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

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**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 blockage 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; Hase et al., 2009). 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), Jak/Sta- and JNK-dependent apoptotic pathways in *Drosophila* (Balakireva et al., 2006; Ghiglione et al., 2008), and vertebrate tumor cell survival (Camonis and White, 2005; Chien et al., 2006). Here, we describe a specific role for Ral in PCP-dependent Notch signaling that patterns the *Drosophila* eye.

The *Drosophila* eye exhibits PCP in the arrangement of its ommatidia, or facets (Wolf and Ready, 1993). There are two chiral forms of ommatidia, dorsal and ventral, reflected through the dorsal/ventral midline, or equator. Ommatidial polarity is governed by the Fz/PCP signaling pathway, which has common core components in vertebrates and *Drosophila* (Strutt and Strutt, 2005; Klein and Mlodzik, 2005; Lawrence et al., 2007; Strutt and Strutt, 2009; Wu and Mlodzik, 2009; Axelrod, 2009; Simons and Mlodzik, 2008). Ommatidial chirality is defined by a pair of photoreceptors, R3 and R4, at the apex of a trapezoidal arrangement of eight photoreceptors. The presumptive R3 is closer to the equator, and thus, early in eye development, has higher levels of Fz activation than the presumptive R4. Fz asymmetry results in the pre-R3 cell, via the ligand Delta, activating the Notch receptor in the pre-R4 cell (Fanto and Mlodzik, 1999; Cooper and Bray, 1999; Tomlinson and Struhl, 1999). Asymmetric Notch activation in the R3/R4 pair ultimately determines the chirality of the ommatidium. Two different mechanisms, which are not necessarily mutually exclusive, have been proposed to explain how the difference in Fz activation leads to asymmetric Delta/Notch signaling. In one model, elevated Fz activation in pre-R3 leads directly to elevated transcription of *Delta* and *neuralized* (*neur*), which promote Delta signaling in R3 (Fanto and Mlodzik, 1999; del Alamo and Mlodzik, 2006). *neur* encodes a ubiquitin ligase that ubiquitylates Delta, and is required for Delta signaling. Subsequently, Notch activation in pre-R4 suppresses *Delta* and *neur* expression, which leads to less Notch activation in pre-R3 via a feedback loop. Alternatively (or in addition), Fz activation polarizes cells by localizing a Fz/Disheveled complex to one side of the plasma membrane (Strutt et al., 2002) (see also Tomlinson 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<sup>EE1</sup> (FBal0197295); Ral<sup>PG90</sup> (FBal0130802); Ral<sup>PG90</sup> (FBal0130801); f<sup>231</sup> (FBal0004937); f<sup>232</sup> (FBal0102708); f<sup>A108</sup> (FBal0031258); neur<sup>1</sup> (FBal0012940); D<sup>IP1</sup> (FBal0002474); N<sup>419</sup> (FBal0000564); lg<sup>FDD1</sup> (FBal0104483); and Act<sup>C-gal4</sup> (FBal012293); ey-gal4 (FBal0012711); ro-gal4 (E. Overstreet, PhD thesis, University of Texas at Austin, 2005); GMR-gal4 (FBal0072862); Act<sup>2>stop>gal4</sup> (from N.-S. Moon, McGill University, Quebec, Canada); tub-gal80 (from G. Struhl, Columbia University, New York, USA); tub-gal80 (FBal0012693, FBal0012683); UAS-flp (FBal0012285); UAS-Ral<sup>wt</sup> (FBal0101574); UAS-Ral<sup>CA</sup> (FBal0101576); UAS-Ral<sup>WE1</sup> (VDRC# 105296); UAS-ngfp (on X, FBal0012492, FBal0012493); UAS-nlacz (FBpt0001611); ubi-ngfp (FBal0015575, FBal0016102); sev-fz (FBal0082914); mi<sup>lac-Z</sup> (FBpt0010977); and ro-gfp (Overstreet et al., 2004); hs-N<sup>e-GV3</sup> (FBal0090683); hs-N<sup>e-Bal2-GV3</sup> (Struhl and Adachi, 1998); FRT194 (FBal0008570); FRT82B (FBal0002074); FRT24 (FBal0002046); eyFLP (FBal0015984, FBal0015982); cyFLP2 (from B. Dickson, IMP, Vienna, Austria); hs-FLP (FBal0002044); and FM7, gfp (FBal0005193).

ro-gal4

gal4 DNA sequences were amplified from GMR-gal4 flies using primers that inserted Ascl sites upstream of the start codon and downstream of the stop codon: 5'-gggceggceTAAGAGCTACTGTCCTCTATCG-3' and 3'-gggceggceTTACCTTTTGGGTGTTTG-3'. The 2.7 kb amplification product was ligated into pGEM (Stratagene, Santa Clara, CA, USA) used at 1:200. Antibody dilutions used were: Alexa568-anti-rabbit, Alexa568-anti-mouse, Alexa488-anti-rabbit, Alexa488-anti-mouse, Alexa568-anti-rabbit, and Cy5-anti-rabbit (Santa Cruz Biochem) at 1:2000. Signals were quantified with NIH Image J software.

RESULTS

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

Three Ral alleles were used in this work: Ral<sup>EE1</sup>, Ral<sup>PG90</sup> and Ral<sup>PG69</sup>. Ral<sup>EE1</sup> is a miss-sense mutation that alters a nucleotide-binding site (Ser<sup>154</sup>→Leu<sup>154</sup>) (Eun et al., 2007). Ral<sup>PG69</sup> and Ral<sup>PG90</sup> are P-element insertions (gal4-expressing enhancer traps) in the 5'-UTR and the first intron of Ral, respectively (Giglioli et al., 2008). Ral protein expression levels from the P alleles are reduced relative to wild type.

Ral<sup>EE1</sup>/Y males or Ral<sup>EE1</sup> homozygous females are viable with morphological abnormalities, including reduced rough eyes, curved wings, and missing hairs and bristles (Eun et al., 2007). Ral<sup>EE1</sup> behaves like a hypomorphic allele. All aspects of the Ral<sup>EE1</sup> mutant phenotype in hemizygous males are complemented by Act<sup>C-gal4</sup>; UAS-Ral<sup>wt</sup> (Eun et al., 2007). In addition, heterozygotes for Ral<sup>EE1</sup> and either of the lethal hypomorphic alleles Ral<sup>PG69</sup> or Ral<sup>PG90</sup> have a mutant phenotype similar to Ral<sup>EE1</sup> hemizygoties or homozygotes, that is complemented by Ral<sup>PG90</sup> and UAS-Ral<sup>wt</sup> (data not shown). Moreover, flies that express Ral<sup>BNA</sup> in the eye have defects similar to those in Ral<sup>EE1</sup> 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 Ral<sup>EE1</sup>/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 Ral<sup>EE1</sup>/Y eyes had a variety of defects, including loss of R cells, loss of R3/R4 asymmetry and defects in orientation with respect to the equator (Fig. 1B,C). Adult eyes of Ral<sup>EE1</sup>/Ral<sup>PG69</sup> and Ral<sup>EE1</sup>/Ral<sup>PG90</sup> were similar to Ral<sup>EE1</sup>/Y (data not shown). We examined Ral<sup>EE1</sup>/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 Ral<sup>EE1</sup>/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 Ral<sup>EE1</sup>/Ral<sup>PG69</sup> and Ral<sup>EE1</sup>/Ral<sup>PG90</sup> eye discs (data not shown). We conclude that Ral is required for patterning early in eye disc development.
**Ral blocks ligand-independent Notch signaling**

**Ral** mutant eye phenotype. (A) An apical tangential section through a wild-type adult eye is shown at the top. The orange line marks the equator. The diagram beneath indicates the facet orientations. (B) Sections of wild-type and *Ral* mutant eyes. The diagrams beneath indicate facet orientations and mutant phenotypes. Symbols are defined in C. (C) Quantification of classes of *Ral* mutant ommatidia. Reverse orientation facets were not scored because in the context of all of the aberrant ommatidia, it was often difficult to locate the equator. (D) Diagram of five R-cell pre-clusters in a third instar larval eye disc and rotation with respect to the equator. (E,F) Third instar larval eye discs expressing GFP in R2/5 and R3/4, and immunolabeled with anti-Elav (R-cell nuclei) and phalloidin (actin). The genotypes are *ro-gfp* (wild-type) and *RalEE1/Y; ro-gfp*. The dotted line is the equator, and the morphogenetic furrow is leftward. Numbers indicate R2/5 and R1/R6. Asterisks are ommatidia in which one of the R1/R6 pair are absent. The lines indicate misrotated ommatidia. Scale bar: in A, 20 μm for A,B; in F, 10 μm for E,F.

**Fig. 1.**

**RAL eye defects and genetic interactions suggest that Ral regulates Notch signaling**

*RalEE1* 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 *RalEE1* 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, *N5419E* (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) (see Fig. S2 in the supplementary material). Second, the *RalEE1* 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,
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Fig. 2. Genetic interactions between Ral and Notch pathway mutants. (A) External adult eyes of the genotypes indicated are shown. Ral heterozygous eyes are like wild type (smooth, left), lqf eyes are rough and although this is not obvious in the photographs, eyes with both mutations are more like wild type than like lqf. The graph shows quantitative analysis of the wild-type and mutant ommatidia observed in tangential adult eye sections. For each genotype, the data were obtained from five eyes and 600-900 ommatidia. The symmetric ommatidia were scorable only in ommatidia with normal numbers of R cells. ***P<0.0001, *P<0.02; unpaired t-test. Data are mean + s.e.m. (B) External adult eyes of the indicated genotypes are shown. Ral hemizygous eyes (left) are rough. They are made smoother by heterozygous loss-of-function of lqf, and rougher by heterozygous loss-of-function of Dl or neur. The suppressive effect of lqf is quantified on the right. Ral/Y data are from Fig. 1 and Ral/Y; lqfAG/lqf+ data were obtained from tangential sections of 732 ommatidia in four eyes. ***P<0.0005, **P<0.003; unpaired t-test. Data are mean + s.e.m.

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 Ral mutants (Fig. 1B), suggesting that in Ral mutants, the Notch pathway is generally overactive.

In contrast to the suppressive interactions between Ral and lqf, we were surprised to find that the Ral 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 Ral and transgenes that overexpress different forms of the Notch receptor, called N-GV3 and N-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 N (icd, intracellular domain) that travels to the nucleus and derepresses target gene transcription (Bray, 2006). N-GV3 contains Gal4/VP16 within its N fragment (N-GV3). N-GV3 functions in the same way as wild-type Notch in that the transgene complements N mutations, and also N-GV3 activates transcription of UAS-lacZ in response to ligand binding (Struhl and Adachi, 1998). N-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 Ral rough eye, and we found that it does (Fig. 3B). Remarkably, we also found that N-GV3 overexpression had a similar effect (Fig. 3B). These results suggest that in Ral cells, both N-GV3 and N-GV3 receptors are activated. To test this, we assayed the expression of UAS-ngfp in Ral or Ral larvae that express either N-GV3 or N-GV3. We observed little or no GFP in Ral larvae (Fig. 3C, parts c,e), but in Ral 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 RaPG69; UAS-ngfp larvae (Fig. 3C, part a). We conclude that Ra blocks ligand-independent activation of Notch.
We have shown above that activation in R4 activity in R3 promotes asymmetric Notch signaling. Notch activation in the R3/R4 pair may be monitored by expression of a transgene called m\(\alpha\)-lacZ, which becomes R4 (Cooper and Bray, 1999). To determine whether Ral\(+\) activity in either the equatorial (pre-R3) or polar cell (pre-R4) affects Notch activation (m\(\alpha\)-lacZ expression), we generated developing ommatidia mosaic for RalEE1, m\(\alpha\)-lacZ expression was often symmetrical or reversed. We conclude that Ral\(+\) activity in the R3/R4 pair does affect Notch activation. In addition, the pattern of m\(\alpha\)-lacZ expression was often symmetrical (absent or at low levels in both cells), or occasionally the pattern of m\(\alpha\)-lacZ expression was reversed, meaning that the equatorial cell expressed m\(\alpha\)-lacZ and the polar cell did not. These results indicate that Ral\(+\) activity in the equatorial (pre-R3) and the polar cell (pre-R4) pair was either symmetrical or wild type.

**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 m\(\alpha\)-lacZ, in which the transcriptional control sequences of the Notch target gene E(spl)m\(\delta\) drives expression of lacZ (Cooper and Bray, 1999). In wild-type third instar larval eye discs, m\(\alpha\)-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 (m\(\alpha\)-lacZ expression), we generated developing ommatidia mosaic for RalEE1, gfp+/Ral\(-\) cells by inducing gfp Ral\(+\) clones (RalEE1, RalEE1P/G89 or RalEE1P/G89) in gfp+ Ral\(+\) eye discs. We scored m\(\alpha\)-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 the other was Ral\(-\) (Fig. 5B-D). We found that when both the equatorial and polar cells were Ral\(+\), m\(\alpha\)-lacZ was expressed in R4. By contrast, when both cells of the R3/R4 pair were Ral\(-\), m\(\alpha\)-lacZ expression was often symmetrical (absent or at low levels in both cells), or occasionally the pattern of m\(\alpha\)-lacZ expression was reversed, meaning that the equatorial cell expressed m\(\alpha\)-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 m\(\alpha\)-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\(+\), m\(\alpha\)-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.
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 than pre-R3 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'UTR of $Ral$, driving expression of UAS-ngfp. We found that GFP was expressed in all R-cells, but in the

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Fig. 6. Ral expression in eye discs. (A) An eye disc is shown on the left that expresses $m\alpha$-lacZ and ngfp under the control of a $Ral$ enhancer trap, and immunolabeled with anti-$\beta$-gal and phalloidin. The genotype is: $RaLP^{G69}/+$; $m\alpha$-lacZ/UAS-ngfp. Enlargements of the three different classes of ommatidia indicated are on the right. Scale bars: in the large panel and in B, 20 $\mu$m; in the small panels, 5 $\mu$m. Arrow indicates morphogenetic furrow. (B) A $RaLP^{G69}$/Ral+ eye disc containing a clone of $RaLP^{G69}$/Ral^{G69} cells (outlined at top), immunolabeled with anti-Ral. The genotype is $RaLP^{G69}$/FRT19A/lubi-6gf; FRT19A; ey-gal4, UAS-flp/+ . (C) Wild-type eye discs and eye discs that overexpress Ral and ngfp in R2/R5 and R3/R4 under the control of ro-gal4 are shown. The genotype is: ro-gal4/+/UAS-Ral/UAS-ngfp. The discs are immunolabeled with anti-Ral and phalloidin. Scale bar: 50 $\mu$m in a,b; 5 $\mu$m in a',a'b',b'. Arrows indicate morphogenetic furrow. (D) A z-section of a developing ommatidium. A, apical membrane; b, basal membrane; c, cone cell; numbers are R-cells. The horizontal line represents the depth of the xy images on the right. A $Ral^+$ (wild-type) eye disc that expresses $m\alpha$-lacZ and an eye disc that also overexpresses $Ral$ under control of a $Ral$ enhancer trap ($RaLP^{G69}; UAS-Ral$) are shown. The genotypes are: $m\alpha$-lacZ/+ (wild type) and $RaLP^{G69}/+$, UAS-Ral/m$\alpha$-lacZ. Each is immunolabeled with anti-$\beta$-gal, anti-Ral and phalloidin. The numbers are R3 and R4. We counted the number of R3/R4 pairs in which there were more $Ral^+$ puncta in R3 (R3>R4), where the numbers were similar (R3~R4), and where there were more R4 (R4>R3) in wild-type and $RaLP^{G69}$, UAS-Ral eye discs. In five wild-type discs: R3>R4 (211/290), R3~R4 (31/290), R4>R3 (48/290). In six $RaLP^{G69}$, UAS-Ral discs: R3>R4 (161/219), R3~R4 (21/219), R4>R3 (37/219). Scale bar: 5 $\mu$m.
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). We also observed non-autonomous repressive effects of \(fz^{--}\) clones on Ral expression outside of the clones (Fig. 7A, parts a-b). 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-Ral<sup>PG69</sup> under Ral<sup>P6G69</sup> control complements Ral<sup>-</sup> mutants, the activity of Ral<sup>P6G69</sup> 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 Ral<sup>P6G69</sup> 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-Ral<sup>pm</sup>, 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 Ral<sup>P6G69</sup>; 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 Ral<sup>P6G69</sup>; UAS-Ral<sup>pm</sup>, 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 Ral<sup>P6G69</sup>; UAS-ngfp expression was disrupted in eye discs expressing sev-fz (Fig. 7B,C). Ral<sup>P6G69</sup>; 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.

**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<sup>+</sup> cell clone (outlined in A’) that overexpresses Ral. The eye disc also expresses m<sup>-</sup>lacZ and is immunolabeled with anti-Svp and anti-β-gal. The arrows indicate Ral-overexpression (gfp<sup>+</sup>) R4s (β-gal+), the R3s of which (β-gal-) do not overexpress Ral (gfp<sup>-</sup>). The genotype is hs-flp; tub-gal4, UAS-gfp<sup>+</sup>; UAS-Ral<sup>pm</sup>-lacZ; 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 Ral<sup>CA</sup>-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<sup>+</sup>; UAS-Ral<sup>CA</sup>-lacZ. Scale bar: 10 μm.

**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, m<sup>-</sup>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, m<sup>-</sup>lacZ and Ral<sup>P6G69</sup>; UAS-ngfp were often expressed in the same cell (Fig. 7D). Moreover, there was no tendency for cells that express m<sup>-</sup>lacZ not to express Ral<sup>P6G69</sup>; UAS-ngfp (Fig. 7D). We also generated discs in which the R3/R4 pair both express constitutively active Notch (sev-N<sup>mics</sup>), which renders them symmetrical (Fanto and Mlodzik, 1999; Cooper and Bray, 1999). Although the pattern of Ral<sup>P6G69</sup>; 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<sup>+</sup> cell always becomes R3 (Tomlinson and Struhl, 1999). The small effects observed for Ral could result, at least in part, from the Ral<sup>-</sup> function remaining in the three hypomorphic mutants we used. However, similar weak effects were observed in analogous experiments using a strong neur loss-of-function allele (del Alamo and Mlodzik, 2006). This suggests that Ral<sup>EE1</sup> 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 Ral<sup>CA</sup>, not shown) in both R3 and R4 in
clones of otherwise wild-type eye disc cells had no effect on the pattern of Notch activation; mα-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 Ral was overexpressed in pre-R4 and not in pre-R3, mα-lacZ was nearly always expressed in the polar cell (Fig. 8B). Similarly, Ral or RalCA overexpression in both R3 and R4 with ro-gal4; 2XUAS-Ralwt resulted in only a very subtle effect on R3/R4 asymmetry in the adult eye (see Table S1 in the supplementary material). If Ral functioned in the sole pathway or in all Fz-dependent pathways for R3 specification, we would expect reversal of the normal Ral expression pattern in mosaics to reverse R3/R4 polarity, and equalization of pathways downstream of Fz to result in R3/R4 symmetry. These effects were observed but they were very subtle. Strikingly similar results were obtained in analogous experiments with neur (del Alamo and Mlodzik, 2006). Moreover, we find that in fz cell clones, where all the pathways downstream of Fz are blocked, Ral transcription in the equatorial cell is enriched in the equatorial cell. 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 N\textsuperscript{Ied} by Presenilin cleavage. Ligand-independent Notch activation may also occur normally in the lysosomal membrane (Wilkin et al., 2008; Fortini and Bilder, 2009). Ral GTase 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). 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 N\textsuperscript{Ied} by Presenilin cleavage. Ligand-independent Notch activation may also occur normally in the lysosomal membrane (Wilkin et al., 2008; Fortini and Bilder, 2009). Ral GTase 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). 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COMPETING INTERESTS STATEMENT
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