

## RESEARCH ARTICLE

# RPE specification in the chick is mediated by surface ectoderm-derived BMP and Wnt signalling

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## ABSTRACT

The retinal pigment epithelium (RPE) is indispensable for vertebrate eye development and vision. In the classical model of optic vesicle patterning, the surface ectoderm produces fibroblast growth factors (FGFs) that specify the neural retina (NR) distally, whereas TGF $\beta$  family members released from the proximal mesenchyme are involved in RPE specification. However, we previously proposed that bone morphogenetic proteins (BMPs) released from the surface ectoderm are essential for RPE specification in chick. We now show that the BMP- and Wnt-expressing surface ectoderm is required for RPE specification. We reveal that Wnt signalling from the overlying surface ectoderm is involved in restricting BMP-mediated RPE specification to the dorsal optic vesicle. *Wnt2b* is expressed in the dorsal surface ectoderm and subsequently in dorsal optic vesicle cells. Activation of Wnt signalling by implanting Wnt3a-soaked beads or inhibiting GSK3 $\beta$  at optic vesicle stages inhibits NR development and converts the entire optic vesicle into RPE. Surface ectoderm removal at early optic vesicle stages or inhibition of Wnt, but not Wnt/ $\beta$ -catenin, signalling prevents pigmentation and downregulates the RPE regulatory gene *Mitf*. Activation of BMP or Wnt signalling can replace the surface ectoderm to rescue MITF expression and optic cup formation. We provide evidence that BMPs and Wnts cooperate via a GSK3 $\beta$ -dependent but  $\beta$ -catenin-independent pathway at the level of pSmad to ensure RPE specification in dorsal optic vesicle cells. We propose a new dorsoventral model of optic vesicle patterning, whereby initially surface ectoderm-derived Wnt signalling directs dorsal optic vesicle cells to develop into RPE through a stabilising effect of BMP signalling.

**KEY WORDS:** Cell fate, Eye, Gsk3 $\beta$ , Mitf, Optic vesicle patterning, Optic cup, Retinal development, Smad, Vsx2

## INTRODUCTION

During vertebrate eye development, the optic neuroepithelium is specified into three domains: retinal pigment epithelium (RPE), neural retina (NR) and optic stalk. Once these domain specifications have occurred, the surface ectoderm overlying the presumptive NR thickens to form the lens placode. Subsequently, the distal portion of the optic vesicle (ov) invaginates and a double-layered optic cup develops, connected to the brain by the optic stalk. The external layer of the optic cup forms the single-layered, pigmented RPE and the inner layer develops into the multilayered NR (reviewed by Fuhrmann, 2010).

Little is known about the cellular and molecular mechanisms that lead to the subdivision of the ov into an RPE and NR domain (reviewed by Adler and Canto-Soler, 2007; Fuhrmann, 2010). Members of the transforming growth factor  $\beta$  (TGF $\beta$ ) superfamily, the hedgehog (HH) and fibroblast growth factor (FGF) families appear to be involved in domain specification by modulating the expression of transcription factors (reviewed by Clegg et al., 2008; Fuhrmann, 2010). The boundaries between the RPE and NR domains are established by the *Microphthalmia-associated transcription factor* (*Mitf*) and the paired-like homeodomain transcription factor *Vsx2*, also known as *Chx10* (Rowan et al., 2004; Horsford et al., 2005). Disruption of the *Mitf* gene in mice leads to a hyperproliferating, unpigmented RPE (reviewed by Bharti et al., 2006). Besides MITF, members of the *orthodenticle-related family of transcription factors*, *Otx1/Otx2*, the paired domain protein *Pax6* and the homeodomain proteins *Vax1/Vax2* and *Lhx2* are involved in RPE development (reviewed by Bharti et al., 2006; Bharti et al., 2011; Bharti et al., 2012; Fuhrmann, 2010; Nishihara et al., 2012; Ou et al., 2013). In both mouse and chick, *Mitf* expression is observed in ov cells before NR commitment. In the mouse, the entire ov initially expresses *Mitf*, whereas in the chick *Mitf* expression is initially restricted to distal ov cells (Nguyen and Arnheiter, 2000; Müller et al., 2007; Ishii et al., 2009). FGFs released from the overlying surface ectoderm appear to repress RPE specification by inducing *Vsx2* expression (Horsford et al., 2005; Nguyen and Arnheiter, 2000). Once NR specification has occurred in the distal ov, *Mitf* expression becomes restricted to the dorsal ov (Nguyen and Arnheiter, 2000; Nakayama et al., 1998; Müller et al., 2007; Ishii et al., 2009). At this stage, the chick ov is divided into a dorsal RPE and ventral NR domain (Kagiya et al., 2005; Hirashima et al., 2008; Kobayashi et al., 2010).

Activins and BMPs belong to the TGF $\beta$  superfamily and appear to be involved in RPE specification (reviewed by Fuhrmann, 2010; Layer et al., 2010). Activin can substitute for the mesenchyme to induce/maintain RPE-specific gene expression in ov explants (Fuhrmann et al., 2000). During early eye development BMP application inhibits NR and optic stalk development, and instead induces RPE (Ohkubo et al., 2002; Hyer et al., 2003; Müller et al., 2007; Kobayashi et al., 2010). By contrast, inhibition of BMP signalling downregulates RPE-specific genes and results in loss of pigmentation (Adler and Belecky-Adams, 2002; Müller et al., 2007).

In several vertebrates, including humans, Wnt family members and their receptors are expressed during the initial stages of eye development, suggesting that Wnt signalling is involved in ov patterning. Overexpression of *Wnt2b* induces *Bmp7* expression and results in thinning of the NR (Ohta et al., 2011), whereas BMP application at ov stages induces *Wnt2b* and *Wnt8b* expression (Müller et al., 2007; Teraoka et al., 2009). By contrast, NR specification requires suppression of *Wnt8b* in the mouse (Liu et al., 2010). However, at the time the RPE and NR are specified, the

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Wnt/ $\beta$ -catenin signalling pathway is not active in ov cells (reviewed by Fuhrmann, 2010).

Although several growth factors and transcriptional regulators have been identified, we still do not know which tissues are involved in RPE specification and how the different signalling pathways integrate to regulate cell fate decisions within the ov. We previously proposed that the BMP-expressing surface ectoderm rather than the mesenchyme is involved in RPE specification (reviewed by Clegg et al., 2008; Layer et al., 2010). *Mitf* expression is induced in the distal ov just underneath the BMP-expressing surface ectoderm at a time when mesenchymal cells are absent (Müller et al., 2007). Here, we show that BMPs and Wnts are released from the surface ectoderm to specify the RPE. *Wnt2b* is expressed at the right time and place to be involved in directing dorsal ov cells towards an epithelial cell fate. We provide gain-of-function evidence that Wnt-mediated GSK3 $\beta$  inhibition elicits RPE development *in vivo*. Accordingly, interfering with Wnt signalling prevents MITF expression and hence RPE development. Finally, we show that BMPs and Wnts cooperate at the level of pSmad to induce MITF expression in ov cells. We present a new model in which Wnts, via a GSK3 $\beta$ -dependent but  $\beta$ -catenin-independent pathway, stabilise BMP signalling to specify the RPE in dorsal ov cells.

## RESULTS

### Molecular signals involved in optic vesicle patterning

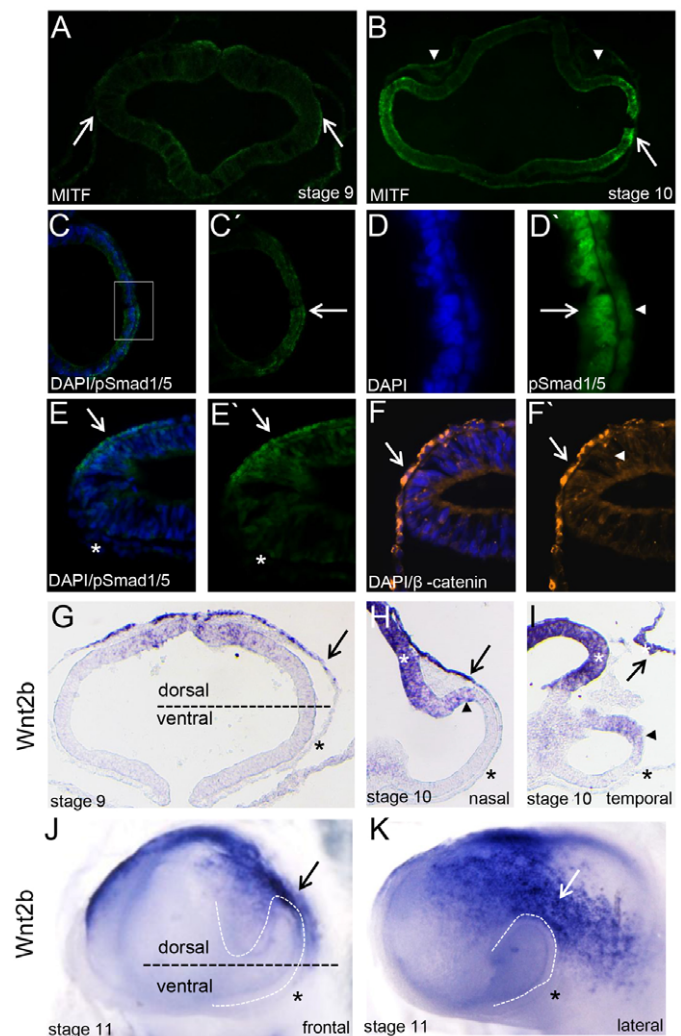
In the chick, *Mitf* transcripts were first detected in the distal ov underneath the BMP-expressing surface ectoderm at stage 9 (Müller et al., 2007), whereas MITF protein expression was not yet observed (Fig. 1A). At stage 10, MITF protein expression was restricted to the distal ov adjacent to the overlying surface ectoderm (Fig. 1B).

Activation of the BMP signalling pathway results in receptor-mediated phosphorylation (p) of Smad1, 5 and/or 8 (pSmad), an indicator for BMP signalling. We compared the distribution of pSmad and MITF. At stage 10, pSmad was strongly detected in the surface ectoderm and in the nuclei of the entire distal ov (Fig. 1C–D'). At stage 10/11, pSmad labelling appeared to be stronger in the dorsal ov (Fig. 1E,E'). Thus, at ov stages the pattern of pSmad correlates well with the *Mitf*/MITF expression pattern.

At stage 10/11, when the distal ov is divided into a dorsal *Mitf*- and ventral *Vsx2*-expressing domain, *Bmp4* and *Bmp7* transcripts are still detected throughout the surface ectoderm (Crossley et al., 2001; Müller et al., 2007). This suggests that another signal is involved in progressively restricting *Mitf* expression to the dorsal ov. The Wnt signalling pathway directs dorsal cell fate decisions during embryonic development (reviewed by Klaus and Birchmeier, 2008). Therefore, we next studied the expression patterns of various Wnt family members, such as *Wnt2b*, *Wnt3a* and *Wnt8b* (see also Garcia-Lopez et al., 2004; Rossi et al., 2007; Quinlan et al., 2009; Van Raay and Vetter, 2004). Of the analysed Wnt ligands, only *Wnt2b* transcripts were restricted to the dorsal surface ectoderm and dorsal ov at the time the RPE is specified (Fig. 1G–K). At this time the Wnt/ $\beta$ -catenin signalling is not active in ov cells (Fig. 1F,F'). Co-localisation of MITF and nuclear  $\beta$ -catenin was detected only in a few cells beginning at the late optic vesicle stage (stage 12/13; supplementary material Fig. S1A–D). Taken together, the spatial and temporal expression pattern of *Wnt2b* and  $\beta$ -catenin suggests that perhaps Wnt signalling, via a  $\beta$ -catenin-independent signalling pathway, is involved in RPE specification.

### The surface ectoderm is required for RPE specification

It has been supposed that the surface ectoderm does not play a role in RPE specification (Fuhrmann et al., 2000; Nguyen and Arnheiter,



**Fig. 1. Signals involved in early optic vesicle patterning in the chick.** (A) At stage HH 9, MITF protein is not detected in the ov (arrows). (B) At stage 10, MITF protein is present throughout the distal ov (arrow). Mesenchymal cells (arrowheads) are located dorsally. (C,C') At stage 10, pSmad1/5 is detected in the entire distal ov (arrow). (D,D') Higher magnification of C, showing pSmad1/5 labelling in neuroepithelial cells (arrow) and in the surface ectoderm (arrowhead). (E,E') Stronger accumulation of pSmad1/5 is observed in the dorsal ov (arrows) compared with the ventral region (asterisks). (F,F') Parallel section of E. Strong  $\beta$ -catenin labelling in the entire surface ectoderm/prospective lens ectoderm (arrow). Nuclear  $\beta$ -catenin is not observed in the ov (arrowhead). (G–I) *Wnt2b* expression during the initial stages of eye development. *Wnt2b* expression is restricted to the dorsal surface ectoderm (arrow), dorsal ov (arrowhead) and dorsal prosencephalon (white asterisks). *Wnt2b* transcripts are not detected in the ventral surface ectoderm and ov (asterisks). (J,K) Frontal and lateral view of a stage 10/11 chick head. *Wnt2b* transcripts in the ectoderm (arrows) covering the dorsal ov (white dashed line). Expression is not detected ventrally (asterisks). In all panels, dorsal is up.

2000). Because surface ectoderm removal in these studies was carried out after *Mitf* expression had been initiated, we removed the surface ectoderm before or at the time *Mitf* expression is initiated. One day following surface ectoderm removal at stages 7 to 9 (Fig. 2A,B), optic cup and lens formation was not observed (Fig. 2C–H', Fig. 3C,C'). Despite the presence of Pax7-positive neural-crest-derived mesenchymal cells, MITF protein expression and pSmad labelling was only weak or not detected (12/13 cases; Fig. 2E–H'; supplementary material Fig. S1F–J,Q–R").



Additional experiments confirmed that the surface ectoderm is required for RPE specification. Optic vesicles from stage 9 and 10 chick embryos were isolated, the surface ectoderm removed and transplanted back to the chicken embryo (Fig. 2I,J). Optic vesicles, where the surface ectoderm had been removed at stages 9 and 10 (ten somites), completely lacked pigmentation 3 days after the operation (3/4 cases, compare Fig. 2K-M). Some pigmentation was observed when the surface ectoderm was removed at late stage 10 (11 and 12 somites; 17/26 cases; data not shown). Reduced pigmentation was also observed in cultured ov explants when surface ectoderm removal was carried out up to the ten-somite stage (36/37 cases; data not shown). Our data demonstrate that the surface ectoderm is required for RPE specification during the initial stages of ov patterning.

### BMPs and Wnts can replace the function of the surface ectoderm to initiate MITF expression

If it is correct that BMPs and/or Wnts from the surface ectoderm are responsible for RPE specification, then replacing the removed surface ectoderm with BMP- or Wnt3a-soaked beads should at least in part restore MITF expression. Following surface ectoderm removal at stage 8 to 10, a BMP7-soaked bead was placed either into the lumen of the ov or temporal to it and the embryos were allowed to develop until the late ov or early optic cup stage (stages 13 to 15; Fig. 3D,E,H,I). One day following the operation, we observed strong MITF and pSmad labelling in the distal ov or outer optic cup (Fig. 3F-G,J-K'). Remarkably, in the absence of the surface ectoderm, BMP7 application rescued optic cup and lens formation in 47% (9/19 cases; Fig. 3I-K').

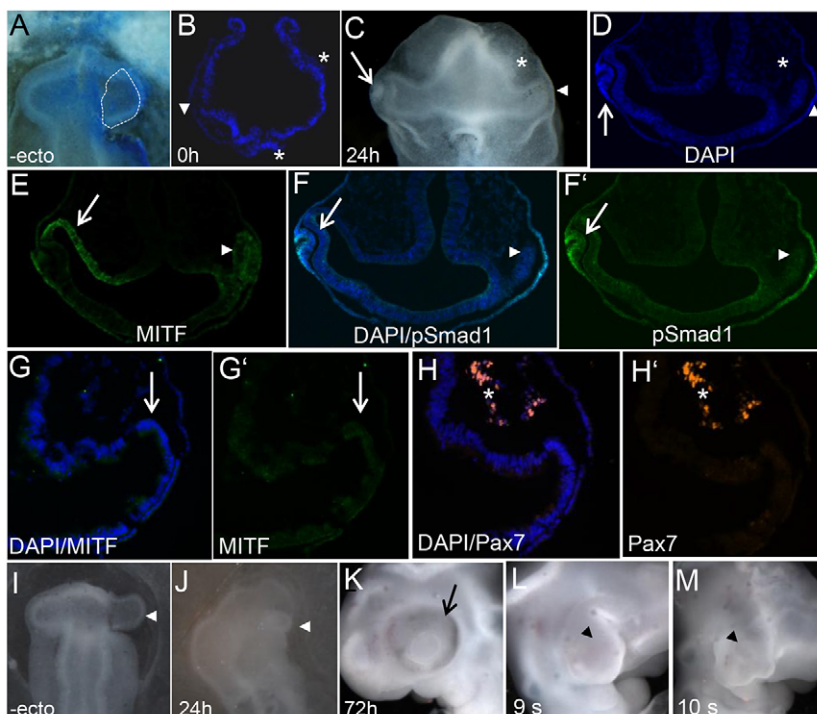
Next, we removed the surface ectoderm at ov stages and activated the Wnt signalling pathway by implanting Wnt3a-soaked beads (Fig. 3L,O). Like Wnt2b, Wnt3a is a known agonist of both the canonical and noncanonical Wnt signalling pathways (Abdul-Ghani et al., 2011; Gessert and Kühl, 2010; Qiu et al., 2011; Qu et al., 2013), and in contrast to Wnt2b, functional Wnt3a protein is commercially available. In the absence of the surface ectoderm,

Wnt3a was sufficient to rescue optic cup and lens formation (8/16 cases) and MITF expression one day after the operation (4/8 cases; Fig. 3N,N',Q-R'). Alsterpaullone (AP) is a chemical compound that specifically inhibits GSK3 $\beta$  (Broun et al., 2005), thereby mimicking Wnt signalling. When the surface ectoderm was removed and AP-soaked beads implanted at ov stages, only faint MITF expression was observed (3/3 cases; Fig. 3S-U'). In control experiments, where the surface ectoderm was removed and beads soaked in either phosphate-buffered saline (PBS) or dimethyl sulfoxide (DMSO) were implanted, MITF expression and pSmad labelling was not, or only weakly, detected (Fig. 3A-C; supplementary material Fig. S1). Taken together, these results show that BMP or Wnt signalling can replace the surface ectoderm to direct neuroepithelial cells towards an RPE cell fate and to allow optic cup and lens development.

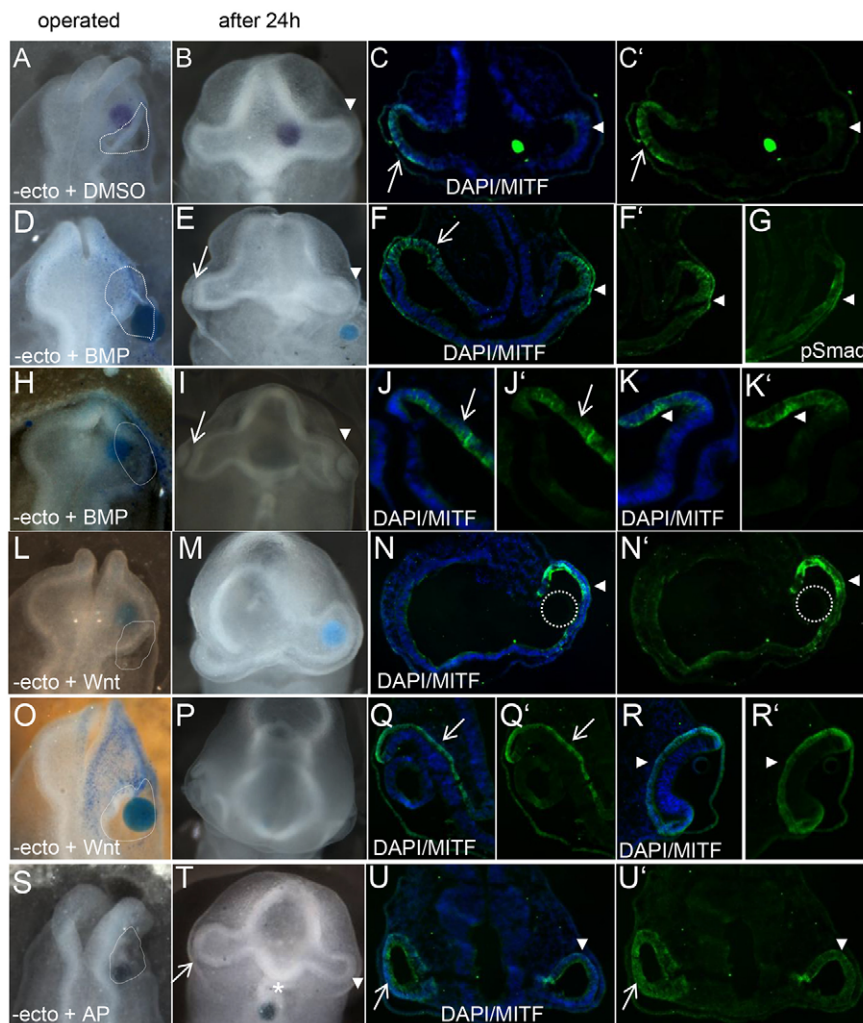
### Wnt signalling is essential for RPE specification

To investigate whether Wnt signalling is required for RPE specification, we interfered with the Wnt signalling pathways at ov stages. Soluble-frizzled-related proteins (sFRP-1) and the Wnt-inhibiting factor-1 (WIF-1) bind to all Wnt ligands, thereby blocking both the canonical and noncanonical Wnt signalling pathways (Kawano and Kypta, 2003). Following treatment with sFRP-1 or WIF-1, some of the embryos were analysed 3 days after the operation to monitor the appearance of pigment granules. The eyes that developed were reduced in size and only faint pigmentation was observed (Fig. 4A,D). A dramatic downregulation or loss of MITF expression in the ov/cup was already observed 1 day after the operation (9/14 cases; Fig. 4B-C',E-F'). The NR-specific marker *Vsx2* was weakly induced in the RPE, which still had RPE-like morphology (supplementary material Fig. S3D,E).

We next carried out experiments that interfered with the Wnt/ $\beta$ -catenin signalling pathway at ov stages. Dickkopf-1 (Dkk1) specifically inhibits the Wnt/ $\beta$ -catenin signalling pathway by binding to low density lipoprotein receptor-related protein 5 or 6 (reviewed by MacDonald et al., 2009). Following implantation of



**Fig. 2. The surface ectoderm is required for RPE specification in the chick.** (A) The surface ectoderm of the right ov of a stage-9 chick embryo was removed (white dashed line). (B) Section stained with DAPI to show the extent of surface ectoderm removal (asterisks) at stage 8/9. The surface ectoderm of the untreated side is indicated by an arrowhead. (C,D) One day later, mesenchymal cells are located dorsally (asterisks) and optic cup and lens development is not observed (arrowheads). The arrows indicate the contralateral lens. (E-F') Surface ectoderm removal results in decreased MITF and pSmad labelling (arrowheads). On the untreated side, MITF and pSmad protein is detected in the presumptive lens (arrows). (G-H') Following surface ectoderm removal, MITF expression (arrows) is dramatically downregulated although Pax7-positive mesenchymal cells are present (asterisks). (I) Following surface ectoderm removal, the ov is transplanted back to the chick embryo (arrowhead). (J) Transplanted ov (arrowhead) one day following the operation. (K) Contralateral side of the operated embryo showing pigmentation at stage 24 (arrow). (L,M) Transplanted ov isolated from a stage 9 (nine somites) and stage 10 (ten somites) embryo completely lack pigmentation (arrowheads) three days following the operation. Note that optic cup formation failed to occur.



**Fig. 3. BMPs or Wnts can replace the function of the surface ectoderm to induce MITF expression in chick optic vesicle cells.** (A) DMSO-soaked bead (blue) in the right ov following surface ectoderm removal (white dashed line) at stage 8. (B) One day later, optic cup and lens development is not observed (arrowhead). The bead is located in a more proximal position. (C,C') MITF expression is downregulated following surface ectoderm removal (arrowheads). The untreated side (arrows) still expresses MITF. (D,H) Surface ectoderm removal (white dashed line) and BMP7 application at stage 8. (E,I) The lens placode developed (arrow) on the unoperated side and on the treated side (arrowhead in I). The bead is attached to the surface ectoderm. (F-G) MITF expression in the presumptive RPE (F, arrow) of the untreated side. Following surface ectoderm removal, BMP application rescued MITF expression and pSmad distally (arrowheads). (J-K') MITF expression in the presumptive RPE of the untreated (J,J', arrows) and BMP-treated side (K,K'). Note that BMP application rescued optic cup and lens formation. (L,O) Surface ectoderm removal and Wnt3a application at stage 9. (M,P) Operated embryos one day after the operation. (N,N') Wnt3a application (white dashed lines) rescued MITF expression (arrowheads) in the absence of the surface ectoderm. (Q-R') MITF expression in the presumptive RPE of the untreated (Q,Q', arrows) and Wnt3a-treated side (R,R', arrowheads). Note that Wnt3a treatment following surface ectoderm removal rescued optic cup formation (arrowheads). (S) Surface ectoderm removal at stage 8, and application of the GSK3 $\beta$  inhibitor Alsterpaullone (AP). (T) One day later, the treated vesicle is smaller (arrowhead) compared with the contralateral side (arrow). The bead is located in the ventral forebrain (asterisk). (U,U') Only faint MITF expression is observed (arrowheads) on the treated side. In the presence of the surface ectoderm, exposure to AP induced MITF expression in the entire ov (arrows).

Dkk1-soaked beads at stages 8 to 10, eye development appeared to be normal (Fig. 4G). Pigmentation and MITF expression was not affected compared with the contralateral eye (6/7 cases; Fig. 4G-I). Thus, we extended previous findings in the mouse (Fujimura et al., 2009; Westenskow et al., 2009) to the ov stage, showing that the Wnt/ $\beta$ -catenin signalling pathway is not required for RPE specification.

To see whether Wnt2b is required for RPE specification, we targeted *Wnt2b* by electroporating Wnt2b siRNA at ov stages. One day later, MITF expression was not affected in the operated eye compared with the contralateral eye (6/6 cases; supplementary material Fig. S2A-D'). This is in agreement with the observation that Wnt2b mutant mice do not exhibit any eye phenotypes (Tsukiyama and Yamaguchi, 2012). However, these data suggest that Wnt signalling, possibly via a Wnt/ $\beta$ -catenin-independent signalling pathway, is required for RPE specification.

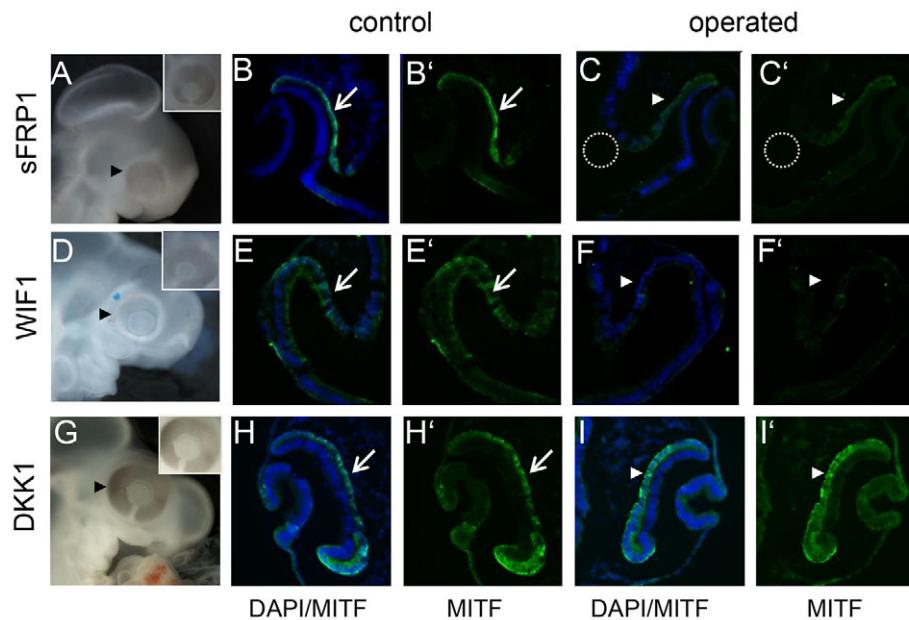
#### Wnt-mediated GSK3 $\beta$ inhibition induces an RPE cell fate in the entire optic vesicle

To test the potential role of Wnt signalling in RPE specification, we carried out gain-of-function experiments and implanted Wnt3a beads at ov stages. Compared with controls (Fig. 5A-E), the Wnt3a-treated embryos developed a small pigmented eye with a rudimentary lens 3 days after the operation (Fig. 5F,G). Histological examination showed that Wnt3a treatment prevented optic cup

formation (Fig. 5G) and that the entire vesicle had RPE-like morphology, being strongly pigmented (11/15 cases; Fig. 5H,I). Moreover, NR development was completely inhibited and the entire ov, including the optic stalk region, expressed MITF (Fig. 5J; data not shown).

In the chick and frog, both BMP and Wnt signalling cooperate to induce an epidermal cell fate (Wilson et al., 2001; Fuentealba et al., 2007; Patthey et al., 2009). Wnt-mediated GSK3 $\beta$  inhibition stabilises BMP signalling by preventing the degradation of pSmad1, thereby inducing an epidermal cell fate (Fuentealba et al., 2007). To test whether a similar mechanism exists during ov patterning, we inhibited GSK3 $\beta$  at these stages (9-14 somites). Following AP treatment, a small, strongly pigmented, MITF-expressing vesicle developed, phenocopying Wnt3a treatment (34/41 cases; compare Fig. 5F-J with 5K-Y). In those vesicles, pSmad labelling (Fig. 5R,W) was observed, colocalised with the RPE-specific marker RPE65 (Fig. 5X,Y). By contrast, the expression of NR markers, such as *Vsx2*, was downregulated or lost (supplementary material Fig. S3A,C). We did not detect any nuclear  $\beta$ -catenin staining in the neuroepithelium of AP-treated vesicles 1-3 days after the operation (6/6 cases; Fig. 5S,T). No changes were observed in control experiments (10/10 cases; supplementary material Fig. S1). Thus, our data suggest that Wnt-mediated GSK3 $\beta$  inhibition can, independently of  $\beta$ -catenin nuclear transfer, induce MITF expression in chick ov cells.





**Fig. 4. Wnt signalling, but not Wnt/ $\beta$ -catenin signalling, is required for RPE specification.** (A-I') Loss-of-function experiments. Following inhibition of Wnt signalling, pigmentation was assayed 3 days after (A,D,G), and MITF expression 1 day after the operation. (A,D) Implantation of a bead soaked in sFrp1 (A) or WIF-1 (D) at ov stages results in optic cup and lens formation, but reduced pigmentation (arrowheads) of the treated eye compared with the contralateral eye (insets) is observed. (B-F') MITF expression with and without DAPI staining. (C,C') Implantation of a bead soaked in sFrp1 (white dashed line) downregulates MITF expression (arrowheads) compared with the contralateral side (B,B', arrows). (F,F') Application of a WIF-1-soaked bead downregulates MITF expression (arrowheads) compared with the contralateral side (E,E', arrows). (G) Following implantation of a Dkk1-soaked bead eye, morphology and pigmentation appears normal (arrowhead) compared with the contralateral eye (inset). (H-I') Dkk1 treatment does not affect MITF expression in the presumptive RPE of both the contralateral (H,H', arrows) and operated eye (I,I', arrowheads). Note that the insets in A, D and G show the contralateral eyes in the same magnification.

#### Wnt-mediated GSK3 $\beta$ inhibition supports BMP/pSmad signalling to initiate MITF expression in optic vesicle cells

BMP application at ov stages induces both ectopic *Wnt2b* and *Mitf* expression in the presumptive NR (Müller et al., 2007). Thus, it is possible that BMP-induced Wnt signalling maintains MITF expression and hence RPE development. Alternatively, Wnt-mediated GSK3 $\beta$  inhibition could stabilise BMP signalling to induce an epithelial phenotype in ov cells (see above). Therefore, we next inhibited BMP signalling by implanting Noggin-soaked beads, simultaneously activating the Wnt signalling pathway.

Exposure to Wnt3a or GSK3 $\beta$  inhibition converts the entire ov into a strongly pigmented and MITF-expressing vesicle (Fig. 5). By contrast, in the absence of BMP signalling, activation of Wnt signalling was no longer able to induce MITF expression (4/5 cases; Fig. 6A-E'). In those embryos, we observed only faint, or no, pSmad labelling in the distal ov and surface ectoderm (Fig. 6F,F').

To investigate whether Wnt signalling is required for BMP-induced MITF expression, we inhibited Wnt signalling by simultaneously activating the BMP signalling pathway. Exposure to BMP alone induces strong MITF/*Mitf* expression in the ov (Fig. 3) (Müller et al., 2007). Following implantation of two beads, soaked in BMP7 and WIF-1 into the ov (Fig. 6G), activation of the BMP signalling pathway was no longer able to induce or maintain MITF expression and only faint or no pSmad staining was observed (6/6 cases; Fig. 6H-L'). In control experiments, pSmad and MITF expression was observed only in the dorsal ov or optic cup and in the dorsal surface ectoderm (5/5 cases; supplementary material Fig. S1K-P).

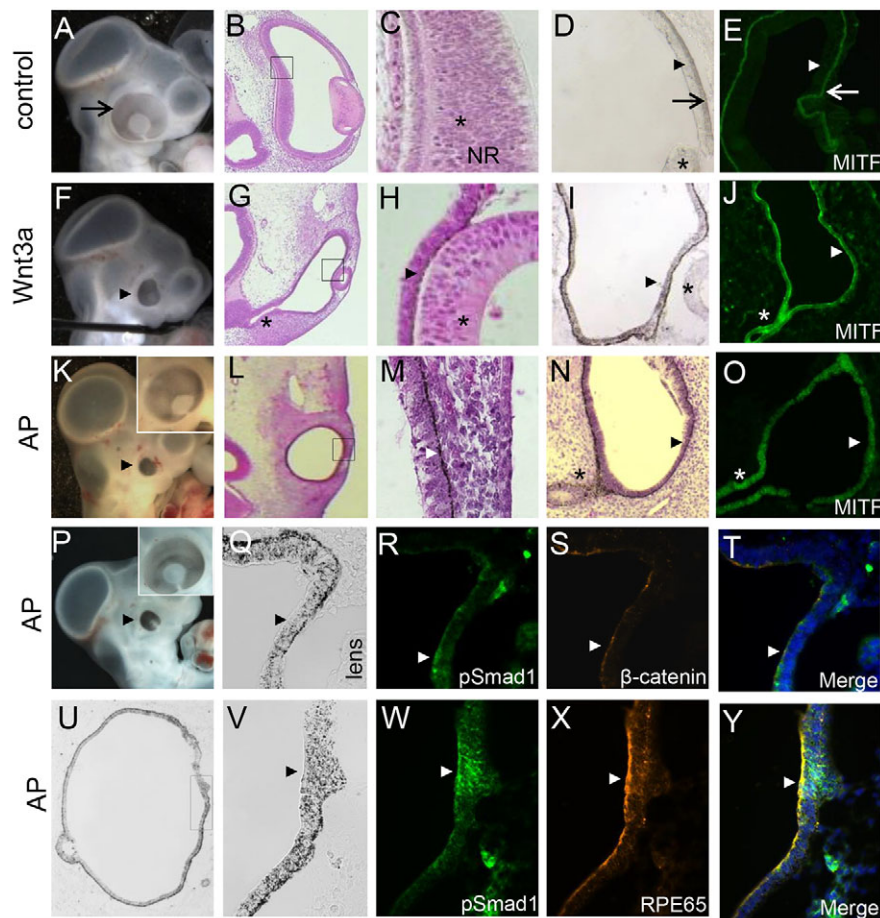
Wnts can signal through Smad1 (Fuentes et al., 2007). Here, GSK3 $\beta$  inhibition prevents degradation of pSmad1, thereby stabilising BMP signalling (reviewed by Eivers et al., 2008). To see

whether a similar mechanism exists during ov patterning, we implanted WIF1-soaked beads in the presence of the GSK3 $\beta$ -inhibitor AP (Fig. 6M). One day after the operation, eye development arrested at the ov stage and nuclear  $\beta$ -catenin labelling was not observed (3/4 cases; Fig. 6N-R'). Strikingly, in the absence of Wnt signalling, GSK3 $\beta$  inhibition was sufficient to stabilise pSmad and rescue MITF expression in both the dorsal and ventral ov (3/4 cases; Fig. 6Q-R'), suggesting that pSmad is sufficient to induce MITF expression in the absence of the surface ectoderm.

#### pSmad mediates RPE specification

To confirm that pSmad mediates RPE specification, we removed the surface ectoderm and electroporated a constitutively active form of human Smad1 (CA-Smad1) (Fuentes et al., 2007) into ov cells (Fig. 7A). In the absence of the surface ectoderm CA-Smad1 was sufficient to rescue MITF expression (6/6 cases; Fig. 7B-C'). In a control experiment, wild-type Smad1 (Fuentes et al., 2007) did not lead to MITF expression in ov cells in the absence of the surface ectoderm (5/5 cases; Fig. 7D,D').

In *Xenopus*, it has been shown that in the absence of Wnt signalling pSmad is degraded, thereby inducing a neuronal cell fate (Fuentes et al., 2007). *Wnt2b* expression is restricted to the dorsal surface ectoderm (Fig. 1), so that a similar mechanism could exist in the ov to allow NR development ventrally. To test this, we tried to inhibit pSmad degradation thereby directing presumptive NR cells towards an RPE cell fate. Application of the proteasomal inhibitor MG132 (Lee and Goldberg, 1998) at ov stages (7-10 somites) induced ectopic MITF expression in the ov/cup (4/7 cases; Fig. 7E-G',J-L'). One to three days following the operation pSmad labelling was increased in the inner optic cup, and downregulation of the NR-specific marker *Vsx2* was observed (Fig. 7H,I,M). Taken



**Fig. 5. Wnt/GSK3 $\beta$ -mediated signalling induces RPE development *in vivo*.** (A) Untreated pigmented eye of a stage-24 chick embryo (arrow). (B) Section of the eye stained with haematoxylin/eosin. (C) Higher magnification of B showing the pseudostratified NR (asterisk) and single-layered RPE. (D) Peripheral region of a stage-24 chick eye with the pigmented RPE (arrow), NR (arrowhead) and lens (asterisk). (E) Dorsal eye cup. MITF is restricted to the RPE (arrow) and is not detected in the NR (arrowhead). (F–O) Wnt gain-of-function experiments. (F) Wnt3a-treated side of the embryo shown in A. The eye is microphthalmic and strongly pigmented (arrowhead, compare with A). (G) Eye morphology following Wnt3a treatment. The optic stalk is indicated by an asterisk. Optic cup formation is prevented and the entire vesicle has RPE-like morphology compared with the untreated side (shown in B). (H) Higher magnification of G. The presumptive NR is respecified and develops into a pigmented layer (arrowhead; asterisk marks the lens vesicle). (I, J) Following Wnt3a treatment, the entire eye vesicle is strongly pigmented (arrowhead; asterisk marks the lens), and the presumptive NR (white arrowhead) and optic stalk region (white asterisk) express MITF. (K) Following treatment with the GSK3 $\beta$  inhibitor AP, a small pigmented eye developed (arrowhead). The inset shows the contralateral eye. (L) The vesicle that developed has RPE-like morphology. (M) Higher magnification of the boxed region in L. The arrowhead indicates basally located pigment granules in the respecified NR. (N, O) Following GSK3 $\beta$  inhibition, the eye vesicle, including the presumptive NR (arrowhead) and optic stalk (asterisk) is pigmented and expresses MITF (white arrowhead and asterisk). (P, Q, U, V) Three days following AP treatment, a strongly pigmented eye vesicle developed (arrowheads). The inset shows the contralateral eye. (R–T) The induced RPE shows pSmad- and  $\beta$ -catenin labelling (arrowheads). The overlay in T shows that  $\beta$ -catenin labelling is restricted to the apical side (arrowhead), whereas pSmad labelling is observed apically and in the nuclei of the induced RPE. (U–Y) Section of the embryo shown in P–T. Following GSK3 $\beta$  inhibition, the neuroepithelium is pSmad-positive and expresses the RPE-specific differentiation marker RPE65 (arrowheads).

together, our data are consistent with a model, according to which Wnt-mediated GSK3 $\beta$  inhibition stabilises BMP signalling, thereby restricting RPE development to the dorsal ov.

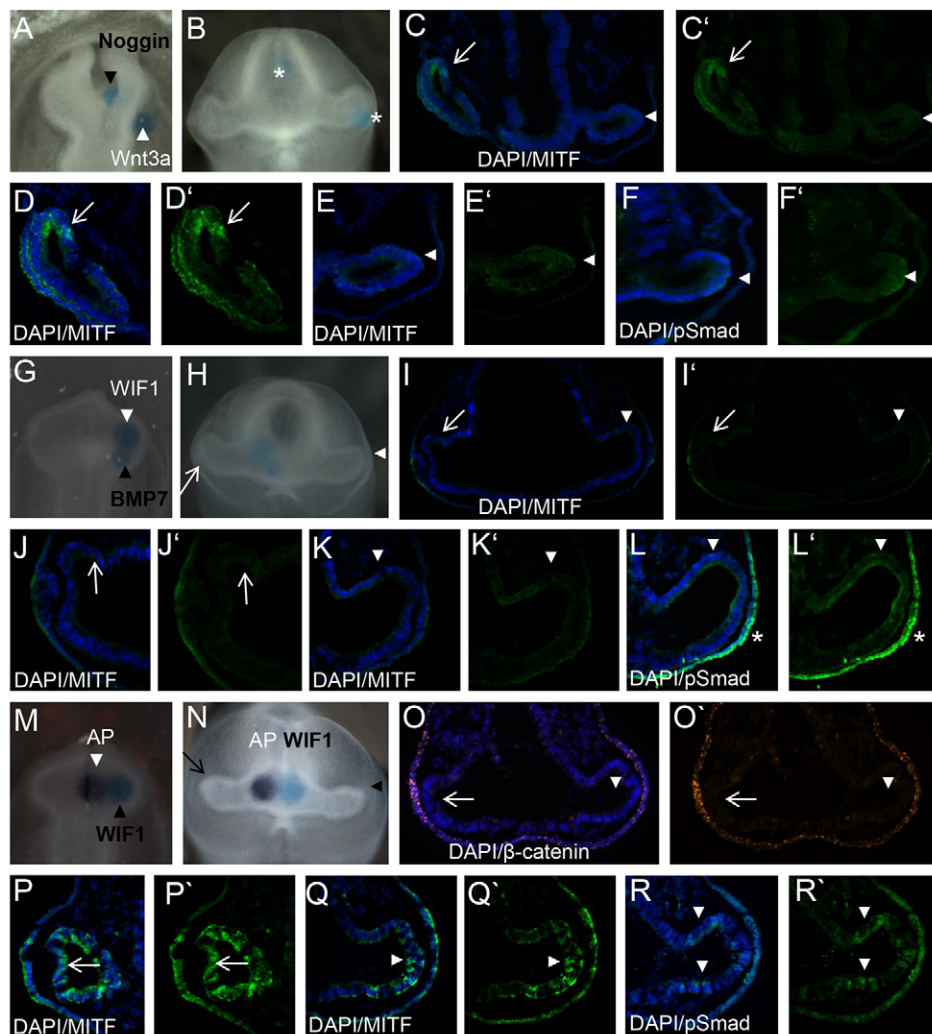
## DISCUSSION

The classical model of ov patterning holds that the distally located surface ectoderm induces NR development, whereas proximally signals released from the mesenchyme elicit RPE development. In this study, we show that signals released from the surface ectoderm, and not from the mesenchyme, promote RPE specification.

### BMP and Wnt ligands expressed in the surface ectoderm specify the RPE

Fate-mapping studies in the chick showed that the distalmost part of the ov at stages 9 and 10 contributes to the eye, whereas the

dorsalmost region of the ov consists of telencephalic precursors (Garcia-Lopez et al., 2009; Pomero and Martinez, 2009). Consistent with this, RPE specification initiates distally underneath the surface ectoderm, but soon the MITF domain becomes restricted to the dorsal ov and optic cup (Fig. 1) (Müller et al., 2007; Ishii et al., 2009). Similarly, polarisation of active BMP signalling to dorsal ov cells is observed in both the chick and mouse (this study; Monteiro et al., 2004). However, at this time point BMPs are still expressed in the entire surface ectoderm (Furuta et al., 1997; Müller et al., 2007), suggesting that another signal(s) is involved in restricting MITF expression to the dorsal ov/cup. BMP and Wnt family members are often co-expressed in dorsal tissues and co-regulate vertebrate pattern formation (e.g. Abu-Khalil et al., 2004; Szeto and Kimelman, 2004; Ramel et al., 2005; Zechner et al., 2007; Ohta et al., 2011). Here, we identified Wnt2b as a candidate gene involved in RPE specification.

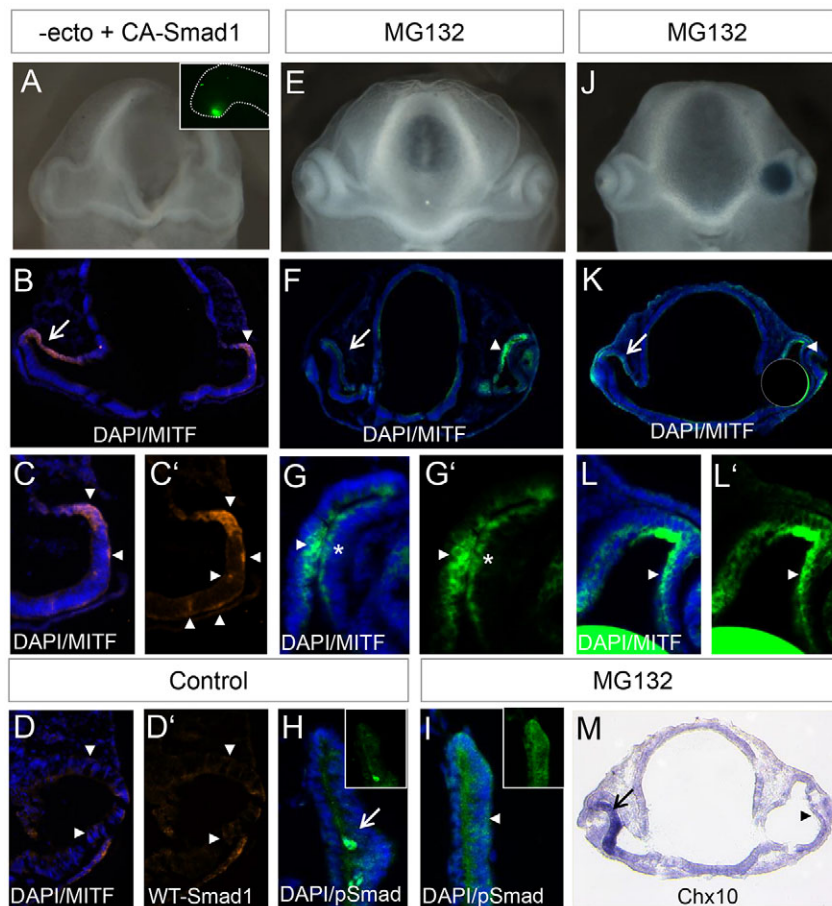


**Fig. 6. Initiation of MITF expression requires the activation of both the BMP and Wnt/GSK3 $\beta$  signalling pathways.** (A-F') Activation of the Wnt signalling pathway in the absence of BMP signalling at ov stage. (A) Two beads, one soaked in the BMP inhibitor Noggin (black arrowhead) and one soaked in Wnt3a (white arrowhead), were implanted. (B) One day following the operation, optic cup formation is not observed (asterisks indicate the localisation of beads). (C,C') In the absence of BMP signalling, activation of the Wnt signalling pathway is no longer capable of inducing MITF expression (arrowheads). The contralateral side still expresses MITF (arrows). (D-E') Higher magnification of the untreated (D,D') and treated side (E,E') shown in C. MITF expression is downregulated (arrowheads) compared with the untreated side (arrows) and to Wnt activation alone (Fig. 5J). (F,F') Following activation of the Wnt signalling pathway and Noggin treatment, pSmad is only weakly detected (arrowheads; parallel section of E). (G-L') Activation of the BMP signalling pathway in the absence of Wnt signalling at ov stage. (G) Two beads, one soaked in the Wnt inhibitor WIF1 (white arrowhead) and one soaked in BMP7 (black arrowhead) were implanted into the ov. (H) One day later at stage 14/15, optic cup formation is not observed on the treated (arrowhead) and contralateral side (arrow). Note that the beads have moved towards the brain. (I-K') MITF expression is downregulated or lost in the presumptive RPE of both the contralateral side (I-J', arrows) and treated side (I,I',K,K', arrowheads). (L,L') On the treated side, pSmad is weakly or is absent in the ov (white arrowheads), but strongly detected in the surface ectoderm (asterisks). (M-R') In the absence of Wnt signalling, GSK3 $\beta$  inhibition is sufficient to stabilise pSmad and MITF expression. (M) Two beads, one soaked in the GSK3 $\beta$  inhibitor AP (white arrowhead) and one in WIF-1 (black arrowhead) were implanted into the ov. (N) One day later at stage 14, optic cup formation is not observed on the contralateral (arrow) and operated side (arrowhead). (O,O') Following GSK3 $\beta$  inhibition, no nuclear accumulation of  $\beta$ -catenin is observed in the optic vesicle on the treated (arrowheads) and contralateral side that had been exposed to AP (arrows). Staining of  $\beta$ -catenin in the surface ectoderm appeared to be unaffected. (P,P') The contralateral side that had been exposed to AP expresses MITF in the entire ov (arrows). (Q,Q') Following WIF-1 mediated inhibition of Wnt signalling, GSK3 $\beta$  inhibition was sufficient to induce MITF expression (arrowheads) in the treated ov (compare with K,K' and Fig. 4I'). (R,R') Parallel section of Q shows that as a consequence of GSK3 $\beta$  inhibition pSmad is stabilised in the absence of Wnt signalling in both the dorsal and ventral region of the ov (arrowheads; compare with L,L'). Note that for clarity, all the embryos have been oriented, so that the treated side is always shown on the right side.

Expression of *Wnt2b* is detected in the dorsal surface ectoderm, dorsal ov and/or optic cup in several species (this study) (Zakin et al., 1998; Cho and Cepko, 2006; Müller et al., 2007; Veien et al., 2008; Grocott et al., 2011), and Wnt signalling can induce an RPE cell fate *in vitro* and *in vivo* (this study; Aoki et al., 2006; Eiraku et al., 2011). Targeting *Wnt2b* by electroporating *Wnt2b* siRNA at ov stages did not affect MITF expression (this study) and, although a large number

of genes associated with Wnt signalling are expressed during vertebrate eye development, none of the single Wnt mutant mice, including the *Wnt2b* knockout mice, displays any eye phenotypes (van Amerongen and Berns, 2006; Tsukiyama and Yamaguchi, 2012). However, similar to BMP inhibition (Adler and Belecky-Adams, 2002; Müller et al., 2007) we show that Wnt signalling is required for RPE development. Targeting several Wnt ligands by





**Fig. 7. pSmad1 signalling mediates RPE specification.**

(A) Chick embryo that developed one day after surface ectoderm removal and electroporation of a constitutively active form of Smad1 (CA-Smad1). The inset shows the GFP-positive ov. (B–C') Following surface ectoderm removal, pSmad signalling is sufficient to rescue MITF expression in both the dorsal and ventral ov (arrowheads). The arrow indicates MITF expression in the untreated eye. (D, D') Optic vesicle of a control experiment. Following surface ectoderm removal and electroporation of wild-type Smad1 (WT-Smad1), MITF expression is not maintained in the neuroepithelium (arrowheads). (E, J) Embryos that developed one day following implantation of the proteasomal inhibitor MG132 at ov stages. (F, K) Optic cup and lens formation is observed on both the treated (arrowheads) and untreated side (arrows). The bead is indicated by a white dashed line. (G, G', L, L') Higher magnification of the dorsal eye cup showing strong MITF expression in both the RPE (arrowheads in G, G') and NR (asterisks in G, G' and arrowheads in L, L'). (H) At stage 26, single positive pSmad cells are present in the ciliary margin zone (arrow). (I) In the MG132-treated embryo, pSmad labelling is detected throughout the ciliary margin zone (arrowhead). (M) Following proteasomal inhibition, expression of the NR-specific marker *Chx10/Vsx2* is lost (arrowhead), compared with the untreated eye (arrow).

inhibiting Wnt receptor-mediated signalling results in reduced pigmentation and downregulation of MITF expression (Fig. 4). Moreover, we show that during a narrow time window, the BMP- and Wnt- expressing surface ectoderm is required to mediate RPE specification. The loss of MITF expression following surface ectoderm removal can be rescued by the activation of the BMP or Wnt signalling pathways.

#### In the absence of the surface ectoderm BMP or Wnt signalling can rescue optic cup formation

During vertebrate development tissue interactions are required for proper organ formation. We confirm previous findings that the surface ectoderm is required for optic cup formation (Fig. 2) (Hyer et al., 1998; Hyer et al., 2003; Nguyen and Arnheiter, 2000). In the absence of the surface ectoderm, an optic cup fails to form, independently of whether mesenchymal cells remain dorsally or cover the entire ov. Interestingly, in the absence of the surface ectoderm, BMPs or Wnts can rescue optic cup and lens formation (Fig. 3). However, recent *in vitro* studies suggest that tissue interactions are not required for optic cup formation (reviewed by Sasai et al., 2012; Sasai, 2013). Embryonic stem cell (ESC) reagggregates cultured on matrigel are patterned into NR and RPE in a self-autonomous way (Eiraku et al., 2011; Nakano et al., 2012). It should be noted, that we observed optic cup formation only if optic vesicle explants were cultured on growth factor-containing matrigel, but not in the presence of collagen (Ichie Steinfeld, unpublished observations). It is possible that matrigel releases proteins (Hughes et al., 2010) that allow optic cup formation in the absence of the surface ectoderm. For example, during matrigel-induced hepatocyte

differentiation,  $\beta$ -catenin undergoes multifactorial regulation (Monga et al., 2006). Interestingly, patterning into NR and RPE of ESC reagggregates is only observed in the presence of neuroectodermal epithelium or in the presence of both Wnt3a and high concentrations of matrigel (Eiraku et al., 2011). Further work is required to identify the exact cellular and molecular mechanisms that direct optic cup formation in vertebrates.

#### Wnt signalling, via a GSK3 $\beta$ -dependent but $\beta$ -catenin-independent pathway, is involved in RPE specification

Wnt signals are context-dependently transduced via the noncanonical or canonical signalling pathways (reviewed by Kikuchi et al., 2007; Katoh and Katoh, 2007). Here, we show that RPE specification is mediated by a Wnt-signalling pathway that is GSK3 $\beta$ -dependent but  $\beta$ -catenin-independent. Firstly, in agreement with previous studies (see Introduction), we did not detect any nuclear  $\beta$ -catenin in the neuroepithelium at the time the RPE is specified (Fig. 1). Secondly, canonical Wnt/ $\beta$ -catenin signalling requires GSK3 $\beta$  inhibition. Following GSK3 $\beta$  inhibition the NR and optic stalk are respecified to develop into a strongly pigmented RPE, independent of nuclear  $\beta$ -catenin accumulation (Fig. 5). Thirdly, following inhibition of Wnt signalling, GSK3 $\beta$  inhibition is sufficient to rescue MITF expression, and this is again independent of nuclear  $\beta$ -catenin accumulation (Fig. 6). Lastly, specific inhibition of the Wnt/ $\beta$ -catenin signalling pathway at optic vesicle stages did not affect MITF expression and pigmentation (Fig. 4). In support of this, eye pigmentation appears normal in mutant mice, where the co-receptors of the Wnt/ $\beta$ -catenin signalling pathway, Lrp5 and 6, are mutated (Fujino et al., 2003; Zhou et al., 2008). Moreover, ectopic



activation of the Wnt/ $\beta$ -catenin signalling pathway alone is not sufficient to induce MITF expression (Westenskow et al., 2010) and instead eye development and optic cup formation is disrupted (Smith et al., 2005; Fu et al., 2006; Miller et al., 2006; Fujimura et al., 2009). In particular,  $\beta$ -catenin overexpression within the RPE itself results in a complete loss of pigmentation and *Mitf* expression (Fujimura et al., 2009). *Dkk1*, a specific antagonist of the Wnt/ $\beta$ -catenin signalling pathway, is expressed in the chick ov (GEISHA chicken embryo gene expression database: <http://geisha.arizona.edu>) and in *Dkk1* mutant mice, eyes or anterior head structures fail to develop (MacDonald et al., 2004; Mukhopadhyay et al., 2001). This suggests that at ov stages, repression of Wnt/ $\beta$ -catenin signalling is required for proper vertebrate eye development. Accordingly, during the process of stepwise differentiation of human or mouse ESCs towards a retinal cell fate, *Dkk1* is initially supplemented to the medium (Osakada et al., 2009).

### Initiation of MITF expression requires both the BMP and Wnt/GSK3 $\beta$ signalling pathways

The status of Wnt signalling regulates neural and epidermal cell fates in chick and frog embryos (Wilson et al., 2001; Fuentealba et al., 2007; Patthey et al., 2009). In *Xenopus*, the phosphorylation status of Smad1 determines whether a cell acquires an epidermal or neuronal cell fate (Fuentealba et al., 2007; Eivers et al., 2008). A neuronal cell fate is induced in the absence of Wnt signalling. Here, Smad-1 receives inhibitory phosphorylations by GSK3 $\beta$  and by the FGF/MAPK, which leads to proteasomal degradation of pSmad-1 (Fuentealba et al., 2007). By contrast, in the presence of Wnt signalling, GSK3 $\beta$  is inhibited and pSmad-1 can now induce an epidermal cell fate (Fuentealba et al., 2007). A similar mechanism appears to exist during ov patterning to restrict the RPE domain to the dorsal ov. On the one hand, activation of the Wnt signalling pathway at ov stages does not rescue MITF expression lost from BMP inhibition (Fig. 6). On the other hand, in the absence of Wnt signalling, activation of the BMP signalling pathway can no longer induce MITF expression in ov cells. In both cases, we observed no, or only weak, pSmad staining in ov cells. Following inhibition of Wnt signalling, GSK3 $\beta$  inhibition is sufficient to stabilise pSmad in ov cells and to rescue MITF expression and hence RPE development (Fig. 6). Moreover, overexpression of a constitutively active form of Smad1 rescues MITF expression in the absence of the surface ectoderm. Accordingly, inhibition of proteasomal pSmad degradation maintains MITF and results in the loss of *Vsx2* expression (Fig. 7).

Based on these findings, we propose a dorsoventral model for the initial stages of ov patterning. Members of the BMP, FGF and Wnt families are released from the surface ectoderm to pattern the ov transiently into a dorsal RPE and ventral NR domain. Initially, the BMP-expressing surface ectoderm initiates *Mitf* expression through pSmad in the entire distal ov (Fig. 8A). Wnt2b and as yet unidentified Wnt ligand(s) localised to the dorsal surface ectoderm and dorsal ov inhibit GSK3 $\beta$ , thereby stabilising pSmad and maintaining *Mitf* expression in the dorsal ov and optic cup. In the absence of Wnt signalling, pSmad is degraded through MAPK/ERK and GSK3 $\beta$  phosphorylation, which then leads to NR specification/*Vsx2* expression in the ventral ov (Fig. 8B). Once RPE and NR specification occurs, *Vsx2* expression is stabilised distally, whereas RPE development will continue in the dorso-proximal region, where mesenchymal cells now become involved in maintaining RPE-specific gene expression.

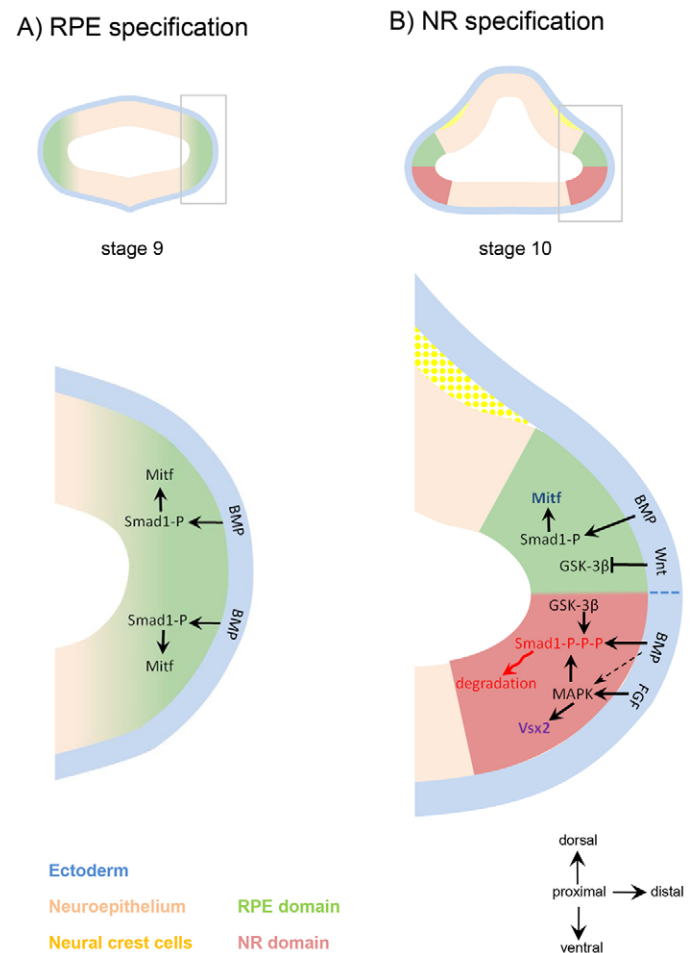
The molecular interplay between several pathways at the level of pSmad during cell fate specification in the ov may provide the basis for directed differentiation of human ESCs towards functional RPE

cells that in the future could be used for cell replacement of RPE-mediated blinding diseases (reviewed by Clegg et al., 2008; Bharti et al., 2011).

## MATERIALS AND METHODS

### In vivo manipulations of the developing chick embryo

Fertilised chicken eggs (*Gallus gallus*, Linnaeus) were obtained from LSL Rhein-Main (Dieburg, Germany). For all experiments the eggs were incubated at 38°C and 60% humidity, until they reached the desired stages (stage 7 to 11) according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). The eggs were then windowed and the embryonic membranes removed. A small incision was made into the midline of the forebrain, or temporally to the ov using sharpened tungsten needles. One bead (see below) was then transferred into the egg, inserted through the slit and positioned into the ov. The eggs were then sealed and left to develop at 38°C until they reached the desired stage. Owing to the turning of the chick embryo at stage 12/13, some



**Fig. 8. Dorsoventral model of optic vesicle patterning in the chick embryo, integrating BMP and Wnt actions.** (A,B) This model suggests that at stage 9 (A), BMPs released from the surface ectoderm initiate RPE specification through phosphorylation of Smad-1, -5 and/or -8, which leads to *Mitf* expression in distal ov cells. At stage 10 (B), Wnt signalling from the dorsal surface ectoderm inhibits GSK3 $\beta$ , which in turn stabilises BMP signalling. The transcription factor pSmad can translocate to the nucleus to initiate *Mitf* expression, thereby directing dorsal ov cells towards an epithelial cell fate (RPE). In the absence of Wnt ligands within the ventral surface ectoderm, Smad-1/5/8 is triple phosphorylated by BMPs released from the surface ectoderm, by GSK3 $\beta$  (not inhibited by Wnts) and possibly by FGF signalling (not studied here). This results in proteasomal degradation of Smad-1/5/8 in the ventral ov. The inhibition of BMP signalling now allows FGF/MAPK signalling to induce *Vsx2* expression, thereby directing the ventral region of the ov towards a neuronal cell fate.

beads placed into the right ov moved into the brain vesicle one day after the operation. The embryos were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C for 2 to 48 hours. Embryos to be used for whole-mount *in situ* hybridisation (ISH) were dehydrated and stored in 100% methanol. Those intended for ISH on sections were cryoprotected overnight in 10% sucrose in diethylpyrocarbonate-H<sub>2</sub>O at 4°C and transferred into 20% and 30% sucrose for at least 1 hour each. Consecutive 10–14 µm thick sections were analysed by ISH or immunohistochemistry. Control experiments were carried out by either implanting PBS- and/or DMSO-soaked beads into the ov of stage 9/10 chick embryos according to the protocol described above.

### Bead preparation

A 2 µl drop of the designated protein or chemical was placed in a Petri dish and about eight drops (10 µl each) of PBS were placed around it. About 15 beads (see below) were added to the solution and incubated for a minimum of 1 hour. Agarose beads (AffiGel Blue Beads, Bio-Rad) were soaked either in 0.5 or 1 µg/µl BMP5 and BMP7 protein (R&D Systems), 0.5 or 1 µg/µl Wnt3a (R&D Systems), 1 µg/µl DKK1 (R&D Systems), 2 µg/µl sFRP1 (PreProTech) or 1 µg/µl human WIF-1 protein (R&D Systems), 1 µg/µl Noggin (R&D Systems). Alsterpaullone (Sigma-Aldrich) and MG132 (Merck) was dissolved in DMSO (Roth) and used at a concentration of 1 µg/µl using AG1-X2 resin beads (Bio-Rad).

### Surface ectoderm removal

Two per cent Nile Blue A sulfate (Sigma-Aldrich) dissolved in agarose was used to support the removal of the surface ectoderm at stages 7 to 10 using fine glass needles (Geetha-Loganathan et al., 2010).

### Transplantation experiments

The right ov was removed from stage 9 (nine somites) or stage 10 chick embryos (10–12 somites), treated with 0.05% collagenase for 30 minutes to loosen the surface ectoderm, which then was removed using sharpened tungsten needles. Using a glass pipette, the remaining ov was then transplanted back to the same chick embryo. Embryos were placed into the incubator and left to develop for another 72 hours, fixed in 4% PFA and processed as described above. *In vitro* experiments using chick explant cultures were carried out as described before (Kobayashi et al., 2010).

### In vivo electroporation

The ov of stage 9–10 chick embryos were usually injected with a green fluorescent protein (GFP) expression construct (1–3 µM) mixed with 1.5–2 µg/µl plasmids or siRNA (see below) containing 0.01% Fast Green. Using a TSS20 Ovodyne Electroporator (Intracel), two pulses (7V, 30-millisecond duration) in 970-millisecond intervals were delivered, using 0.5 mm diameter, tungsten and platinum electrodes. The anode was positioned lateral to the right ov and the cathode was placed into the brain. The DNA/RNA constructs used were Wnt2b siRNA and siRNA-A (both Santa Cruz), pC S2 hSmad1-WT (Addgene plasmid 22989) and pC S2 hSmad1-GM (Addgene plasmid 22992; Edward de Robertis; Pera et al., 2003; Fuentealba et al., 2007). The embryos were fixed 1 day later and assayed for MITF and GFP expression.

### Assaying gene expression in chick embryos and ov explants by ISH and immunohistochemistry

RNA ISH on cryostat sections and whole mounts was performed as previously described (Müller et al., 2007). Antisense RNA probes specific for chicken *Rx* (T. Ogura, Tohoku University, Aoba, Japan), *Mitf* (M. Mochii, University of Hyogo, Japan), *Vsx2* and *Wnt3a*, *Wnt4*, *Wnt5*, *Wnt5b*, *Wnt7b*, *Wnt8b* (D. Schulte, Goethe University of Frankfurt/M., Germany) and *Wnt2b* (H. Roelink, University of Washington, Seattle, WA), were used.

In explant cultures of ov, MITF expression was carried out as previously described (Kagiyama et al., 2005; Kobayashi et al., 2010). Human MITF antibody (Sigma-Aldrich, HPA003259), PY489-β-catenin (DSHB), pSmad1/5, pSMAD1/5/8 (Cell Signaling, 9516, 9511S), Retinal pigment epithelial-specific protein 65 kDa (Novus Biologicals, 4018BD113D9) and mouse Pax7 (MAB1675) were used on cryostat sections.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

A.V.-H., P.G.L. and M.A. designed the experiments and gave advice. J.S., I.S. and N.C. carried out embryological manipulations and gain- and loss-of-function studies and analysed the data. M.-L.H. carried out the proteasomal inhibition experiment. J.S., P.G.L. and A.V.-H wrote the manuscript.

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### Supplementary material

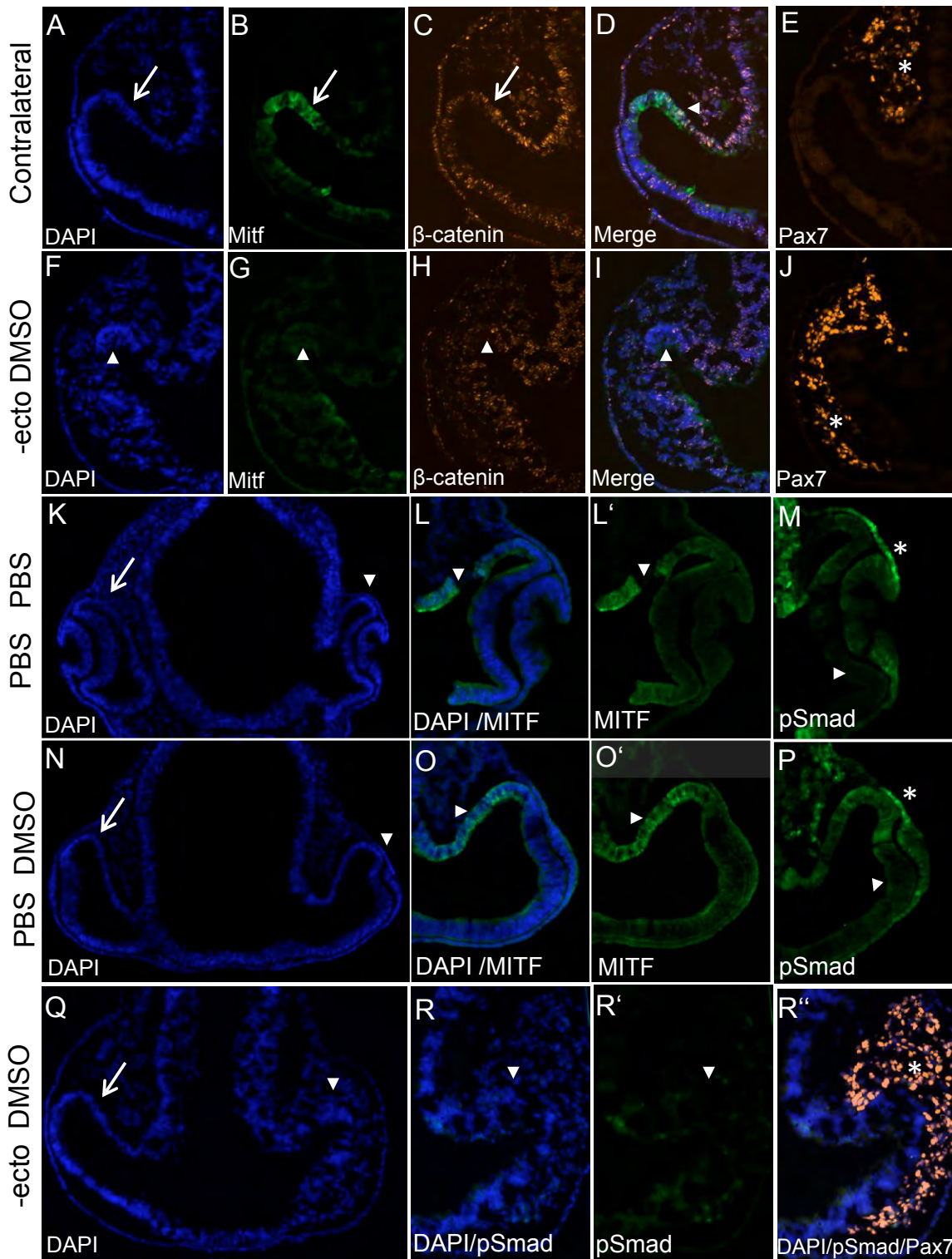
Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.096990/-/DC1>

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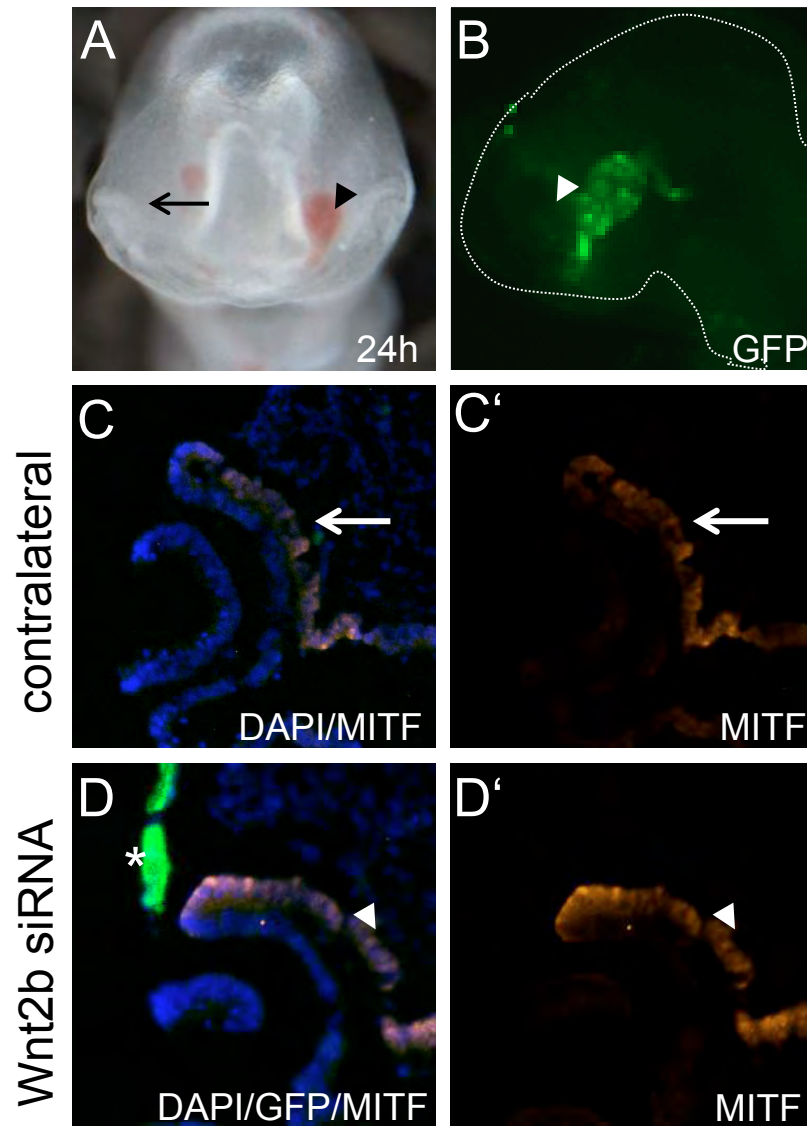


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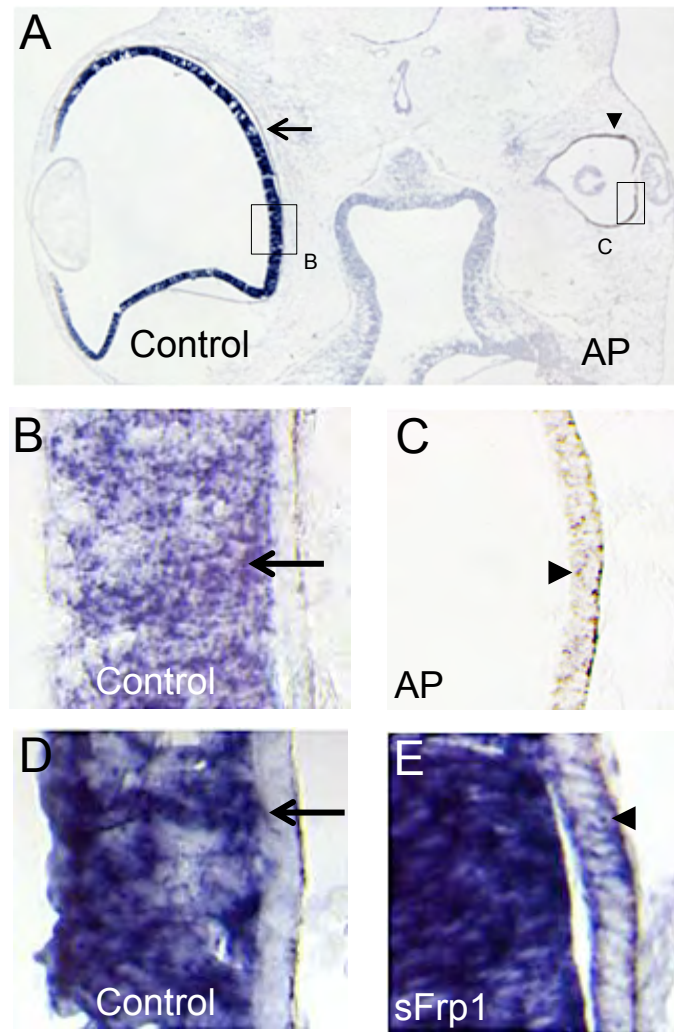


**Fig. S1. Expression of proteins following implantation of PBS- and/or DMSO-soaked beads in the presence or absence of the surface ectoderm.** (A-E) Contralateral ov (arrows) at stage 12/13, showing MITF (B),  $\beta$ -catenin (C) and Pax7 (E) expression following removal of the surface ectoderm and implantation of a DMSO-soaked bead. The overlay shown in (D) shows that at this stage nuclear staining of  $\beta$ -catenin starts to co-localise with a few MITF-positive cells (arrowhead). (E) In a parallel section Pax7 marks the neural crest-derived mesenchyme dorsally to the ov (asterisk). (F-J) Expression of MITF (G),  $\beta$ -catenin (H), and overlay (I) following surface ectoderm removal and implantation of a DMSO-soaked bead. MITF expression is lost (arrowhead in G), although in this case Pax-7 positive mesenchymal cells are surrounding the entire ov (asterisk in J). (K-P) Effects on protein expression at early optic cup stages, following implantation of two beads soaked in PBS (K-M), or one in PBS and one in DMSO (N-P). MITF expression is detected in the dorsal RPE (arrowheads in L, L'; O, O') and pSmad1 labelling is not detected ventrally (arrowheads in M and P). Note that pSmad labelling is detected in the presumptive RPE, dorsal NR and dorsal surface ectoderm (asterisks). (Q-R'') Effect on protein expression following surface ectoderm removal and implantation of two beads, one soaked in PBS and one soaked in DMSO. In the absence of the surface ectoderm, pSmad is not detected in the ov (arrowheads in R-R''), although Pax-7 positive mesenchymal cells (asterisk) are surrounding the optic vesicle.





**Fig. S2. Electroporation of Wnt2b siRNA does not affect MITF expression.** (A) Frontal view of an embryo one day following electroporation of Wnt2b siRNA at ov stages. On the treated (arrowhead) and untreated (arrow) side an eye cup developed. (B) The electroporated ov of this embryo is GFP-positive (arrowhead). (C, C') MITF expression in the RPE of the untreated side (arrow). (D, D') MITF expression (arrowheads) appears to be unaffected following electroporation of Wnt2b siRNA at ov stages. The asterisk marks the GFP-positive surface ectoderm.



**Fig. S3. Effects on *Vsx2* expression following activation and inhibition of Wnt signalling at ov stages.** (A) Expression of *Vsx2*, 3 days following GSK-3 $\beta$  inhibition at ov stages. On the Alsterpaullone (AP)-treated side, a small microphthalmic and pigmented eye vesicle (arrowhead) develops with decreased *Vsx2* expression, when compared to the untreated side (arrow). (B) Higher magnification of the untreated eye showing *Vsx2* expression in the NR (arrow). (C) Following GSK-3 $\beta$  inhibition, *Vsx2* expression is dramatically downregulated and the distal ov is pigmented and has got RPE-like morphology (arrowhead). (D) *Vsx2* expression of a stage 25 chick eye. *Vsx2* expression is restricted to the NR and expression is not detected in the RPE (arrow). (E) Following implantation of beads soaked with the Wnt inhibitor sFrp1, faint *Vsx2* expression is detected in the RPE (arrowhead).