Ral GTPase promotes asymmetric Notch activation in the Drosophila eye in response to Frizzled/PCP signaling by repressing ligand-independent receptor activation

Bomsoo Cho and Janice A. Fischer*

SUMMARY

Ral is a small Ras-like GTPase that regulates membrane trafficking and signaling. Here, we show that in response to planar cell polarity (PCP) signals, Ral modulates asymmetric Notch signaling in the Drosophila eye. Specification of the initially equivalent R3/R4 photoreceptor precursor cells in each developing ommatidium occurs in response to a gradient of Frizzled (Fz) signaling. The cell with the most Fz signal (R3) activates the Notch receptor in the adjacent cell (R4) via the ligand Delta, resulting in R3/R4 cell determination and their asymmetric positions within the ommatidium. Two mechanisms have been proposed for ensuring that the cell with the most Fz activation sends the Delta signal: Fz-dependent transcriptional upregulation in R3 of genes that promote Delta signaling, and direct blockade of Notch receptor activation in R3 by localization of an activated Fz/Disheveled protein complex to the side of the plasma membrane adjacent to R4. Here, we discover a distinct mechanism for biasing the direction of Notch signaling that depends on Ral. Using genetic experiments in vivo, we show that, in direct response to Fz signaling, Ral transcription is upregulated in R3, and Ral represses ligand-independent activation of Notch in R3. Thus, prevention of ligand-independent Notch activation is not simply a constitutive process, but is a target for regulation by Ral during cell fate specification and pattern formation.

KEY WORDS: Ral GTPase (Rala), Notch/Delta signaling, Drosophila eye, Planar cell polarity

INTRODUCTION

Functions for Ral (RalA – FlyBase), a small Ras-like GTPase, are only beginning to be discovered. Ral has a well-characterized role in secretion (Moskalenko et al., 2001; Sugihara et al., 2002), and is also implicated in other membrane trafficking and remodeling events (Feig, 2003; van Dam and Robinson, 2006; Chen et al., 2006; Cascone et al., 2008; Wu et al., 2008; Spiczka and Yeaman, 2008; Lalli, 2009; Hase et al., 2009). Ral also regulates Rheb-dependent nutrient sensing in vertebrate cells (Maehama et al., 2008; Lalli, 2009; Wu and Mlodzik, 2009; Axelrod, 2009; Simons and Struhl, 1999). Disheveled (Dsh) is a cortical cytoplasmic protein required for transducing the Fz signal. At the interface between pre-R3 and pre-R4, Fz/Dsh is at the pre-R3 plasma membrane, where Dsh may directly inhibit Notch receptor activation in R3. The asymmetrically localized Fz/Dsh complex may also amplify the difference in Fz activation between the two cells through a feedback loop.

Here, we discover a unique Ral-dependent pathway by which Fz/PCP signaling leads to asymmetric Notch activation in R4. We show that in direct response to Fz activation, Ral expression in pre-R3 represses Notch activation, thereby biasing pre-R3 to become the Delta signaling cell. Moreover, we found unexpectedly that Ral prevents Notch activation that occurs independent of ligand
binding. Thus, Ral regulates Notch signaling, and ligand-independent Notch activation is a target of regulation during cell patterning.

MATERIALS AND METHODS

Drosophila strains

The following alleles were used in this work. FlyBase id numbers, when available, are in parentheses. Chromosomes used are indicated in the figure legends. *Ral*E1 [FBal0197295]; *RalP69* [FBal0130802]; *RalP69* [FBal0130801]; f2341 [FBal009437]; f2351 [FBal012708]; fafP68 [FBal0031258]; neur1 [FBal0012940]; DPP [FBal0002474]; N419 [FBal0005654]; IglP69D [FBal014483]; IglP69G [FBal0104486]; Act5C-gal4 [FBti0012711]; ro-gal4 (E. Overstreet, PhD thesis, University of Texas at Austin, 2005); GMR-gal4 [FBti0072862]; Act->stop>gal4 (from N-S. Moon, McGill University, Quebec, Canada); tub-gal80 (from G. Struhl, Columbia University, New York, USA); tub-gal80 [FBti0012693, FBti0012683], UAS-flp [FBti0012285]; UAS-RalE1 [FBal0101574]; UAS-RalC [FBal0101576]; UAS-RalP69A (VDRCl# 105296); UAS-ngfp (on X, FBti0012492, FBti0012493); UAS-nlacZ [FBpt0001611]; ubi-ngfp [FBti0015575, FBti0016102]; sev-fz [FBal0082914]; mi-lacZ [FBpt0010977]; ro-gfp (Overstreet et al., 2004); hs-N-Gal4 [FBal0090683]; hs-N-Gal4 [FBal0090683]; hs-Nfl[+]-Gal4 (Struhl and Adachi, 1998); FRT194 [FBti0008870]; FRT82B [FBti0002074]; FRT2A [FBti0002046]; eyFLP [FBti0015984, FBti0015982]; eyeFLP2 (from B. Dickson, IMP, Vienna, Austria); hs-FLP [FBos0062044]; and FM7, gfp [FBos0005193].

ro-gal4

gal4 DNA sequences were amplified from GMR-gal4 flies using primers that inserted Act5C sites upstream of the start codon and downstream of the stop codon: 5'-gggsgcATAGAAGCTAGCTTCTTCTAG-3' and 5'-gggssgctATATCGTGTGTTTCGG-3'. The 2.7 kb amplification product was ligated into pGEM (Stratagene, Santa Clara, CA, USA) used at 1:200 were: Alexa568-anti-rabbit, Alexa568-anti-mouse, Alexa488-anti-rabbit, Alexa488-anti-mouse, Alexa555-anti-rat, Cy5-anti-rabbit. Plastic sections of adult eyes was as described (Tomlinson and Ready, 1987). Eye sections were photographed with a Zeiss Axioplan and Axiocam HRC. Adult external eyes were photographed with an Olympus SZX12 microscope equipped with a SPOT camera (Diagnostic Instruments, Sterling Heights, MI, USA) digital camera. Larvae expressing GFP were viewed with a Leica M165FC microscope and photographed with a Leica DFC420C camera. Images were processed with Adobe Photoshop CS3. Statistical analysis of eye sections was using the unpaired t-test, Prism 3.0 software.

RESULTS

*Ral* is required for R-cell specification and PCP in the eye

Three *Ral* alleles were used in this work: *RalE1*, *RalP69* and *RalP69*. *RalE1* is a miss-sense mutation that alters a nucleotide-binding site (Ser154→Leu154) (Eun et al., 2007). *RalP69* and *RalP69* are P-element insertions (gal4-expressing enhancer traps) in the 5'-UTR and the first intron of *Ral*, respectively (Ghiglione et al., 2008). Ral protein expression levels from the P alleles are reduced relative to wild type.

*RalE1/*Y males or *RalE1* homozygous females are viable with morphological abnormalities, including reduced rough eyes, curved wings, and missing hairs and bristles (Eun et al., 2007). *RalE1* behaves like a hypomorphic allele. All aspects of the *RalE1* mutant phenotype in hemizygous males are complemented by Act5C-gal4; UAS-RalE1 (Eun et al., 2007). In addition, heterozygotes for *RalE1* and either of the lethal hypomorphic alleles *RalP69* or *RalP69* have a mutant phenotype similar to *RalE1* hemizygotes or homozygotes, that is complemented by *RalP69*, UAS-RalE1 (data not shown). Moreover, flies that express RalRNAi in the eye have defects similar to those in *RalE1* flies, and the defects are rescued to wild-type by overexpression of wild-type *Ral* (see Fig. S1 in the supplementary material).

We analyzed the eyes of *RalE1/*Y flies in detail. Wild-type adult eyes have ~800 facets, or ommatidia, each with eight photoreceptors (R cells) arranged in a trapezoid that is asymmetrical owing to the positions of R3 and R4 (Fig. 1A,D). In wild-type eyes, the trapezoids are perfectly aligned with one another. There are two chiral forms of ommatidia, mirror-image symmetrical through the equator that divides the eye into dorsal and ventral halves (Fig. 1A). Adult ommatidia of *RalE1/*Y eyes had a variety of defects, including loss of R cells, loss of R3/R4 symmetries and defects in orientation with respect to the equator (Fig. 1B,C). Adult eyes of *RalE1/RalP69* and *RalE1/RalP69* were similar to *RalE1/*Y (data not shown). We examined *RalE1/*Y eye discs to determine whether or not the adult eye abnormalities were due to defects in early development. The eye disc is a monolayer epithelium in which ommatidia assemble stepwise posterior to the morphogenetic furrow as it travels across the disc from posterior to anterior (Wolff and Ready, 1993). Five R-cell precursors (R8, R2/5, R3/4) emerge as a pre-cluster, and then R1/6 and R7 are recruited from the remaining pool of undifferentiated cells (Fig. 1D). Assembling ommatidia normally rotate in mirror-image reflection with respect to the equator (Fig. 1D). In *RalE1/*Y eye discs, many ommatidia rotate either too much or too little, and R1 or R6 are frequently absent (Fig. 1E,F). Similar observations were made with *RalE1/RalP69* and *RalE1/RalP69* eye discs (data not shown). We conclude that *Ral* is required for patterning early in eye disc development.

GFP protein blot

For quantitation of GFP (see Fig. S3 in the supplemental material), animals were grown at 29°C, heat shocked as third instar larvae for 1 hour at 37°C, and allowed to recover for 2 hours at 29°C. To generate protein extracts, five males were homogenized in 100 μl ice-cold PBS and 100 μl of 2X loading buffer was added. The extract was boiled for 10 minutes and then microfuged for 10 minutes. Supernatants (13 μl) were subjected to 10% SDS-PAGE and western blotted. Primary antibodies were rabbit anti-GFP (Abcam) at 1:5000 and mABE7 (DSHB) at 1:1000. Secondary antibodies were HRP-anti-mouse and HRP-anti-rabbit (Santa Cruz Biochem) at 1:2000. Signals were quantified with NIH Image J software.
**Ral eye defects and genetic interactions suggest that Ral regulates Notch signaling**

*Ral* was identified in a mutagenesis screen for dominant enhancers of the rough eye caused by epsin overexpression (Eun et al., 2007). Epsin is an endocytic protein required in Notch signaling cells for ligand endocytosis and signaling (Overstreet et al., 2004; Wang and Struhl, 2004; Wang and Struhl, 2005). Loss-of-function alleles of genes acting in Notch signaling both positively and negatively were identified in the screen (Eun et al., 2007). To determine whether *Ral* plays a role in Notch signaling normally, we tested for genetic interactions between *Ral* and *lqf* loss-of-function mutations. *Ral* mutations were dominant suppressors of *lqf* hypomorphic eye phenotypes (Fig. 2A), and the *Ral* eye phenotype was suppressed by *lqf* (Fig. 2B). These results imply that *Ral* regulates Notch signaling, and further suggest that *Ral* activity opposes Notch activation.

Two additional observations supported the idea that *Ral* is a negative regulator of Notch signaling. First, *N5419* (a null allele) was a dominant enhancer of the eye defects caused by expression of constitutively active Ral (Sawamoto et al., 1999) during eye development (*ey-gal4, GMR-gal4; UAS-RalCA*). Second, the *RaLE1* ommatidial defects described above are similar to those observed when there is too much Notch activity. Normal R3/R4 asymmetry (and rotation) results from Notch activation in the R4 precursor by Delta in the R3 precursor (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). Notch activation in both R3/4 precursors results in equivalent symmetric R3/4 cells (Fanto and Mlodzik, 1999; Cooper and Bray, 1999), and overactive Notch in R1/6 precursors results in their failure to differentiate as R cells (Cooper and Bray, 1999). Too little Notch activation early in eye development likewise results in symmetric R3/4 cells (Fanto and Mlodzik, 1999; Cooper and Bray, 1999).
and also extra R3/4 cells due to the failure of Notch activation in surplus precluster cells (Cagan and Ready, 1989; Overstreet et al., 2004). Extra R3/4 cells are not observed in RalEE1 ommatidia (Fig. 1B), suggesting that in Ral mutants, the Notch pathway is generally overactive.

In contrast to the suppressive interactions between RalEE1 and lqf, we were surprised to find that the RalEE1 eye roughness was dominantly enhanced by loss-of-function mutations in two other Notch pathway genes, Dl and neur (Fig. 2B). These results suggest that Ral promotes Notch signaling. Taken together, the genetic interactions led us to conclude that Ral regulates Notch signaling, but in a complex manner. Further experiments described below that illuminated the role of Ral in Notch signaling also suggested resolutions to this paradox, and this will be explained below.

### Ral prevents ligand-independent Notch activation

To examine further the idea that Ral represses Notch activation, we tested for genetic interactions between RalEE1 and transgenes that overexpress two different forms of the Notch receptor, called N+-GV3 and NSev11-GV3, under heat-shock control (Struhl and Adachi, 1998) (Fig. 3A). When Notch is activated, two proteolytic cleavages occur; an ADAM metalloprotease cleaves the extracellular domain, and then Presenilin cleaves within the transmembrane domain to generate a cytoplasmic fragment called Nicd (icd, intracellular domain) that travels to the nucleus and derepresses target gene transcription (Bray, 2006). N+-GV3 contains Gal4/VP16 within its Nicd fragment (Nicd-GV3). N+-GV3 functions in the same way as wild-type Notch in that the transgene complements N– mutations, and also Nicd-GV3 activates transcription of UAS-lacZ in response to ligand binding (Struhl and Adachi, 1998). NSev11-GV3 is an altered version of N+-GV3, in which the Notch extracellular and transmembrane domains were replaced by a truncated version of those domains of the Sevenless receptor. NSev11-GV3 thus cannot bind Notch ligands, and therefore it does not normally activate UAS-lacZ (Struhl and Adachi, 1998). We used both forms of the Notch receptor in order to determine whether the interaction between Ral and Notch required the Notch extracellular domain, and, thus, ligand binding.

If Ral represses Notch activation, then N+-GV3 overexpression would be expected to enhance the RalEE1 rough eye, and we found that it does (Fig. 3B). Remarkably, we also found that NSev11-GV3 overexpression had a similar effect (Fig. 3B). These results suggest that in RalEE1 cells, both N+-GV3 and NSev11-GV3 receptors are activated. To test this, we assayed the expression of UAS-ngfp in RalEE1 cells, and they were typical of N+-GV3 or NSev11-GV3. We observed little or no GFP in RalEE1 larvae (Fig. 3C, parts c,e), but in RalEE1 larvae, there were high levels of GFP in the midgut (Fig. 3C, parts d,f) (see also Fig. S3 in the supplementary material). Ral expression is reportedly elevated in the larval gut (Tweedie et al., 2009), and Ral is required for intestinal antibacterial immunity (Cronin et al., 2009). Consistent with these reports, we observed
high levels of GFP in the midguts of Ral
PG09; UAS-ngfp larvae (Fig. 3C, part a). We conclude that Ral blocks ligand-independent activation of Notch.
RAL activity in R3 promotes asymmetric Notch activation in R4

We have shown above that Ral activity in R3 influences R3/R4 asymmetry, but does it do so through an effect on Delta/Notch signaling? Notch activation in the R3/R4 pair may be monitored by the expression of a transgene called mΔ-lacZ, in which the transcriptional control sequences of the Notch target gene E(spl)mΔ drives expression of lacZ (Cooper and Bray, 1999). In wild-type third instar larval eye discs, mΔ-lacZ is expressed mainly in the polar cell, which becomes R4 (Cooper and Bray, 1999) (Fig. 5A). To determine whether Ral activity in either the equatorial (pre-R3) or polar cell (pre-R4) affects Notch activation (mΔ-lacZ expression), we generated developing ommatidia mosaic for RalEE1 and RalPG69, or where one of the R3/R4 pair was Ral−/Ral− or Ral+/Ral− (Fig. 5B-D). We scored mΔ-lacZ expression in mosaic facets in which the polar and equatorial cells were both Ral+, both Ral−, or where one of the R3/R4 pair was Ral+ and one was Ral− (Fig. 5B-D). We found that when both the equatorial and polar cells were Ral+, mΔ-lacZ was expressed in R4. By contrast, when both cells of the R3/R4 pair were Ral−, mΔ-lacZ expression was often symmetrical (absent or at low levels in both cells), or occasionally the pattern of mΔ-lacZ expression was reversed, meaning that the equatorial cell expressed mΔ-lacZ and the polar cell did not. These results indicate that Ral activity in the R3/R4 pair does affect Notch activation. In addition, the pattern of mΔ-lacZ expression was almost always wild-type when the equatorial cell was Ral+ and the polar cell Ral−. By contrast, when the equatorial cell was Ral− and the polar cell Ral+, mΔ-lacZ expression was often symmetrical or reversed. We conclude that Ral activity in the equatorial cell (pre-R3) promotes asymmetric Notch activation in the polar cell (pre-R4).

The role of Ral in R3 clarifies how different loss-of-function mutations in genes that promote Delta signaling, Delta and neur versus lqf, can have opposite effects on the Ral mutant phenotype. Ral, Delta and neur are all required in the pre-R3 cell, where they bias pre-R3 to become the Delta signaler. Pre-R3 is sensitive to the levels of activity of all three genes, and so the observation that Delta or neur mutations enhance Ral mutations makes sense in this...
context. Why does lqf interact with Ral in the opposite way? One possibility is that pre-R4 is more sensitive to Lqf levels than pre-R3 is, and so the major effect of lqf mutation is not in pre-R3, but in pre-R4. Unlike Delta and neur, lqf is not upregulated in pre-R3 (B.C. and J.A.F., unpublished observations). Perhaps the lower levels of Delta and Neur in pre-R4 render pre-R4 more sensitive to the levels of Lqf. If so, the negative effect of lowering the lqf gene dose on the ability of a cell to become the signaler would be more significant in pre-R4 than in pre-R3. In this scenario, Ral+ or lqf− mutations would have opposite effects on R3/R4 asymmetry, and would be expected to suppress each other. Alternatively, Lqf might antagonize Ral activity directly in R3 by promoting ligand-independent Notch activation. The latter possibility may be tested with additional experiments. If the role of Ral in other cell fate decisions in the eye is similar to its role in R3/R4, then this kind of logic could explain the effects on overall eye roughness observed in various mutant combinations.

**Ral expression is enriched in R3 and depends on Frizzled near the morphogenetic furrow**

The results above indicate a requirement for Ral+ in the equatorial cell (pre-R3). We were curious to know whether Ral expression is ubiquitous or spatially restricted. To monitor Ral transcriptional activation, we used RalPG69, the gal4-expressing enhancer trap in the 5'/H11032-UTR of Ral, driving expression of UAS-ngfp. We found that GFP was expressed in all R-cells, but in the
majority of ommatidia, GFP was enriched in R3 beginning at approximately row 4 posterior to the furrow (Fig. 6A). In ~12% of ommatidia, GFP levels were higher in R4 than in R3 (Fig. 6A), but all of these ommatidia were posterior to row 7. As m\(\alpha\)-lacZ expression in R4, which is indicative of R3/R4 specification, normally begins at row 3 or 4 (Fanto and Mlodzik, 1999; Cooper and Bray, 1999), RalPG69 activity is generally elevated in pre-R3 at the time when R3 and R4 are specified. (We expect a delay of about one row in RalPG69 due to the Gal4 intermediate.)

Specification of the equatorial cell as R3 depends on Fz activation (Zheng et al., 1995; Tomlinson and Struhl, 1999; Fanto and Mlodzik, 1999; Cooper and Bray, 1999). In fz– eyes, ommatidia are symmetrical, or their chirality is randomized (wild-type or reversed). Fz activation increases transcription of Delta and neur (Fanto and Mlodzik, 1999; del Alamo and Mlodzik, 2006) and may also repress Notch receptor activation in the equatorial cell (Strutt et al., 2002). To determine whether RalPG69 activity depends on Fz signaling, we monitored \(\beta\)-galactosidase (\(\beta\)-gal) in fz– clones with RalPG69; UAS-nlacZ.

\(\beta\)-Gal expression was reduced in fz– clones, most severely near the morphogenetic furrow, where R3 and R4 are first distinguished (Fig. 7A, parts a-b/H11032). We also observed non-autonomous repressive effects of fz– clones on Ral expression outside of the clones (Fig. 7A, parts a-b/H11032). Moreover, at the borders of clones, when one cell of a mosaic (fz+/fz–) R3/R4
pair expressed β-galactosidase, it was usually the fz+ cell (15/21 pairs in eight clones) (Fig. 7A, parts b,b′), and the six exceptions were at the posterior of the eye disc.

As expression of UAS-RalGsp under RalGsp control complements Ral− mutants, the activity of RalGsp observed probably mirrors, at least in part, the normal Ral transcription pattern. To test this assumption, we examined Ral protein in eye discs using a polyclonal antibody to human RalB [Drosophila Ral and human RalB are identical in 148/201 amino acids, and a different antibody to human Ral B was used to recognize Drosophila Ral in ovaries and on protein blots (Balakireva et al., 2006; Ghiglione et al., 2008)]. In wild-type eye discs, the antibody labeled puncta posterior to the furrow (Fig. 6C, parts a,a′). Although a Ral protein null allele to use as a control is unavailable, several experiments lead us to conclude that the antibody recognizes Ral specifically in the eye. First, the antibody signal was strikingly lower in RalGsp homozygous clones than in surrounding heterozygous tissue (Fig. 6B). Second, in eye discs that overexpress Ral in a subset of R cells (R2/5, R3/4) using ro-gal4; UAS-RalGsp, highly elevated signal was detected in R2/5 and R3/4 (Fig. 6C, parts b,b′). Third, the pattern of antibody labeling resembles closely the pattern of expression of GFP from RalGsp, UAS-ngfp. Ral antibody signal begins posterior to the furrow in approximately row 3 or 4 (Fig. 6C, parts a), and appears generally elevated in R3 (Fig. 6D). In wild-type eye discs, or in eye discs that overexpress Ral using RalGsp, UAS-RalGsp, Ral protein is in basal puncta (Fig. 6C, parts a,a′), that in most ommatidia appear concentrated in R3 (Fig. 6D and legend). The results above indicate that near the morphogenetic furrow, Ral expression is controlled by Fz. We tested this idea further by overexpressing fz in the R3/R4 pair using a sevenless expression vector construct, sev-fz. Expression of sev-fz results in R3/4 symmetry or random chirality, because the equalization of and/or excess of Fz activity in the R3/4 pair disrupts asymmetric Notch activation in the polar cell (pre-R4) (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). We found that the normal pattern of RalGsp, UAS-ngfp expression was disrupted in eye discs expressing sev-fz (Fig. 7B,C). RalGsp, UAS-ngfp expression was enriched in the equatorial cell (pre-R3) less often than in wild-type discs, and enriched in the polar cell (pre-R4) more often than in wild type (Fig. 7C). We conclude that Ral transcriptional control is downstream of Fz.

**Frizzled control of asymmetric Ral expression is not through Notch.**

Enrichment of Ral transcription in R3 versus R4 near the furrow could be a direct effect of Fz signaling, and a reflection of more Fz signaling in R3 than R4. Alternatively, asymmetric Ral expression could be downstream of Notch activation. If so, Ral enrichment in R3 could reflect that Notch activation in R4 represses Ral transcription directly in R4 and/or that Notch activation in R4 activates Ral transcription in R3 through a feedback mechanism. One observation suggests that Ral expression could be controlled by Notch; in sev-fz discs, mo-lacZ (Notch activation) was generally depressed (Fig. 7B), and Ral had an increased tendency to be expressed in the polar cell (pre-R4) (Fig. 7D). However, we observed further that in sev-fz discs, mo-lacZ and RalGsp, UAS-ngfp were often expressed in the same cell (Fig. 7D). Moreover, there was no tendency for cells that express mo-lacZ not to express RalGsp, UAS-ngfp (Fig. 7D). We also generated discs in which the R3/R4 pair both express constitutively active Notch (sev-N1c), which renders them symmetrical (Fanto and Mlodzik, 1999; Cooper and Bray, 1999). Although the pattern of RalGsp, UAS-ngfp is disrupted somewhat,

**Ral-mediated Notch inhibition is one of several Fz-dependent pathways that control R3/R4 asymmetry.**

The effects on R3/R4 asymmetry of losing Ral in one or both cells are small; symmetry defects were observed in these experiments in 10-30% of the R3/R4 pairs (Fig. 1C; Fig. 4; Fig. 5). In contrast to the results with Ral (Fig. 5), in ommatidia where R3/R4 are mosaic for Delta, the Delta+ cell always becomes R3 (Tomlinson and Struhl, 1999). The small effects observed for Ral could result, at least in part, from the Ral+ function remaining in the three hypomorphic mutants we used. However, similar weak effects were observed in analogous experiments using a strong neurol loss-of-function allele (del Alamo and Mlodzik, 2006). This suggests that RalEE1 may have an incompletely penetrant effect on R3/R4 asymmetry primarily because Ral works in only one or a subset of distinct Fz-dependent pathways that bias the pre-R3 cell to become the Delta signaler. Consistent with this interpretation, we find that overexpression of Ral (or RalCA, not shown) in both R3 and R4 in

---

Fig. 8. Ral overexpression in pre-R4 has only a subtle effect on R3/R4 determination. (A, A′) An eye disc is shown containing a gfp+ cell clone (outlined in A′) that overexpresses Ral. The eye disc also expresses mo-lacZ and is immunolabeled with anti-Svp and anti-β-gal. The arrows indicate Ral-overexpressing (gfp+) R4s (β-gal+), the R3s of which (β-gal−) do not overexpress Ral (gfp−). The genotype is hs-flp, tub-gal4, UAS-gfp/+; UAS-RalCA/+; FRT82B/FRT82B tub-gal80. Larvae (2nd and 3rd instar) were heat-shocked for 1 hour at 37°C. (B) An analysis of R3/R4 determination in pairs mosaic for wild-type and RalCA-overexpressing cells. Pooled results from six mosaic eye discs are shown. EQ, equatorial cell; PO, polar cell. The genotype is hs-flp; Act5C>stoP>gal4, UAS-gfp/+; UAS-RalCA/+; mo-lacZ. Scale bar: 10 µm.

Ral expression is not depressed (Fig. 7E), and Ral has a greater tendency than in wild type to be expressed asymmetrically (Fig. 7C). Finally, we find that Ral mutations suppress sev-fz defects, but fail to suppress (and in fact enhance) sev-N1c defects (Fig. 7F). This means that Fz in the R3/4 pair requires Ral even when Notch signaling is depressed and the R3/4 pair are symmetrical, but Notch does not. We conclude that Fz activation controls Ral transcription directly, not through Notch activation.
clones of otherwise wild-type eye disc cells had no effect on the pattern of Notch activation; \( \text{m} \text{a-lacZ} \) is still expressed specifically in the polar cell (pre-R4) (Fig. 8A, A'). Even in mosaic R3/R4 pairs at the clone border in which \( \text{Ral}^{\text{CA}} \) is overexpressed in pre-R4 and not in pre-R3, \( \text{m} \text{a-lacZ} \) was nearly always expressed in the polar cell (Fig. 8B). Similarly, \( \text{Ral} \) or \( \text{Ral}^{\text{CA}} \) overexpression in both R3 and R4 with \( \text{ro-gal4; 2XUAS-Ral} \) had only a very subtle effect on R3/4 asymmetry in the adult eye (see Table S1 in the supplementary material).

The eye disc also expresses \( \text{m} \text{a-lacZ} \), at reduced levels compared with wild-type R4 (the cells are both R4 or some fate in between R3 and R4) (Fig. 9A-B). \( \text{Ral} \) overexpression in \( f z^2\) R3/4 precursor pairs abolished \( \text{m} \text{a-lacZ} \) expression (both cells are now R3) (Fig. 9C-D'). We conclude that \( \text{Ral} \) functions in one of several \( f z \)-dependent pathways that control R3/R4 asymmetry.

DISCUSSION

The results presented support the model for \( \text{Ral} \) function shown in Fig. 10. \( \text{Ral} \) transcription is upregulated in response to \( f z \) activation. As \( f z \) is activated more in the equatorial cell than the polar cell, \( \text{Ral} \) is enriched in the equatorial cell. \( \text{Ral} \) activity represses ligand-independent Notch activation, and thus biases the equatorial cell to become R3. One way in which ligand-independent Notch activation occurs is an accident when normal Notch trafficking is disrupted (Fortini and Bilder, 2009; Fortini, 2009). Notch receptor undergoes endocytosis and endosomal trafficking continually and mutations that block trafficking of late endosomes to the lysosome block Notch degradation and result in endosomal accumulation of Notch and ligand-independent activation (Fortini and Bilder, 2009; Fortini, 2009). One possibility is that the endosomal environment promotes production of \( \text{N}^{\text{Ied}} \) by Presenilin cleavage. Ligand-independent Notch activation may also occur normally in the lysosomal membrane (Wilkin et al., 2008; Fortini and Bilder, 2009). \( \text{Ral} \) GTPase activity might block ligand-independent Notch activation by regulating Notch trafficking to the lysosome, or by inhibiting another process, such as endosomal acidification (Yan et al., 2009), \( \text{N}^{\text{Ied}} \) production or nuclear translocation. The punctate appearance of \( \text{Ral} \) protein suggests the possibility that \( \text{Ral} \) may play a role in endosomal trafficking. Although further experiments are required to determine the precise mechanism of \( \text{Ral} \) function, we have shown that \( \text{Ral} \), a protein that prevents ligand-independent Notch activation, is a target for regulation during pattern formation. \( f z/\text{PCP} \) signaling upregulates \( \text{Ral} \) expression to ensure that ligand-independent Notch activation does not tip the scales in favor of pre-R3 becoming the signal receiving cell. Moreover, we have shown that prevention of ligand-independent Notch activation is not simply a constitutive process, but one that is modulated to ensure specific developmental outcomes.

Acknowledgements

We thank M. Balakireva, S. Bray, J. Camonis, B. Dickson, M. Fortini, H. Kramer, M. Mlodzik, N.-S. Moon, K. Sawamoto, G. Struhl, the Bloomington \( \text{Drosophila} \) Stock Center and the Vienna \( \text{Drosophila} \) Resource Center for fly strains, and R. Cripps for anti-Svp. We are grateful to Paul Macdonald and Steve Vokes for use of their microscopes. This work was supported by a grant to J.A.F. from the US National Institutes for Child Health and Human Development (R01-HD30680). Deposited in PMC for release after 12 months.

Fig. 9. \( \text{Ral} \) controls R3/R4 cell fate in \( f z^2 \) cells. (A-A') Two separate \( \text{gfp}^+ \) \( f z^2 \) clones (outlined) in an eye disc are shown in A-A' and B-B'. The eye disc also expresses \( \text{m} \text{a-lacZ} \) and is immunolabeled with anti-\( \beta \)-gal and anti-Svp. The genotype is \( \text{hs-flp tub-gal4; UAS-gfp}^+; \text{m} \text{a-lacZ}^{\text{trh}}, f z^{2\text{C}}, \text{FRT2A/tub-gal80 FRT2A} \). (C-D') Two separate \( \text{gfp}^+ \) \( f z^2 \) clones that overexpress \( \text{Ral} \) (outlined) in eye discs are shown in C-C' and D-D'. The eye disc also expresses \( \text{m} \text{a-lacZ} \), and is immunolabeled with anti-\( \beta \)-gal and anti-Svp. The genotype is \( \text{hs-flp tub-gal4; UAS-gfp}^+; \text{m} \text{a-lacZ}^{\text{trh}}, f z^{2\text{C}}, \text{FRT2A/tub-gal80 FRT2A} \). Scale bar: 10 \( \mu \text{m} \). To generate clones, larvae (2nd and 3rd instar) were heat-shocked for 1 hour at 37°C.

Fig. 10. Model for \( \text{Ral} \) function in R3/R4 cell fate decision. \( f z \) activation in the equatorial cell results in asymmetric Notch activation through two proposed mechanisms: (1) promotion of Delta signaling through transcriptional upregulation of \( \text{Delta} \) and \( \text{neur} \), and their repression in the polar cell, and (2) direct repression of the Notch receptor through relocation of a \( f z/\text{Dsh} \) complex to the side of the equatorial cell plasma membrane adjacent to the polar cell. We have presented evidence for a distinct \( \text{Ral} \)-dependent mechanism in the equatorial cell. \( \text{Ral} \) transcription is upregulated in response to \( f z \) activation, and \( \text{Ral} \) activity represses ligand-independent Notch activation. Notch activation in R4 does not repress \( \text{Ral} \) transcription in the polar cell.
Lalli, G.

Klein, T. J. and Mlodzik, M.


References

Supplementary material for this article is available at Supplementary material

The authors declare no competing financial interests.

Competing interests statement

Ghiglione, C., Devergne, O., Cerezo, D. and Noselli, S.


Cagan, R. L. and Ready, D. F.


Feig, L. A.

Cooper, M. T. D. and Bray, S. J.

Chien, Y., Kim, S., Bumeister, R., Loo, Y.-M., Kwon, S. W., Johnson, C. L., Fanto, M. and Mlodzik, M.

del Alamo, D. and Mlodzik, M.

Chen, X. W., Inoue, M., Hsu, S. C. and Salltiel, A. R.

Chen, X., Zhang, B. and Fischer, J. A.

Balakireva, M. G., Romeo, Y., Kopelovich, L., Gale, M., Jr et al.

Camonis, J.

(2009). Genome-wide RNAi screen identifies genes involved in intestinal

Tomlinson, A. and Struhl, G.

Tomlinson, A. and Struhl, G.

Spiczka, K. S. and Yeaman, C.

Simons, M. and Mlodzik, M.

DEVELOPMENT