BMP antagonists and a DV gradient of FGF signalling (Ramel and Hill, 2013). During gastrulation, this gradient contributes to ectodermal patterning, with discrete levels of BMP activity along the morphogen gradient defining the position of ectodermal derivatives such as the preplacodal ectoderm (PPE), neural crest (NC) and neural plate (NP) (Nguyen et al., 1998). The NC is a transient population of progenitor cells that gives rise to diverse cell types such as neurons, cells of the peripheral nervous system, pigment cells, smooth muscle cells and craniofacial cartilage and bone (Huang and Saint-Jeannet, 2004). The PPE, by contrast, differentiates into several sensory placodes that develop into structures such as the lens, the inner ear and the olfactory epithelium (Baker and Bronner-Fraser, 2001). During gastrulation, the NC and the PPE arise at the border between the neural and non-neural ectoderm at intermediate levels of BMP signalling, with the PPE thought to require slightly higher levels of BMP activity (Neave et al., 1997; Wilson et al., 1997; Nguyen et al., 1998; Tucker et al., 2008; Schumacher et al., 2011).

However, a gradient of BMP signalling is not sufficient to explain ectodermal patterning after gastrulation. This is particularly evident...
in the case of PPE specification. In PPE formation, BMP signalling is required only during late blastula stages, but is no longer necessary after the onset of gastrulation (Kwon et al., 2010). In fact, full attenuation of BMP signalling is required during late gastrulation, presumably through localised expression of BMP antagonists. Indeed, ectopic sources of BMP inhibitors result in expansion of PPE marker expression (Glavic et al., 2004; Ahrens and Schlosser, 2005; Litsiou et al., 2005; Kwon et al., 2010). Thus, the requirement of BMP activity in PPE development differs over time, whereas NC is thought to require continuous BMP signalling (Steventon et al., 2009). However, it is not known whether distinct domains of BMP signalling are specified at the neural plate border (NPB), nor how the different requirements for BMP signalling of the distinct progenitor populations at the NPB are implemented.

Progress in answering these key questions has been hampered by difficulties in visualising active BMP signalling during embryogenesis. Staining for phosphorylated Smad1/5 (PSmad1/5) readily detects strong signals, such as the BMP activity gradient during gastrulation (Tucker et al., 2008; Ramel and Hill, 2013), but might not detect weaker staining at later stages. In addition, much of the available data have been generated from phenotypic analyses of BMP mutants, such as the zebrafish swirl (bmp2b), snailhouse (bmp7) and somitabun (smad5) mutants (Mullins et al., 1996; Nguyen et al., 1998). These mutants, however, lack the early BMP signalling that is required for the initiation of DV patterning and can therefore only provide limited information on the specific requirements of NC and PPE for BMP activity during later stages.

To overcome some of these limitations we have made use of a transgenic Tg(BRE:mRFP) zebrafish line that expresses monomeric red fluorescent protein (mRFP) in response to BMP signalling, and thereby allows direct visualisation of BMP activity dynamics (Wu et al., 2011; Ramel and Hill, 2013). We describe the evolution of two distinct BMP signalling domains at the NPB. We demonstrate that the pro-BMP factor crossveinless 2 (cvl2, also called bmpper) is expressed in domains of BMP activity during NC induction and maintenance, and is regulated by BMP signalling. We show, using a loss-of-function approach, that Cvl2 is essential for the localised BMP activity and for NC development. Using double-fluorescent staining (DFISH) we show unequivocally that BMP activity and for NC development. Using double-fluorescent probes were used: mRFP (Wu et al., 2011), sox10 (Dutton et al., 2001), knox20 (eger2 – Zebrafish Information Network) (Oxtoby and Jowett, 1993), bmp2b (Kishimoto et al., 1997), bmp7a (Schmid et al., 2000), cvl2 (Rentzsch et al., 2006), snail1b (Thiess et al., 1995), ncad (cdh2 – Zebrafish Information Network) (Lele et al., 2002), dix3b (Akimenko et al., 1994) and bambi-b (IMAGE clone ID 7038842). Probes for bmp6, gdf6a, eya1 and sixl.1 (six4 – Zebrafish Information Network) were cloned into pgEMT (Promega) using standard PCR methods and correspond to the following nucleotides (relative to the start of transcription): bmp6 (+630 to +1560), gdf6a (+440 to +1248), eya1 (+288 to +1199), sixl.1 (+886 to +1732). Alcian Blue staining was as described (van Boxtel et al., 2011).

Quantitative real-time PCR (qRT-PCR)
RNA was extracted and qRT-PCR was performed as described (Wu et al., 2011). All results were normalised to efa1 (efa1 – Zebrafish Information Network) expression. Primers are listed in supplementary material Table S2.

Tissue culture, cell treatments and in vitro transcription/translation
HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% foetal calf serum. Cells were transiently transfected using FUGENE HD (Promega). LDN-193189 (a gift from Paul Yu, Department of Medicine, Brigham and Womens Hospital, Boston, USA) was dissolved in DMSO and used at a final concentration of 1 µM. BMP4 (R&D Systems) was dissolved in 5 mM HCl/1 mg/ml BSA and used at concentrations ranging from 2 ng/ml to 20 ng/ml. Synthetic bambi-b mRNA was generated from IMAGE clone ID 7038842 (Source BioScience) and translated in reticulocyte lysate (Promega) (Howell and Hill, 1997).

Plasmids
Sequence encoding full-length zebrafish FLAG-Dlx3b was cloned into pCS2+, introducing an N-terminal FLAG tag. FLAG-Bambi-b was generated using a similar approach, introducing a C-terminal FLAG tag. HA-ALK3 was as described (Daly et al., 2008). The control plasmid used in transfections was pcDNA3 (Invitrogen).

Bandshift assay
Bandshift assays were performed as described (Germain et al., 2000). Two potential binding sites for zebrafish Dlx3b were identified in the bambi-b gene and flanking sequence using Predicted Motifs for Transcription Factors (PreMoTF) (Christensen et al., 2012). One was in the first intron at region +199 to +204 (AAATTG) and the other was in the upstream region on the opposite strand at –851 to –846 (TAATTC). Probes containing these sites were generated by PCR. Probe 1 corresponds to +167 to +230 and probe 2 to –877 to –818 relative to the start of transcription. For the mutant probes the central AAT was mutated to CCG. FLAG-tagged Dlx3 was expressed transiently in HEK293T cells using the pCS2+–FLAG-Dlx3b plasmid. Cell lysates were prepared as described (Germain et al., 2000). Anti-FLAG antibody (Sigma, F7425) was used in the bandshift assays.

Western blot analysis and immunoprecipitations
Embryos were snap frozen and whole cell extracts were prepared as described (Dorey and Hill, 2006). Whole cell extracts from tissue culture cells and immunoprecipitations were as described (Germain et al., 2000; Levy et al., 2007), using anti-FLAG antibody (Sigma, F7425) coupled to protein A beads. Western blotting was performed using standard techniques. The antibodies used were: mouse anti-GFP (Roche, 11814460001), rabbit anti-PSmad1/5 (Cell Signaling Technology, 9511L), rabbit anti-Smad5 (GeneTex, GTX124559), rat anti-tubulin (Roche, ab6160), anti-FLAG-HRP (Sigma, A8592), anti-HA-HRP (Roche, 12013819001), anti-MCM6 (Santa Cruz, sc-9843) and anti-actin (Sigma, A3853).

RESULTS
Distinct domains of BMP activity at the NPB
To investigate the dynamic temporal and spatial regulation of BMP signalling during ectodermal patterning that leads to the specification of distinct progenitor cells at the NPB we used the Tg(BRE:mRFP) transgenic zebrafish line (Wu et al., 2011; Ramel and Hill, 2013). We visualised mRFP mRNA, which has a half-life...
of substantially less than 20 minutes (Ramel and Hill, 2013). As expected, ISH for mRFP mRNA revealed a ventral-to-dorsal gradient of BMP activity during epiboly stages (Fig. 1A, left panel) (Ramel and Hill, 2013). During gastrulation, as morphogenetic movements shape the embryo and cells converge to the dorsal side, this gradient is lost, and, by the onset of somitogenesis, BMP signalling is transformed into a distinct horseshoe-shaped domain at the NPB (Wu et al., 2011). This domain then evolves further into an outer and an inner domain (Fig. 1A). DFISH, using probes directed against mRFP and sox10 (NC marker), revealed that the inner BMP activity domain coincides with the NC cell population (Fig. 1A, right panel).

We next determined exactly when BMP signalling is required for NC development using a transgenic zebrafish line expressing a dominant-negative BMP type I receptor (dnBMPR1a) in response to heat shock [Tg(hsp70l:dnXla.Bmpr1a-GFP)] (Pyati et al., 2005). This line enabled us to perturb BMP activity at specific stages. Robust inhibition of BMP activity was confirmed by the loss of PSmad1/5 as detected by western blot analysis (Fig. 1B, right panel). Induction of dnBMPR1a expression in Tg(hsp70l:dnXla.Bmpr1a-GFP) × Tg(BRE:mRFP) embryos at bud stage also resulted in reduction of mRFP staining at the 3-somite stage (supplementary material Fig. S1A). Early inhibition of BMP signalling from 30% epiboly, when the BMP gradient is being established (Ramel and Hill, 2013), resulted in a loss of ventral tissues and in an expansion of the neuroectoderm (shown by the hindbrain rhombomere 3 and 5 marker krox20) and NC (Fig. 1B).

By contrast, embryos that were heat shocked at early gastrula stages (60% epiboly) displayed only a slight expansion of the krox20 expression domain. Expression of sox10, however, was reduced. Most importantly, inhibition of BMP signalling from early somitogenesis onwards also led to a decrease in sox10 expression, demonstrating a requirement for BMP activity in the maintenance of the NC cell population (Fig. 1B).

The BMP ligands bmp2b and bmp7a are expressed at the NPB and have been implicated in NC induction in several organisms (reviewed by Stuhlmiller and García-Castro, 2012). However, neither bmp2b nor bmp7a mRNA was detected in the inner domain of BMP activity during NC maintenance (supplementary material Fig. S1B), suggesting the involvement of alternative ligands. BMP6, BMP7, GDF5 and GDF6 can all induce differentiation of rat cortical neural stem cells into various NC lineages (Gajavelli et al., 2004).
To examine a possible function for these ligands in zebrafish NC development, we assessed their temporal and spatial expression patterns at different developmental stages. gdf5 is only expressed from 40 hpf onwards (Bruneau et al., 1997) and was therefore not investigated further. By contrast, bmp6 and gdf6a were both detected by qRT-PCR at the end of gastrulation (bud stage) (supplementary material Fig. S1C), when the horseshoe-shaped domain of BMP activity at the NPB is first formed. ISH analysis confirmed the presence of the ligands during somitogenesis (Fig. 1C). gdf6a is expressed in the NC and the anterior NPB at the 6-somite stage, as confirmed by DFISH using probes against sox10 and gdf6a (Fig. 1D). bmp6 is expressed at the border with the epidermis (Fig. 1C), similar to the outer domain of BMP signalling. Thus, bmp6 and gdf6a are likely candidates to be involved in the localised regulation of BMP signalling.

To test this hypothesis, bmp6 and gdf6a were knocked down using morpholinos (MOs). Injection of two different gdf6a MOs (Sidi et al., 2003) (supplementary material Fig. S1D) resulted in loss of BMP activity in the inner NC domain and substantially reduced the number of sox10-positive cells (Fig. 1E). By contrast, bmp6 morphants did not display a phenotype, indicating that Bmp6 activity is not essential for the formation of the outer horseshoe-shaped domain of BMP signalling (Fig. 1F; supplementary material Fig. S1E).

Taken together, these data provide direct evidence for remodelling of the BMP gradient after gastrulation. Two distinct domains of BMP activity are formed at the NPB. The inner domain of BMP signalling, which corresponds to the NC, requires the BMP/GDF ligand Gdf6a, which in turn is essential for NC development.

cvl2 expression coincides with the BMP activity domains

ISH analysis of bmp2b and bmp7a mRNA indicates their presence at the NPB. However, both ligands are expressed throughout the entire non-neural ectoderm, whereas BMP activity is tightly restricted to the NPB (Fig. 2A; supplementary material Fig. S1B). This suggests that a mechanism exists to concentrate the activity of these ligands at the NPB by the onset of somitogenesis.

To determine the spatial regulators that might promote localised BMP signalling, we reviewed the expression of BMP modulators, and identified the secreted BMP-binding factor Cvl2 as a possible candidate. Cvl2 is a known positive regulator of BMP signalling during zebrafish gastrulation (Conley et al., 2000; Rentzsch et al., 2006). Functionally, Cvl2 is thought to locally enhance BMP activity predominantly by increasing the availability of BMP proteins for receptor activation (Rentzsch et al., 2006; Serpe et al., 2008).

We observed that the expression pattern of cvl2 overlapped with domains of BMP activity in a ventral-to-dorsal gradient during epiboly stages (Fig. 2B) (Rentzsch et al., 2006). Similarly, ISH revealed that cvl2 mRNA is present in an outer horseshoe-shaped domain and an inner NC-like domain during somitogenesis (Fig. 2A, B), very reminiscent of the pattern of BMP activity described above. We confirmed that cvl2 was expressed in the horseshoe-shaped BMP signalling domain by performing DFISH using probes against mRFP and cvl2 (Fig. 2C). In addition, DFISH showed that the inner cvl2 expression territory is localised to the NC (Fig. 2D).

These findings demonstrate that cvl2 expression overlaps with domains of BMP activity during ectodermal patterning. Given the described function of Cvl2, this suggested that Cvl2 might stabilise domains of BMP signalling by trapping ligands in the vicinity of the receptors at early somitogenesis stages. As a result, despite the broad expression of bmp2b and bmp7a, BMP signalling would only be active in a discrete domain at the NPB.

cvl2 is required for localised BMP activity and NC development

The expression of cvl2 in the NC suggested a role for Cvl2 in BMP-dependent NC development. We therefore carried out loss-of-function experiments using a translation-blocking MO against cvl2 (MO1). At both MO doses used there was a substantial loss of mRFP expression in Tg(BRE:mRFP) embryos, as well as reduced levels of PSmad1/5 and reduced expression of the NC markers sox10 and snai1b (Fig. 3A, B; supplementary material Fig. S2A). Importantly, these effects were not due to an early defect in DV patterning (Rentzsch et al., 2006), as the krox20 expression domain was not substantially ventrally expanded at the 4 ng dose of MO1 (Fig. 3A). Interestingly, expression of the bmp2b ligand was unaffected by Cvl2 knockdown, supporting a role for Cvl2 at the level of Bmp2b protein (Fig. 3A). The effects on mRFP, snai1b and sox10 were also present with two additional cvl2 MOs (supplementary material Fig. S2B). Note that BMP activity was reduced not only in the anterior, but also in the posterior, consistent with a posterior cvl2 expression domain (data not shown). We also showed that, as in other...
systems (Ramel and Hill, 2012), the expression of cvl2 is regulated in a positive-feedback loop. Inhibition of BMP signalling in Tg(hsp70l:dnXla.Bmpr1a-GFP) transgenic embryos at bud stage substantially reduced cvl2 mRNA levels at the 4-somite stage (Fig. 3C).

To ensure that the loss of NC markers was not due to a developmental delay, cvl2 morphants were also assayed at later times for NC derivatives. Alcian Blue staining on 5-day-old embryos revealed a decrease in craniofacial cartilage, confirming inhibition of NC development (supplementary material Fig. S2C,D). Thus, we demonstrate a requirement for Cvl2 in NC formation, presumably by locally enhancing BMP signalling activity in distinct domains at the NPB.

**Temporal and spatial analysis of the formation of distinct BMP signalling domains**

Ectodermal patterning requires discrete levels of BMP signalling for the induction of cell fates such as the PPE and NC at the NPB. Specification of the PPE necessitates complete inhibition of BMP activity after gastrulation (Kwon et al., 2010). This inhibition of BMP signalling has previously been postulated to be mediated by an antagonistic role of Cvl2 on BMP signalling (Esterberg and Fritz, 2009). That study suggested that the functionally redundant Distal-less transcription factors Dlx3b and Dlx4b, which are PPE markers, activate the expression of cvl2 in the PPE. By contrast, our data point to Cvl2 being a positive regulator of BMP activity during ectodermal patterning (Fig. 3A,B). In addition, we hypothesise that cvl2 and dlx3b are likely to be co-expressed during epiboly stages (Fig. 2B) (Akimenko et al., 1994). To clarify this and to analyse the establishment of distinct cell populations after gastrulation, we monitored dlx3b/cvl2 expression at four stages from the end of gastrulation to the formation of the first somite (Fig. 4B). At 95% epiboly, cvl2 and dlx3b are expressed in the same domains, although it is difficult to determine whether they are expressed in the same cells or in intermingled cells. At later times, however, as dlx3b expression sharpens to the NPB, the cvl2 and dlx3b expression domains become increasingly separated, until two clearly distinct domains are visible at the 1-somite stage (Fig. 4B). Analysis of Tg(BRE:mRFP) embryos using DFISH to detect mRFP and dlx3b showed that formation of the outer mRFP expression domain follows a similar pattern to that of the outer cvl2 expression domain (supplementary material Fig. S3A). A dorsal anterior view of 1-somite stage embryos revealed that, compared with cvl2 and dlx3b, the mRFP horseshoe-shaped domain at the NPB was transiently slightly broader, with the outer edge colocalising with cvl2 expression and the inner edge overlapping with dlx3b (supplementary material Fig. S3B). At later stages, however, mRFP colocalised exclusively with cvl2 (Fig. 2C).

Taken together, these data show that although cvl2, dlx3b and mRFP are initially expressed in the same territories during gastrulation, distinct expression domains form by the onset of neurulation. The PPE separates the two domains of BMP activity, confirming an absence of BMP signalling in the PPE at somitogenesis stages.

**dlx3b/4b are required for BMP activity in the NC**

The expression of dlx3b between the two domains of BMP activity/cvl2 expression and the known requirement for Dlx3 in NC induction in Xenopus (Pieper et al., 2012) prompted us to investigate the role of Dlx3b in regulating BMP activity at the NPB. Knockdown of dlx3b in conjunction with the functionally redundant dlx4b (Solomon and Fritz, 2002) resulted in reduced sox10 expression (Fig. 5A). Contrary to previous reports we did not...
observe a general reduction in cvl2 mRNA levels, but a specific loss of the inner cvl2 expression domain (Fig. 5A). Similarly, we no longer detected the NC domain of BMP activity in dlx3b/4b morphants of Tg(BRE:mRFP) embryos (Fig. 5A). This is not due, however, to an expansion of neural tissue, as shown by DFISH of cvl2 and ncad (Fig. 5B). Moreover, ISH for other PPE markers (eya1, six4.1) showed that they were still present in dlx3b/4b morphants (Fig. 5C), suggesting a direct effect of dlx3b/4b knockdown on BMP activity rather than a loss of PPE per se. Our results therefore show that dlx3b/4b in the PPE are essential for formation of the neighbouring inner BMP signalling domain and hence the NC.

Previous data have suggested that expression of dlx3b depends on BMP signalling during early stages, as bmp2b mutant embryos lack dlx3b mRNA expression (Nguyen et al., 1998). The observation that dlx3b/4b morphants lacked NC therefore prompted us to re-analyse the cvl2 morphants to exclude the possibility that the NC phenotype observed in these embryos was due to inhibition of early BMP activity and a concomitant loss of dlx3b expression. However, levels of dlx3b were not diminished in the cvl2 morphants, demonstrating a direct effect of Cvl2 on NC (Fig. 5D).

dlx3b regulates expression of the BMP inhibitorambia-b in the PPE
cvl2 and dlx3b are expressed in similar domains during gastrulation but then become mutually exclusive at the onset of somitogenesis, with dlx3b situated between the outer and inner domains of cvl2 expression/BMP activity. This observation suggests an active
inhibition of BMP signalling in the PPE, as the process of domain formation is rapid and spatially tightly regulated. Such an inhibition could be achieved by the localised expression of BMP antagonists in the PPE. As Dlx3b is a transcription factor, its loss-of-function phenotype might be explained by the loss of a localised BMP inhibitor that is under the transcriptional control of dlx3b at the onset of neurulation.

Analysis of the expression patterns of potential BMP inhibitors led us to identify BMP and activin membrane-bound inhibitor b (bambi-b) as being expressed at the right time and place to locally attenuate BMP signalling at the onset of neurulation. This putative membrane-spanning protein is highly similar to Xenopus BAMBI, which has been shown to function as a pseudoreceptor, inhibiting BMP activity in a cell-autonomous manner (Onichtchouk et al., 1999). qRT-PCR analysis revealed the onset of bambi-b expression at the end of gastrulation (Fig. 6A). At bud stage, bambi-b is weakly expressed in the non-neural ectoderm. From the onset of somitogenesis a new stripe of bambi-b mRNA expression was detected in a similar territory to dlx3b expression, and this expression domain was still present at the 4-somite stage. To confirm that zebrafish Bambi-b inhibits BMP signalling we overexpressed FLAG-tagged Bambi-b in HEK293T cells and showed that it inhibited BMP-induced phosphorylation of Smad1/5, particularly when cells were exposed to low levels of BMP4 (Fig. 6B). Furthermore, Xenopus BAMBI has been shown to function by forming a non-productive signalling complex with the BMP type I receptors. Similarly, we demonstrated that Bambi-b interacted with the BMP type I receptor ALK3 (Fig. 6C).

To test the requirement for bambi-b during embryonic development we performed loss-of-function studies using a translation-blocking MO (Fig. 6D, bottom panel). The expression of the NC marker sox10 was significantly reduced in the bambi-b mutants. From these experiments, we conclude that bambi-b is required for BMP signalling at the onset of neurulation.
morphants and the inner NC cvl2 expression domain could no longer be detected (Fig. 6D). This phenotype is similar to that observed for dlx3b/4b morphants, suggesting that bambi-b might be downstream of dlx3b. We therefore tested whether dlx3b is required for induction of the stripe of bambi-b expression. Knockdown of dlx3b/4b led to a loss of this specific bambi-b expression domain (Fig. 6E). Importantly, expression in the non-neural ectoderm, where dlx3b/4b are not expressed, was unaffected. Finally, we investigated whether Dlx3b might be directly responsible for regulating bambi-b expression. We used PreMoTF (Christensen et al., 2012) to predict the DNA-binding specificity of Dlx3b, which was identified as TAATTG/A. We found two possible sites in the bambi-b gene and flanking region: one in the first intron (+199 to +204 bp, probe 1) and the other upstream of the start of transcription (−851 to −846 bp, probe 2). In a bandshift assay we demonstrated direct binding of FLAG-tagged Dlx3b to both of these sites, and these complexes were supershifted with an anti-FLAG antibody. The binding was abolished by specific mutation of the binding sites (Fig. 6F).

In conclusion, Bambi-b, which is expressed after bud stage under the control of Dlx3b, is an excellent candidate to inhibit BMP activity in these Dlx3b-expressing cells and consequently enable them to be specified as PPE.

**DISCUSSION**

**Identification of dynamic BMP signalling domains at the NPB during ectodermal patterning**

Here we have used a transgenic zebrafish BMP reporter line to identify distinct territories of BMP activity at the NPB. We show that, at the end of gastrulation, BMP activity concentrates into a horseshoe-shaped domain at the NPB, which then resolves into two distinct domains: an outer domain abutting the epidermis and an inner domain. Using DFISH, we show that this inner domain corresponds to the NC, and thus provide direct evidence for ongoing BMP activity in the NC cell population. We further show that BMP signalling is absolutely required for the maintenance of NC progenitors, as inhibition of BMP signalling at the onset of neurulation reduced the expression of NC markers.

We went on to determine which ligands contribute to the localised BMP activity and show that, in addition to bmp2b and bmp7a, bmp6 is expressed in the horseshoe-shaped domain of BMP activity that abuts the epidermis. Bmp6 alone, however, is not solely responsible for the formation of this domain, but is likely to reinforce and sharpen the signalling domain in concert with Bmp2b and Bmp7a. We further identify Gdf6a as the BMP ligand required for BMP signalling activity in the NC and also for NC cell fate, thus solving the conundrum of how BMP signalling is activated in the NC given the absence of bmp2b, bmp4 and bmp7a expression. How transcription of gdf6a is induced in this specific domain remains to be determined.

**Cvl2 locally enhances BMP activity at the NPB and is required for NC development**

The reshaping of BMP activity at the end of gastrulation from a gradient to sharply defined domains at the NPB prompted us to search for spatial regulators of BMP signalling. We identified the secreted BMP-binding protein Cvl2 as playing a crucial role in locally enhancing BMP activity in the two distinct domains at the NPB. Cvl2 has been described as both an activator and inhibitor of BMP signalling (Conley et al., 2000; Moser et al., 2003; Binnerts et al., 2004; Kamimura et al., 2004; Ambrosio et al., 2008). Like many other proteins that interact with BMPs, such as the BMP antagonist Chordin, Cvl2 contains cysteine-rich domains (Conley et al., 2000). Indeed, Cvl2 has been shown to interact with Bmp2, Bmp4 and Bmp7, as well as with Chordin and type I BMP receptors (Rentzsch et al., 2006; Ambrosio et al., 2008; Serpe et al., 2008; Zhang et al., 2010). Different mechanisms have been proposed for its activating effect on BMP signalling. In the posterior crossvein of the *Drosophila* pupal wing it is thought to act at short range to promote binding of BMPs to receptors (Serpe et al., 2008). In zebrafish, Cvl2 has been shown to act during gastrulation in BMP gradient formation by stabilising BMP signalling territories, at least partly by competing with Chordin for binding to BMPs (Rentzsch et al., 2006). That study also suggested that proteolytic cleavage converts Cvl2 from an anti- to a pro-BMP factor, with the cleaved form being predominantly present during zebrafish development. We now show that, at neurula stages, cvl2 expression recapitulates patterns of BMP activity and is also under BMP control. We further suggest a pro-BMP function of Cvl2 at these stages and demonstrate its requirement for BMP domain formation at the NPB, thus identifying a novel role for Cvl2 in NC development. Our experiments indicate that Cvl2 and BMP signalling act in a positive-feedback loop, and the non-diffusible nature of the BMP ligands probably contributes to this (Ramel and Hill, 2013). We propose that Cvl2 may locally enhance BMP activity in the discrete domains at the NPB via a mechanism analogous to that suggested to operate at the *Drosophila* posterior crossvein, ensuring robust levels of BMP activity while the territories of BMP signalling are being reshaped.

**The PPE is specified in the region that lacks BMP activity**

Previous work has indicated that PPE specification requires full inhibition of BMP signalling at the end of gastrulation (Kwon et al., 2010). Here we directly demonstrate the lack of BMP signalling in the PPE by DFISH. Using dlx3b as a marker for the PPE, we then reveal the process of separation of dlx3b and cvl2/mRFP-positive cells into distinct domains at the NPB. Taking advantage of the high resolution of DFISH, we carefully analysed how cvl2 and dlx3b expression changes from the end of gastrulation to the 1 Somite stage. cvl2 and dlx3b are expressed in similar domains during gastrulation. However, by the 1-Somite stage dlx3b and cvl2 are expressed in mutually exclusive domains. This separation occurs as the cells converge dorsally as a result of gastrulation movements (Schier and Talbot, 2005). We could not definitively determine whether cvl2 and dlx3b are co-expressed in the same cells or are expressed uniquely in intermingled cells. However, the observation that the formation of the distinct dlx3b and cvl2 expression domains is very rapid (~1 hour) would support a mechanism whereby intermingled cells are separated by cell sorting, with dlx3b-expressing cells migrating slightly more dorsally than the cvl2-expressing cells. It will be important in the future to determine how this is achieved.

**Expression of Bambi-b under control of Dlx3b/4b inhibits BMP activity in the PPE**

Our DFISH analysis indicated rapid clearance of BMP activity from dlx3b-expressing cells by the 1 Somite stage. This suggested that active inhibition of BMP signalling occurs in the dlx3b-positive cells and hence predicted the expression of a cell-autonomous BMP inhibitor in the PPE. We identified Bambi-b as an excellent candidate to fulfil this role. We have shown that bambi-b morphants lack the inner domain of cvl2/BMP activity and thus display a similar phenotype to dlx3b/4b morphants. This strongly suggested
that *bambi-b* expression is regulated by *dlx3b/4b*, which we were able to demonstrate. However, *Dlx3b* expression alone is not sufficient to induce *bambi-b* transcription, as *bambi-b* mRNA can only be detected from bud stage onwards, whereas *dlx3b* is already expressed during gastrula stages. Moreover, *bambi-b* is not only present in the PPE, but also in the non-neural ectoderm, which does not express *dlx3b*, suggesting that *bambi-b* transcription is subject to a different mode of regulation in these cells.

**A two-step model for controlling cell fate decisions during ectodermal patterning**

Differential tissues require distinct levels of BMP signalling at specific times during ectodermal patterning. NC requires intermediate to low levels of BMP signalling for induction (Schumacher et al., 2011) and, as we show here, continues to require BMP activity for its maintenance. PPE, by contrast, only requires BMP activity during its inductive phase, but is devoid of BMP signalling after gastrulation (Kwon et al., 2010). The data that we present here provide direct evidence for a two-step model of ectodermal patterning (Fig. 7). We show that the early BMP DV gradient that is present during gastrulation is remodelled into distinct BMP signalling domains at the NPB. We demonstrate active BMP signalling in the NC and at the epidermal border, whereas BMP activity is completely inhibited in the intervening PPE. The ligand responsible for the inner BMP activity domain is Gdf6a, and the activity is reinforced in this domain by coincident expression of Cvl2. The ligands responsible for the outer BMP activity are Bmp2b, Bmp7a and Bmp6. Again, our data indicate a crucial role for Cvl2 acting in a positive-feedback loop to locally enhance BMP activity at the epidermal border. We show that the region between these two domains of BMP activity is specified as the PPE, which expresses the homeobox transcription factor Dlx3b. We demonstrate that Dlx3b is responsible for inducing the expression of the BMP inhibitor Bambi-b, thereby allowing BMP repression in the presumptive PPE. Thus, our model suggests that the dynamic expression of BMP inhibitors and activators explains the differential requirements of NC and PPE for BMP activity at different developmental stages.

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**Competing interests statement**

The authors declare no competing financial interests.

**Author contributions**

S.R. and C.S.H. designed the research. S.R. performed the experiments with additional help from R.A.R. S.R. and C.S.H. analysed the data and wrote the paper.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.098707/-/DC1

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