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Ral inhibits ligand-independent Notch signaling in Drosophila

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We discovered recently that the Drosophila Ral GTPase regulates Notch signaling and thereby affects cell patterning in the eye. Although Ral functions in the ligand signaling cells, Ral does not stimulate ligand signaling directly. Rather, in cells that express both Notch receptor and ligand, Ral activity promotes a cell to become the signaler by inhibiting Notch receptor activation in that cell. Moreover, Ral inhibits a particular pathway of Notch activation—receptor activation that occurs independent of ligand binding. In this commentary, we discuss the phenomenon of ligand-independent Notch receptor activation and how this event might be regulated by Ral.

Keywords: Ral, Drosophila eye, Notch signaling, presenilin cleavage, endosome

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Introduction

Ral proteins are Ras-like GTPases whose known roles are mainly in regulation of membrane trafficking.[1] The best-characterized Ral effectors are RalBP1 (aka RLIP76) [2,3] and the exocyst complex proteins Sec5 and Exo84.[4] Through RalBP1, Ral coordinates endocytosis and mitochondrial fission with mitosis.[5-8] Through the exocyst, Ral regulates many cellular processes including secretion,[4] cytokinesis,[9] cytoskeletal remodeling,[10,11] membrane remodeling,[12] and apoptosis.[13,14]

There are two separate *Ral* genes in vertebrates, *RalA* and *RalB*, which produce proteins comparable in amino acid sequence (81% identical and 89% similar) and organization of functional units.[1,15] RalA and RalB have different cellular functions through RalA-specific and RalB-specific RalGEFs.[9] Drosophila has a single *Ral* gene and a single Ral protein that resembles RalA more closely than RalB in overall amino acid sequence, but has amino acids specific to both vertebrate Ral proteins.[10]

Most of what we know about the cellular functions of Ral and its effectors is from studies in vertebrate cell culture. Genetic and biochemical studies in Drosophila suggest that Ral function is largely conserved between flies and vertebrates.[16] Moreover, Drosophila provides a multicellular context in which to understand the significance of Ral activity. Before Drosophila *Ral* loss-of-function mutants were generated, experiments were performed with transgenic flies that overexpressed wild-type, constitutively active, or dominant negative Ral proteins. This work implicated Ral as a regulator of the cytoskeleton to effect cell shape.[10,17] Subsequent analysis of *Ral* loss-of-function mutants showed that Ral, through regulation of Jak/Stat signaling, controls the fate and survival of so-called polar cells in the ovary that control oocyte polarization.[18] Another study using *sec5* mutants showed that through the exocyst effector, Ral regulates JNK signaling and p38 MAPK to prevent apoptosis in sensory organ precursor cells.[13] Although there has not yet been a genetic analysis of RalBP1 effector function, Drosophila Ral does bind in vitro to RalBP1 as well to Sec5.[16]

We recently discovered a specific role for Drosophila Ral in Notch signaling.[19] We identified *Ral* mutants originally in a screen for mutations that modify the eye defects caused by overexpression of Drosophila Epsin.[20] Epsin is an endocytic protein that is required specifically for Notch signaling in Drosophila,[21,22] *C. elegans*,[23] and vertebrates.[24] Epsin is required in signal-sending cells in order for ligand to activate the Notch receptor

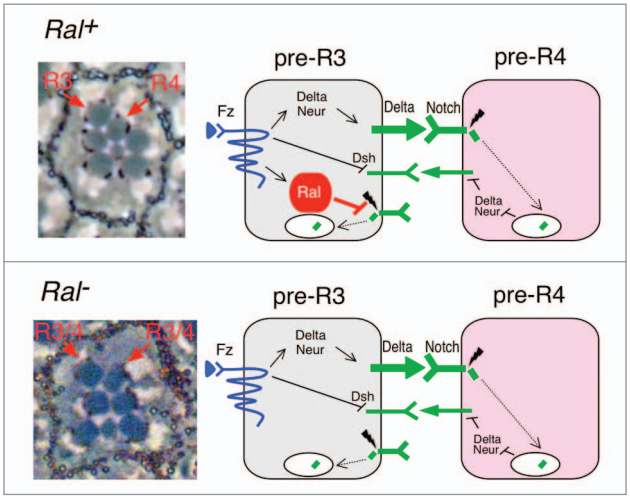

Figure 1. Model for Ral function in R3/R4 specification. At left are light microscope images of a single ommatidium. At right are diagrams of the signaling events that distinguish R3 and R4. The top box is wild type (*Ral*$^{+}$). Photoreceptors in the wild-type adult Drosophila eye (top) are arranged in a trapezoid with R3 at the apex. The asymmetric arrangement of the originally equivalent R3/R4 pair is due to pre-R3 experiencing more Frizzled (Fz) activation than does pre-R4. This difference in Fz activity is amplified by subsequent Notch activation in pre-R4.[28-30] Fz activation results in increased transcription of *Delta* and *neuralized* (*neur*) [28-31] and also relocalization of a Fz/Dsh (Disheveled) complex that represses Notch receptor at the pre-R3 plasma membrane that faces pre-R4.[32] Thus pre-R3 becomes the Delta signaling cell and Notch is activated in pre-R4. Ral defines a unique pathway for insuring that pre-R3 becomes the Delta signaling cell.[19] *Ral* transcription also depends on Fz, and Ral activity prevents activation of Notch receptor that is not bound to ligand.[19] Loss of *Ral* activity (bottom) results in symmetric R3/R4 pairs and ligand-independent Notch activation.[19]

in adjacent signal-receiving cells.[21-23] The Notch receptor and its ligands (Delta and Serrate in Drosophila) are transmembrane proteins.[25] Notch is unusual in its mechanism of activation; ligand binding leads to cleavage of the Notch receptor intracellular domain (NICD), which travels to the nucleus and derepresses transcription of target genes.[25] Most evidence suggests that Epsin is required after ligand/receptor binding to internalize ligand into the signal-sending cells thereby exerting mechanical force on the receptor that leads to its activation.[26,27]

We determined that we identified *Ral* mutants in our screen because Ral is a negative regulator of Notch signaling.[19] We do not understand the mechanism by which Epsin overexpression disrupts eye development and so we cannot say precisely why loss of *Ral* activity enhances the eye defects. Nevertheless, we determined that loss of *Ral* activity results in Notch receptor activation independent of ligand binding.[19] The main evidence for this lies in four observations. First, the specific defects in *Ral* mutant eyes were typical effects of too much Notch activity. Second, *Ral* interacts genetically with several Notch pathway genes in a manner consistent with the idea that *Ral* inhibits Notch activation. For example, Notch overexpression worsens the eye defects caused by loss of *Ral* activity. Third, we observed that overexpression of a hybrid Notch protein with its extracellular domain replaced by that of a different receptor had the same effect of worsening the defects in *Ral* mutant eyes. As the hybrid Notch protein cannot bind Notch ligand, this result suggests that in the absence of Ral Notch might be activated without binding ligand. Finally, we observed that this is indeed the case. In each overexpressed Notch protein NICD was fused to Gal4. Activation of the Notch/Gal4 receptor allows the cleaved NICD/Gal4 fragment to enter the nucleus and activate a *UAS-gfp* reporter transgene. We detected GFP in animals that overexpressed either normal or hybrid Notch/Gal4 and only in *Ral* mutants.

We wanted to determine whether or not the idea that Ral inhibits ligand-independent Notch activation made sense in a developmental context. One of the defects in *Ral* mutant eyes typical of Notch pathway dysfunction was the symmetry of two photoreceptor cells, R3 and R4. These two photoreceptors are normally positioned asymmetrically, and as the molecular mechanism by which Notch signaling mediates R3/R4 specification is understood at least in part, we decided to investigate a possible role for Ral in this process. The ~800 identical ommatidia (or facets) of the fly eye each has eight photoreceptors (R1-R8) arranged in a central trapezoid, with R3 at its apex (**Fig. 1**). The difference between R3 and R4 is established early during eye development in a five-cell ommatidial precluster that includes adjacent R3/R4 precursor cells. The R3/R4 precursor closest to the dorsal/ventral midline (equator) always becomes R3, and the other cell of the pair becomes R4. R3/R4 specification depends on Frizzled and Notch signaling (**Fig. 1**).[28-30] Although each R3/R4 precursor expresses both Notch and the ligand Delta, Notch activation is asymmetric in R3/R4. Because it is closer to the equator, the pre-R3 cell experiences more Frizzled activation and thus activates Notch in the adjacent pre-R4 cell. Frizzled activation is thought to result in asymmetric Notch activation in pre-R4 through either or both of two pathways. Frizzled signaling leads to elevated transcription in pre-R3 of the ligand Delta and also the Ubiquitin-ligase Neuralized.[28,31] Neuralized ubiquitylates Delta, a prerequisite for Delta endocytosis by pre-R3 and activation of Notch in pre-R4.[27] Alternatively or in addition, activated Frizzled relocalizes in a complex with a cortical protein called Disheveled to the R3/R4 interface where the complex prevents Notch receptor activation directly in pre-R3.[30,32]

We found that Ral defines a distinct pathway by which R3 is biased to become the Delta signaling cell.[19] In response to Frizzled activation, elevated Ral activity in R3 prevents ligand-independent Notch activation in pre-R3, thereby helping to tip the scales in favor of pre-R3 becoming the signaling cell. The main evidence for this idea is 2-fold. First, we analyzed mosaic eyes in which one of the R3/R4 pair was *Ral*$^{+}$ and the other was *Ral*$^{-}$. We

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found that when pre-R3 was *Ral*-, the R3/R4 pair was often symmetrical or reversed and Notch was often activated in pre-R3 or else weakly in both pre-R3 and pre-R4. In contrast, loss of *Ral* activity in R4 had no effect. Second, *Ral* transcription in R3/R4 precursors was dependent on Frizzled and elevated in R3 relative to R4.

It should be noted that the effects of Ral on R3/R4 asymmetry while consistent were weak; most ommatidia in *Ral*- eyes had normally asymmetric R3/R4 pairs.[19] We used three different *Ral* loss-of-function mutations in these experiments, none of which are null alleles. Therefore, one possible explanation for the preponderance of ommatidia in *Ral* mutants with normally differentiated R3/R4 pairs is the presence of residual Ral activity in the R3 cells. Alternatively, the fact that Ral defines just one of several independent parallel pathways for biasing pre-R3 to become the signaling cell could explain why loss of Ral activity has a limited effect on R3/R4 specification. We are presently generating a *Ral* null allele that should allow us to distinguish between these two alternatives.

The big question that remains is: how does Ral block ligand-independent activation of Notch? We will discuss below the phenomenon of ligand-independent Notch activation and speculate about how Ral might inhibit it.

How is Notch Activated Without Binding Ligand?

Notch receptor not bound to ligand undergoes endocytosis and endosomal trafficking continually.[26,33] Internalized Notch in an early endosome may be recycled to the plasma membrane or routed to the lysosome for degradation (**Fig. 2**). One way in which ligand-independent Notch activation is thought to occur is as an accident when the flow of Notch through the endosomal pathway is blocked.[26] This idea comes from analysis of mutants in which genes required for efficient routing of Notch to the lysosome are inactive. In Drosophila, these genes include *Suppressor of Deltex (Su(Dx))*,[34] which encodes a Ubiquitin-ligase thought to ubiquitylate Notch, genes for ESCRT complex proteins,[35-39] which sort ubiquitylated cargos into intralumenal vesicles of multivesicular bodies (MVBs), *lethal giant discs* (*lgd*),[40-42] which encodes a cytosolic lipid-binding protein, and *Kurtz (Krz)*,[43] a gene for a β-arrestin required to route Notch to the lysosome. When Notch is activated by ligand binding, the Notch extracellular domain is cleaved by a metalloprotease, which allows the subsequent γ-secretase cleavage that releases NICD.[25] Cleavage of ligand-activated Notch by γ-secretase may occur in endosomes normally, although this point is controversial.[26,33,35,44] Nevertheless, ligand-independent Notch activation in mutants where endosomal flow is blocked requires γ-secretase.[35,42] It is thought that accumulation of Notch in the acidic environment of endosomes may alter Notch protein conformation and thereby allow γ-secretase cleavage of NICD without ligand binding.[26] This idea is supported by the observation that ligand-independent Notch signaling also occurs in mutants where endosome acidity is increased. These mutants are in the gene for an aquaporin called Bigbrain (Bib),[26,33] a gene for a subunit of the vacuolar proton pump V-ATPase,[45] and genes for Rbcn-3A and Rbcn-3B, two V-ATPase regulators.[45] In addition, γ-secretase activity is enhanced by an acidic environment,[46] which also could play a role in endosomal activation of unliganded Notch.

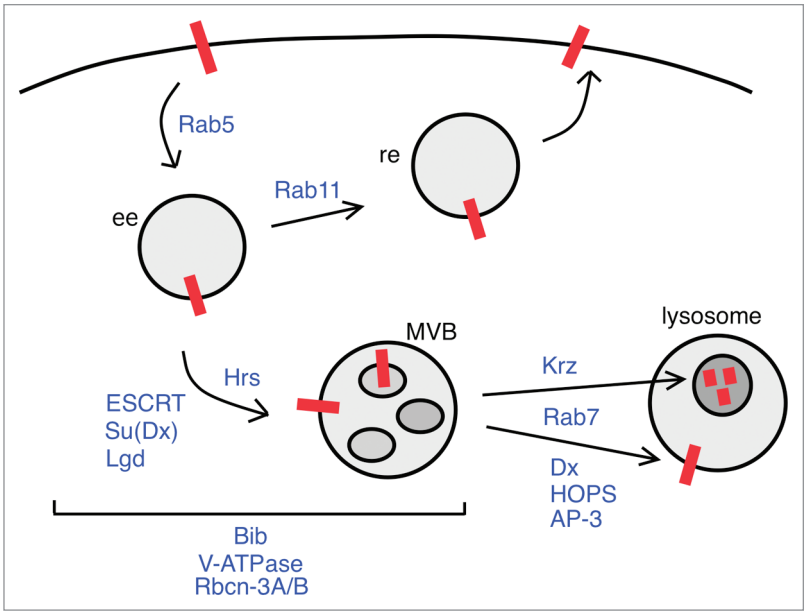

Figure 2. Endosomal trafficking of unliganded Notch receptor. A diagram of the known endosomal routes of the Notch receptor that was not activated by ligand binding. The precise step in the endocytic pathway where the Ubiquitin ligases Su(Dx) and Dx function is not clear. Their locations in the diagram are meant to indicate the process for which each is required. See text for explanation of diagram. ee = early endosome, re = recycling endosome, Hrs protein is required to deliver proteins to MVBs. Other abbreviations are defined in the text.

Ligand-independent Notch activation may also occur normally in the lysosomal membrane. A pathway antagonistic to Krz and that requires the Ubiquitin-ligase Deltex (Dx), the HOPS (homotyic fusion and vacuole protein sorting) complex and the endocytic adaptor complex AP-3 is thought to route Notch from MVBs to the lysosomal membrane.[47] How low-level Notch signaling from the lysosome might affect cell function is unclear.

How Might Ral Prevent Ligand-Independent Notch Activation?

The observation that a hybrid Notch receptor lacking the normal extracellular domain is activated specifically in *Ral* mutants indicates that Ral functions at or downstream of NICD cleavage by γ-secretase.[19] Given our present understanding of ligand-independent Notch activation, Ral might regulate endosome pH or endosomal trafficking of unliganded Notch. Immunofluorescence

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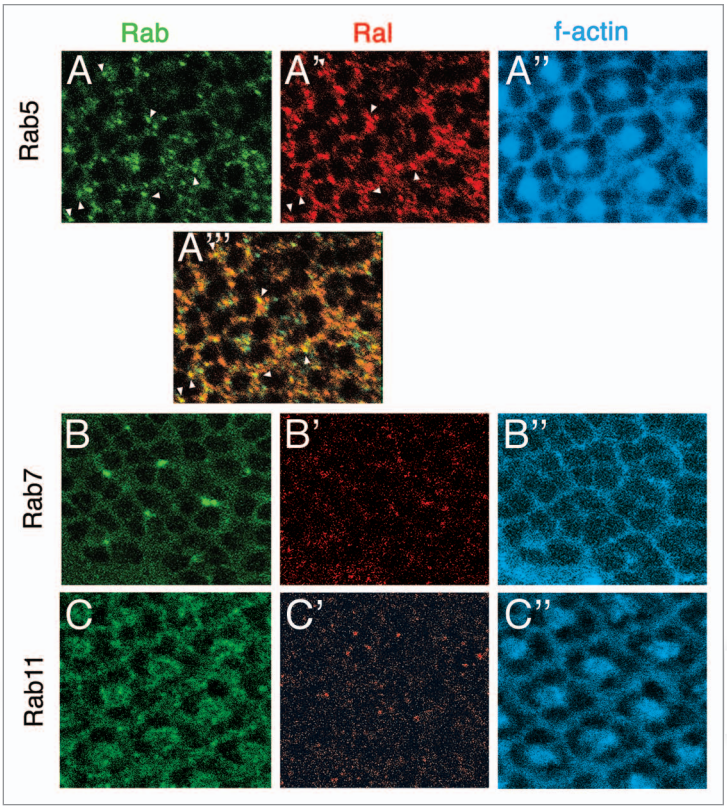

Figure 3. Ral does not colocalize with Rab5, Rab7, nor Rab11. Confocal microscope images of part of three developing eyes (third instar larval eye discs) are shown. All three discs were labeled with anti-Ral[49] and phalloidin (binds f-actin at the plasma membrane). (A–A"') A YFP-Rab5 expressing disc is shown. The genotype is *ey-gal4, GMR-gal4/UAS-yfp-rab5*. (A"') is a merge of (A and A'). The white arrows indicate the few puncta that are simultaneously YFP-Rab5-positive and Ral-positive. The focal plane of this disc image is more basal than that of the other two discs shown below. (B–B") A Rab7-GFP expressing disc is shown. The genotype is *Actin5C-gal4, UAS-rab7-gfp*. At the apical focal plane at which there are Rab7-GFP-positive puncta (B), Ral puncta are absent (B'). (C–C") A wild-type disc also labeled with anti-Rab11 is shown. At the apical focal plane at which Rab11 resides, no Ral is detected (C').

experiments with Ral antibodies showed that in developing Drosophila eyes most Ral resides in intracellular puncta.[19] Although this observation tempts us to speculate that Ral is endosomal, we have not yet been able to identify the Ral-positive puncta, which are basal in photoreceptor cells. We asked whether or not the Ral puncta colocalize with Rab5, Rab7 or Rab11, markers for early, late, and recycling endosomes, respectively.[48] YFP-Rab5 [49] expressed in the developing eye using the Gal4/UAS expression system is punctate like Ral but mainly apical (**Fig. 3A**). There are Rab5 puncta that are as basal as Ral but they colocalize with Ral very little (**Fig. 3A–A"**). Rab7-GFP[50] expressed using Gal4/UAS is apical and there is essentially no overlap between Rab7-GFP and Ral along the apical/basal axis of photoreceptor cells (**Fig. 3B–B"**). Similarly, Rab11 detected with an antibody[51] is in puncta that are more apical than those containing Ral (**Fig. 3C–C"**).

The analysis of Ral-positive puncta above is only preliminary and the results should be taken in that light. Yet, the results are surprising as they suggest that the majority of Ral is not present in early or late endosomes defined by Rab5 or Rab7, nor in the recycling endosomes defined by Rab11. Additional experiments of this type with other markers may help to identify the Ral-positive vesicles. Also, it would be useful to examine the Ral puncta in mutants where endocytosis in general is inhibited or specific endosomal pathways are blocked. Another aspect of this analysis that must remain in mind is that the Ral-positive puncta represent only the location of the majority of Ral protein at any given time. This may or may not reflect the Ral protein involved in repression of ligand-independent Notch signaling.

Another means to uncover the critical function of Ral in the eye is to determine which effector is involved. One experiment we did along these lines was to ask if Drosophila Ral proteins with mutations that specifically inhibit binding of two effectors, RalBP1 (Ral^{D46N}) or the exocyst protein Sec5 (Ral^{D46E}), could substitute for endogenous Ral in the fly eye. Ral proteins with these identical mutations have been characterized biochemically using vertebrate RalA.[3,4] We expect that they would have the same effect in Drosophila Ral because fly Ral binds RalBP1 and Sec5,[16] and because the amino acid sequence of the effector binding region in Drosophila Ral and vertebrate Ral A are identical. Normal *Ral* (Ral^{WT}), Ral^{D46N} or Ral^{D46E} were expressed in flies under Gal4 control. When expressed using the *eyeless-gal4* driver, either Ral^{WT} or Ral^{D46E} (binds RalBP1) rescued the eye defects of Ral^{EE1} mutants to wild type (**Fig. 4**). In contrast, Ral^{D46N} (binds exocyst) rescued the eye defects only slightly. Similar results were obtained in the eye with the *Actin5C-gal4* driver (**Fig. 4**). These results suggest that in the eye, *Ral* works through RalBP1, consistent with a role in endocytosis. One caveat, however, to this interpretation is that the difference in rescuing activity between Ral^{D46E} and Ral^{D46N} could potentially be explained by Ral^{D46E} protein accumulating to higher levels than Ral^{D46N}. For example, different levels of transcription could result from position effects, or the two mutant Ral proteins could be differentially stable. While this explanation for the results is possible, we think it is unlikely. First, we know that Ral^{D46N} is stably produced in the fly because when their expression is driven by *eyeless-gal4*, Ral^{D46E} and Ral^{D46N} each rescue the notal bristle defects of Ral^{EE1} completely (**Fig. 4**). Second, when Ral^{D46N} expression is driven by either *eyeless-gal4* or *Actin5C-gal4*, a similar small amount of rescue activity is observed in the eye. The *Actin5C-gal4* driver is much more active than *eyeless-gal4*, and so if the small amount of activity of Ral^{D46N} were due

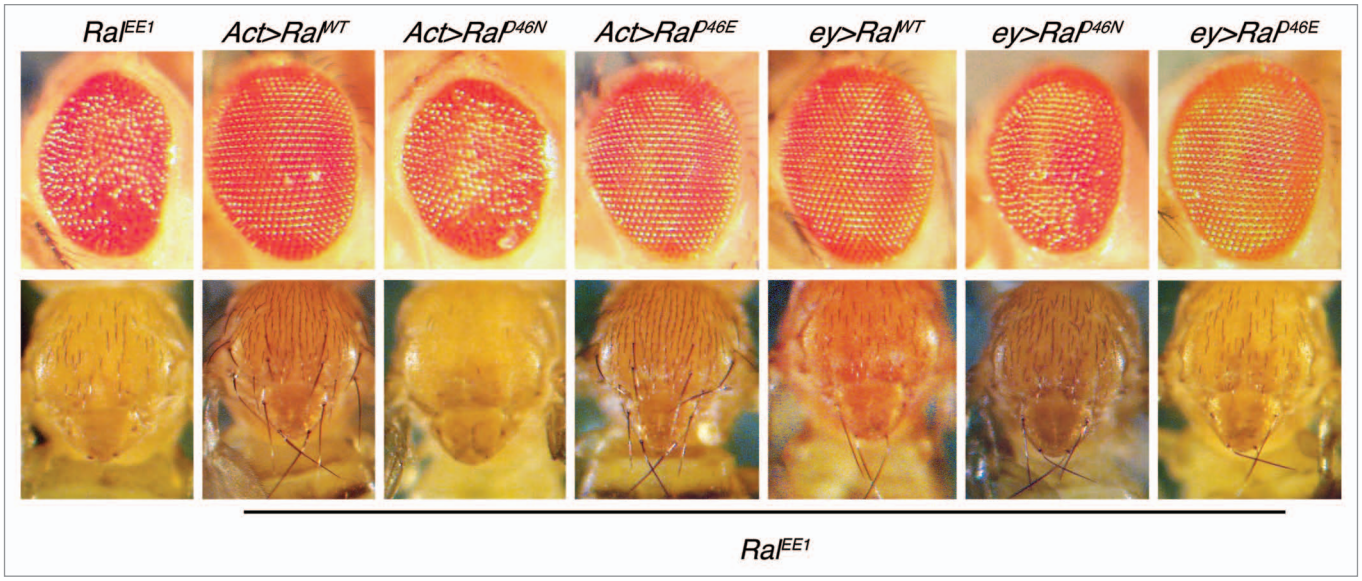

Figure 4. Rescue of *Ral*^{EE1} eye organization and notal bristle defects by wild-type and mutant *Ral* transgenes. Adult eyes and nota of flies of *Ral*^{EE1} flies with no transgene (leftmost), or that express the indicated *Ral* transgenes using the Gal4/UAS system are shown. *Act* is the *Actin5C-gal4* driver and *ey* is the *eyeless-gal4* driver. *UAS-Ral*^{WT} has been described in reference 17. Details of how we constructed *UAS-Ral*^{D46N} and *UAS-Ral*^{D46E} are available upon request.

to low expression, more rescue of the eye defects would be expected with *Actin5C-gal4*. We saw evidence that *Actin5C-gal4* is likely driving high levels of *Ral*^{D46N} expression in our flies. When expressed with *eyeless-gal4*, *Ral*^{D46N} rescues the bristle loss in the notum (**Fig. 4**). In contrast, when overexpressed with *Actin5C-gal4*, *Ral*^{D46N} not only fails to rescue the bristle development defect, it makes it worse presumably by acting in a dominant negative fashion.

Although the tentative conclusion from these preliminary experiments is that in its role in Notch signaling Ral may function through RalBP1, to be more secure in this interpretation, careful quantitation of protein levels in the different tissues is necessary. In addition, genetic studies using mutations in the effectors genes could be illuminating.

Conclusions

There is presently some evidence suggestive of an endosomal function for Ral in the Notch pathway. Ral inhibits ligand-independent Notch activation, all known forms of which take place in endosomes. Moreover, Ral localizes mainly to intracellular puncta suggestive of endosomes. Also, the failure of Ral^{D46N} and the ability of Ral^{D46E} to function in the eye suggests that the key Ral effector for Notch signaling is not the exocyst and may be RalBP1 which functions in endocytosis. The role of RalBP1 in endocytosis, however, is poorly defined. Future experiments are needed to identify the Ral puncta and determine if they are associated with Ral function in Notch signaling, and to identify the key Ral effector and how it works. It will also be informative to observe the effect of *Ral* mutation, if any, on endosome pH and morphology. The discovery of the Ral pathway in pre-R3 revealed that ligand-independent Notch activation is regulated to achieve specific developmental outcomes. It seems likely that more surprises await unraveling the mechanism by which Ral blocks Notch activation.

Acknowledgments

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References

1. van Dam EM, Robinson PJ. Ral: mediator of membrane trafficking. Int J Biochem Cell Biol 2006; 38:1841-7; PMID:16781882; http://dx.doi.org/10.1016/j.biocel.2006.04.006.
2. Jullien-Flores V, Dorseuil O, Romero F, Letourneur F, Saragosti S, Berger R, et al. Bridging Ral GTPase to Rho pathways. RLIP76, a Ral effector with CDC42/Rac GTPase-activating protein activity. J Biol Chem 1995; 270:22473-7; PMID:7673236.
3. Bauer B, Mirey G, Vetter IR, García-Ranea JA, Valencia A, Wittinghofer A, et al. Effector recognition by the small GTP-binding proteins Ras and Ral. J Biol Chem 1999; 274:17763-70; PMID:10364219; http://dx.doi.org/10.1074/jbc.274.25.17763.
4. Moskalenko S, Henry DO, Rosse C, Mirey G, Camonis JH, White MA. The exocyst is a Ral effector complex. Nat Cell Biol 2002; 4:66-72; PMID:11740492; http://dx.doi.org/10.1038/ncb728.
5. Jullien-Flores V, Mahé Y, Mirey G, Leprince C, Meunier-Bisceuil B, Sorkin A, et al. RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. J Cell Sci 2000; 113:2837-44; PMID:10910768.
6. Rossé C, L'Hoste S, Offner N, Picard A, Camonis J. RLIP, an effector of the Ral GTPases, is a platform for Cdk1 to phosphorylate epsin during the switch off of endocytosis in mitosis. J Biol Chem 2003; 278:30597-604; PMID:12775724; http://dx.doi.org/10.1074/jbc.M302191200.
7. Kashatus DF, Lim KH, Brady DC, Pershing NLK, Cox AD, Counter CM. RALA and RALBP1 regulate mitochondrial fission at mitosis. Nat Cell Biol 2011; 13:1108-15; PMID:21822277; http://dx.doi.org/10.1038/ncb2310.
8. Nakashima S, Morinaka K, Koyama S, Ikeda M, Kishida M, Okawa K, et al. Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors. EMBO J 1999; 18:3629-42; PMID:10393179; http://dx.doi.org/10.1093/emboj/18.13.3629.

9. Cascone I, Selimoglu R, Ozdemir C, Del Nery E, Yeaman C, White M, et al. Distinct roles of RalA and RalB in the progression of cytokinesis are supported by distinct RalGEFs. EMBO J 2008; 27:2375-87; PMID:18756269; http://dx.doi.org/10.1038/emboj.2008.166.
10. Sawamoto K, Yamada C, Kishida S, Hirota Y, Taguchi A, Kikuchi A, et al. Ectopic expression of constitutively activated Ral GTPase inhibits cell shape changes during Drosophila eye development. Oncogene 1999; 18:1967-74; PMID:10208418; http://dx.doi.org/10.1038/sj.onc.1202522.
11. Sugihara K, Asano S, Tanaka K, Iwamatsu A, Okawa K, Ohta Y. The exocyst complex binds the small GTPase RalA to mediate filopodia formation. Nat Cell Biol 2002; 4:73-8; PMID:11744922; http://dx.doi.org/10.1038/ncb720.
12. Hase K, Kimura S, Takatsu H, Ohmae M, Kawano S, Kitamura H, et al. M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex. Nat Cell Biol 2009; 11:1427-32; PMID:19935652; http://dx.doi.org/10.1038/ncb1990.
13. Balakireva M, Rossé C, Langevin J, Chien YC, Gho M, Gonzy-Treboul G, et al. The Ral/exocyst effector complex counters c-Jun N-terminal kinase-dependent apoptosis in *Drosophila melanogaster*. Mol Cell Biol 2006; 26:8953-63; PMID:17000765; http://dx.doi.org/10.1128/MCB.00506-06.
14. Chien Y, Kim S, Bumeister R, Loo YM, Kwon SW, Johnson CL, et al. RalB GTPase-mediated activation of the IkappaB family kinase TBK1 couples innate immune signaling to tumor cell survival. Cell 2006; 127:157-70; PMID:17018283; http://dx.doi.org/10.1016/j.cell.2006.08.034.
15. Feig LA. Ral-GTPases: approaching their 15 minutes of fame. Trends Cell Biol 2003; 13:419-25; PMID:12888294; http://dx.doi.org/10.1016/S0962-8924(03)00152-1.
16. Mirey G, Balakireva M, L'Hoste S, Rossé C, Voegeling S, Camonis J. A Ral guanine exchange factor-Ral pathway is conserved in *Drosophila melanogaster* and sheds new light on the connectivity of the Ral, Ras and Rap pathways. Mol Cell Biol 2003; 23:1112-24; PMID:12529414; http://dx.doi.org/10.1128/MCB.23.3.1112-24.2003.
17. Sawamoto K, Winge P, Koyama S, Hirota Y, Yamada C, Miyao S, et al. The Drosophila Ral GTPase regulates developmental cell shape changes through the Jun NH(2)-terminal kinase pathway. J Cell Biol 1999; 146:361-72; PMID:10427090; http://dx.doi.org/10.1083/jcb.146.2.361.
18. Ghiglione C, Devergne O, Cerezo D, Noselli S. Drosophila RalA is essential for the maintenance of Jak/Stat signalling in ovarian follicles. EMBO Rep 2008; 9:676-82; PMID:18552769; http://dx.doi.org/10.1038/embor.2008.79.
19. Cho B, Fischer JA. Ral GTPase promotes asymmetric Notch activation in the Drosophila eye in response to Frizzled/PCP signaling by repressing ligand-independent receptor activation. Development 2011; 138:1349-59; PMID:21350007; http://dx.doi.org/10.1242/dev.056002.
20. Eun SH, Lea K, Overstreet E, Stevens S, Lee JH, Fischer JA. Identification of genes that interact with Drosophila liquid facets. Genetics 2007; 175:1163-74; PMID:17179082; http://dx.doi.org/10.1534/genetics.106.067959.
21. Overstreet E, Fitch E, Fischer JA. Fat facets and Liquid facets promote Delta endocytosis and Delta signaling in the signaling cells. Development 2004; 131:5355-66; PMID:15469967; http://dx.doi.org/10.1242/dev.01434.
22. Wang W, Struhl G. Drosophila Epsin mediates a select endocytic pathway that DSL ligands must enter to activate Notch. Development 2004; 131:5367-80; PMID:15469974; http://dx.doi.org/10.1242/dev.01413.
23. Tian X, Hansen D, Schedl T, Skeath JB. Epsin potentiates Notch pathway activity in Drosophila and *C. elegans*. Development 2004; 131:5807-15; PMID:15539484; http://dx.doi.org/10.1242/dev.01459.
24. Chen H, Ko G, Zatti A, Di Giacomo G, Liu L, Raiteri E, et al. Embryonic arrest at midgestation and disruption of Notch signaling produced by the absence of both epsin 1 and epsin 2 in mice. Proc Natl Acad Sci USA 2009; 106:13838-43; PMID:19666558; http://dx.doi.org/10.1073/pnas.0907008106.
25. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 2006; 7:678-89; PMID:16921404; http://dx.doi.org/10.1038/nrm2009.
26. Fortini ME. Notch signaling: the core pathway and its posttranslational regulation. Dev Cell 2009; 16:633-47; PMID:19460341; http://dx.doi.org/10.1016/j.devcel.2009.03.010.
27. Weinmaster G, Fischer JA. Notch ligand ubiquitylation: what is it good for? Dev Cell 2011; 21:134-44; PMID:21763614; http://dx.doi.org/10.1016/j.devcel.2011.06.006.
28. Fanto M, Mlodzik M. Asymmetric Notch activation specifies photoreceptors R3 and R4 and planar polarity in the Drosophila eye. Nature 1999; 397:523-6; PMID:10028968; http://dx.doi.org/10.1038/17389.
29. Cooper MTD, Bray SJ. Frizzled regulation of Notch signalling polarizes cell fate in the Drosophila eye. Nature 1999; 397:526-30; PMID:10028969; http://dx.doi.org/10.1038/17395.
30. Tomlinson A, Struhl G. Decoding vectorial information from a gradient: sequential roles of the receptors Frizzled and Notch in establishing planar polarity in the Drosophila eye. Development 1999; 126:5725-38; PMID:10572048.
31. del Alamo D, Mlodzik M. Frizzled/PCP-dependent asymmetric neuralized expression determines R3/R4 fates in the Drosophila eye. Dev Cell 2006; 11:887-94; PMID:17141162; http://dx.doi.org/10.1016/j.devcel.2006.09.016.
32. Strutt D, Johnson R, Cooper K, Bray S. Asymmetric localization of frizzled and the determination of notch-dependent cell fate in the Drosophila eye. Curr Biol 2002; 12:813-24; PMID:12015117; http://dx.doi.org/10.1016/S0960-9822(02)00841-2.
33. Fortini ME, Bilder D. Endocytic regulation of Notch signaling. Curr Opin Genet Dev 2009; 19:323-8; PMID:19447603; http://dx.doi.org/10.1016/j.gde.2009.04.005.
34. Wilkin MB, Carbery AM, Fostier M, Aslam H, Mazaleyrat SL, Higgs J, et al. Regulation of notch endosomal sorting and signaling by Drosophila Nedd4 family proteins. Curr Biol 2004; 14:2237-44; PMID:15620650; http://dx.doi.org/10.1016/j.cub.2004.11.030.
35. Vaccari T, Lu H, Kanwar R, Fortini ME, Bilder D. Endosomal entry regulates Notch receptor activation in *Drosophila melanogaster*. J Cell Biol 2008; 180:755-62; PMID:18299346; http://dx.doi.org/10.1083/jcb.200708127.
36. Moberg KH, Schelble S, Burdick SK, Hariharan IK. Mutations in erupted, the Drosophila ortholog of mammalian tumor susceptibility gene 101, elicit non-cell-autonomous overgrowth. Dev Cell 2005; 9:699-710; PMID:16256744; http://dx.doi.org/10.1016/j.devcel.2005.09.018.
37. Thompson BJ, Mathieu J, Sung HH, Loeser E, Rørth P, Cohen SM. Tumor suppressor properties of the ESCRT-II complex component Vps25 in Drosophila. Dev Cell 2005; 9:711-20; PMID:16256745; http://dx.doi.org/10.1016/j.devcel.2005.09.020.
38. Vaccari T, Bilder D. The Drosophila tumor suppressor *vps25* prevents nonautonomous overproliferation by regulating notch trafficking. Dev Cell 2005; 9:687-98; PMID:16256743; http://dx.doi.org/10.1016/j.devcel.2005.09.019.
39. Herz HM, Chen Z, Scherr H, Lackey M, Bolduc C, Bergmann A. vps25 mosaics display non-autonomous cell survival and overgrowth, and autonomous apoptosis. Development 2006; 133:1871-80; PMID:16611691; http://dx.doi.org/10.1242/dev.02356.
40. Childress JL, Acar M, Tao C, Halder G. Lethal giant discs, a novel C2-domain protein, restricts notch activation during endocytosis. Curr Biol 2006; 16:2228-33; PMID:17088062; http://dx.doi.org/10.1016/j.cub.2006.09.031.
41. Gallagher CM, Knoblich JA. The conserved c2 domain protein lethal (2) giant discs regulates protein trafficking in Drosophila. Dev Cell 2006; 11:641-53; PMID:17084357; http://dx.doi.org/10.1016/j.devcel.2006.09.014.
42. Jaekel R, Klein T. The Drosophila Notch inhibitor and tumor suppressor gene *lethal (2) giant discs* encodes a conserved regulator of endosomal trafficking. Dev Cell 2006; 11:655-69; PMID:17084358; http://dx.doi.org/10.1016/j.devcel.2006.09.019.
43. Mukherjee A, Veraksa A, Bauer A, Rosse C, Camonis J, Artavanis-Tsakonas S. Regulation of Notch signalling by non-visual β-arrestin. Nat Cell Biol 2005; 7:1191-201; PMID:16284625; http://dx.doi.org/10.1038/ncb1327.
44. Yamamoto S, Charng WL, Bellen HJ. Endocytosis and intracellular trafficking of Notch and its ligands. Curr Top Dev Biol 2010; 92:165-200; PMID:20816395; http://dx.doi.org/10.1016/S0070-2153(10)92005-X.
45. Yan Y, Denef N, Schüpbach T. The vacuolar proton pump, V-ATPase, is required for notch signaling and endosomal trafficking in Drosophila. Dev Cell 2009; 17:387-402; PMID:19758563; http://dx.doi.org/10.1016/j.devcel.2009.07.001.
46. Pasternak SH, Bagshaw RD, Guiral M, Zhang S, Ackerley CA, Pak BJ, et al. Presenilin-1, nicastrin, amyloid precursor protein, and γ-secretase activity are co-localized in the lysosomal membrane. J Biol Chem 2003; 278:26687-94; PMID:12736250; http://dx.doi.org/10.1074/jbc.M304009200.
47. Wilkin M, Tongngok P, Gensch N, Clemence S, Motoki M, Yamada K, et al. Drosophila HOPS and AP-3 complex genes are required for a Deltex-regulated activation of notch in the endosomal trafficking pathway. Dev Cell 2008; 15:762-72; PMID:19000840; http://dx.doi.org/10.1016/j.devcel.2008.09.002.
48. Zerial M, McBride H. Rab proteins as membrane organizers. Nat Rev Mol Cell Biol 2001; 2:107-17; PMID:11252952; http://dx.doi.org/10.1038/35052055.
49. Zhang J, Schulze KL, Hiesinger PR, Suyama K, Wang S, Fish M, et al. Thirty-one flavors of Drosophila rab proteins. Genetics 2007; 176:1307-22; PMID:17409086; http://dx.doi.org/10.1534/genetics.106.066761.
50. Entchev EV, Schwabedissen A, González-Gaitán M. Gradient formation of the TGF-beta homolog Dpp. Cell 2000; 103:981-91; PMID:11136982; http://dx.doi.org/10.1016/S0092-8674(00)00200-2.
51. Bogard N, Lan L, Xu J, Cohen RS. Rab11 maintains connections between germline stem cells and niche cells in the Drosophila ovary. Development 2007; 134:3413-8; PMID:17715175; http://dx.doi.org/10.1242/dev.008466.

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