Spalt transcription factors are required for R3/R4 specification and establishment of planar cell polarity in the *Drosophila* eye

Pedro M. Domingos[1], Marek Mlodzik[2], César S. Mendes[1], Samara Brown[1], Hermann Steller[1] and Bertrand Mollereau[1,*]

[1]Howard Hughes Medical Institute, Strang Laboratory of Cancer Research, The Rockefeller University, Box 252, 1230 York Avenue, New York, NY 10021, USA
[2]Brookdale Department of Molecular, Cell and Developmental Biology, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, USA
*Author for correspondence (e-mail: bertrand.mollereau@rockefeller.edu)

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Summary

The establishment of planar cell polarity in the *Drosophila* eye requires correct specification of the R3/R4 pair of photoreceptor cells. In response to a polarizing factor, Frizzled signaling specifies R3 and induces *Delta*, which activates Notch in the neighboring cell, specifying it as R4. Here, we show that the *spalt* zinc-finger transcription factors (*spalt major* and *spalt-related*) are part of the molecular mechanisms regulating R3/R4 specification and planar cell polarity establishment. In mosaic analysis, we find that the *spalt* genes are specifically required in R3 for the establishment of correct ommatidial polarity. In addition, we show that *spalt* genes are required for proper localization of Flamingo in the equatorial side of R3 and R4, and for the upregulation of *Delta* in R3. These requirements are very similar to those of *frizzled* during R3/R4 specification. We show that *spalt* genes are required cell-autonomously for the expression of *seven-up* in R3 and R4, and that *seven-up* is downstream of *spalt* genes in the genetic hierarchy of R3/R4 specification. Thus, *spalt* and *seven-up* are necessary for the correct interpretation of the Frizzled-mediated polarity signal in R3. Finally, we show that, posterior to row seven, *seven-up* represses *spalt* in R3/R4 in order to maintain the R3/R4 identity and to inhibit the transformation of these cells to the R7 cell fate.

Key words: *spalt*, *seven-up*, *Drosophila*, Planar cell polarity, Eye development

Introduction

Planar cell polarity (PCP), also known as tissue polarity, occurs when epithelial cells are polarized along the plane of the epithelium (perpendicular to the apical-basal axis of the cell). PCP is evident in many different biological systems, such as the coordinated orientation of bristles in invertebrates, scales in fish, or feathers in birds. Recent work has suggested that the molecular mechanisms that establish PCP are conserved throughout evolution (reviewed by Fanto and McNeill, 2004; Keller, 2002; Mlodzik, 2002; Veeman et al., 2003).

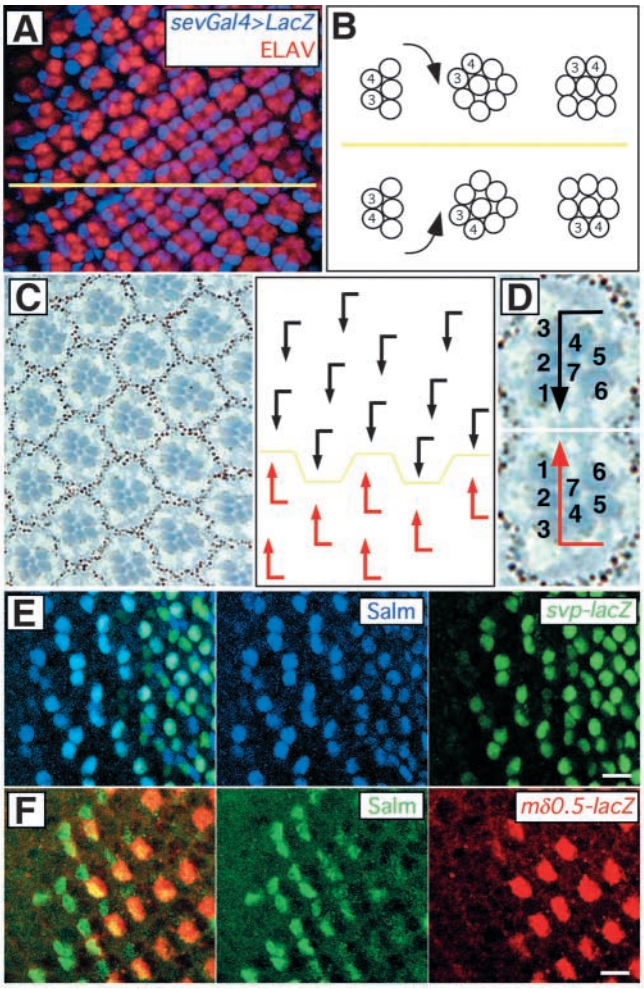
In the *Drosophila* eye, PCP is manifested by the distinct specification of the photoreceptor (PR) fate of R3 and R4, and the rotational movement performed by the developing ommatidia. In the adult eye, each ommatidium contains six outer PRs (R1-R6), which are positioned in a trapezoidal arrangement, and two inner PRs (R7 and R8), which are located in the center of this trapezoid. The trapezoidal arrangement comes in two chiral shapes (generated through the asymmetric positioning of R3 and R4) that form a mirror-image symmetry on either side of the dorsoventral (DV) midline, also called the equator (Fig. 1) (reviewed by Tomlinson, 1988).

PCP is generated during the third larval instar, when the R3 and R4 precursors are specified and the developing ommatidial clusters rotate 90° in opposite directions in the dorsal and ventral halves of the eye (clockwise in the dorsal and anticlockwise in the ventral; Fig. 1). The direction of rotation and the chirality adopted by the ommatidia are a direct consequence of the specification of the R3/R4 pair. The cell of the R3/R4 precursor pair closest to the equator adopts the R3 fate, while the other cell of the pair takes the R4 fate. The specification of R3 is mediated by the activation of the Frizzled/PCP (Fz/PCP) pathway in the R3 precursor, and leads to the activation of Notch signaling in the other cell of the pair, and its specification as R4. Gain- and loss-of-function experiments with several members of Fz/PCP or Notch signaling pathways lead to the random specification of R3 and R4, or the establishment of symmetric ommatidia, where both cells acquire either the R3 or the R4 fate (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999; Zheng et al., 1995). Several other genes are also involved in the establishment of PCP in the *Drosophila* eye and in other tissues, and are generally referred to as 'core PCP' genes (Fanto and McNeill, 2004). Among these are *dishevelled* (*dsh*) (Theisen et al., 1994), *strabismus* (*stbm*, also known as *Van Gogh*) (Taylor et al., 1998; Wolff and Rubin, 1998), *flamingo* (*fmi*, also known as *starry night*) (Chae et al., 1999; Das et al., 2002; Usui et al., 1999), *diego* (*dgo*) (Feiguin et al., 2001) and *prickle/spiny legs* (*pk*) (Gubb et al., 1999).

The *spalt* (*sal*) gene complex encodes two related

transcription factors, *spalt major* (*salm*) and *spalt-related* (*salr*), which are required for the differentiation of the inner PRs (R7 and R8) (Mollereau et al., 2001). In *sal* null mutant (*sal*$^{-}$) retinas, the morphology of the rhabdomeres (the light-sensing structure of the PR), and the expression patterns of *rhodopsins* in R7 and R8, change to become identical to those of the outer PRs (Mollereau et al., 2001). More recently, we found that *sal* is required for R7 differentiation in the third instar larva, as the expression of several R7 markers is lost in *sal* null mutant clones (Domingos et al., 2004). In this last study, we found that the expression of *Enhancer of split mδ0.5-lacZ* (*mδ0.5-lacZ* – a direct target of Notch signaling in R4 and R7) (Cooper and Bray, 1999; Cooper and Bray, 2000) is lost in *sal*$^{-}$ clones both in R4 and R7. This result was an indication that *sal* could also be required for R3/R4 specification and PCP establishment.

Here, we demonstrate that *sal* is required for the establishment of proper ommatidial chirality. We show that the PCP defects observed in *sal*$^{-}$ clones are due to incorrect specification of the R3/R4 cells, as several R3/R4 markers are not correctly expressed. We find that *sal* is required for R3/R4 specification upstream of *seven-up* (*svp*), a gene that is also required for R3/R4 specification and PCP establishment (Fanto et al., 1998; Mlodzik et al., 1990). Finally, we show that, posterior to row seven, *svp* represses *sal* in R3/R4 in order to maintain R3/R4 identity and to inhibit the transformation of these cells to an R7 cell fate.



Materials and methods

Fly stocks and mosaic analysis

The following transgenic and mutant fly stocks were used: *Df(2L)32FP5, FRT40A/CyO* (deficiency spanning both *salm* and *salr*) (Barrio et al., 1999); *FRT82B, svp*^{e22} (Fanto et al., 1998); *svp*^{rA028} (Hoshizaki et al., 1994); *E(spl)mδ0.5* (Cooper and Bray, 1999); *Dl-lacZ 1282* (Fanto and Mlodzik, 1999); *sev-svp* (Hiromi et al., 1993); and *sev-N*^{act} (Fortini et al., 1993).

Clones of mutant eye tissue were generated by the Flp/FRT technique (Golic, 1991). Flipase expression was induced under the control of the *eyeless* (Newsome et al., 2000) or heat-shock promoters (larvae were heat-shocked at 37°C for 1 hour, 48 hours after egg laying).

Immunohistochemistry and histology

Third instar larval eye discs were dissected in 1×PBS, fixed in 1×PBS + 4% formaldehyde for 20 minutes at room temperature, and washed 3 times with PBX (1×PBS + 0.3% Triton X-100). Primary antibodies were incubated in BNT (1×PBS, 1% BSA, 0.1% Tween 20, 250 mM NaCl) overnight at 4°C. Primary antibodies were as follows: rabbit anti-Salm (Kuhnlein et al., 1994), anti β-gal (Cappel), rat anti-ELAV (DSHB), mouse anti-Ro (DSHB), rabbit anti-BarH1 (Higashijima et al., 1992) and mouse anti-Fmi (Usui et al., 1999). Samples were washed 3 times with PBX and incubated with appropriate secondary antibodies (Cy3, Cy5, FITC from Jackson Immuno-Research Laboratories) for 2 hours at room temperature. Samples were mounted in Vectashield (Vector Laboratories) and analyzed on a Zeiss LSM 510 confocal microscope. Tangential sections of adult eyes were performed as described (Tomlinson and Ready, 1987).

Results

Salm expression in the R3/R4 precursor pair is concomitant with R3/R4 specification

In the third instar eye imaginal disc, *salm* is expressed in the R3/R4 precursor pair, starting in row three posterior to the

Fig. 1. Salm is expressed in R3 and R4 when planar polarity is established. Anterior is to the left and dorsal is up. (A) Third larval instar eye imaginal disc, stained with anti-ELAV (red) and anti-β-galactosidase (blue), in which *lacZ* is expressed under *sev-Gal4* control to show the R3/R4 position (lower staining levels are also observed in R1, R6 and R7) and reveal the rotation of the developing ommatidia. Close to the morphogenetic furrow (MF, to the left) the R3/R4 pair is perpendicular to the DV midline, the equator (yellow line). By row six, ommatidia have rotated 45°, in a clockwise or counter-clockwise direction in the dorsal and ventral halves, respectively. In the posterior part of the eye disc, the 90° rotation of the ommatidia is almost complete, and the R3/R4 pair is parallel to the equator. (B) Schematic illustrating ommatidial rotation in the imaginal disc. (C) Tangential section of an adult eye (left) and corresponding schematic drawing (right). The section is at the level of R7. R8 is not visible as it is localized below the R7 plane. Ommatidia in the adult eye are arranged as two opposite chiral forms separated by the equator (yellow line), as a consequence of R3/R4 specification and the following 90° rotation. Ommatidia in the dorsal half are represented with black arrows and in the ventral half with red arrows. (D) Magnification of one dorsal (top) and one ventral (bottom) ommaditium; arrows as in C. Numbers indicate the identities of the photoreceptors. (E) Eye imaginal disc stained for Salm (blue) and *svp-lacZ* (green). The initiation of Salm expression in R3/R4 precedes *svp-lacZ* by one row. Posterior to row seven, Salm expression in R3/R4 starts to fade, whereas *svp-lacZ* continues to be expressed in R3/R4 and also, at lower levels, in R1/R6. (F) Salm (green) expression in R3/R4 precedes the onset of *mδ0.5-lacZ* (red) expression in R4 by one to two rows. Scale bars: 10 μm.

morphogenetic furrow and progressively fading in these cells from row seven onwards (as determined by co-staining with mAb22C10) (Domingos et al., 2004). A direct consequence of the R3/R4 specification is the induction of *mδ0.5-lacZ* expression in R4 (Cooper and Bray, 1999), as well as the initial 45° ommatidial rotation that occurs by row six (Strutt et al., 2002). The onset of Salm expression in R3/R4 precedes the expression of *svp* in R3/R4 by one row (Fig. 1E), and precedes the expression of *mδ0.5-lacZ* in R4 by one to two rows (Fig. 1F). Although the *svp-lacZ* and *mδ0.5-lacZ* lines may not faithfully report the expression of these genes because of the perdurance of the β-galactosidase protein, the expression of *sal* in R3/R4 is concomitant with the specification of R3/R4 and establishment of PCP, and is consistent with the possibility that *sal* is part of the molecular mechanisms that regulate these cellular processes.

sal is required in R3 for establishment of correct ommatidial chirality

In *sal*$^{-}$ ommatidia, the rhabdomeres of both R7 and R8 acquire the typical 'large' morphology of outer PRs (Mollereau et al., 2001). This leads to the disruption of the normal position of rhabdomeres in each ommatidium, thus making the evaluation of PCP defects impossible. However, on the borders of *sal*$^{-}$ clones, it is possible to detect mosaic ommatidia with wild-type R7 and R8, allowing the analysis of PCP defects.

To investigate the role of *sal* in R3/R4 specification and PCP generation, we analyzed a large number of such mosaic *sal*$^{-}$ ommatidia and scored them for PCP defects. Within 1391 mosaic ommatidia, we could identify 29 different mosaic configurations with a normal number of PRs, where it was possible to score polarity. Fig. 2A shows examples of *sal*$^{-}$ clones, containing mosaic ommatidia, which display typical PCP defects with chirality inversions and mis-rotations. Interestingly, this analysis of mosaic clusters reveals a requirement of *sal* in R3 for PCP establishment. In the 16 configurations that always adopt the correct chiral form, R3 is always *sal*$^{+}$ (Fig. 2B). In mosaic ommatidia adopting the wrong chiral form, the cell in the R4 position is invariably *sal*$^{-}$ (Fig. 2C). Presumably, in such ommatidia, the *sal*$^{-}$ precursor for R3 developed incorrectly as an R4. In ommatidia where only the R3 precursor was *sal*$^{-}$, we found seven cases that adopted the wrong chirality (an example is shown in Fig. 2A, top panel), and eight cases with the correct chirality (data not shown). These results demonstrate randomization of the R3/R4 chirality choice when the R3 precursor is *sal*$^{-}$. We also found 15 ommatidia with symmetric R4/R4 (14) or R3/R3 (1) configurations (data not shown). In each of these 15 symmetric ommatidia, at least one cell of the R3/R4 pair was *sal*$^{-}$. Thus, the PCP requirement of *sal* is similar to that of *fz* (Tomlinson and Struhl, 1999; Zheng et al., 1995), and demonstrates that *sal* is required in R3 for correct ommatidial chirality and PCP establishment.

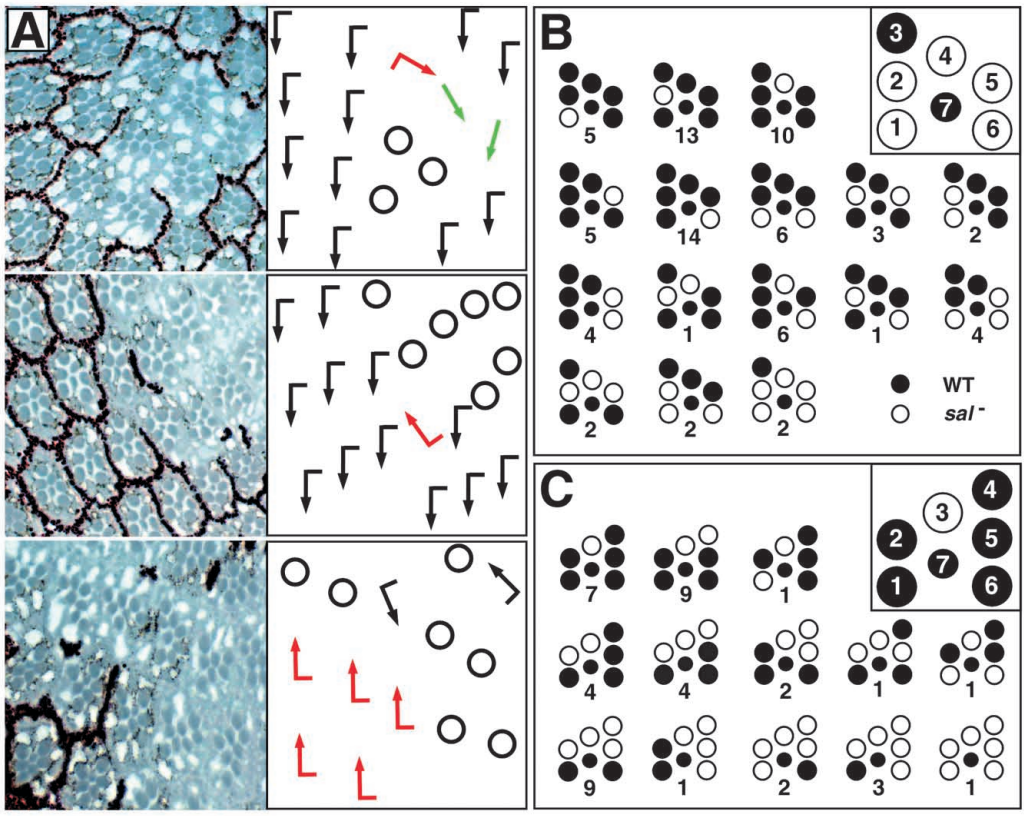


Fig. 2. *sal* is required in R3 for the establishment of correct chirality. (A) Tangential sections of adult eyes containing *sal* null mutant clones [in all experiments we used a small chromosomal deficiency – Df(2L)32FP5 – covering only *salm* and *salr* (Barrio et al., 1999)]. *sal* mutant (*sal*$^{-}$) cells are shown by the absence of the pigment (*w*) marker (dark dots at the base of each rhabdomere and in pigment cells). In schematic drawings, black arrows represent dorsal and red arrows ventral orientation. Green arrows represent ommatidia where it is possible to identify R1/R6 and R7, but not R3 or R4. Black circles represent ommatidia where it is impossible to score orientation, because R7 or R8 are transformed into outer PRs, or they contain extra photoreceptors. Top panel: note that in the ommatidium with (wrong) ventral chirality, only the presumptive R3 precursor is *sal*$^{-}$ and has acquired an R4 fate. (B) Statistical analysis of mosaic ommatidia that always present correct chirality. *sal*$^{-}$ cells are represented as white circles and non-mutant cells as black circles. The number of ommatidia is indicated below each configuration. The inset at the top right corner represents the common feature of these configurations, which is that R3 always has the *sal*$^{+}$ genotype. The numbers inside the circles represent the identity of each PR. (C) Statistical analysis of mosaic ommatidia exhibiting chirality inversions. The common feature of these configurations is that the cell in the R4 position always has the *sal*$^{-}$ genotype. This R4 mutant cell corresponds to a R3 precursor that made the wrong chiral choice.

sal is required for R3/R4 specification and PCP establishment during the third larval instar

To determine at which stage of development the PCP defect in *sal* mutants occurred, we analyzed orientation of ommatidial rotation in *sal*$^{-}$ clones in larval third instar eye discs. The BarH1 antibody (Higashijima et al., 1992), which labels R1 and R6, allows the visualization of the progressive rotational movement of developing ommatidia in the eye disc. In *sal*$^{-}$ clones, 17.9% (*n*=212) of the ommatidia display rotational errors (Fig. 3A). However, this may be an underestimation of the number of affected ommatidia, as from the BarH1 staining it is not possible to distinguish between ommatidia with a correct or flipped chirality. Thus, we conclude that PCP defects in *sal* mutants are present from the early time of R3/R4 specification. The R3 and R4-specific expression of Rough (Kimmel et al., 1990) and the neuronal marker Elav are unaffected in *sal*$^{-}$ clones (Fig. 3), indicating that neuronal specification and certain aspects of R3/R4 subtype identity do not require the *sal* genes.

Next, we investigated at which level of the PCP pathway *sal* is required. We have shown that *sal* is required in the R3 precursor for correct establishment of chirality (Fig. 2). According to current models, PCP signaling in the eye is a two tiered process: (1) activation of Fz/PCP signaling and upregulation of *Delta* (*Dl*) in the R3 precursor; and (2) activation of Notch signaling in the neighboring R4 cell. The atypical cadherin Flamingo (Fmi) is thought to play a dual role during the establishment of PCP (Das et al., 2002; Strutt et al., 2002). Initially, from row three to five, Fmi is asymmetrically localized in the equatorial side of R3 and R4, and promotes Fz/PCP signaling in R3. Subsequently, Fmi is enriched predominantly in R4, where it functions to downregulate *Dl* expression and antagonize Fz/PCP signaling (Das et al., 2002). In *sal*$^{-}$ clones, as with *fz*$^{-}$ or *dsh*$^{-}$ clusters (Das et al., 2002), neither the asymmetric localization of Fmi in the equatorial side of R3/R4 nor the subsequent enrichment in R4 are observed. Instead, Fmi is present on all sides of the apical membrane cortex of R3/R4 (Fig. 3B). We have also observed a defect in the asymmetric localization of Fz-GFP and Dgo in R3/R4 in *sal*$^{-}$ clones (data not shown).

Activation of Fz/PCP signaling leads to the transient transcriptional upregulation of *Dl* in R3 within approximately two to three ommatidial rows, which can be observed by in situ hybridization (Parks et al., 1995), or with a *Dl* enhancer detector line (Fanto and Mlodzik, 1999). During this period, most *sal*$^{-}$ ommatidia fail to upregulate *Dl* in R3, and both cells of the R3/R4 pair show a low level of expression (Fig.

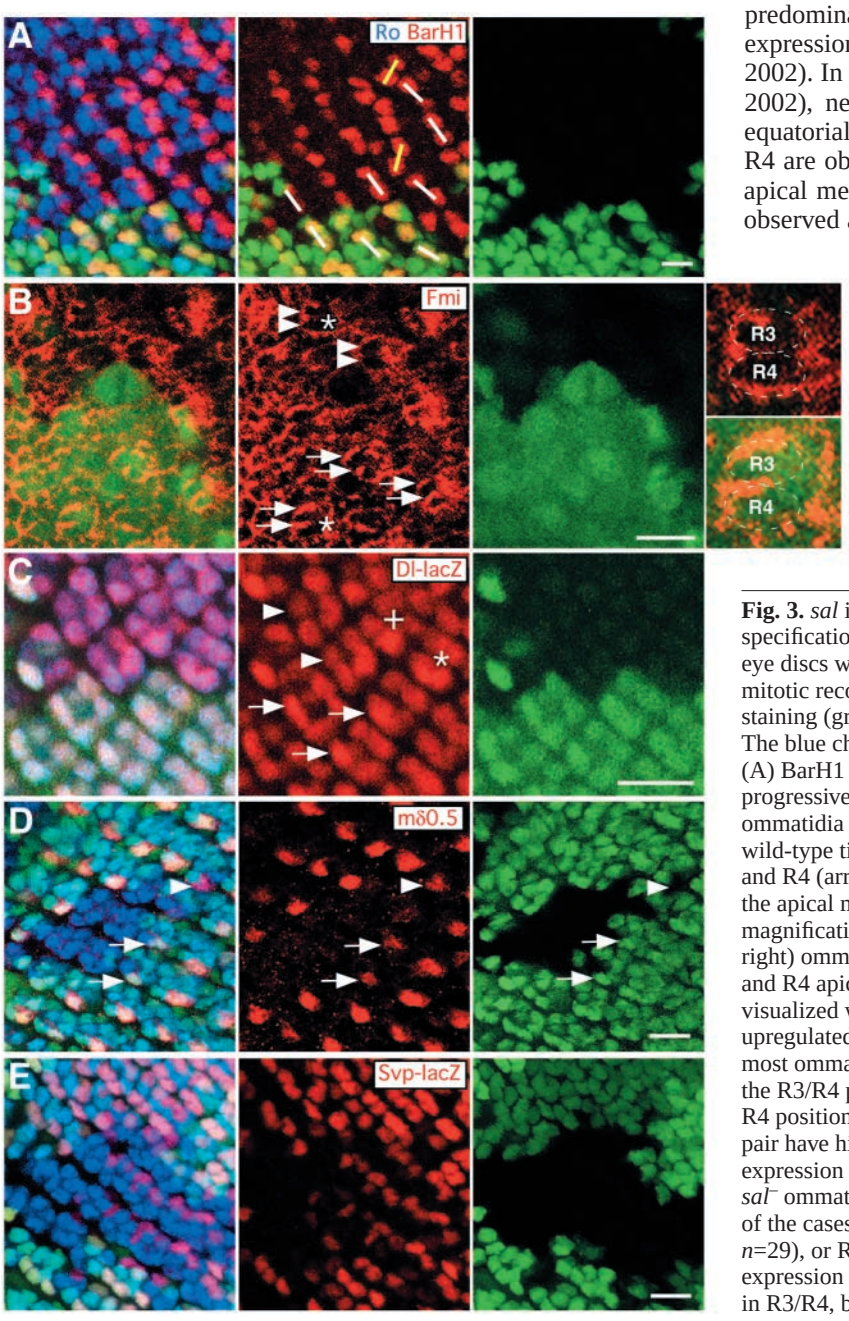


Fig. 3. *sal* is required for the correct expression of R3/R4 specification and polarity markers. All panels represent third instar eye discs where *sal*$^{-}$ clones were induced by Flipase-mediated mitotic recombination and are labeled by the absence of ubi-GFP staining (green). Anterior is to the left and the equator is at the top. The blue channel shows Ro in A and ELAV in all other panels. (A) BarH1 (red) stains R1/R6 and allows the visualization of the progressive ommatidial rotation. White and yellow bars indicate ommatidia with correct and incorrect rotation, respectively. (B) In wild-type tissue, Fmi (red) localizes in the equatorial side of R3 and R4 (arrows). In *sal*$^{-}$ ommatidia, Fmi is present in all sides of the apical membrane of R3 and R4 (arrowheads). High magnification images of *sal*$^{-}$ (top, right) and wild-type (bottom, right) ommatidia (asterisk) show the localization of Fmi in the R3 and R4 apical membrane (dashed line). (C) *Dl* expression (red), visualized with the enhancer trap line *Dl-lacZ1282*, is transiently upregulated in R3 (arrows) in two to three rows. In *sal*$^{-}$ tissue, most ommatidia show low levels of *Dl* expression in both cells of the R3/R4 pair (arrowheads). In some ommatidia, the cell in the R4 position has stronger staining than R3 (+), or both cells in the pair have high levels of *Dl* expression (asterisk). (D) The expression of *mδ0.5-lacZ* (red) in R4 is lost in 91% (*n*=218) of *sal*$^{-}$ ommatidia. Some residual expression is still observed in 9% of the cases. In mosaic ommatidia where R3 but not R4 (arrows, *n*=29), or R4 but not R3 (arrowhead, *n*=21) is *sal*$^{-}$, *mδ0.5-lacZ* expression is reduced. (E) The expression of *svp-lacZ* (red) is lost in R3/R4, but not R1/R6, in *sal*$^{-}$ clones. Scale bars: 10 μm.

3C). We can also find clusters where the cell in the R4 position has higher levels than R3, or where both cells in the R3/R4 pair have increased levels of *Dl* expression (Fig. 3C). These results suggest that *sal* is required for the correct interpretation of the Fz/PCP-mediated polarity signal and for the upregulation of *Dl* expression in R3.

The expression of *mδ0.5-lacZ*, a marker of Notch signaling activation in R4 (second tier), is lost in *sal*$^{-}$ ommatidia (Fig. 3D) (Domingos et al., 2004). Interestingly, in mosaic ommatidia, where only one cell of the R3/R4 pair is *sal*$^{-}$, either R3 or R4, we observe reduced expression of *mδ0.5-lacZ*. This indicates that *sal* is required for normal levels of *mδ0.5-lacZ* expression, both non-cell-autonomously in R3 (arrows in Fig. 3D), and cell-autonomously in R4 (arrowhead in Fig. 3D), which is surprising given the specific genetic requirement in R3 only for ommatidial polarity (Fig. 2). The non-cell autonomous requirement of *sal* in R3, for normal *mδ0.5-lacZ* expression in R4, is consistent with the deficient upregulation of *Dl* in R3 (Fig. 3C). It is also possible that the lack of asymmetric localization of PCP proteins, as seen in the case of Fmi in *sal*$^{-}$ (Fig. 3B), is responsible for the Notch activity modulation, as previously proposed (Strutt et al., 2002). The autonomous requirement of *sal* in R4 for *mδ0.5-lacZ* expression either could be due to a defective activation of Notch signaling, or *sal* may be required for transcriptional activation of *E(spl)mδ* in parallel to Notch signaling (see also Discussion).

sal acts upstream of *svp* during R3/R4 specification

To further investigate the role of *sal* in R3/R4 specification, we asked whether *sal* is required for *svp* expression in R3/R4. It was previously shown that *svp* is also required in R3 for proper R3/R4 specification and PCP establishment in the eye (Fanto et al., 1998). Our data indicate that *sal* genes are cell-autonomously required for the expression of *svp* (*svp*^{rA28}; *svp-lacZ*) (Hoshizaki et al., 1994) in R3 and R4 (Fig. 3F). Conversely, in *svp*$^{-}$ (*svp*^{e22}) clones, the initiation of *salm* expression in R3/R4 is normal, although *salm* is not repressed in more posterior rows (Fig. 4A). As in *sal*$^{-}$ clones, in *svp*$^{-}$ clones Fmi is not properly localized in R3/R4 (Fig. 4B) and the expression of *mδ0.5-lacZ* is lost (Fig. 4C). These results suggest that *sal* acts upstream of *svp* during R3/R4 specification.

To test this hypothesis, we attempted to rescue the expression of *mδ0.5-lacZ* in *sal*$^{-}$ clones by the exogenous expression of *svp* in the R3/R4 pair [under the control of the *sevenless (sev)* promoter – *sev-svp* (Hiromi et al., 1993)]. Strikingly, *sev-svp* can induce *mδ0.5-lacZ* expression in at least one cell of the pair, in many cases the one in the R4 position (Fig. 4D). This result indicates that exogenous *svp* expression can rescue *mδ0.5-lacZ* expression in *sal*$^{-}$ clones, and thus *svp* acts downstream of *sal* in this context. In addition, exogenous expression in R3/R4 of a constitutively active form of Notch (*sev-N*^{act}) (Fortini et al., 1993) upregulates *mδ0.5-lacZ* expression in both R3 and R4, independently of

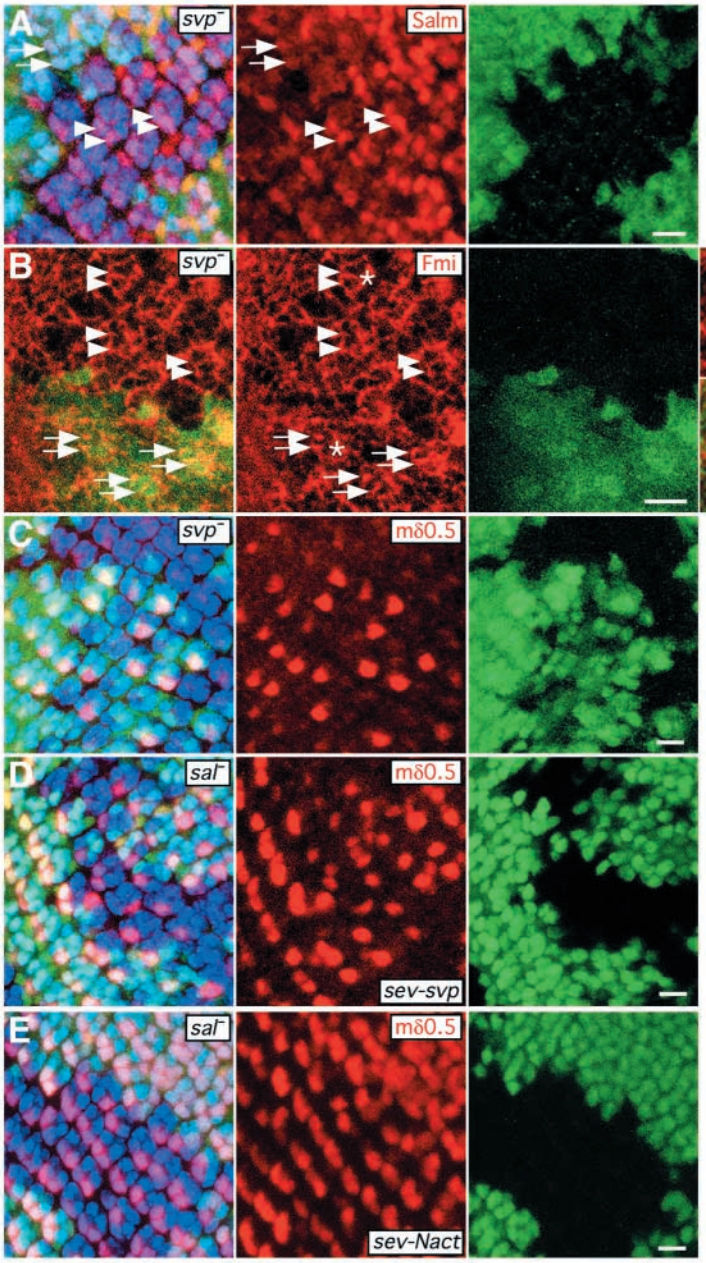


Fig. 4. *sal* acts upstream of *svp* during R3/R4 specification. All panels represent third instar eye discs where *svp*$^{-}$ clones (A-C, *svp*^{e22} – transcript null allele) or *sal*$^{-}$ clones (D,E) were induced by Flipase-mediated mitotic recombination and are labeled by the absence of ubi-GFP staining (green). Anterior is to the left and the equator is at the top. The blue channel shows ELAV. (A) Salm (red) expression in R3/R4 is not repressed after row seven in the *svp*$^{-}$ area. In wild-type ommatidia, Salm expression is progressively repressed in R3/R4 after row seven (arrows). In *svp*$^{-}$ ommatidia, Salm expression persists in R3/R4 in more posterior rows (arrowheads). (B) In *svp*$^{-}$ clones, Fmi (red) is present in all sides of the apical membrane of R3/R4 (arrowheads). In wild-type ommatidia, Fmi is localized in the equatorial side of R3 and R4 (arrows). High magnification of *svp*$^{-}$ (top, right) and wild-type (bottom, right) ommatidia (asterisk) show the localization of Fmi in the R3 and R4 apical membrane (dashed line). (C) In *svp*$^{-}$ clones, the expression of *mδ0.5-lacZ* (red) is lost in R4. (D) In *sal*$^{-}$ ommatidia, *sev-svp* rescues *mδ0.5-lacZ* (red) expression in one cell of the pair, in many cases the one in the R4 position. In the wild-type ommatidia, *sev-svp* leads to *mδ0.5-lacZ* expression in both R3 and R4. (E) *sev-N*^{act} induces *mδ0.5-lacZ* (red) in R3 and R4, both in *sal*$^{-}$ and non-mutant ommatidia. Scale bars: 10 μm.

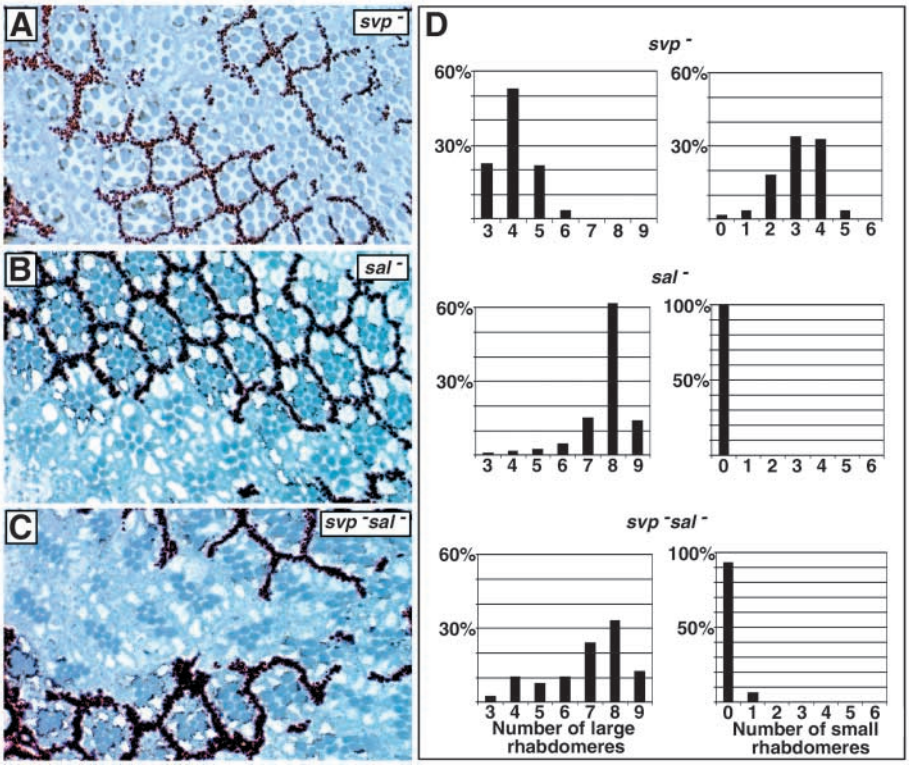


Fig. 5. *sal* is required for the transformation of R3/R4 into R7 in *svp* mutants. (A-C) Tangential cross-sections of adult eyes containing clones for *svp*$^{-}$ (A), *sal*$^{-}$ (B) and *svp*$^{-}$/*sal*$^{-}$ double (C) mutants. The mutant cells are visualized by the absence of the *w* marker, and the *svp*^{e22} transcript null allele was used. (D) Quantitative analysis of the number of photoreceptors with large or small rhabdomeres in *svp*$^{-}$, *sal*$^{-}$ and *svp*$^{-}$/*sal*$^{-}$ mutants. The numbers under the columns represent the number of large (diagrams on the left) or small (diagrams on the right) rhabdomeres observed for each individual ommatidium. The number of ommatidia with a particular number of large or small rhabdomeres is indicated as a percentage of the total number of ommatidia analyzed (116 in *svp*$^{-}$, 127 in *sal*$^{-}$ and 79 in *svp*$^{-}$/*sal*$^{-}$).

their *sal* genotype (Fig. 4E). Thus, although *sal* is normally required for *mδ0.5-lacZ* expression, constitutively activated Notch can overcome the *sal* requirement, demonstrating that Notch activation acts downstream of or in parallel to *sal* to regulate *E(spl)mδ* expression.

Repression of *sal* in R3/R4 by *svp* is required for inhibition of R7 cell fate

In *svp* mutants, R3, R4, R1 and R6 fail to adopt their normal fate and are transformed into the R7 fate (Mlodzik et al., 1990). The PCP defects observed in *svp* mutant ommatidia were attributed to a failure of the R3/R4 cells to interpret the Fz/PCP signal, because of their transformation to R7 (Fanto et al., 1998). Consistent with this, we have observed that in *svp*$^{-}$ clones in the larval eye disc, both R3 and R4 express the R7 marker *prospero* (data not shown). In addition, in *svp*$^{-}$ clones, *salm* is not repressed in R3/R4 by row seven, but continues to be expressed in more posterior rows (Fig. 4A) [*sal* normally starts to be expressed in R7 by rows seven to nine, and is both required and sufficient for R7 differentiation during larval stages (Domingos et al., 2004)]. Thus, it is likely that, in *svp*$^{-}$ clones, the ectopic expression of *salm* in R3/R4 posterior to row seven is responsible for their transformation into R7.

To test this hypothesis, we have analyzed the number of large (R1-R6) and small (R7 and R8) rhabdomeres in *svp*$^{-}$/*sal*$^{-}$ double mutants (Fig. 5). In *svp*$^{-}$ clones, most ommatidia have three to five cells with small rhabdomeres because of the transformation of R3, R4, R1 and R6 into R7 (Fig. 5A,D) (Mlodzik et al., 1990). In *sal*$^{-}$ clones, most ommatidia have eight large and no small rhabdomeres, due to the transformation of R7 and R8 to the outer PRs subtype (Fig. 5B,D) (Mollereau et al., 2001). Strikingly, *svp*$^{-}$/*sal*$^{-}$ double mutant ommatidia have the same appearance as single *sal*$^{-}$ mutant clusters (Fig. 5C,D). This result demonstrates that *sal* is required, downstream of *svp* mutation, for the transformation of R3/R4 into R7. In conclusion, *sal* is required upstream of *svp* during R3/R4 specification (rows three to seven), but repression of *sal* by *svp* posterior to row seven is required to avoid the transformation of these cells into R7 (Fig. 6 and Discussion).

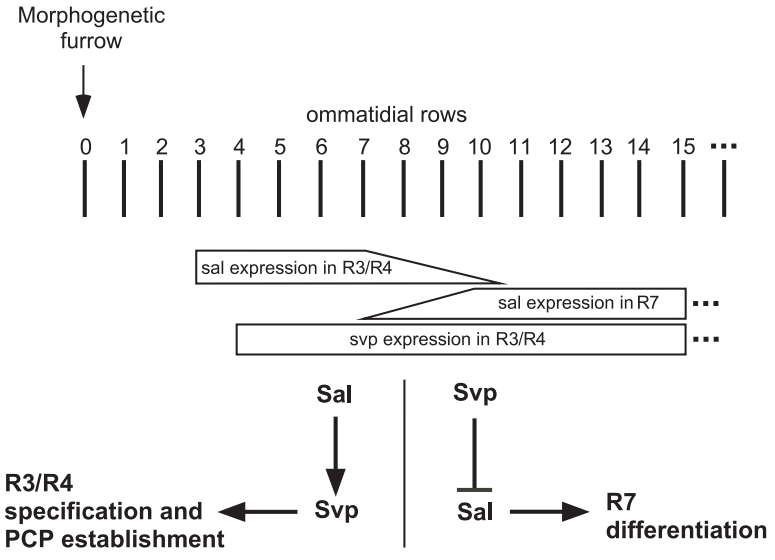


Fig. 6. Model of the roles of *sal* and *svp* in the specification of R3/R4 versus R7 [based on our present findings and on Domingos et al. (Domingos et al., 2004)]. *sal* is expressed in R3/R4 from row three to row seven, after which it is progressively repressed. *sal* expression in R7 starts from row seven to nine. Expression of *svp* in R3/R4 starts in row four. From row three to row seven, *sal* is required for *svp* expression in R3/R4, for R3/R4 specification and for PCP establishment. After rows seven to nine, *sal* is necessary and sufficient for R7 differentiation. Repression of *sal* by *svp* in R3/R4 is necessary for the maintenance of R3/R4 identity and the inhibition of R7 fate.

Discussion

Relation between *sal* and Fz/PCP signaling during R3/R4 specification

PCP establishment in the *Drosophila* eye requires the correct specification of the R3/R4 pair of cells. It is thought that R3/R4 specification occurs as consequence of a higher level of Fz signaling in the equatorial cell of the R3/R4 precursor pair, which specifies the R3 fate. This leads to the upregulation of *Dl* in R3 and activation of Notch signaling in the polar cell of the pair, specifying it as R4 (Cooper and Bray, 1999; Fanto and Mlodzik, 1999; Tomlinson and Struhl, 1999; Zheng et al., 1995).

We show that *sal* is also required for PCP establishment in the *Drosophila* eye. The analysis of *sal*$^{-}$ mosaics reveals that *sal* is specifically required in R3 for establishment of ommatidial chirality (Fig. 2). The analysis of *sal*$^{-}$ clones in the larval eye reveals that *sal* is required for the asymmetric localization of Fmi in the R3/R4 precursor pair (Fig. 3B) and upregulation of *Dl* in R3 (Fig. 3C). In a similar manner to *sal*, *fz* is required in R3 for the establishment of ommatidial chirality (Tomlinson and Struhl, 1999), and in *fz* mutants, unlike in *stbm* or *dgo* mutants, Fmi is not localized asymmetrically in R3/R4 (Das et al., 2002). However, *fz* mutants also have a non-autonomous effect, disrupting PCP in ommatidia located outside the mutant clone (Zheng et al., 1995), which is not observed in *sal* mutants. Thus, *sal* is required for the correct interpretation of the *fz*-mediated polarity signal in R3, but not for the propagation of the polarity signal across the equatorial-polar axis. This also indicates that the expression of Fz should not be affected in *sal*$^{-}$ clones. Finally, in *sal*$^{-}$ clones, all PCP proteins tested (Fmi, Fz, Dgo) exhibit a defect in their asymmetric localization (Fig. 3B and data not shown), but their overall expression remains unaffected. A possible interpretation of these results is that *sal* transcription factors induce the expression of a yet unidentified factor, which is required for the asymmetric localization of PCP genes.

Therefore, our results suggest that *sal* is required upstream or in parallel to the Fz/PCP pathway for R3/R4 specification. Also, in support of this model, *sal* expression is not affected in R3/R4, either in gain- or loss-of-function experiments with members of the Fz/PCP and Notch signaling pathways [*fz*^{R52} clones – data not shown; *dsh*^{1}, *sev-Gal4/uas-Dsh* and *sev-Gal4/uas-N*^{icd} (Cooper and Bray, 1999); *sev-Fz* and *sev-N** (Fanto and Mlodzik, 1999); *fmi*$^{-}$ clones and *sev-Fmi* (Das et al., 2000)].

We show that *sal* is required cell-autonomously in R4 for normal levels of *mδ0.5-lacZ* expression (Fig. 3D). This requirement of *sal* in R4 could be due to a defect in the activation of Notch signaling (e.g. *sal* may be required for the expression of *Notch* or *Su(H)*). Alternatively, *sal* may be required for transcriptional activation of *E(spl)mδ*, in parallel to Notch signaling. We favor the latter possibility, as the expression of a transgenic line, where *lacZ* is under the regulation of 12 *Suppressor of Hairless* multimerized-binding sites [*12Su(H)-lacZ* (Go et al., 1998)], is not affected when R4 is *sal*$^{-}$ (data not shown). The *12Su(H)-lacZ* transgenic line is a reporter for Su(H)-dependent Notch signaling, and thus, *sal* is not required for the expression or activation of Notch, Su(H) or other components required for signaling. In addition, exogenous expression of a constitutively activated Notch (*sev-N*^{act}) can rescue *mδ0.5-lacZ* expression in *sal*$^{-}$ clones (Fig. 4E). Altogether, these results suggest that *sal* acts in parallel to Notch signaling for the transcriptional activation of *E(spl)mδ*. Finally, although there is a reduction of *E(spl)mδ* expression when R4 is *sal*$^{-}$, this does not correspond to chirality defects in mature ommatidia (Fig. 2). This suggests that other genes may be redundant to *sal* in R4 for PCP establishment.

sal and *svp* in R3/R4 versus R7 specification

Several pieces of evidence demonstrate that *sal* is required upstream of *svp* for R3/R4 specification: (1) *sal* is required for *svp* expression in R3/R4 (Fig. 3F); (2) both *sal* and *svp* are required in R3 for the establishment of proper ommatidial chirality (Fig. 2) (Fanto et al., 1998); (3) in both *sal* and *svp* mutants Fmi is not asymmetrically localized in R3/R4 (Fig. 3B, Fig. 4B) and *mδ0.5-lacZ* expression is lost in R4 (Fig. 3D, Fig. 4C); and (4) exogenous expression of *svp* in R3/R4 (*sev-svp*) can rescue the expression of *mδ0.5-lacZ* in *sal*$^{-}$ clones (Fig. 4D).

In addition, we show that, posterior to row seven, *svp* is required to repress *sal* expression in R3/R4 (Fig. 4A), and *sal* is responsible for the transformation of R3/R4 into R7 in *svp* mutants (Fig. 5). Based on our current and previous results, which demonstrate that *sal* is both necessary and sufficient for R7 differentiation posterior to row seven (Domingos et al., 2004), we propose a model for the action of *sal* and *svp* during R3/R4 specification (Fig. 6): from rows three to seven, *sal* is required for *svp* expression in R3/R4 and for R3/R4 specification; posterior to rows seven to nine, repression of *sal* by *svp* in R3/R4 is necessary for the maintenance of R3/R4 identity and the inhibition of R7 fate. This dual regulation between *sal* and *svp* helps to understand the complex *sal*$^{-}$ phenotype in R3/R4. Strikingly, although *svp* expression is lost in *sal*$^{-}$ R3/R4, these cells do not get transformed into R7, but keep an outer PR identity. Thus, in the absence of *sal*, the presumptive R3/R4 remain as outer PRs with an unspecified subtype identity.

In conclusion, our results demonstrate that *sal* is required in R3 to allow normal Fz/PCP signaling to specify the R3 and R4 cell fates. Ommatidia mutant for *sal* show defects that are very similar to those observed in *fz* and *dsh* mutants, as judged by the loss of asymmetric Fmi localization at the equatorial side of the R3/R4 precursors, and by the lack of *Dl* and *E(spl)mδ* upregulation within the R3/R4 pair. In addition, *sal* is required upstream of *svp* for normal R3/R4 specification. Finally, our results show that, posterior to row seven, *svp* represses *sal* in R3/R4 in order to maintain R3/R4 identity and to inhibit transformation of these cells to the R7 cell fate.

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