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BMP signaling orchestrates photoreceptor specification in the zebrafish pineal gland in collaboration with Notch

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SUMMARY

A variety of signaling pathways have been shown to regulate specification of neuronal subtype identity. However, the mechanisms by which future neurons simultaneously process information from multiple pathways to establish their identity remain poorly understood. The zebrafish pineal gland offers a simple system with which to address questions concerning the integration of signaling pathways during neural specification as it contains only two types of neurons – photoreceptors and projection neurons. We have previously shown that Notch signaling inhibits the projection neuron fate. Here, we show that BMP signaling is both necessary and sufficient to promote the photoreceptor fate. We also demonstrate that crosstalk between BMP and Notch signaling is required for the inhibition of a projection neuron fate in future photoreceptors. In this case, BMP signaling is required as a competence factor for the efficient activation of Notch targets. Our results indicate that both the induction of a photoreceptor fate and the interaction with Notch relies on a canonical BMP/ Smad5 pathway. However, the activation of Notch-dependent transcription does not require a canonical Smad5-DNA interaction. Our results provide new insights into how multiple signaling influences are integrated during cell fate specification in the vertebrate CNS.

KEY WORDS: Notch, Bone morphogenetic proteins, Signal integration

INTRODUCTION

Cell fate diversification is a prerequisite for the formation of a functional vertebrate nervous system and a growing number of mechanisms have been described concerning the specification of neuronal subtype identity. In one such mechanism, gradients of signals, or morphogens, pattern the neural tube along the dorsoventral and anteroposterior axis. Among these morphogens, BMPs (bone morphogenetic proteins) secreted from the ectoderm and the dorsal-most compartment of the neural tube (the so-called roof plate) play an instrumental role in the specification of the dorsal cell types. BMPs are TGFβ (transforming growth factor β) molecules that bind to heterotetramers of type I and type II serine-threonine kinase receptors. Upon ligand binding, the type I receptors phosphorylate the Smad 1/5/8 effectors that, together with Smad4, enter the nucleus to activate transcription (for a review, see Liu and Niswander, 2005). The combined activities of dorsal (BMPs and Wnt) and ventral morphogens (such as sonic hedgehog) define progenitor domains that express different combinations of transcription factors belonging to the homeodomain and bHLH (basic helix-loop-helix) families. Within these progenitor domains, cells choose between several distinct possible identities (for a review, see Wilson and Maden, 2005). However, although the mechanisms that establish progenitor domains are beginning to be deciphered, it is not yet clear what triggers the definitive neuronal subtype choice within a given progenitor domain. Recently, the Notch pathway has been implicated in binary choices within specific progenitor domains in the vertebrate spinal cord. In these contexts, Notch has been proposed to act as a binary switch in that it is instructive for one fate and inhibitory for the other (Batista et al., 2008; Cau and Blader, 2009; Del Barrio et al., 2007; Peng et al., 2007; Shin et al., 2007).

The zebrafish epiphysis or pineal gland provides a powerful model for the study of cell fate choice, owing to its simplicity. Indeed, the epiphysial vesicle, which is located in the dorsal diencephalon, contains only two types of neurons: photoreceptors (PhRs) and projection neurons (PNs) (Masai et al., 1997). Previous studies have shown that gradients of BMP and Wnt activity are responsible for the positioning of the presumptive pineal territory along the dorsoventral and anteroposterior axis, respectively, leading to the restricted expression of the homeodomain transcription factor *flh* (Barth et al., 1999; Masai et al., 1997). Flh defines the progenitor domain of the pineal gland and is required for the expression of the bHLH transcription factors Ascl1a and Ngn1, which are, in turn, necessary for neuronal production. However, neither of these proneural factors is required for the establishment of neuronal identity (Cau and Wilson, 2003; Masai et al., 1997).

We have previously shown that activation of the Notch pathway inhibits the PN fate but is insufficient to promote the PhR fate. This led us to propose a model in which the specification of PhRs relies on two events: inhibition of a PN program by Notch on the one hand and the induction of a PhR fate by an unknown PhR inducing signal on the other (Cau and Blader, 2009; Cau et al., 2008). Here, we show that BMP signaling plays an important role as a PhR-inducing signal. We also show that BMP signaling acts as a competence factor for Notch inhibition of PN identity and that both roles of BMP rely on a canonical Smad-dependent pathway. Finally, although a canonical BMP/Smad pathway is required for an efficient Notch-induced transcriptional response, surprisingly this activity does not appear to require a Smad/DNA interaction.

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Based on these results, we propose a model in which BMP signaling activates the appropriate genetic program for PhR specification, while crosstalk between the Notch and the BMP pathways ensures the simultaneous inhibition of PN traits in the same cells.

MATERIALS AND METHODS

Cloning of the dominant negative and constitutive active form of the BMP receptor 1a

The CMV promoter/enhancer of pCS2:CFP (a gift from Dr U. Strähle, Karlsruhe Institute of Technology, Germany) was replaced by a PCR fragment containing the previously described *hsp70* promoter (Halloran et al., 2000). Subsequently, the extracellular and transmembrane domain of the human BMP receptor type 1a (BMPR1a) was amplified by PCR and the resulting fragment inserted in frame immediately upstream of the CFP-coding region (ten Djike et al., 1993). The hsp70:dnBMPR1a-CFP cassette was then transferred into *p*I-*Sce*I which has been described as enhancing the frequency of transgenesis (Thermes et al., 2002).

The constitutive active form of the Bmp receptor 1a (Nikaido et al., 1999) was PCR amplified with the primers 5′-CGCGATCGAT-CAATTTGACAATGCGTCAGC-3′ and 5′-CCGGGGATCCGATTT-TAATGTCTTGAG-3′, and then cloned into the pME-MCS vector using *Cla*I and *Bam*HI sites. The following oligos: 5′-ATCGTACCCTTAC-GACGTGCCTGACTACGCTTGAT-3′ and 5′-TAGATCAAGCG-TAGTCAGGCACGTCGTAAGGGTAC-3′ were hybridized as to generate a double-stranded oligo coding for an HA tag. This tag was cloned in frame to the C terminus end of the receptor using *Bam*HI/*Xba*I sites. We used the tol2 kit to generate a UAS:ca-Bmpr1a-HA transgene flanked with Tol2 terminal inverted repeats and containing the transgene marker cmlc2:egfp-polyA (Kwan et al., 2007).

Generation of a stable transgenic line

100 pg of *p*I-*Sce*I hsp70:dnBMPR1a-CFP was injected into one-cell stage embryos. I-*Sce*I meganuclease was co-injected with the DNA to maximize the number of integration events (0.5× I-*Sce*I buffer, 10% I-*Sce*I enzyme). The F1 generation was screened for heat shock-inducible CFP fluorescence. In this manner, three independent transgenic founder fish were identified displaying varying levels of germline transmission to their offspring.

To generate stable transgenic lines, Tol2-based constructs were co-injected with transposase into one-cell staged embryos according to standard procedures (Kwan et al., 2007). Embryos expressing GFP in their heart were identified at 2 dpf and then transferred into the fish facility until sexual maturity. Individual founder fish were crossed with *Tg(hs:Gal4)* transgenic fish for examination of ventralized embryos in the offspring. A founder fish containing a functional constitutive active form of Bmp receptor 1a, as well as a GFP-positive heart was outcrossed to establish the stable *Tg(UAS:caBmpr1a)* transgenic line.

Strains and developmental conditions

Embryos were reared at 28.5°C and staged according to standard protocols (Kimmel et al., 1995). *Tg(flh:GFP)*, *Tg(HuC:GFP)*, *Tg(AANAT2:GFP)*, *Tg(hsp70:noggin3)*, *Tg(hsp70:bmp2b)*, *Tg(hs:Gal4)*, *Tg(UAS:Nintra)* and *Tg(TP1:GFP)* transgenic lines have been described previously (Chocron et al., 2007; Concha et al., 2003; Gothilf et al., 2002; Park et al., 2000; Parsons et al., 2009; Scheer et al., 2001). The conditions of heat shock were as follows: *Tg(hs:Gal4); Tg(UAS:caBmpr1a)*, 30 minutes at 39°C; *Tg(hs:dnBmpr1a-CFP)*, *Tg(hsp70:noggin3)* and double *Tg(hsp70:noggin3); Tg(hs:dnBmpr1a-CFP)* transgenic embryos, 1 hour at 38°C; and *Tg(hsp70:bmp2b)*, 30 minutes at 37°C. *Tg(hs:dnBmpr1a-CFP)+/–* transgenic embryos were identified using CFP fluorescence; *Tg(hsp70:noggin3)+/–* embryos were genotyped using a Flag antibody as well as morphological features (Chocron et al., 2007). *Tg(hsp70:bmp2b)+/–* embryos were identified morphologically owing to the small eye-size induced by the transgene or using a Flag antibody (Chocron et al., 2007). *Tg(hs:Gal4); Tg(UAS:caBmpr1a)* double heterozygous embryos were either identified using morphological criteria or genotyped by PCR. Details of the procedure are available upon request.

bmp2a MO (5′-TGGACGAGACCATGATGATCTCTGC-3′), *bmp2a* MOII (5′-AACCGGACAGATCACTGACGAAGGA-3′) and *bmp2a*-mismatch (5′-TGGACCACACCATCATCATCTCTCC-3′) were injected at 12.5 mg/ml, 2.5 mg/ml and 0.83 mg/ml, respectively. Sequence and conditions for the use of the *smad5* MO have been described previously (McReynolds et al., 2007).

To perform DAPT treatments, embryos were raised in embryo medium containing DAPT (Calbiochem) at 100 μM and DMSO (1%), as previously described (Geling et al., 2002). Control embryos were incubated in an equivalent concentration of DMSO. Transplantation was performed as previously described (Masai et al., 1997).

Birthdating of neurons with 5-bromo-2-deoxyuridine

Embryos were incubated in embryo medium with 10 mM BrdU and 8% DMSO for 20 minutes on ice followed by 2 hours at 28.5°C. BrdU incorporation was detected by immunohistochemistry using an anti-BrdU antibody (G3G4, 1/1000, Developmental Studies Hybridoma Bank).

In situ hybridization

In situ hybridization was performed as described previously (Cau et al., 2008). The following antisense riboprobes were used: *neurod1* (Blader et al., 1997), *otx5* (Gamse et al., 2002), *her4* (Pasini et al., 2004), *her15* (Shankaran et al., 2007) and *bmp2a* (IMAGE SPCLONE number 9037347). Finally, a partial *her2* cDNA was cloned using the following oligonucleotides: 5′-CGCGGAATTCATGCGCAGAGATCGCATC-3′ and 5′-CGCGCAATTGCACAATCCATGCTTGGCG-3′. The resulting cDNA was used as a template.

Immunostaining

Antibody staining was performed as previously described (Cau et al., 2008). The rabbit Phospho-Smad1/5/8 antibody (α-PSmad 1/5/8) was used at a 1/100 dilution (Cell Signaling). For anti-Phospho-Smad1/5/8 staining, all solutions were supplemented with phosphate disodium salt hydrate at 50 mg/ml (Sigma). To reveal transplanted cells after transplantation experiments, we used either Streptavidine-TRITC (1/50) or Streptavidine-Alexa 647 (1/100) (Molecular Probes).

Image acquisition and counts

Confocal acquisition was performed using a Leica (SP5) and ImageJ software was used for cell counting. For each condition a minimum of five embryos was analyzed.

RESULTS

BMP activity is necessary for PhR specification

Our previous work on pineal neuron specification led us to postulate the existence of a PhR-inducing signal (Cau and Blader, 2009; Cau et al., 2008), which we began to search for using a candidate approach. To examine whether the BMP signaling pathway is involved in the production of PhRs, we generated a transgenic line carrying a heat shock-inducible, dominant-negative form of Bmpr1a, *Tg(hs:dnBmpr1a-CFP)*, to reduce BMP activity. This dominant-negative form of Bmpr1a lacks the intracellular kinase domain of the protein and is therefore expected to be unable to trigger Smad phosphorylation. A similar line has previously been shown to induce a strong reduction of BMP activity when misexpressed in zebrafish embryos (Pyati et al., 2005). Using this tool, we analyzed the effects of reducing BMP activity on general neuronal production, using an antibody against islet 1 (Isl1), as well as on the production of specific neuronal subtypes: PhRs [using the *Tg(AANAT2:GFP)* transgene] and PNs [using the *Tg(HuC:GFP)* transgene] (Cau et al., 2008). When induced at 9 or 12 hours post-fertilization (hpf), reduction of BMP activity leads to a reduction of the total number of Isl1+ neurons (see Fig. S1A in the supplementary material). The decrease in the number of neurons affects both PhRs and PNs, as judged by the expression of the PhR-specific and PN-specific transgenes and markers (see Fig. S1B,C

in the supplementary material). Birthdating experiments suggest that at these early stages BMP activity is required for the proliferation of pineal progenitors (see Fig. S1D,E in the supplementary material). To circumvent the effect of BMP on proliferation, we induced the *Tg(hs:dnBmpr1a-CFP)* transgene at later stages (from 14 hpf to 22 hpf). Interestingly, although at these later stages the reduction of BMP activity no longer affected the total number of neurons formed, it reduces the number of PhR cells, as judged by the expression of *Tg(AANAT2:GFP)* and a second PhR marker, FRet43 (Fig. 1C,D; see Fig. S1A,B and Fig. S2A in the supplementary material). This reduction of PhR numbers is not caused by cell death, as judged by the expression of activated-caspase 3 (see Fig. S1F in the supplementary material).

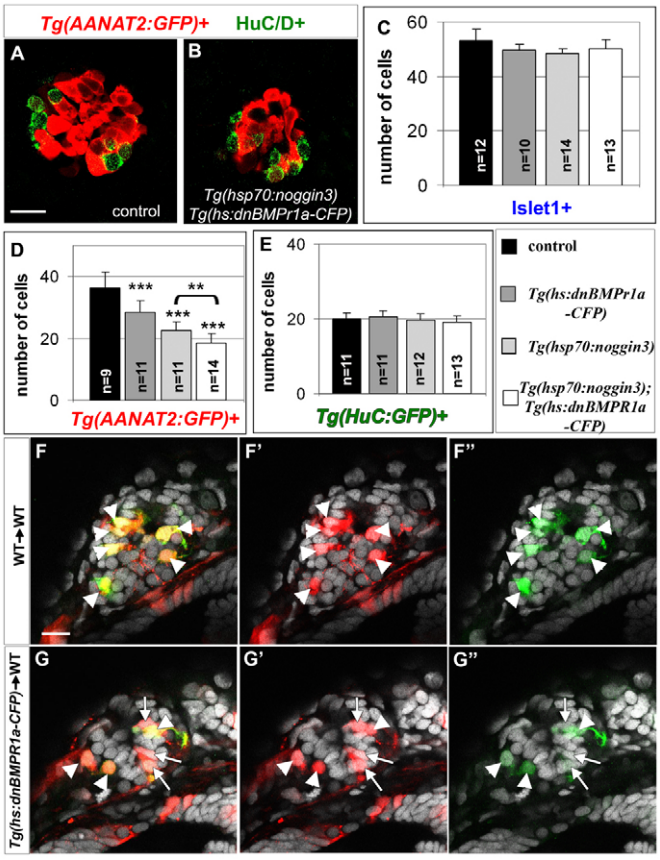

Fig. 1. BMP activity is necessary for PhRs specification. (**A**,**B**) Confocal sections of control (A) and *Tg(hs:dnBmpr1a-CFP);Tg(hsp70:noggin3)* double transgenic (B) embryo. Pineal glands are double labeled with the *Tg(AANAT2:GFP)* transgene (in red) and a HuC/D antibody (in green) at 48 hours. Anterior is upwards. Scale bar: 16 μm. (**C-E**) Average numbers of Isl1+ neurons, *Tg(AANAT2:GFP)*+ and *Tg(HuC:GFP)*+ cells per pineal gland in control, *Tg(hs:dnBmpr1a-CFP)* transgenic, *Tg(hsp70:noggin3)* transgenic and *Tg(hs:dnBmpr1a-CFP);Tg(hsp70:noggin3)* double-transgenic embryos at 48 hours. Error bars represent s.d. $^{**}P<0.001$; $^{***}P<0.0005$ using a *t*-test. The number (*n*) of embryos analyzed is noted for each case. (**F-G″**) Confocal images of the pineal gland of wild-type host embryos that have received cells transplanted from wild type (F-F″) or *Tg(hs:dnBmpr1a-CFP)* (G-G″) donors. *Tg(AANAT2:GFP)*+ PhRs are in green and the transplanted cells are shown in red. White arrowheads show transplanted cells with a PhR identity; white arrows indicate transplanted cells not expressing GFP from the *Tg(AANAT2:GFP)* transgene.

In addition, upon 'late' heat shock (16 hours) the percentage of PhRs that have not yet exited their last S-phase at 19 hours is similar in *Tg(hs:dnBmpr1a-CFP)* transgenic embryos when compared with wild-type siblings (see Fig. S1D in the supplementary material), which suggest that the multiplication of PhR progenitors is not affected. We conclude that a reduction of BMP activity induced at later stages causes a defect in the specification of PhRs. To analyze whether this diminution in the number of PhRs is translated into a corresponding increase in the number of PNs, we looked at the expression of the PN markers HuC/D+, *Tg(HuC:GFP+)* and *lhx3*+; we also looked at the expression of Pax6, but as Pax6 is expressed both in PNs and neural progenitors, we used a double immunohistochemistry against Pax6 and Isl1 to distinguish between Pax6+ PNs and Pax6+ neural progenitors (Cau et al., 2008). As the number of PNs remains unchanged with all these markers (Fig. 1E; see Fig. S1C and Fig. S2B-D in the supplementary material), we conclude that, upon late reduction of BMP activity, a proportion of Ist1+ neurons apparently fail to acquire either a PhR or PN identity by 48 hours.

To confirm our results, we repeated these loss-of-BMP signaling experiments using the previously described *Tg(hsp70:noggin3)* transgenic line; Noggin3 is an endogenous BMP antagonist (Chocron et al., 2007). Furthermore, we analyzed the activities of the *Tg(hsp70:noggin3)* transgene either alone or in combination with the *Tg(hs:dnBmpr1a-CFP)*, after heat shock at 16 hours. In this manner, we found that double-transgenic embryos show a stronger phenotype compared with each individual transgene in terms of the number of PhRs (Fig. 1A,B,D; see Fig. S2A in the supplementary material), a result that is consistent with a stronger inactivation of the BMP pathway as judged by anti-phospho Smad1/5/8 (α-PSmad 1/5/8) staining (see Fig. S3A-D′ in the supplementary material). As for the single transgenic lines, embryos carrying both constructs displayed no effects on the total number of neurons or on the specification of PNs after induction at late stages (Fig. 1C,E; see Fig. S2B-D in the supplementary material).

To identify BMP ligands involved in PhR specification, we screened an expression pattern database for BMPs transcribed in the presumptive pineal territory (http://zfin.org). *bmp2a* appeared to be the only candidate BMP expressed in the pineal anlagen and its onset of expression immediately precedes α-PSmad 1/5/8 immunoreactivity (Fig. 2A-H′). To address the function of *bmp2a*, we knocked down its activity using an antisense morpholino approach. Interestingly, although *bmp2a* morphant embryos exhibit a decrease in the number of PhRs, no effect on the number of PNs or on the total number of post-mitotic neurons was detected (Fig. 2I-O). This *bmp2a* morphant phenotype was confirmed using a second non-overlapping morpholino (*bmp2a* MO II), while no detectable phenotype was obtained upon injection of a mismatch-morpholino (see Fig. S4A,B in the supplementary material). Finally, we tested whether BMP activity on PhR fate depends on a canonical Smad-dependent pathway. Smad5 is involved in the transduction of canonical BMP signaling (Liu and Niswander, 2005) and is expressed in the pineal anlagen (http://zfin.org). However, complete knock down of Smad5 activity has a strong effect on the establishment of the general DV axis of the embryo, as indicated in studies using the antimorphic *smad5* mutant *somitabun* (Barth et al., 1999). We, thus, reasoned that it would not be possible to use a complete loss of *smad5* function in our studies. As such, we used a low dose of a *smad5* morpholino (McReynolds et al., 2007) in order to produce normally patterned embryos. Nonetheless, embryos injected with low doses of *smad5* MO show

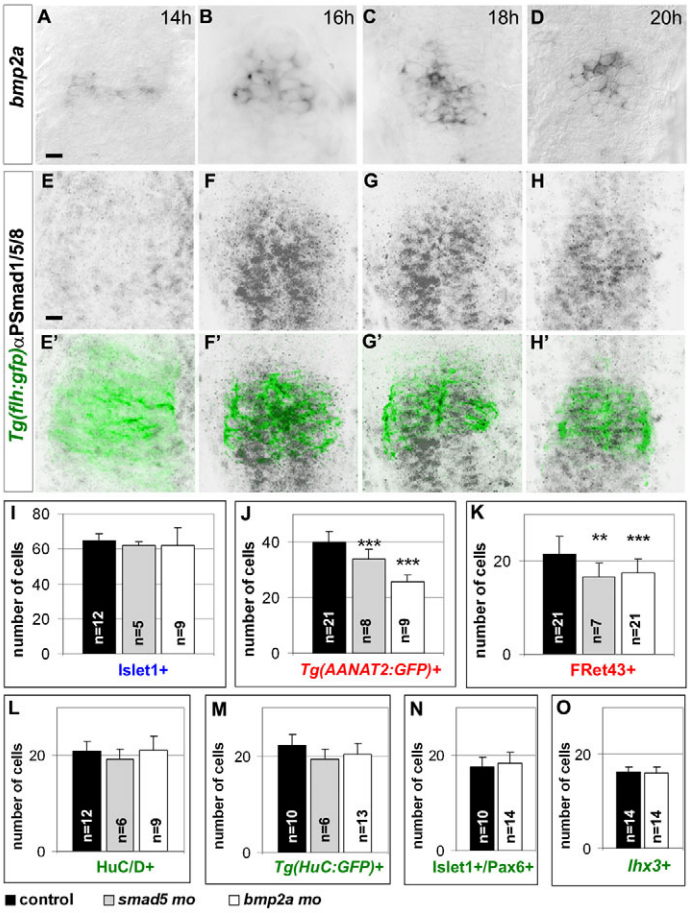

Fig. 2. Bmp2a is required for PhR specification.
(**A-D**) Expression of *bmp2a* in the wild-type pineal at 14, 16, 18 and 20 hours. (**E-H′**) Activation of the BMP pathway as judged by labeling with an α-PSmad 1/5/8 antibody shown on confocal projections. The presumptive pineal territory is delineated by GFP expression from the *Tg(flh:gfp)* transgenic line (Concha et al., 2003). Anterior is upwards. Scale bars: 16 μm. (**I-O**) Average numbers of Isl1+ neurons, *Tg(AANAT2:GFP)*+, FRet43+, HuC/D+, *Tg(HuC:GFP)*+, Isl1+/Pax6+ and *lhx3*+ cells per pineal gland at 48 hours in control, *bmp2a* and *smad5* morpholino-injected embryos. The number (*n*) of embryos analyzed is noted for each case. Error bars represent s.d. $^{**}P<0.001$, $^{***}P<0.0005$ using a *t*-test.

a mild but consistent reduction in the number of PhRs, coherent with a role for Smad5 in the specification of pineal neurons (Fig. 2I-O). The reduction in the number of PhRs can be rescued by injecting synthetic *smad1* mRNA (see Fig. S4C in the supplementary material), suggesting that the morphant phenotype is specific to loss-of-function of Smads regulated by BMP signaling and ruling out the possibility of specific Smad5 versus Smad1 functions.

Finally, we asked whether BMP activity is required cell autonomously within pineal progenitors for them to adopt a PhR fate. For this, we transplanted either wild type, *Tg(AANAT2:GFP)* or *Tg(hs:dnBmpr1a-CFP); Tg(AANAT2:GFP)* cells into wild-type hosts, performed a heat shock at 16 hpf and analyzed the proportion of cells located in the pineal gland that adopted a PhR fate [as judged by the expression of the *Tg(AANAT2:GFP)* transgene]. In this manner, we obtained pineal gland containing two to 28 transplanted cells; no difference was observed in the numbers of transplanted cells found in the pineal using wild-type versus *Tg(hs:dnBmpr1a-CFP)* donors (P=0.145 using a *t*-test). Although 69.2% of wild-type transplanted cells located in the pineal gland adopt a PhR fate (92/133 cells from 10 embryos), only 36.8% of *Tg(hs:dnBmpr1a-CFP)* cells adopt this fate (45/122 cells from 13 embryos) (Fig. 1F-G″); the difference in the behavior of wild-type and *Tg(hs:dnBmpr1a-CFP)* cells was statistically significant ($P=2.4\times10^{-7}$ using a χ-square test). As cells impaired in their capacity to activate the BMP pathway adopt a PhR fate less efficiently than wild-type cells, we conclude that activation of the BMP pathway is required autonomously for PhR fate.

Altogether, these results suggest that activation of a canonical BMP/Smad5 pathway is cell-autonomously required within pineal progenitors to adopt a PhR fate and that Bmp2a is required for this specification activity.

BMP activity is sufficient to promote the PhR fate

We next addressed whether the BMP pathway is sufficient for the induction of a PhR fate. First, we used a *Tg(hsp70:Bmp2b)* line to examine the consequences of globally increasing ligand levels (Chocron et al., 2007). On induction, this transgene induces strong and ubiquitous activation of the BMP pathway, as measured by α-Psmad 1/5/8 immunoreactivity (see Fig. S3E-F′ in the supplementary material). Furthermore, *Tg(hsp70:Bmp2b)* embryos exhibit increased numbers of pineal neurons when heat shock was performed at 16 or 18 hours (Fig. 3D). Interestingly, however, all the additional neurons induced appear to adopt a PhR fate, as the number of PhRs is increased while the number of PNs is unchanged (Fig. 3A,B,E-I). Finally, when induced at 21 hours, a stage when the vast majority of pineal progenitors have exited their last S phase (Cau et al., 2008), induction of the *Tg(hsp70:Bmp2b)* transgene is still able to induce an increase in the number of PhRs without any change in the total number of Isl1+ neurons or in the number of PNs (Fig. 3D-I). We conclude that at this stage the BMP pathway is able to force Isl1+ neurons that have not yet chosen an identity to adopt a PhR fate.

We next generated a *Tg(UAS:caBmpr1a)* transgenic line to address the consequences of the misexpression of a constitutively active form of BMP receptor 1a (caBmpr1a) (Nikaido et al., 1999). *Tg(UAS:caBmpr1a)* transgenic carriers were crossed to

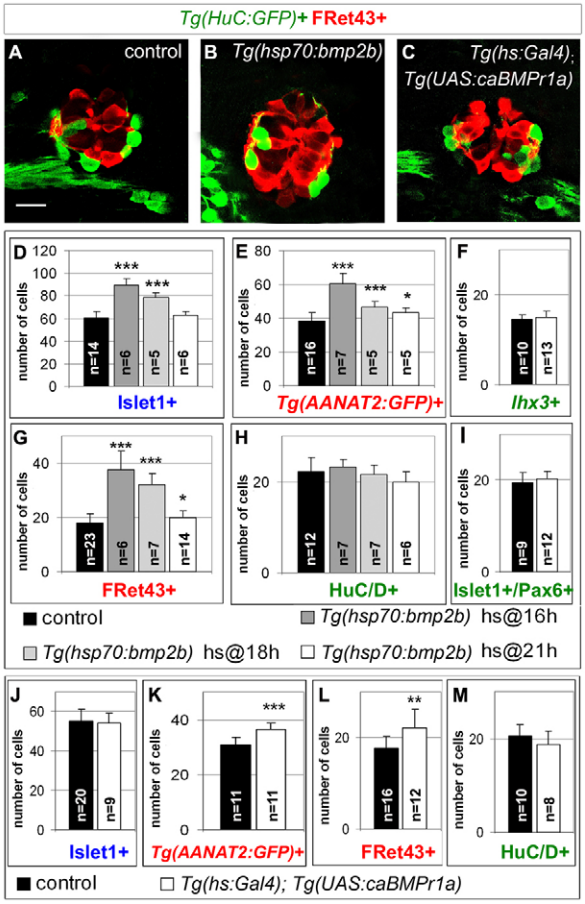

Fig. 3. BMP activity is sufficient to promote the PhR fate. (**A-C**) Confocal sections of control, *Tg(hsp70:bmp2b)* transgenic and *Tg(hs:Gal4);Tg(UAS:caBmpr1a)* double transgenic embryos at 48 hours. Pineal glands are double-stained for *Tg(HuC:GFP)* (in green) and FRet43 (in red). Anterior is upwards. Scale bar: 16 μm. (**D-I**) Average numbers of Isl1+ neurons, *Tg(AANAT2:GFP)*+, *Ihx3*+, FRet43+, HuC/D+ and Isl1+/Pax6+ cells in the pineal gland of control and transgenic *Tg(hsp70:bmp2b)* embryos heat-shocked at various stages and analyzed at 48 hours. In E and G, *Tg(hs:bmp2b)* embryos heat-shocked at 21 hours show a relatively mild but statistically significant effect (P=0.03 and P=0.037, respectively). (**J-M**) Average numbers of Isl1+ neurons, *Tg(AANAT2:GFP)*+, Fret43+ and HuC/D+ cells in the pineal gland of control and *Tg(hs:Gal4); Tg(UAS:caBmpr1a)* double-transgenic embryos at 48 hours. Heat shocks were performed at 16 hours. Error bars represent s.d. *P<0.05, **P<0.001, ***P<0.0005 using a t-test.

Tg(hs:Gal4) animals as to generate double transgenic embryos (Scheer et al., 2001). Heat-shocked *Tg(hs:Gal4);Tg(UAS:caBmpr1a)* transgenic embryos exhibited increased BMP activity in the pineal area, although the effect is more modest than that induced in *Tg(hsp70:bmp2b)* embryos (see Fig. S3E-G′ in the supplementary material). However, although this milder activation of the BMP pathway appears insufficient to trigger an increased number of Isl1+ neurons, it is still sufficient to promote an increase in the number of PhRs (Fig. 3A,C,J-M; see Fig. S5 in the supplementary material).

Altogether, these results suggest that ectopic activation of the BMP pathway, either via the misexpression of ligands or a constitutively active receptor, is sufficient to promote the PhR fate.

BMP activity controls transcriptional regulators of the PhR fate

To look further into the mechanism by which BMP signaling promotes PhR fate specification, we examined how loss- and gain-of-function of the pathway affects the expression of transcription factors important for PhR differentiation. The transcription factor Neurod1 (Neurod – Zebrafish Information Network) is the earliest pineal PhR-specific marker known to date (Fig. 4A-B′,E-H). In the retina of both zebrafish and mice, Neurod1 is required for the survival and differentiation of PhRs (Morrow et al., 1999; Ochocinska and Hitchcock, 2009; Pennesi et al., 2003); Neurod1 also regulates gene expression in the mouse pinealocytes, which are highly reminiscent of zebrafish PhRs (Munoz et al., 2007). Expression of *neurod1* in the pineal anlage is virtually abolished in *Tg(hs:dnBmpr1a-CFP);Tg(hsp70:noggin3)* and appreciably increased in *Tg(hsp70:bmp2b)* embryos 3 hours after transgene induction (Fig. 4M-P). The homeodomain transcription factor *otx5* is required in the zebrafish pineal gland for the establishment of circadian rhythms through its regulation of genes such as *rev-erb-α* (Gamse et al., 2002; Nishio et al., 2008). We observed that the onset of *otx5* expression is concomitant with that of *neurod1* and that *otx5* is mainly expressed in PhRs, although a few PNs express this gene at 24 hours but not at 48 hours (Fig. 4C-D′,E-L; data not shown). Nonetheless, as for *neurod1*, *otx5* expression is significantly reduced in *Tg(hs:dnBmpr1a-CFP);Tg(hsp70:noggin3)* and augmented in *Tg(hsp70:bmp2b)* embryos (Fig. 4Q-T). These results suggest that BMP signaling acts to promote PhR identity through the regulation of transcription factors important for various aspects of PhR differentiation.

BMP is required for Notch-induced inhibition of the PN fate

The correct specification of the PhR fate requires the simultaneous activation of a PhR program by BMP (this study) and the inhibition of a PN fate by the Notch signaling pathway (Cau et al., 2008). We hypothesized that these two pathways could interact such that their activation always occurs in the same cells. To address this possibility, we tested whether Notch signaling regulates BMP activity using *Tg(hsp70:bmp2b)* embryos raised in the γ-secretase inhibitor DAPT. Under such conditions, the number of *Tg(AANAT2:GFP)*+ cells was not significantly different from that of mock-treated *Tg(hsp70:bmp2b)* embryos, indicating that loss of Notch has no effect on the ability of BMP signaling to promote the PhR fate (see Fig. S6C in the supplementary material). Next, we tested whether inhibition of the PN fate by mild misexpression of the intracellular domain of Notch requires BMP signaling. Remarkably, although *bmp2a* knockdown has no effect on PN numbers in wild-type embryos, it significantly rescues their development in the double *Tg(hs:Gal4);Tg(UAS:Notch^{intra})* transgenic context (Fig. 5A-D). Similarly, knock down of *smad5* partially rescues Notch-mediated inhibition of the PN fate (Fig. 5E). Thus, canonical BMP/Smad signaling not only acts to promote PhR identity but also interacts with Notch signaling during the repression of the PN fate. Furthermore, as loss-of-BMP function rescues the effects of misexpression of Notch^{intra}, the two pathways must interact downstream of the activation of Notch.

BMP regulates the competence of Notch targets to respond to Notch^{intra}

One possibility to explain how BMP signaling interacts with the Notch pathway downstream of the release of Notch^{intra} might be that it is required for an efficient Notch transcriptional response. To

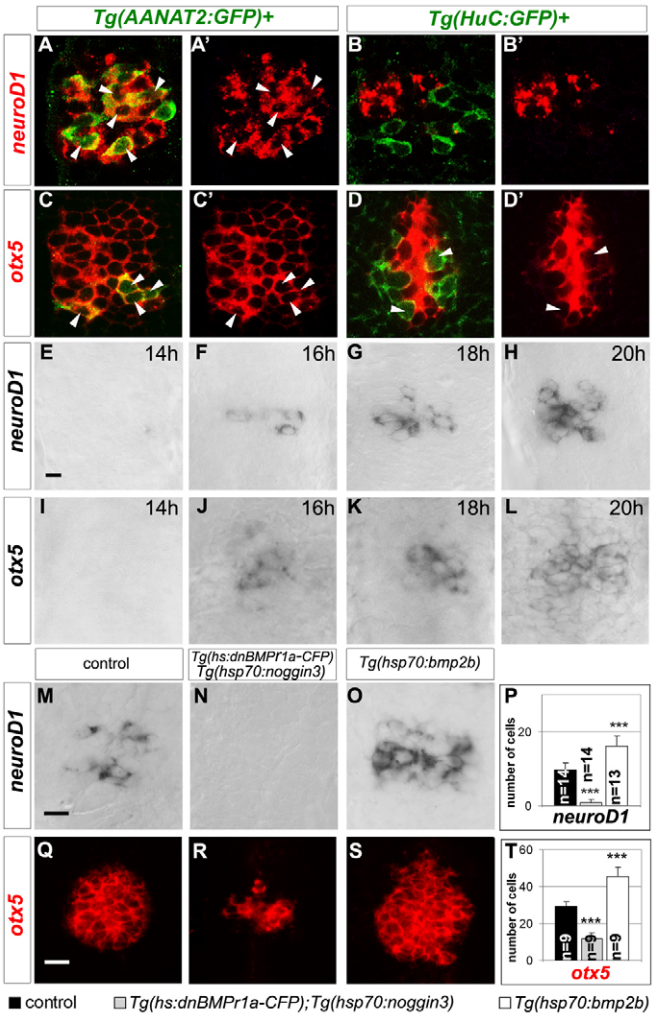

Fig. 4. BMP activity is necessary and sufficient for the expression of regulators of the PhR fate. (**A-D′**) Confocal sections of pineal glands, showing expression of *neurod1* or *otx5* (in red) and GFP (in green) from *Tg(AANAT2:GFP)* (A,A′,C,C′) and *Tg(HuC:GFP)* (B,B′,D,D′) transgenic embryos at 24 hours. White arrowheads indicate double-labeled cells. *neurod1* and *Tg(AANAT2:GFP)* are co-expressed in the majority of cells, although single labeled cells are also present. The observation of *neurod1*+/*Tg(AANAT2:GFP)*– cells most probably reflects the earlier onset of expression for *neurod1* compared with that of the *Tg(AANAT2:GFP)* transgene (Gothilf et al., 2002). Conversely, *neurod1*–/*Tg(AANAT2:GFP)*+ could be PhRs that do not express *neurod1* during their life or alternatively more mature cells that have already turned off the gene. Similarly, although all *Tg(AANAT2:GFP)*+ cells are also *otx5* positive, a number of single-labeled *otx5*+ cells are observed, which is probably due to the early onset of *otx5* (see below) compared with the *Tg(AANAT2:GFP)* transgene. (**E-L**) Dorsal view of pineal glands stained for *neurod1* (E-H) or *otx5* (I-L) by in situ hybridization. Both genes start to be expressed at 16 hours. (**M-O**) Dorsal view of pineal gland from wild-type (M), *Tg(hs:dnBmpr1a-CFP)*; *Tg(hsp70:noggin3)* double transgenic (N) and *Tg(hs:bmp2b)* embryos (O) at 24 hours stained for *neurod1*. (**P**) Quantification of the data represented in M-O. (**Q-S**) Dorsal view of pineal glands from wild-type (Q), *Tg(hs:dnBmpr1a-CFP)*; *Tg(hsp70:noggin3)* double transgenic (R) and *Tg(hs:bmp2b)* embryos (S) at 24 hours stained for *otx5*. (**T**) Quantification of the data represented in Q-S. In M-T, *Tg(hs:dnBmpr1a-CFP); Tg(hsp70:noggin3)* transgenics were heat shocked at 16 hours, while *Tg(hs:bmp2b)* were heat shocked at 21 hours. The number (*n*) of embryos analyzed is noted for each case in P and T. Error bars represent s.d. ***P<0.0005 using a *t*-test. Anterior is upwards. Scale bars: 16 μm.

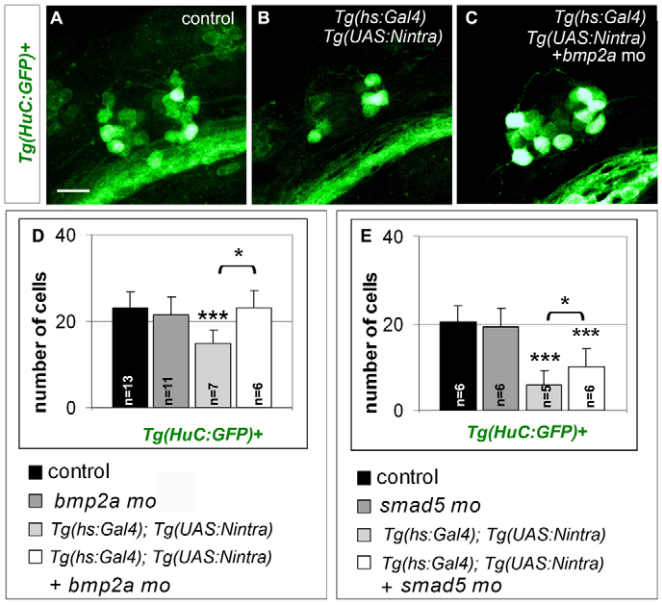

Fig. 5. BMP regulates the competence to respond to Notch. (**A-C**) Confocal projections of control (A), *Tg(hs:Gal4);Tg(UAS:Notch^{intra})* transgenic (B) and *Tg(hs:Gal4);Tg(UAS:Notch^{intra})* transgenic *bmp2a*-morphant (C) embryos at 48 hours. Heat shock was performed at 14 hours. The effects of the various treatments were monitored by GFP immunostaining in the *Tg(HuC:GFP)* background. (**D**,**E**) Average numbers of *Tg(HuC:GFP)*+ cells in 48 hour control, *Tg(hs:Gal4);Tg(UAS:Notch^{intra})* transgenic, *Tg(hs:Gal4);Tg(UAS:Notch^{intra})* transgenic *bmp2a*-morphant and *bmp2a*-morphant (D) embryos or *smad5*-morphant embryos (E). Heat shocks were performed at 14 hours. The number (*n*) of embryos analyzed is noted for each case in D and E. Error bars represent s.d. *P<0.05, ***P<0.0005 using a *t*-test. Anterior is upwards. Scale bar: 16 μm.

address this, we analyzed the expression of known Notch targets in the loss-of-BMP context. Members of the *her* (*hairy and enhancer of split related*) family of genes are regarded as canonical targets of Notch signaling activity (Davis and Turner, 2001). Although five *her* genes are expressed in the developing pineal gland, only *her2*, *her4* and *her15* appear to be controlled by Notch activity (Fig. 6A,B,D,E,H,I; see Fig. S7A in the supplementary material). We tested how reducing BMP activity affects the expression of these Notch targets in double *Tg(hsp70:noggin3);Tg(hs:dnBmpr1a-CFP)* transgenics and found that the expression of *her2*, *her4* and *her15* is significantly reduced (Fig. 6C,F,I; see Fig. S7A in the supplementary material). To test whether the effect of BMP on the expression of Notch targets is cell-autonomous, we transplanted wild-type and *Tg(hs:dnBmpr1a-CFP)* cells into wild-type hosts and analyzed the proportion of pineal cells that express *her4* at 24 hpf after a heat shock at 16 hpf. Although 59.9% of wild-type transplanted cells in the pineal gland express *her4* (97/162 cells from 16 embryos) only 36.8% *Tg(hs:dnBmpr1a-CFP)* cells do so (35/95 cells from 11 embryos) (Fig. 6M-N′). The difference between wild-type and *Tg(hs:dnBmpr1a-CFP)* cells in their ability to express *her4* was found to be statistically significant (P=1.1×10^{-31}, using a χ-square test) suggesting that the BMP pathway has a cell-autonomous effect on the activation of Notch targets.

The effect on *her* expression suggests that the BMP pathway synergizes with Notch during the transcriptional regulation of Notch targets. However, it is not clear whether this synergy requires

recruitment of BMP activated Smads to BMP response elements in Notch responsive loci or whether it involves the modulation of the transcriptional activity of Notch^{intra} alone. Upon activation of the Notch pathway, the intracellular domain of Notch is released from the cell membrane and translocates to the nucleus, where, together with its co-factor RBP-Jκ, it activates transcription. RBP-Jκ is the canonical DNA-binding factor responsible for Notch-induced transcriptional activity and, as such, the transgenic line *Tg(Tp1bglob:eGFP)* (in which eGFP is under the control of a synthetic promoter containing only RBP-Jκ-binding sites) has proven to be a faithful reporter for Notch-induced transcriptional activity (Parsons et al., 2009). *egfp* transcription driven from this synthetic promoter is robust and Notch dependent in the pineal gland (Fig. 6J,K). Interestingly, *egfp* expression from *Tg(Tp1bglob:eGFP)* was severely reduced in the loss-of-BMP context, including when *smad5* was knocked down (Fig. 6L; see Fig. S7B in the supplementary material). As the synthetic promoter in the *Tg(Tp1bglob:eGFP)* transgene does not contain Smad-binding sites, we conclude that BMP/Smad signaling is able to modulate the activity of Notch^{intra} without an activated Smad-DNA interaction being required.

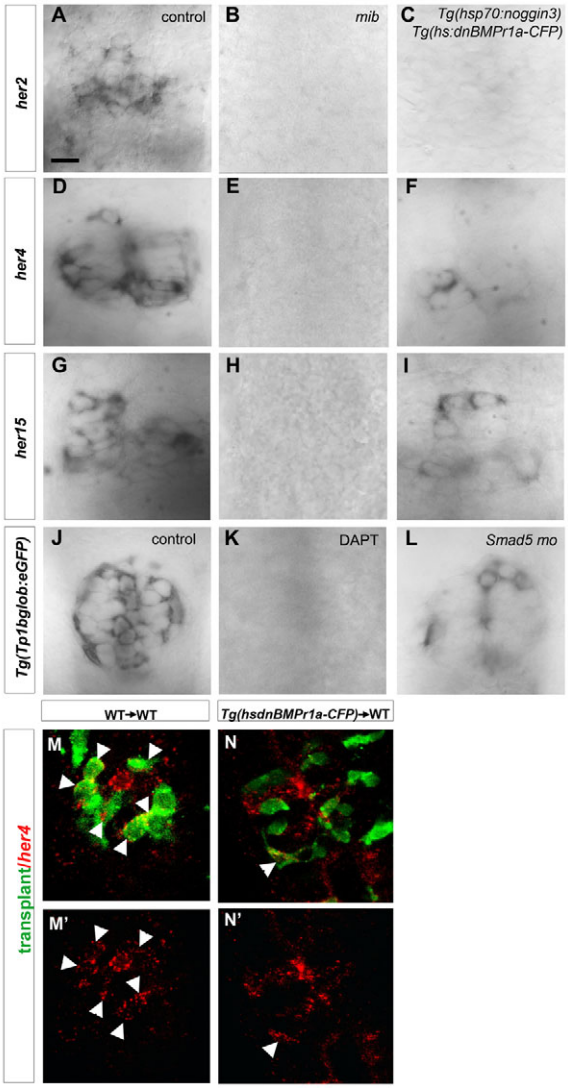

Fig. 6. BMP regulates Notch target gene expression. (**A-I**) Dorsal view of pineal glands showing expression of *her* genes in control (A,D,G), *mib* (B,E,H) and *Tg(hs:dnBmpr1a-CFP);Tg(hsp70:noggin3)* transgenic embryos (C,F,I) at 20 hours. For *Tg(hs:dnBmpr1a-CFP);Tg(hsp70:noggin3)* transgenic embryos, heat shocks were performed at 16 hours. (**J-L**) Dorsal view of pineal glands from mock-treated (J), DAPT-treated (K) or *smad5*-morphant (L) embryos. The effects of the various treatments were monitored by in situ hybridization against *gfp* transcripts in a Tg(Tp1bglob:eGFP) background. Anterior is upwards. Scale bar: 16 μm. (**M-N'**) Confocal sections of pineal glands of wild-type host having received cells transplanted from wild-type (M-M′) or *Tg(hs:dnBmpr1a-CFP)* donors (N-N′). Transplanted cells are shown in green, while expression of *her4* mRNA is shown in red. White arrowheads highlight transplanted cells expressing *her4*.

DISCUSSION

We have previously shown that Notch signaling is required for the repression of the projection neuron (PN) fate in the developing zebrafish pineal gland (Cau et al., 2008). In this study, we show that BMP signaling plays a dual role during the specification of the second neuronal type in the pineal gland, the photoreceptors (PhR). On the one hand, BMP activity is required to induce factors involved in PhR fate specification, while on the other it acts as a competence signal for the regulation of Notch-driven inhibition of a PN fate. Based on these results, we propose a novel model for the integration of BMP and Notch activities during the specification of neuronal subtype identity (see model in Fig. 7). Below, we discuss our results and this model.

A simple system relying on a complex network of signaling pathways

Our studies on Notch and BMP signaling during PhR/PN specification points to a situation that contrasts with previously described binary fate decisions (Batista et al., 2008; Cau and Blader, 2009; Cau et al., 2008; Del Barrio et al., 2007; Peng et al., 2007; Shin et al., 2007). Indeed, a striking feature of our system is that a reduction in the number of one cell type does not translate into an increased number of cells acquiring the alternative fate. For example, although impairing BMP activity reduces the number of PhRs, it does not result in an increase in the number of PNs. Several possibilities can be envisioned as to why the number of PNs is not increased in such conditions. First, even if BMP activity

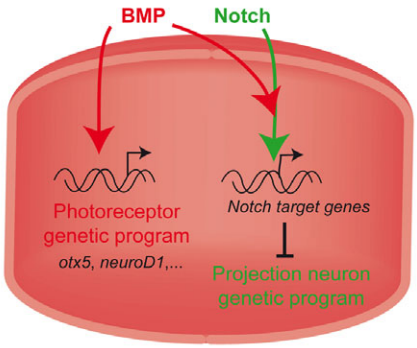

Fig. 7. Schematized interactions between Notch and BMP during the specification of pineal neurons. Specification of the PhRs (pink cell) involve both the activation of a PhR-specific program which is triggered by the BMP signaling pathway (in red) and the inhibition of a PN fate. This last event involves cooperation between Notch (in green) and BMP on target genes. Whether the action of BMP and its interaction with Notch occurs in a dividing or postmitotic cell remains to be determined.

is strongly diminished, a sufficient level of Notch activity could remain to inhibit the projection neuron fate. However, that the simultaneous inhibition of BMP and Notch activities does not result in an all-PN pineal gland (A.Q., P.B. and E.C., unpublished) argues against this explanation. It is, thus, more likely that specification of the PN fate requires a yet to be discovered PN-promoting signal. Similarly, decreasing the number of PNs using overexpression of Notch^{intra} does not induce an increase in the number of PhR, despite the number of Isl1+ neurons remaining constant (Cau et al., 2008). In light of results from our present study, we would now interpret this to mean that these naïve, identity-less neurons have not received a BMP signal or are not competent to respond to one and thus cannot take on a PhR fate.

Alternatively, the stability of the number of cells acquiring one fate in the context of a deficit in the second fate in the pineal gland could reflect the coexistence of multiple specification mechanisms acting in parallel, including those other than signaling pathways. One possibility, for example, is that the pineal gland has an early prepattern, which renders specific populations of neural progenitors more competent to adopt a PN or a PhR fate. In such a model, although some progenitors would be predisposed towards a PN fate, other progenitors would be more likely to adopt a PhR fate. This prepattern would be insufficient, however, to achieve the complete specification of pineal neural subtypes and would require fine-tuning via signaling interactions. In this manner, constitutive activation of the BMP pathway would be sufficient to force some neural progenitors towards a PhR fate, even if they were initially biased towards a PN fate; upon reduction of BMP activity, the prepattern effect would prevent the 'PhR-prepatterned' progenitors from adopting a PN fate. Finally, pineal neurons could auto-regulate their own numbers through feedback controls such as those described in the vertebrate retina (Gonzalez-Hoyuela et al., 2001; Kim et al., 2005; Poggi et al., 2005; Reh and Tully, 1986). Indeed, studies in the retina have shown that ablation of a specific cell type such as ganglion or amacrine cells biases neurogenesis towards the specification of the missing population (Gonzalez-Hoyuela et al., 2001; Poggi et al., 2005; Reh and Tully, 1986). Moreover, molecules such as GDF11, which inhibits ganglion cell specification, and NGF, which favors ganglion cell death, are both produced by ganglion cells, therefore providing a mechanistic explanation for this feedback (Gonzalez-Hoyuela et al., 2001; Kim et al., 2005). Such a mechanism allows for a precise control of the number of neurons of each population. Similarly, it is interesting to note that even in a wild-type pineal the number of PNs and PhRs are remarkably stable with a coefficient of variation (s.d./average) inferior to 0.2 (data not shown).

Molecular cross talk between Notch and BMP signaling

Gain of Notch activity in *Tg(hs:Gal4);Tg(UAS:Nintra)* double transgenic embryos is sufficient to inhibit the projection neuron fate; however, this activity is rendered much less efficient upon simultaneous reduction of BMP activity (Cau et al., 2008) (this study). BMP activity also seems necessary for the expression of the Notch target genes *her2*, *her4* and *her15*. Finally, BMP activity is required for the expression of a synthetic Notch-reporter transgene, TP1:GFP. Taken together, these results suggest that BMP regulates the competence of cells to respond to Notch through the co-regulation of Notch target genes. Such crosstalk represents an attractive mechanism by which simultaneous activation of the two pathways can promote the correct specification of photoreceptors (see model in Fig. 7). Importantly, the fact that BMP regulates the TP1:GFP transgene, which only contains binding sites for Notch transcriptional partner RBP-Jκ, suggests that BMP is able to regulate Notch-dependent transcription independently of Smad binding to DNA. However, this does not preclude that Smad-binding sites are required in the endogenous *her2*, *her4* or *her15* regulatory sequences or in other Notch targets.

How might BMP signaling modulate Notch-driven transcription independently of the binding of Smad to DNA? A previous study has shown that Smad1 and N^{intra} form complexes with the histone acetyltransferases p300 and p/CAF (Takizawa et al., 2003). Furthermore, activation of Smad1 by BMP receptors increases the recruitment of histone acetyltransferases to such complexes that are, in turn, instrumental for Notch-mediated transcription. Finally, the formation of such complexes was shown to contribute to the regulation of the murine *hairy* and *Enhancer of Split* related gene *Hes5* in neural cell cultures (Takizawa et al., 2003). Such a mechanism of crosstalk is probably not restricted to the cultured cells from the nervous system, as a co-regulation of Hes genes by both BMP and Notch signalling has also been observed in cell cultures of endothelial and myogenic origin (Blokzijl et al., 2003; Dahlqvist et al., 2003; Itoh et al., 2004). Our study provides evidence that a similar mechanism might be employed in an integrated in vivo context. Given this, we speculate that in the pineal gland complexes of histone acetyltransferases, Smads and N^{intra} are formed and that the recruitment of activated Smad is important for Notch-mediated transcription. This said, the activation of Notch and BMP signaling in cultured cells leading to the synergistic upregulation of *Hes5* to some extent involves binding of both RBP-Jκ and Smads to *Hes5* promoter elements (Takizawa et al., 2003). By contrast, the synergistic interaction between Notch and BMP signaling in the pineal apparently does not require Smad/DNA interactions. Whether these differences reflects subtle modulations of the activity of a Smad/Notch^{intra}/p300-like complex in an in vivo system or a fundamentally different mechanism is not clear.

Regulation of competence versus transcriptional integration

Establishment of cell fate relies on a combination of influences (signaling pathways, prepattern factors, etc.) that synergize or antagonize. The most common integration mechanism occurs at the transcriptional level. In such a mechanism, pre-pattern factors and transcriptional effectors of signaling pathways independently bind to the regulatory sequences of a key actor of cell fate specification (Barolo and Posakony, 2002; Flores et al., 2000; Halfon et al., 2000). This allows for the production of a transcriptional outcome, which is the sum of the negative and positive influences directly exerted on the regulatory sequences of this determinant of cell fate. In the nervous system, the best example is the *Drosophila* eye, where the simultaneous activities of the Runx transcription factor Lozenge, and the EGF and Notch signaling pathways are required for the expression of the cone cell fate regulator *D-pax2* (*sv* – FlyBase) (Flores et al., 2000; Fu and Noll, 1997). We propose that the co-regulation of *her* genes by BMP and Notch activities could represent a new integration mechanism whereby cell specification determinants would be regulated by distinct signaling pathways without a requirement for the binding of some of the effectors of these pathways to DNA.

Conclusion

Notch has previously been proposed to function as a binary switch during the choice between two cell fates in that it both promotes one fate while inhibiting the alternative fate. We have previously

shown that this simple idea does not hold during the choice between the PN and PhR fates in the zebrafish pineal gland, as Notch represses the PN fate but has no effect on PhR specification. Here, we show that BMP regulates both the expression of effectors of PhR specification and the competence for a Notch-driven inhibition of the PN fate, and that this later function is achieved through cooperation at the level of transcriptional targets. Future work will help in understanding whether crosstalk between the BMP and Notch pathways also occurs during other binary fate decisions.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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