Sonic hedgehog is involved in formation of the ventral optic cup by limiting *Bmp4* expression to the dorsal domain

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Summary

Accumulating evidence suggests that Sonic hedgehog (Shh) signaling plays a crucial role in eye vesicle patterning in vertebrates. Shh promotes expression of Pax2 in the optic stalk and represses expression of Pax6 in the optic cup. Shh signaling contributes to establishment of both proximal-distal and dorsal-ventral axes by activating Vax1, Vax2, and Pax2. In the dorsal part of the developing retina, *Bmp4* is expressed and antagonizes the ventralizing effects of Shh signaling through the activation of *Tbx5* expression in chick and Xenopus. To examine the roles of Shh signaling in optic cup formation and optic stalk development, we utilized the *Smoothened* (*Smo*) conditional knockout (*CKO*) mouse line. Smo is a membrane protein which mediates Shh signaling into inside of cells. Cre expression was driven by *Fgf15* enhancer. The ventral evagination of the optic cup deteriorated from E10 in the *Smo-CKO*, whereas the dorsal optic cup and optic stalk develop normally until E11. We analyzed expression of various genes such as *Pax* family (*Pax2/Pax6*), *Vax* family (*Vax1/Vax2)* and *Bmp4*. *Bmp4* expression was greatly upregulated in the optic vesicle by the 21-somite stage. Then *Vax1/2* expression was decreased at the 20-24somite stages. *Pax2/6* expression was affected at the 27-32somite stages. Our data suggest that the effects of the absence of Shh signaling on *Vax1/Vax2* are mediated through increased *Bmp4* expression throughout the optic cup. Also unchanged patterns of *Raldh2* and *Raldh3* suggest that retinoic acid is not the downstream to Shh signaling to control the ventral optic cup morphology.

Introduction

In vertebrates, there are three members of the hedgehog family: Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh) (Ingham and McMahon, 2001). Shh is required for multiple aspects of development in a wide range of tissue types (reviewed in McMahon et al., 2003). Smo is a membrane protein which medicates hedgehog (Hh) signal into the cells (Taipale et al., 2002). In the absence of Hh, Patched (Ptc) represses Smo. Hh binding to Ptc releases Smo, which then transduces the signal intracellularly. Downstream of Smo, a multimolecular network, through interactions with microtubules, transduces the Hh signal to modify the activity of Gli proteins. These zinc-finger motif transcription factors, Gli1, Gli2 and Gli3, play critical roles in the mediation and interpretation of Hh signals through the activation and repression of Hh target genes (Amato et al., 2004).

The eye develops from the optic vesicle which arises as an optic eminence of the neuroepithelium of ventrolateral forebrain at embryonic day (E) 8-8.5 in mice (Rugh et al., 1968; Pei and Rhodin, 1970). As the optic vesicle expands distally, its proximo-distal (P-D) axis is established. Next the distal-most region invaginates to form the optic cup while the proximal region gives rise to the optic stalk. Shh, secreted from the ventral midline, plays important roles in this process. Genetic ablation of Shh in mice leads to severe defects in the anterior neural tube and cyclopia (i.e. the presence of an unseparated optic vesicle) (Chiang et al., 1996). Gain-of-function experiments led to the conclusion that Shh promotes proximal fate and represses distal fate by regulating the expression of Pax genes. In zebrafish and Xenopus, Shh overexpression promotes expression of Pax2, a marker of optic stalk, and represses expression of Pax6, a marker of the retina (Ekker et al., 1995, Macdonald et al., 1995, Perron et al., 2003). In addition, these two genes transcriptionally repress each other, forming a precise boundary between the retina and the optic stalk (Schwarz et al., 2000)

Studies in mice suggest that Shh is also involved in the establishment of eye dorsal-ventral (D-V) axis. Previous studies implicate the paired homeodomain transcription factors, Pax-6

and Pax-2, and the secreted Shh in dorsal-ventral patterning of the optic vesicle. Soon after the evagination of the optic vesicle, the expression of Pax-6 becomes restricted to the cells of the developing optic cup, which include progenitors of the pigment epithelium and the retina (Grindley et al., 1995). The expression domain of Pax-2 first overlaps that of Pax6 in the ventral retinal cells surrounding the choroid fissure. Later, Pax 2 expression is complementary to Pax-6 expression with a sharp boundary between the retina and the optic stalk (Nornes et al., 1990; Schwarz et al., 2000). Loss of Pax-6 function in the small eye (Sey) mouse and rat leads to the absence of the eyes (Grindley et al., 1995, Ohsumi-Yamashita et al., 1997) while loss of Pax-2 results in defects of the optic tract and chiasm (Torres et al., 1996). Furthermore, Pax-6 and Pax-2 expression in the optic vesicle is regulated by Shh. Alterations in Shh activity in zebrafish have been shown to perturb Pax-6 and Pax-2 expression, leading to anomalies of eye development (Macdonald et al., 1995; Ekker et al., 1995).

The optic vesicles receive two antagonistic signals: Shh from the ventral midline and BMP4 from the dorsal part of the optic vesicle. These molecules act in a coordinated manner to pattern the eye along the D-V axis, repressing each other (Ohkubo et al., 2002). It is likely that this mutual repression is achieved by their target genes, *Vax2* and *Tbx5*. *Vax2* is activated in the ventral part of the optic vesicle by Shh (Sasagawa et al., 2002). *Tbx5* is activated in the dorsal part of the optic vesicle by *Bmp4* (Sasagawa et al., 2002, Koshiba-Takeuchi et al., 2000). Their misexpression affects the D-V axis of the eye (Barbieri et al., 1999; Koshiba-Takeuchi et al., 2000). Vax2 drives development of the ventral tissue by inhibiting development of the dorsal tissue (Mui et al., 2005).

Vax1 and *Vax2* are homeobox genes and expressed in the retina primordium. The two genes share the same gene organization (Ohsaki et al., 1999). At E9.5, both *Vax* genes were expressed in the ventral optic vesicles. Between E11.5–E14.5, *Vax1* became restricted to the optic stalk while *Vax2* was expressed in the ventral half of the neural retina anlagen. At E9.5, the optic vesicle had already been patterned along the dorsal-ventral axis through the action of

Shh (Mui et al., 2005). By the study of *Vax1* homozygous mutants, it has been indicated that *Vax1* and *Pax2* expression in the optic stalk requires midline signals, such as Shh (Hallonet et al., 1999). Also, Shh overexpression leads to dorsal expansion of the *Vax2* expression domain (Sasagawa et al., 2002). *Vax2* has been thought to play an important role in eye development because of both its expression patterns and functional studies carried out in frog and chicken (Barbieri et al., 1999; Schulte et al., 1999). In another report, the analysis of *Vax2* mutant mice demonstrates that *Vax2* is essential for normal eye formation and pathfinding of retinal ganglion cell axons (Barbieri et al., 2002).

The previous studies have demonstrated that Shh signaling regulates the above genes during eye development. However, it has not been elucidated whether these genes are the direct targets of Shh signaling. In this study, we examined expression patterns of these genes in *Smo*-conditional knock-out mice. We identified the temporal and spatial changes of expression of these genes. At least at early stages, the effects of Shh signaling on *Vax1/Vax2* expression were mediated through Bmp4, but not through Pax6 and Pax2. It is also possible that Shh signaling does not directly regulate *Vax1/Vax2* expression in the eye field at all stages. Furthermore, Shh signaling is critical for the ventral retinal cell proliferation and survival. Our data also suggest Shh activity is required to maintain the dorsal part of the developing optic cup.

Results

Generation of *Smo*-conditional knock-out mice

To examine the roles of Shh signaling in optic cup development, we utilized the *Smo* conditional allele line (Zhang et al., 2001). To remove the *Smo* conditional allele in the developing optic cup cells, we used the Cre transgenic mice in which Cre expression is driven by the *Fgf15* enhancer (Saitsu et al., 2006). To gain *Fgf15nCre; Smo c/-* mice (*Smo-CKO*), *Fgf15nCre* mice were mated with *Smo +/- mice*. *Fgf15nCre; Smo +/-* mice were mated with *Smo c/c* to produce *Fgf15nCre; Smo c/-* (*Smo-CKO*). *Smo-CKOs* were considered as conditional mutant embryos. *Smo c/+* and *Fgf15nCre; Smo c/+* were considered as Control 1 and 2, respectively.

To clarify Cre expression, coronal sections of eyes were immunostained at the 26-somite stage (E9.75) (Fig.1A-C) and the 36-somite stage (E10.5) (Fig. 1D-F). Cre expression was observed in the distal to ventral walls of the optic vesicle (Fig.1B and C) at the 26-somite stage (E9.75). At the 36-somite stage (E10.5), *Smo-CKOs* and Control 2 expressed Cre (brown) in the dorsal and middle domains of neural retina (Fig. 1E and F). Cre expression in these embryos corresponded to *Fgf15* expression at the same stage. Cre staining was not detected on Control 1 at the 26-somite stage (E9.5) and the 36-somite stage (E10.5) (Fig. 1A and D).

To confirm Cre activity, *Fgf15-Cre* transgenic mice mated with Rosa26 reporter mice, in which lacZ expression is constitutively activated in cells after Cre-mediated recombination. At E9.75 and E10.5 recombination occurred at very high efficiency in optic stalk and optic vesicle (data not shown). These results confirmed that a Cre-mediated recombination had been undergone in optic vesicle and optic stalk of *Smo-CKOs* by the 20-somite stage.

By RNA in situ hybridization (Fig. 1G-1I), *Smo* mRNA expression was completely undetectable in the optic vesicle and optic stalk of *Smo-CKOs* at the 20-somite stage (Fig. 1I). We also examined expression of *Gli1*, which is thought to be the most faithful reporter of Hh

signaling (Corrales et al., 2004). *Gli1* mRNA was not detected at all in the optic vesicle of *Smo-CKOs* (Fig. 1L) while it was detected in that of Controls (Fig. 1J, K). These results indicate that Cre-mediated removal of the *Smo* allele efficiently occurred in the developing optic vesicles.

Craniofacial morphology and eye histology

At the 26-somite stage (E9.75) the optic vesicle was not obviously affected (Fig. 2C and L). At the 30-somite stage, morphology of the optic vesicle still seemed to be almost normal though cell proliferation was decreased (see below). There were several defects in *Smo-CKOs* from the 32-somite stage (Fig. 2D-I, 2M-R). The whole body size of *Smo-CKOs* was smaller than that of Control 1 and 2 after the 26-somite stage (E9.75), and the diencephalon was disproportionately hypotrophic (Fig. 2F; I white arrow). The ventral parts of *Smo-CKO* optic vesicles were lost or did not grow appropriately at the 32-somite stage (E10), the 35-somite stage (E10.5) and the 40-somite stage (E11) (Fig. compare 2F with 2D-E, 2O with 2M-N, 2R with 2P-Q. black arrows). The hypotrophic lens anlagen was observed at the 35-somite stage (E10.5) (Fig. 2O, green arrow). Shortly after, at the 40-somite stage (E11), the lens anlagen and ventral optic cup were not observed at all (Fig. 2R). After this stage, the dorsal optic cup also degenerated. Newborn *Smo-CKOs* had no eye tissue with complete penetrance (Fig. 2I) while Control 1/2 did not show any abnormalities (Fig. 2G, H).

Cell proliferation is decreased and cell death is increased in *Smo-CKOs*

The failure of ventral optic cup development could reflect altered retinal cell proliferation and apoptosis. To examine cell proliferation, we performed BrdU incorporation analysis at 21-somite stage and 30-somite stage (Fig. 3A-F). At the 21-somite stage in *Smo-CKOs*, incorporation in the optic vesicle was not significantly different from Controls (Fig. 3A-C). In contrast, at the 30-somite stage, the ventral optic cup of *Smo-CKOs* showed a significantly

decreased incorporation index compared with Controls (Fig. 3F, G). Thus, a reduced rate of proliferation in optic vesicle precursors at the 30-somit stage, at least partly contributes to the defects of the ventral optic cup. To determine whether cell death was increased in the optic vesicle of *Smo-CKOs*, we performed Caspase-3 immunostaining that marks apoptotic cells. In the optic vesicle of Control 1 and 2 mice, there was few Caspase-3-positive cells at the 24-somite stage (Fig. 3H-I), while *Smo-CKO*s exhibited increased Caspase-3-positive cells in optic vesicle (Fig. 3J). These data indicated that increased cell death also may contribute to the ventral optic cup phenotype.

Pax6 mRNA expression and Pax2 protein distribution in the optic vesicle of _Smo-CKO_s are altered from the 30-somite stage

Previous studies in chick, mouse and Xenopus suggest that *Shh* is also involved in the establishment of the eye dorsal-ventral (D-V) axis (Huh et al., 1999; Zhang et al., 2001; Sasagawa et al., 2002). To confirm *Pax6* expression, we performed RNA in situ hybridization with *Pax6* probe. While *Pax6* was coexpressed with *Pax2* at early stages, they repress each other later to become distinctly expressed in optic cup and optic stalk, respectively (Schwarz et al., 2000; Baumer et al., 2003). At the 27-somite stage, the *Pax6* expression pattern in *Smo-CKOs* was not different from that in Control 1 and 2 (Fig.4A-C). At the 30-somite and 35-somite stages, the ventral optic cup was hypomorphic or degenerated (data not shown, Fig.2O, Fig.3F). Therefore, *Pax6* expression in the ventral part was not detected in *Smo-CKOs* while it remained in that of Controls (Fig. 4D-I). While the ventral tissues itself was not detectable, the *Pax6*-positive hypomorphic lens was still observed at the 35-somite stage (Fig.4I). We also performed double immunostaining of Pax2 and Pax6. Pax6 (red in Fig.4J-K) was coexpressed (yellow) with Pax2 (green) in optic vesicles at the 25-somite stage. Pax2 immunoreactivity showed a similar pattern to Pax6 at the 27-somite stage in all genotypes (Fig. 4J-O), suggesting that increased BMP4 (see below) did not affect the ventral

optic cup and optic stalk at this stage yet. At the 32-somite stage, Pax2 protein was restricted only in the optic stalk of *Smo-CKOs,* though it was detected in the optic cup of Controls as well (Fig. 4P-R). By the 40-somite stage, Pax2 was decreased more obviously, and the Pax2-posotive optic stalk of *Smo-CKOs* became shorter than that of Controls (Fig.4S-U). These findings indicate that the changes of Pax2 and *Pax6* expression patterns happened after the 27-somite stage in *Smo- CKOs*.

Vax1 and _Vax2_ mRNA are repressed earlier than Pax2 and _Pax6_ expression domains are changed

At the 20-somite stage, *Vax1* was expressed normally in *Smo-CKOs* (Fig.5A-C, A'-C'). Similarly, *Vax2* expression did not show any abnormality in *Smo-CKOs* at the 22-somite stage (Fig.5J-L, J'-L'). At the 24-somite stage, *Vax1* and *Vax2* expression patterns in the dorsal optic cup of *Smo-CKOs* were different from those of Controls (Fig. 5D-F, D'-F'; M-O, M'-O'). At the 24-somite stage *Vax1* mRNA and *Vax2* mRNA were coexpressed in almost all cells of both dorsal and ventral parts of the optic vesicle in Controls (*Vax1*: Fig.5D, D', E, E'; *Vax2*: 5M, M', N, N'). However, in *Smo-CKOs, Vax1/2* expression in the dorsal optic vesicle was downregulated almost to the background level (Fig.5F, F', O, O'). The data suggest that the dorsal defects of *Vax1* and *Vax2* expression were caused by the ectopic *Bmp4* expression at the 21-somite stage (Fig.6F, F') as mentioned below. At the 30-somite stage, *Vax1* was not detectable in the ventral optic cup of *Smo-CKOs* because of both low expression level and ventral tissue defects (Fig.5I, I'). At the 38-somite stage, *Vax2* expression was undetectable in *Smo-CKOs* because of ventral tissue defects of the optic cup (Fig.5R, R'). The data show that downregulation of *Vax1* and *Vax2* expression was initiated between the 20- and 24-somite stages in the dorsal optic vesicle of *Smo-CKOs*.

Bmp4 is upregulated in the optic vesicle of _Smo-CKOs_

In chick, overexpression studies indicate that ectopic *Bmp4* expands *Tbx5* expression into the ventral part of the optic vesicle. Tbx5, then, represses ventrally expressed *cVax* (Koshiba-Takeuchi et al., 2000). In mice, *Bmp4* represses the ventral optic cup marker *Vax2* (Behesti et al., 2006). *Smo-CKOs* exhibited slightly increased *Bmp4* expression around the optic pit region at the 18-somite stage (Fig.6C). At the 21-somite stage, *Bmp4* expression was greatly upregulated in the optic vesicle of *Smo-CKOs* (Fig.6F, F'). *Bmp4* was not detectable in Controls at this stage (Fig.6D, D', E, E'). At the 27-somite stage, *Bmp4* was confined to the dorsal optic cup in Controls (Fig.6G, G', H, H') while *Bmp4* expression expanded into the ventral optic cup of *Smo-CKOs* (Fig.6I, I'). *Vax1* and *Vax2* expression defects were initiated in the dorsal optic vesicle of *Smo-CKOs*, but not in the ventral at the 24-somite stage (Fig.5F, F', O, O'). Later, at the 30-somite stage, *Vax1* was also undetectable in the ventral optic vesicle (Fig.5I, I'). At the 38-somite stage, Vax2 was not detectable since the ventral part of the optic cup was absent (Fig.5R'). These results suggest that downregulation of *Vax1* and *Vax2* in the dorsal optic vesicle at the 24-somite stage and in the ventral optic vesicle at the 30-somite stage was caused by the increased concentration of BMP4 and ventral tissue defects in *Smo-CKOs*. *Shh* expression in the ventral midline was not affected (data not shown). To confirm downregulation of *Vax1* and *Vax2* by BMP4, eye culture with and without BMP4 was performed. After 6 hours, *Vax1* and *Vax2* expression was downregulated in the BMP4-positive culture (data not shown), consistent with the previous study (Behesti et al., 2006).

Bmp4 action in the nervous system includes effects on neural induction, cell fate determination, apoptosis and proliferation (Mehler et al., 1997). In the chick retina, *Bmp4* has been implicated in regulating programmed cell death in the dorsal optic cup (Trousse et al., 2001) and in regulating topographic mapping of retinal ganglion cells (Koshiba-Takeuchi et al., 2000). At 21-somite stage the *Bmp4* mRNA was increased abnormally in the optic vesicle of *Smo-CKOs* (red arrow in Fig. 6F). At the 24-somite stage, cell death was increased in the

optic vesicle of *Smo-CKOs* (Fig.3J). Our data suggest that the ectopic expression of *Bmp4* contributed to cell death in the optic vesicle of *Smo-CKOs*.

RA does not act downstream to Shh signaling

Some studies suggest a connection between impaired retinol or retinoc acid (RA) function and developmental eye defects in humans and mice. RA is synthesized in discrete regions of the embryonic eye by three retinaldehyde dehydrogenases (*RALDHs: Raldh1, Raldh2 and Raldh3*) displaying distinct expression patterns (Molotkov et al., 2006). At early stages, *Raldh2* is expressed in the mesenchyme and *Raldh3* is expressed in the retinal pigmented epithelium. RA delivers an essential signal to the neural retina, leading to ventral invagination of the optic cup. *Raldh2–/–; Raldh3–/–* double null embryos exhibit a failure in the ventral invagination of the optic vesicle that defines the junction between the ventral retina and optic stalk. These results show RA is necessary for ventral invagination of the optic cup (Molotkov et al., 2006). Morphologically, this mutant phenotype is very similar to *Smo-CKOs*. Therefore, to see whether RA is involved in the eye phenotype of *Smo-CKOs*, we examined *Raldh2* expression pattern at 24-somite stage and *Raldh3* expression pattern at 25-somite stage in *Smo-CKOs*. As no overt alterations of these genes expression were observed (Fig.7), it is most likely that Shh signaling and RA control eye development independently, consistent with the previous report (Sasagawa et al., 2002).

Discussion

Vax1* and *Vax2* expression patterns might be altered by increased BMP4 in *Smo-CKO

Shh is the dominant ventralizing signal in the developing eye field as it is elsewhere in the embryonic CNS; it patterns the eye field through induction of the *Pax2* gene and repression of the *Pax6* gene proximally, leaving *Pax6* expression distally (Ekker et al. 1995; Macdonald et al. 1995). Our results demonstrate that *Pax6*/Pax2 expression patterns were not affected until

the 27-somite stage (Fig.4C, O). *Vax1* and *Vax2* exhibited the expression changes as early as the 24-somite stage (Fig. 5F, F', O, O'), suggesting that downregulation of *Vax1/2* in *Smo-CKOs* is not mediated by Pax2/6. The *Vax1/Vax2* double mutant mice study indicates Vax1/2 proteins directly inhibit optic stalk expression of Pax6 which acts as a dominant driver of retinal differentiation (Mui et al., 2005). *Vax1* and *Pax2* cooperatively repress the *Pax6* gene (Mui et al., 2005). The *Vax* and *Pax2* genes are coexpressed in the ventral optic primordium and both of them are maintained by *Shh* (Schulte et al. 1999; Take-uchi et al. 2003).

Misexpression of chicken *Vax* results in upregulation of *Pax2* (Schulte et al. 1999; Barbieri et al. 2002). *Vax* gene expression is maintained in *Pax2*-/- mice (Bertuzzi et al., 1999). These observations and our results indicate *Vax1* and *Vax2* are upstream to *Pax2/Pax6* in the developing eye field. The effects of *Shh* on *Vax1/Vax2* are not mediated through *Pax2/Pax6*, whereas it can not be determined if *Vax* genes are the direct targets of Shh signaling.

We examined another important signal, Bmp4, in the developing eye. Shh from the ventral midline and Bmp4 from the dorsal optic vesicle are two antagonistic signals. These molecules act in a coordinated manner to pattern the eye along D-V axis, repressing each other (Ohkubo et al., 2002). *Smo-CKOs* clearly exhibited the increased *Bmp4* mRNA in the optic region at 21-somite stage (Fig. 6C). The *Bmp4* expression pattern was changed earlier than *Vax1/Vax2* and Pax2/*Pax6*. Furthermore, the previous report showed that exogenous BMP4 extends expression of T-box genes, *Tbx2/3/5* in the optic cup and reduces *Vax2* expression in the ventral optic cup (Behesti et al., 2006). In *Drosophila*, Dpp regulates *omb*, a T-box gene critical for formation of fly eyes. BMP4 is a vertebrate homolog of Dpp and *Tbx2/3/5* are vertebrate homologs of *omb*. Several lines of evidence suggest that this regulation is conserved in vertebrates, namely BMP4-soaked beads induce ectopic expression of *Tbx2/3/5* in the developing retina (Behesti et al., 2006).

Our data showed that *Bmp4* was increased in all the optic vesicle regions of *Smo-CKOs* from the 21-somite stage. *Vax1* and *Vax2* expression defects were initiated in the dorsal optic vesicle of *Smo-CKOs* at the 24-somite stage (Fig.5F, F', O, O'). These results suggest that down-regulation of *Vax1/2* was caused, directly or indirectly, by the increased concentration of BMP4 in the optic vesicle of *Smo-CKOs* before the 24-somite stage (Fig. 6C, F, F'). In addition, the 21-24 somite stages, the effects of loss of Shh signaling on *Vax1/Vax2* might be mediated through *Bmp4*, but not through *Pax6* and *Pax2* (Fig.8). *Pax6* is directly repressed by *Vax1* and *Vax2*, and Vax proteins may normally act to maintain expression of Pax2 (Mui et al., 2005). It is also possible that Shh signaling dose not directly upregulate *Vax1/Vax2* expression in the eye field. As shown in Fig.5, *Vax1/2* mRNA was expressed in the whole optic cup of Control embryos at the 24-somite stage (Fig.5D, D', E, E', M, M', N, N'). After the 24-somite stage, the dorsal expression gradually disappeared (Fig.5G, G', H, H', P, P', Q, Q'), suggesting that *Vax1/2* expression is initiated in all regions by unknown factors and that the dorsal expression is repressed by BMP4 (Fig.8).

It has been proposed that *Tbx5* represses *cVax* and vice versa in chick based on overexpression studies (Schulte et al., 1999; Koshiba-Takeuchi etal., 2000; Adler et al., 2002). The downregulation of *Vax1/2* in *Smo-CKOs* by increased *Bmp4* was started from the dorsal optic vesicle. This *Vax1/2* down-regulation might be mediated by Tbx5 and/or unknown factors. Our results from eye culture that *Vax1/2* expression was quickly (within 6 hours) repressed in the presence of BMP4, suggesting that this repression was mediated by very few factors. However, as mentioned above, the repression was most likely mediated, at least partly, by *Tbx5* in the dorsal optic cup. As discussed above, it is most likely that the regulation of *Tbx* genes by BMP4 may be conserved between flies and vertebrates. To elucidate whether repression of *Vax1/2* by BMP4 is direct or indirect, the presence of functional Smad-binding sites in the enhancer of *Vax1/2* genes must be demonstrated.

Shh is essential for formation of the ventral optic cup and maintenance of the developing dorsal optic cup

The detailed coronal sections of cell proliferation analysis showed that ventral invagination of the neural retina did not happen in *Smo-CKOs* at the 30-somite stage (Fig. 3D-F). The disturbed invagination led to the ventral optic cup defect from the 30-somite stage in the *Smo-CKOs*, whereas the dorsal optic cup and optic stalk develop normally until E11. At E9.5, *Raldh2* in the mesenchyme and *Raldh3* in the retinal pigmented epithelium generate RA that delivers an essential signal to neural retina. This signal is required for morphogenetic movements that lead to ventral invagination of the optic cup (Molotkov et al., 2006). We examined the *Raldh2* and *Raldh3* mRNA expression patterns. *Raldh2* and *Raldh3* expression patterns were not obviously different around the optic cup (Fig.7). These results suggest that RA is not the downstream to Shh signaling to control the ventral optic cup morphology.

It has been reported that Shh signaling plays an important role in layer formation of the retina (Yu et al., 2006). *Shh* is expressed in the retinal ganglion cells and the eye-specific knockout of *Shh* resulted in perturbation of layer formation (Wang et al., 2005). This abnormality could not be observed in our *Smo-CKOs* since the dorsal retina/optic cup was degenerated by E11.5. This result also suggests that the ventral midline-derived Shh signaling is essential for maintenance and development of the dorsal retina.

Shh signaling controls cell proliferation and survival

Our morphological and marker analysis indicate that Shh is clearly critical for formation of the ventral optic cup. In *Smo-CKOs*, the ventral half of the optic cup showed a significantly decreased BrdU incorporation rate at the 30-somite stage in comparison with the control embryos (Fig.3G).

Ectopic expression studies have demonstrated that Shh can have a mitogenic role in the developing CNS (Rowitch et al., 1999). In particular in the cerebellum there is good evidence

that Purkinje cell-derived Shh is the principal mitogen for proliferation of cerebellar granule cell precursors (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Moreover, several G1 cyclins, including *Ccnd1*, are transcriptional targets in this mitogenic response (Kenney and Rowitch, 2000). Also, *Shh* mutants demonstrated Shh may play a role in regulating neural precursor proliferation in the diencephalic and midbrain regions (Ishibashi and McMahon, 2002).

Previous studies have showed that high concentrations of recombinant N-terminal Shh (Shh-N) (Lee et al., 1994; Fan et al., 1995; Roelink et al., 1995) in embryonic day (E) 18 mouse pellet cultures caused a marked increase in retinal progenitor cell proliferation and general increases in the accumulation of differentiated cell types (Jensen and Wallace, 1997). Another study showed Shh-N appears to have a transient mitogenic effect, followed by an increase in retinal cell differentiation (Levine et al., 1997).

Also, cell death was increased in the developing spinal cord of *Shh* mutants (Litingtung and Chiang, 2000) and in the cerebral cortex of *Smo$^{c/-}$;Emx1-Cre* mutants (Komada et al., 2008), suggesting requirement of Shh for cell survival. Our results in the optic cup of *Smo-CKOs* are consistent with the previous studies.

However, the previous study with fish embryos demonstrated that increased hh signaling also inhibits eye formation. In embryos of blind cavefish, *shh* and *twhh* expression domain expanded in the anterior midline, compared to surface fish. Consistently, *pax2a* expression was also expanded. Cell death was increased in the developing eye of cavefish, thus the eye primordia degenerated. Injection of *shh* mRNA into surface fish embryos phenocopied cavefish eye defects. Cell death was increased in the injected embryos (Yamamoto et al., 2004). These results suggest that too strong Hh signaling rather deteriorates eye development. It seemed that their results are inconsistent with our results. Our interpretation is that appropriate intensity of Shh signaling from the anterior midline is important for normal eye development. In another words, the balance between ventralizing signals and dorsalizing

signals is crucial for patterning of the eye field.

In conclusion, our results suggest that Shh signaling controls eye morphogenesis by specifying fates along the P-D and D-V axes and by regulating cell proliferation and survival.

Experimental Procedures

Mouse lines

Cre transgenic mice in which Cre expression is driven by the *Fgf15* enhancer (*Fgf15nCre*) were made by Dr. Hirotomo Saitsu (Saitsu et al., 2005). *Fgf15nCre* mice were crossed with *Smo* heterozygous (The Jackson Laboratory, Maine, USA) (Zhang et al., 2001) mutants to generate *Fgf15nCre; Smo*$^{+/-}$ males. These mice were mated with and *Smo*$^{fl/fl}$ (The Jackson Laboratory, Maine, USA) (Long et al., 2001) females to generate *Smo* conditional knockout mice of the informative genotype *Fgf15nCre; Smo*$^{fl/-}$ (*Smo-CKO*). Genotypes of Control embryos were *Smo*$^{c/+}$ and *Fgf15nCre; Smo*$^{c/+}$.

In Situ Hybridization

Whole-mount RNA in situ hybridization of embryos was performed as previously described (Parr et al., 1993). Section in situ hybridization was performed as previously described (Ishii et al., 1997). The probes used in this study were as follows: *Smo* (Akiyama et al., 1997), *Shh*, *Bmp4*, *Pax6* (Ishibashi and McMahon, 2002), *Vax1* and *Vax2* (gifts from Dr. Takahashi) *Raldh2* and *Rakdh3* (from Dr. Okano). Digoxigenin-labeled probes were synthesized using a digoxigenin RNA labeling kit (Roche 1362372).

BrdU incorporation analysis

Pregnant mice at 9.5-10.5-11.5 days of gestation were injected intraperitoneally with BrdU (50μg/g body weight) and were sacrificed 1 hour later. Embryos were fixed in PLP for 3 hours. Then embryos were embedded in paraffin and sectioned at 7 μm for immunohistochemiscal detection of a rat monoclonal anti-BrdU antibody (Sigma B5002-1G).

Immunohistochemistry

Primary antibodies were: anti-Cre (Chemicon MAB3120), anti-Caspase3 (CST Asp175), anti-Pax2 (Zymed 71-6000) and anti-Pax6 (Hybridoma Bank). The ABC avidin/biotin method (Vector) was used. Fluorescent staining was performed for double immunostaining of Pax2 and Pax6.

Acknowledgements

We thank Dr. Motoyama for the plasmid of Smo probe, Dr. Takahashi for Vax1/2 and Dr. Okano for Raldh2/3. We also thank Dr. Komada for technical assistance and Dr. Miura for critical comments. This work is supported by The Japanese Ministry of Education, Culture, Sports Science and Technology (Grant number: 15689004, 18590168, 20590169, 20022020).

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Figure Legends

Fig.1 Expression analysis of nCre protein and *Smo* mRNA

Coronal sections of eyes were immunostained with anti-Cre antibody at the 26-somite stage (A-C) and the 36-somite stage (D-F). Control 2 (B) and *Smo-CKOs* (C) showed nCre immunoreactivity (brown) in the distal to ventral walls of the optic cup. nCre expression (brown) was observed in the neural retina in Control 2 (E) and *Smo-CKOs* (F). In situ hybridization of *Smo* mRNA (G, H, I) at the 20-somite stage (E9.5): *Smo* mRNA expression was completely abolished in the optic vesicle and optic stalk of *Smo-CKOs* (I). In situ hybridization of *Gli1* mRNA (J, K, L) at the 26-somite stage (E9.75): *Gli* mRNA expression was completely abolished on optic cup of *Smo-CKOs* (L). ov: optic vesicle, op: optic cup, os: optic stalk.

Fig.2 Craniofacial morphology and eye histology

Craniofacial morphology: A-I. (A, B, C) The optic vesicle was not obviously different at the 26-somite stage (E9.75). (D, E, F) At the 32-somite stage (E10), *Smo-CKOs* showed the ventral defect of the optic cup (F, black arrow: vop) and the diencephalon was hypotrophic (F, white arrow). (G, H, I) P0: No eyes were observed in *Smo-CKOs* (I, white arrowhead) and the forebrain part was small (I, white arrow), while Contol 1 (G) and Control 2 (H) did not show any abnormality.

Eye histology: J-R. (J, K, L) At the 26-somite stage (E9.75), the optic cup of *Smo-CKOs* was not obviously different from that of Controls. (M, N, O) At the 35-somite stage (E10.5), *Smo-CKOs* displayed the ventral half defect of the optic cup and the hypotrophic lens was detected (O, green arrow). (P, Q, R) At the 40-somite stage (E11), the ventral half of the optic cup and the lens were missing in *Smo-CKOs* (R). Control 1 (P) and Control 2 (Q) did not show any abnormality. vop: ventral optic cup

Fig.3 Cell proliferation and cell death defected in *SmoCKOs*

BrdU incorporation analysis was performed (see Experimental Procedures). (A, B, C) At the 21-somite stage (E9.75), Controls and the mutants showed similar incorporation rates at the 21-somite stages (A-C). (D, E, F) At the 30-somite stage (E10.5), in Control 1 (D, n=3, 64.66±0.98%) the ventral optic cup showed a comparable rate with Control 2 (E, n=3, 66.97±0.34 %) while *Smo-CKOs* (F, n=3, 47.27±0.48%) showed significantly decreased rates of BrdU incorporation (G). (D', E', F') Higher power-views of the ventral optic cups in D-F. (H, I, J) Caspase3 was immunostained. In the optic vesicle of Control 1 (H) and Control 2 (I), there were few Caspase3-positive cells at the 21-somite stage while *Smo-CKOs* exhibited increased Caspase3-positive cells in the optic vesicle (J, black arrows). vop: ventral optic cup. $P<0.01$

Fig.4 *Pax6* and Pax2 expression patterns in the optic vesicle/cup and stalk

(A-I) *Pax6* mRNA expression. (A, B, C) At the 27-somite stage (E9.75), all embryos showed normal expression patterns of *Pax6*. (D, E, F) At the 30-somite stage (E10.25), *Smo-CKOs* showed disturbed expression of *Pax6* in the ventral optic cup (F, vop, red arrowhead). (G, H, I) At the 35-somite stage (E10.5), In *Smo-CKOs* (I) the *Pax6* mRNA was confined to the dorsal optic cup only while *Pax6* mRNA was expressed in both the ventral optic cup and the dorsal optic cup of Control 1 (G) and Control 2 (H). Coronal sections of eye: J-R. (J, K, L) SS25 (E9.75): The double immunostaining of Pax2 (green) and Pax6 (red). Pax2 and Pax6 were coexpressed in the optic vesicles (yellow). (M, N, O) At the 27-somite stage (E9.75), Pax2 expression was not obviously different among all genotypes. (P, Q, R) At the 32-somite stage (E10.25), Pax2 expression domain in the mutant optic stalk (R) was similar to those seen in Control 1 (P) and Control 2 (Q). Pax2 expression of the dorsal optic cup was not detected in the mutants (R, black arrowhead). (S, T, U) At the 40-somite stage (E11), Pax2

expression was reduced in *Smo-CKOs* (U), compared to Control 1 (S) and Control 2 (T) not only in the optic cup but also in the optic stalk. os: optic stalk. dop: dorsal optic cup. vop: ventral optic cup.

Fig.5 *Vax1* and *Vax2* expression patterns in the optic vesicle and cup

(A-R) Side views of whole mount in situ hybridization. (A'-R') In situ hybridization on coronal sections. (A-C, A'-C') At the 20-somite stage (E9.5), the *Vax1* mRNA expression pattern in *Smo-CKOs* was not obviously different from that in Controls. (D-F, D'-F') At the 24-somite stage (E9.75), comparing with Control 1 (D, D') and Control 2 (E, E'), *Vax1* mRNA was not expressed on the dorsal optic cup of *Smo-CKOs* (F, F', red arrow). (G-I, G'-I') At the 30-somite stage (E10), in *Smo-CKOs* (I, I'), *Vax1* mRNA was downregulated in the ventral optic cup (black arrow) while Control 1 (G, G') and Control 2 (H,H') showed *Vax1* expression in the ventral optic cup (black arrow).

(J-R, J'-R') *Vax2* expression patterns were examined. (J-L, J'-L'). At the 22-sfomite stage (9.5), *Vax2* expression patterns in the optic cup were not obviously different in all genotypes. (M-O, M'-O') At the 24-somite stage (E9.75), comparing with Control 1 (M, M') and Control 2 (N, N'), *Vax2* mRNA was not expressed in the dorsal optic cup of *Smo-CKOs* (O, O', red arrow). (P-R, P'-R') At the 38-somite stage (E10), *Vax2*-positive ventral optic cup disappeared completely in *Smo-CKOs* (R', asterisk) while Contrl 1 (P, P') and Control 2 (Q, Q') showed *Vax2* expression in the ventral optic cup (black arrow). dop: dorsal optic vesicle/cup. vop: ventral optic vesicle/cup.

Fig.6 *Bmp4* expression is up-regulated in the *Smo-CKO* eye region

(A-C) At the 18-somite stage (E9.25), the mutant embryos exhibited slightly increased *Bmp4* mRNA in the optic vesicle (C, dotted circle), compared to Controls (A, B). (D-F, D'-F')At the 21-somite stage (E9.5), the *Bmp4* mRNA was greatly increased in the optic vesicle (ov) of the

Smo-CKOs (F, F', red arrow). (G-I, G'-I') At the 27-somite stage (E10), in Control 1(G,G') and Control 2 (H, H'), *Bmp4* mRNA was confined to the dorsal optic cup only, while in *Smo-CKOs* (I) *Bmp4* mRNA was detected in both the ventral optic cup (vop) and the dorsal optic cup (dop).

Fig.7 Raldh2/Raldh3 expression patterns are not altered in the mutants

(A-C) At the 24-somite stage (E9.75), *Raldh2* expression pattern in *Smo-CKOs* (C) was not obviously different from that in Controls (A, B). (D-F) At the 25-somite stage (E9.75), *Raldh3* expression pattern in *Smo-CKOs* (F) was not obviously different from that in Controls (D, E).

Fig.8 Schematic representation of the relationship among Shh, Vax1/2, Pax2/6 and Bmp4

Shh signaling directly or indirectly mediates *Vax1/2* expression in the eye field. *Vax1* and *Vax2* are suppressed by Bmp4 in the dorsal optic vesicle. This suppression might be mediated by Tbx and unknown factors. Vax1 and Vax2 directly inhibit Pax6 expression and maintain Pax2 expression. Pax2 and Pax6 transcriptionally repress each other.

Fig 1

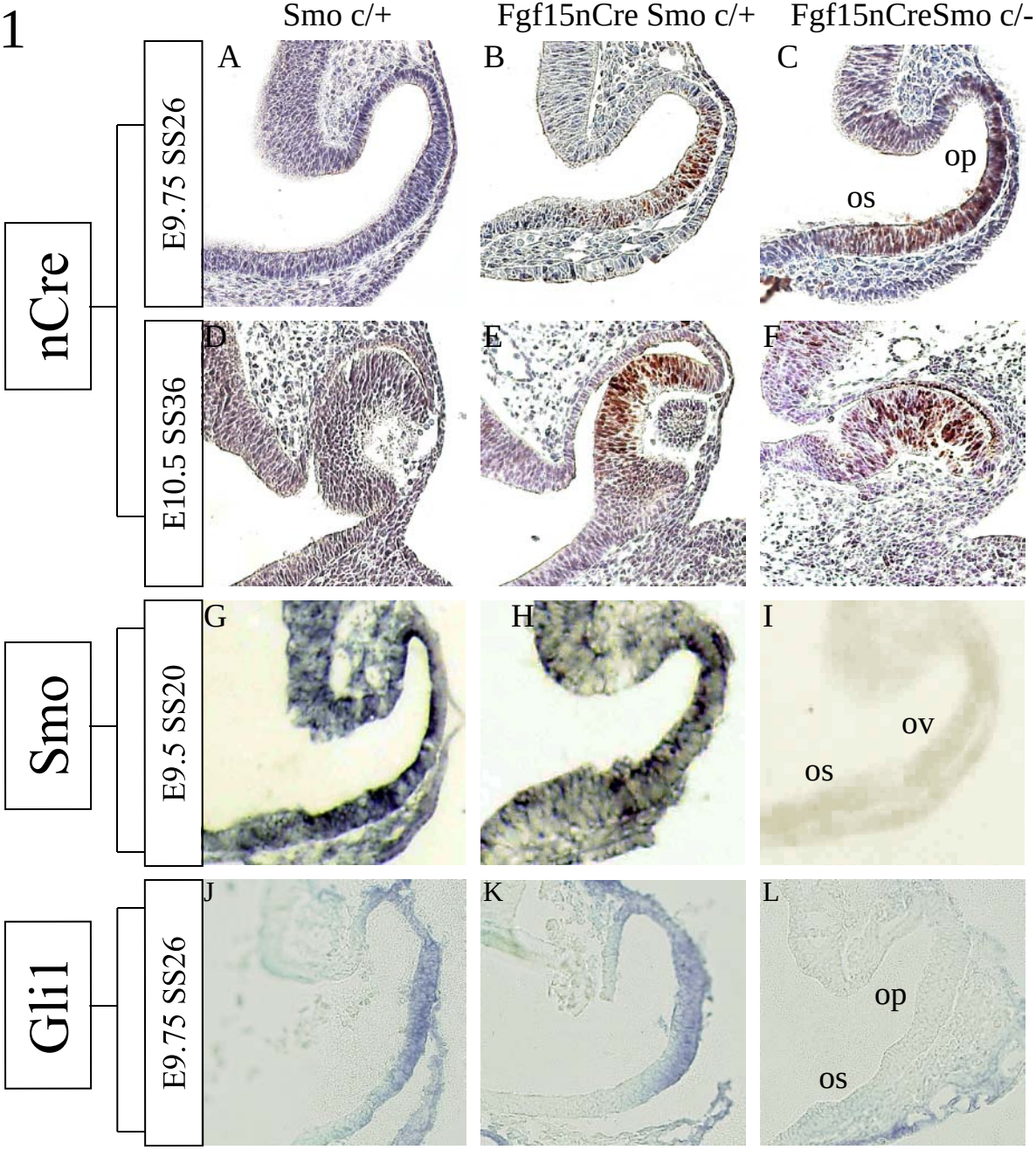
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Fig2

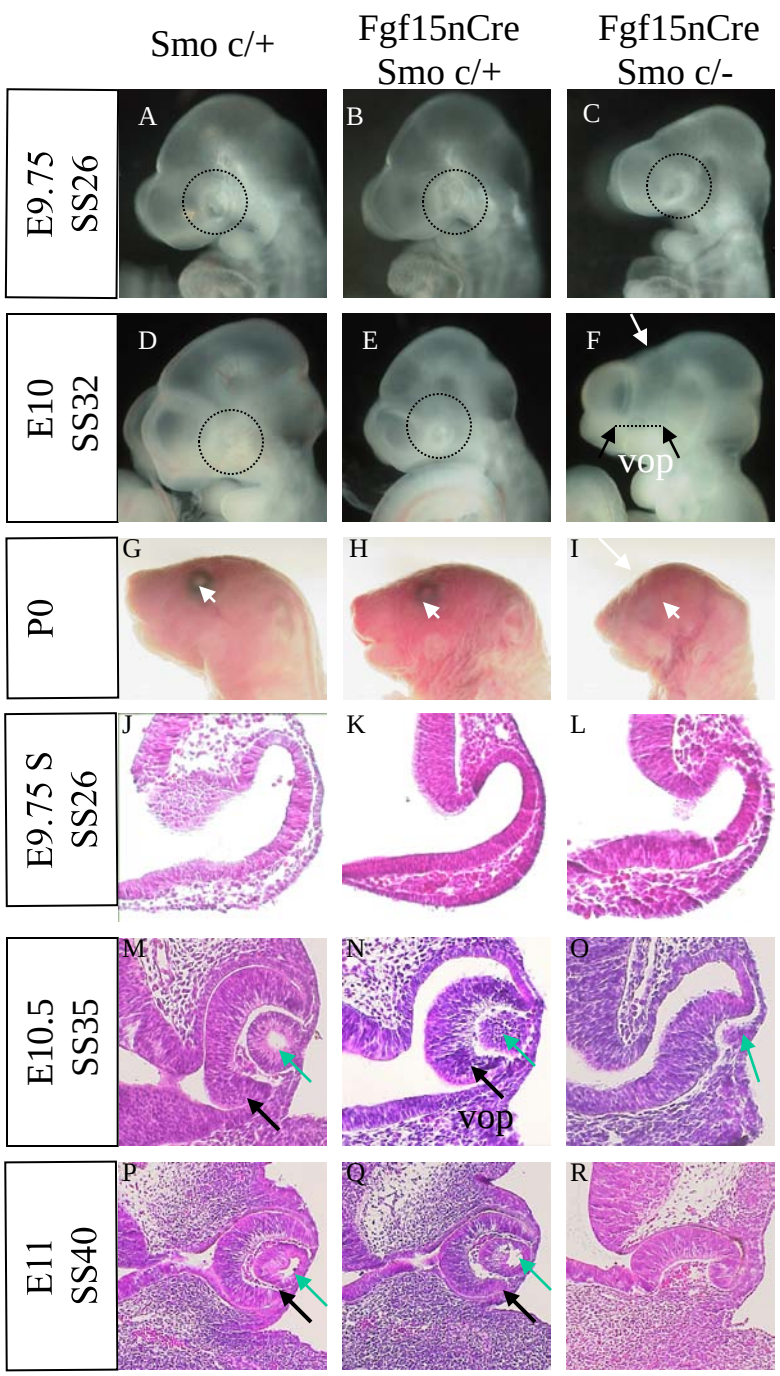
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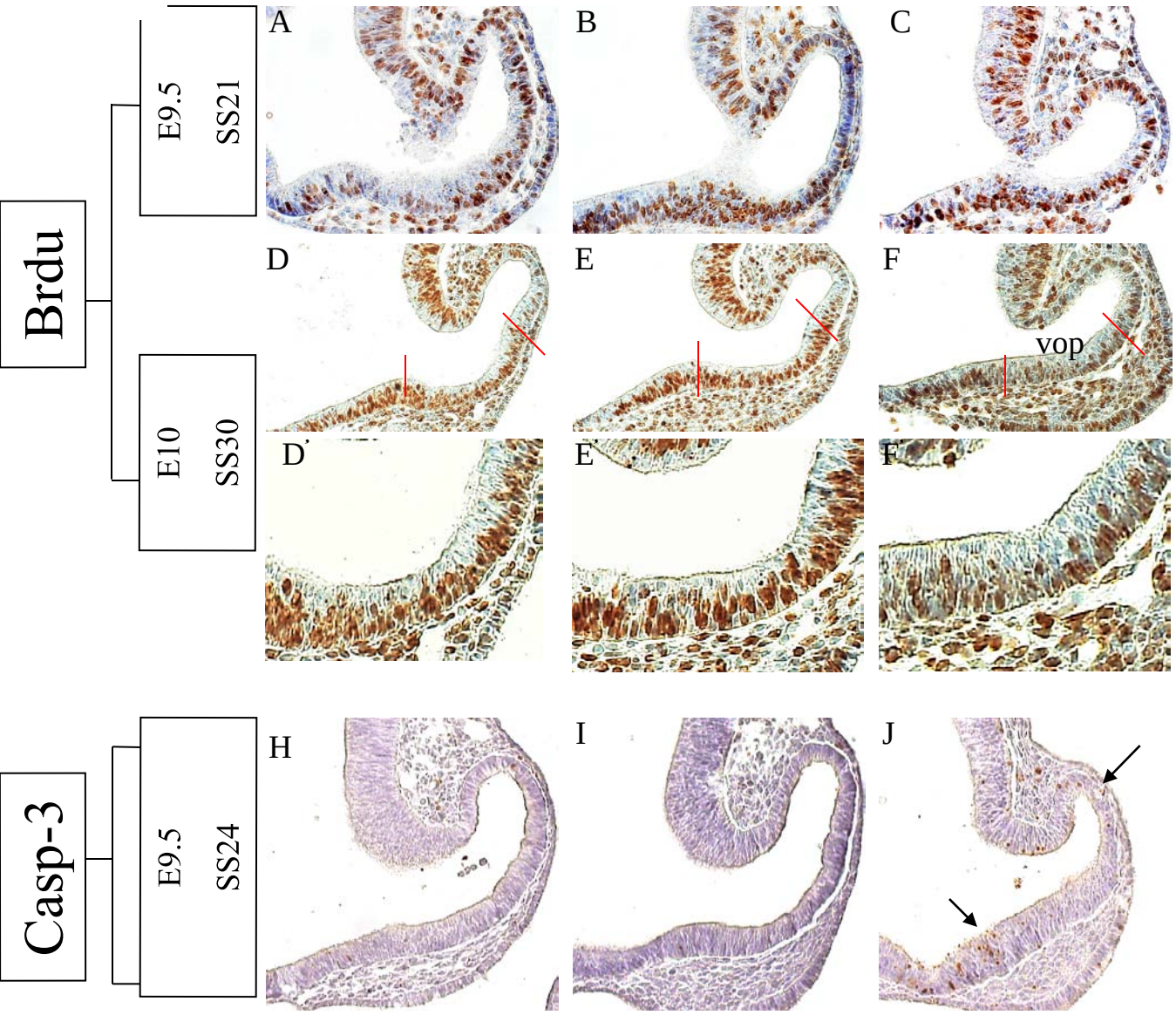
Fig 3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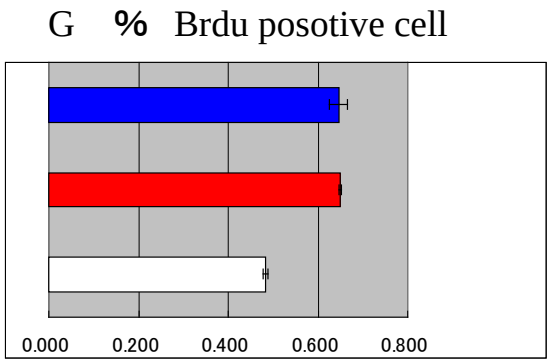
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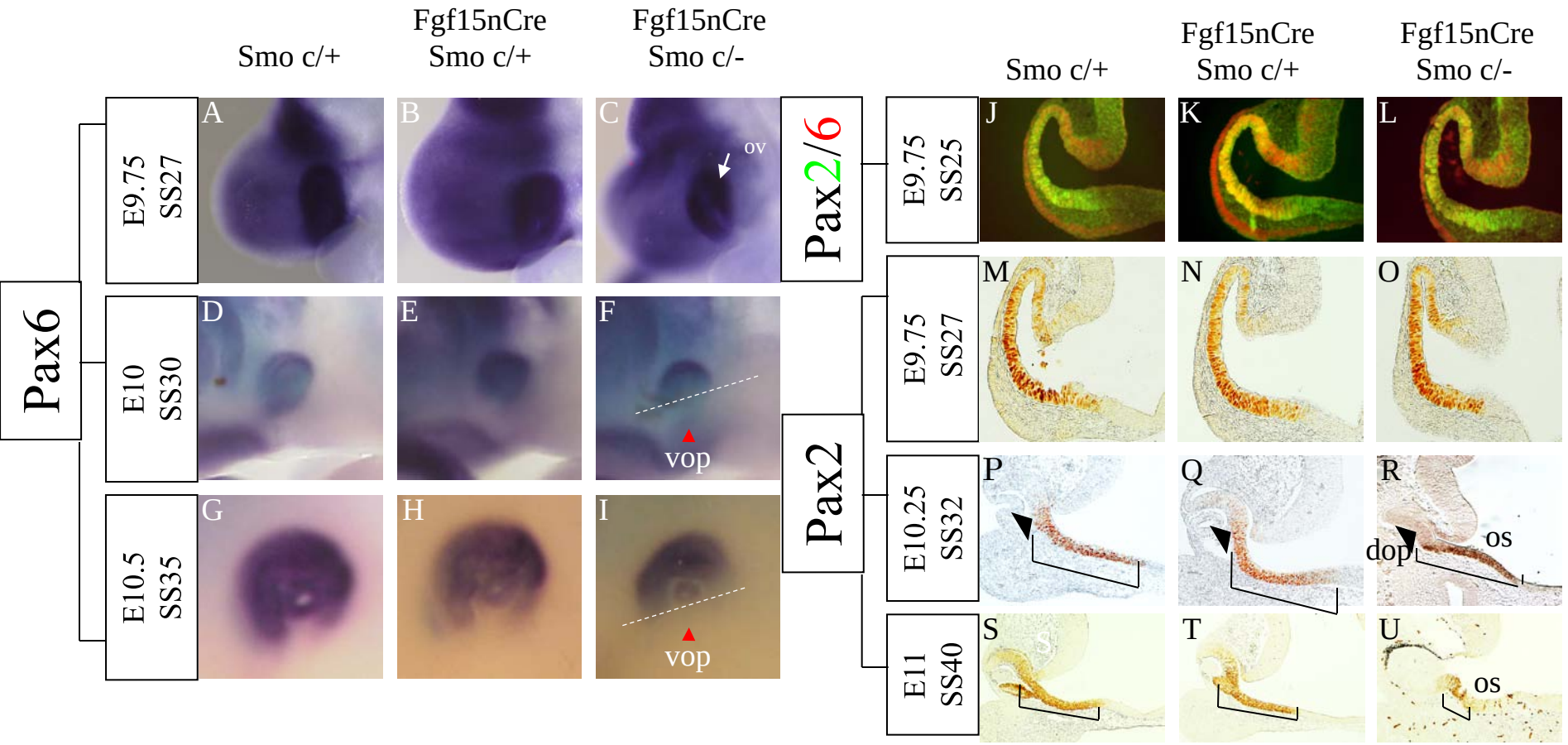
Fig 4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Pax2/6
Pax2
Smo c/+
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Fgf15nCre Smo c/-
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E9.75 SS27
E10.25 SS32
E11 SS40
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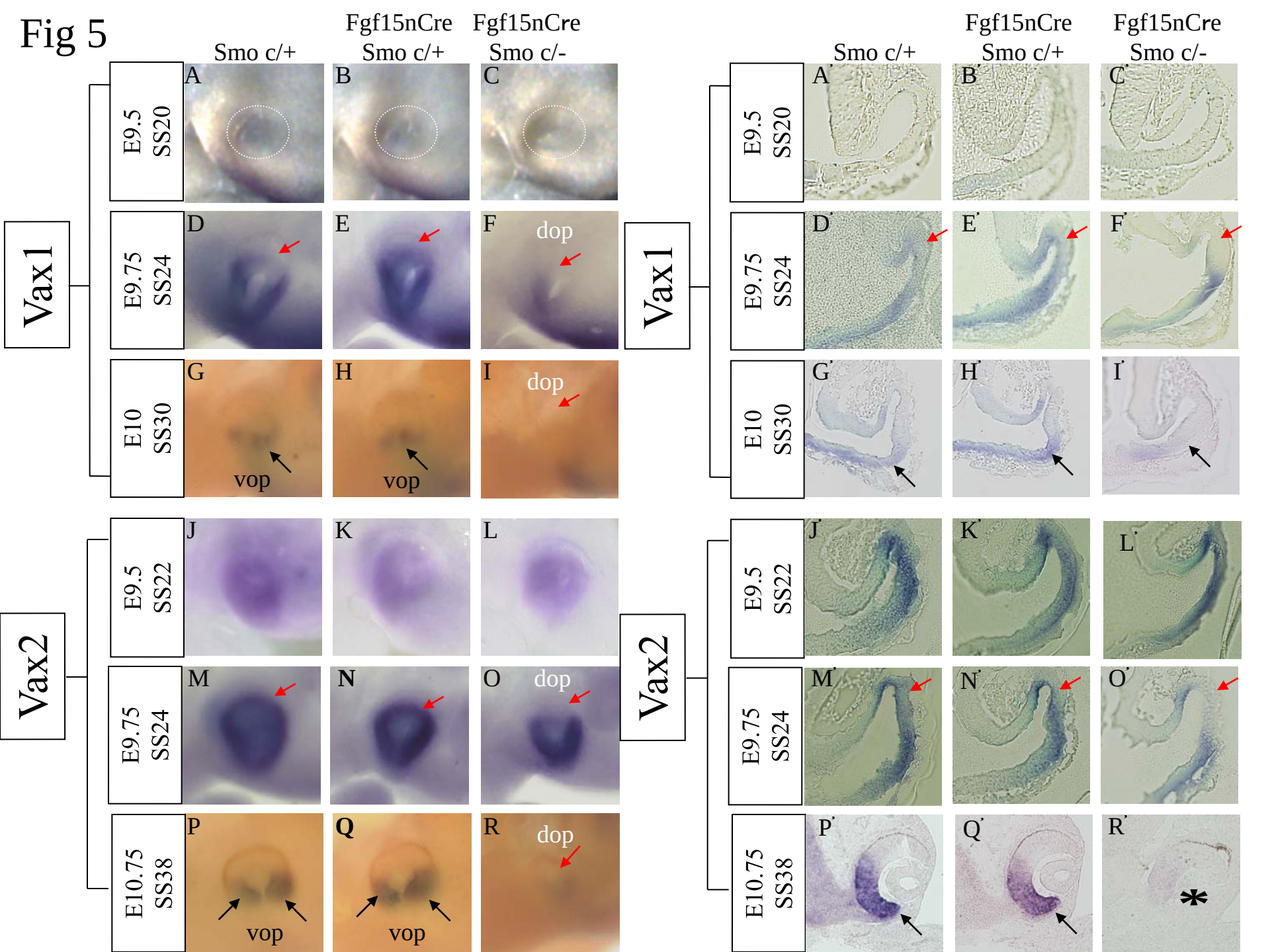


Fig 5
Smo c/+
Fgf15nCre Smo c/+
Fgf15nCre Smo c/-
Vax1
Vax2
E9.5 SS20
E9.75 SS24
E10 SS30
E9.5 SS22
E9.75 SS24
E10.75 SS38
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
dop
vop
A'
B'
C'
D'
E'
F'
G'
H'
I'
J'
K'
L'
M'
N'
O'
P'
Q'
R'
*

Fig 6

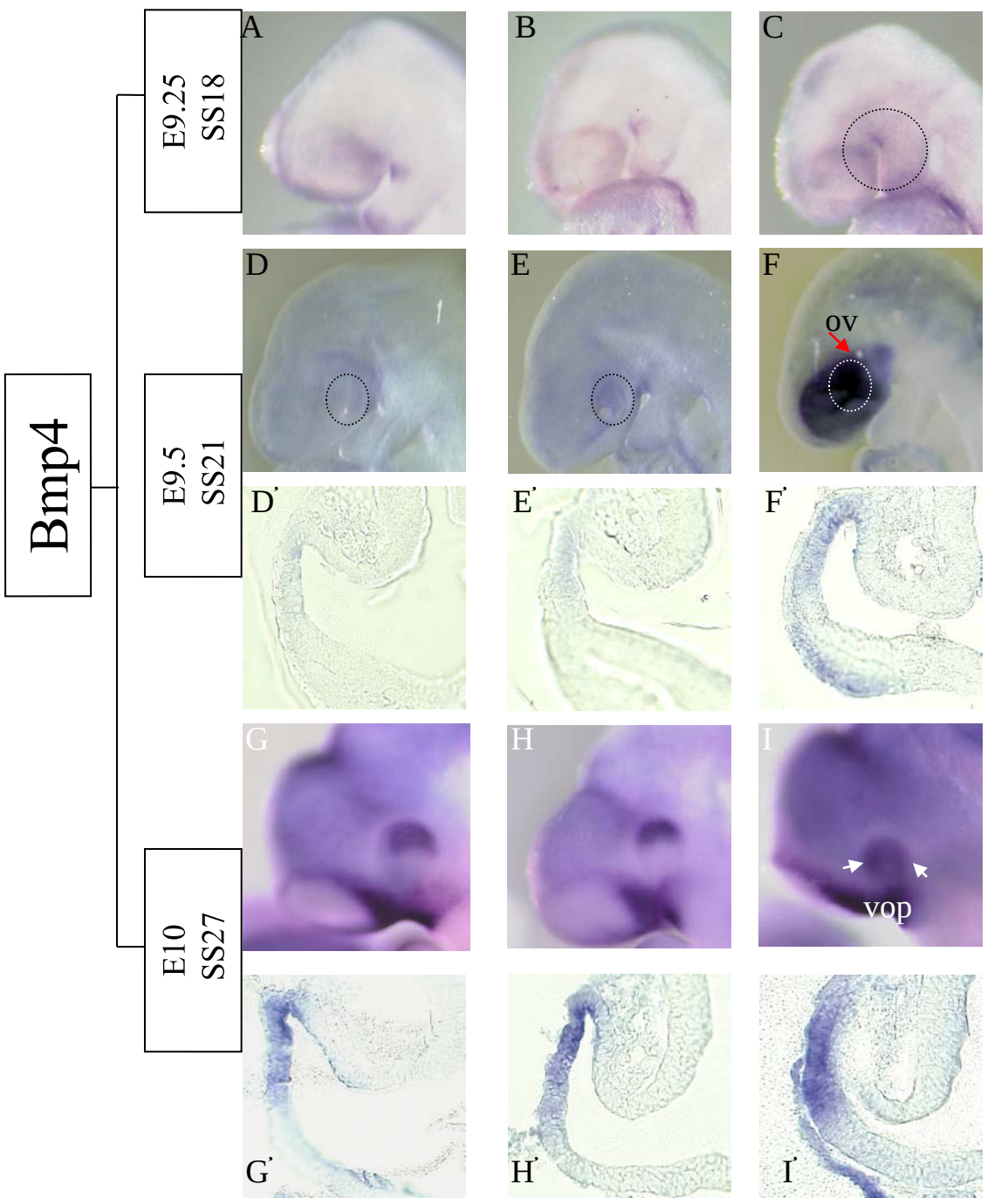

Smo c/+
Fgf15nCre Smo c/+
Fgf15nCreSmo c/-
Bmp4
E9.25 SS18
E9.5 SS21
E10 SS27
A
B
C
D
E
F
ov
D'
E'
F'
G
H
I
vop
G'
H'
I'

Fig 7

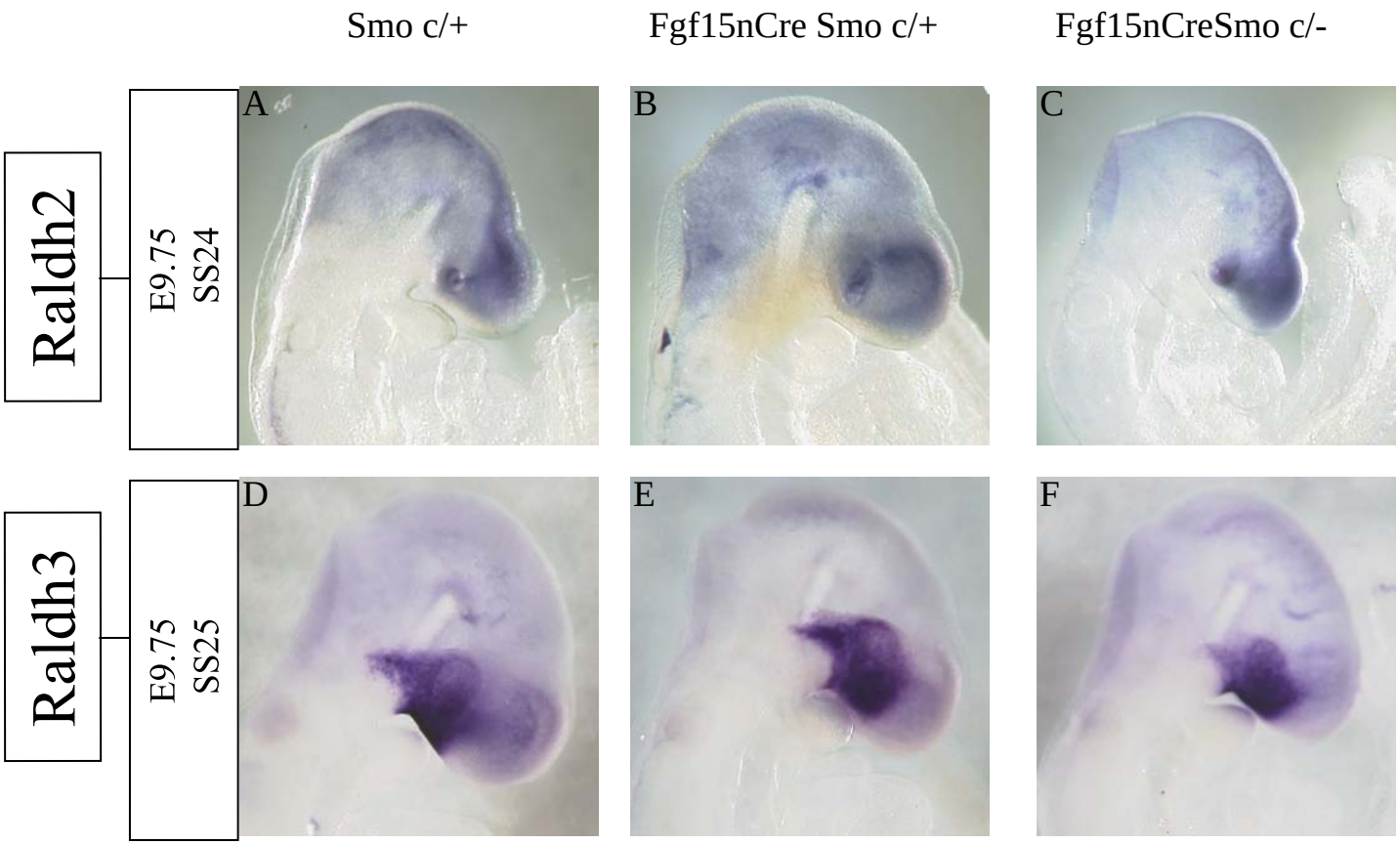
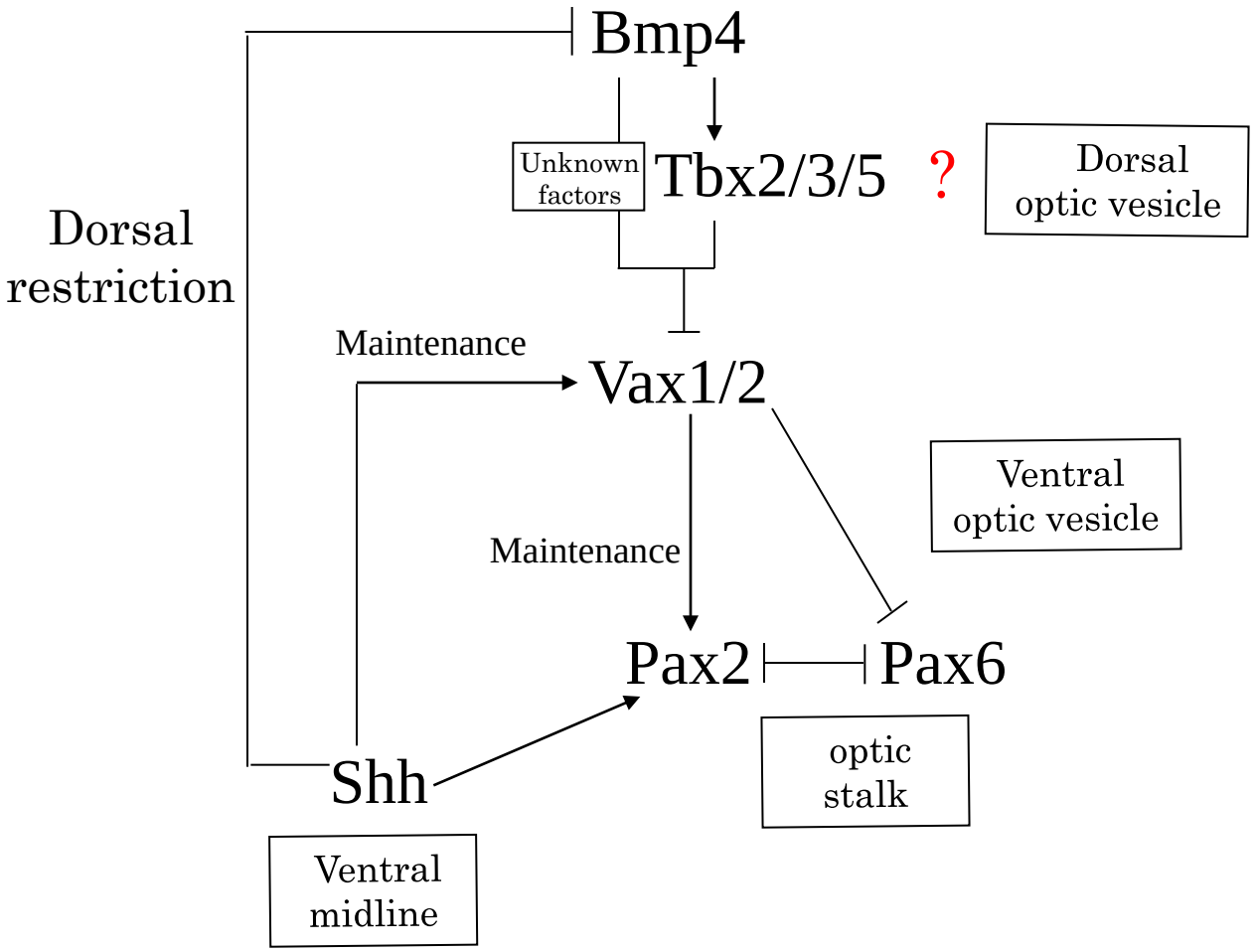

Smo c/+
Fgf15nCre Smo c/+
Fgf15nCreSmo c/-
Raldh2
E9.75
SS24
Raldh3
E9.75
SS25
A
B
C
D
E
F

Fig.8


Bmp4
Unknown factors
Tbx2/3/5
?
Dorsal optic vesicle
Dorsal restriction
Maintenance
Vax1/2
Ventral optic vesicle
Maintenance
Pax2
Pax6
optic stalk
Shh
Ventral midline