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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

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Ras GTPases are central to many physiological and pathological signaling pathways and act via a combination of effectors. In mammals, at least three Ral exchange factors (RalGEFs) contain a Ras association domain and constitute a discrete subgroup of Ras effectors. Despite their ability to bind activated Rap as well as activated Ras, they seem to act downstream of Ras but not downstream of Rap. We have revisited the Ras/Rap-Ral connections in *Drosophila melanogaster* by using iterative two-hybrid screens with these three GTPases as primary baits and a subsequent genetic approach. We show that (i) the Ral-centered protein network appears to be extremely conserved in human and flies, (ii) in this network, RGL is a functional *Drosophila* orthologue of RalGEFs, and (iii) the RGL-Ral pathway functionally interacts with both the Ras and Rap pathways. Our data do not support the paradigmatic model where Ral is in the effector pathway of Ras. They reveal a signaling circuitry where Ral is functionally downstream of the Rap GTPase, at odds with the pathways described for mammalian cell lines. Thus, in vivo data show variations in the connectivity of pathways described for cell lines which might display only a subset of the biological possibilities.

The Ras GTPases contribute to the functioning of several molecular systems that transduce extracellular signals to regulate cell fate. They are involved not only in cell proliferation but also in cell differentiation as well as in oncogenic processes (3, 7). Ras is found in single-cell organisms like *Saccharomyces cerevisiae*, where it uses a unique pathway to convey its function through the control of adenylate cyclase (62). In mammals, Ras functions through a multiplicity of effectors. The use of effector loop mutants of Ras (72) has shown that three effectors account for many, if not all, Ras functions: phosphatidylinositol 3-kinase (PI3K; a preferred effector of the Ras^{Y40C} allele), Raf (a preferred effector of the Ras^{T35S} allele), and Ral guanine nucleotide exchange factors (RalGEFs; preferred effectors of the Ras^{E37G} allele).

The combined contribution of these effectors to cell signaling in a tissue under physiological conditions as well as in oncogenic transformation remains to be clarified. Oncogenic Ras^{G12V} properties can be fulfilled by the concomitant expression of $Ras^{G12V\ T35S}$ and $Ras^{G12V\ E37G}$, and this complementation is accounted for by a cooperation between the Raf and RalGEF pathways (73), although in immortalized primary human fibroblasts, a large part of Ras^{G12V} transforming activity can be fulfilled by $Ras^{G12V\ E37G}$, which can be replaced by an activated RalGEF, Rlf (23). On the other hand, neural differentiation of PC12 cells that can be driven by Ras^{G12V} is antagonized by RalGEFs (21). Another issue in Ras signaling is that the Rap GTPases, which share approximately 50% identity with Ras proteins, bind many Ras effectors but, in most cases, fail to activate them in vivo. Rap binds one of the RalGEFs, RalGDS with a higher affinity than does Ras and is able biochemically to stimulate Ral activation in vitro; however, Rap does not lead to the activation of Ral in mammalian cell lines (31, 75, 82).

Ral proteins were the first Ras-like GTPases discovered by a systematic search for p21-Ras homologues (14). Converging evidence in mammalian cell lines has shown that Ral is activated in a cascade consecutive to the activation of Ras via the RalGEFs RalGDS, RGL, and Rlf (reviewed in reference 76). There are also Ras-independent ways to activate Ral (17, 43, 47, 68, 78), and Ral may act as an integrator of signals from different sources. Once bound to GTP, Ral interacts with one or more of its effectors, and among them, RLIP76 and SEC5 are the best characterized (11, 27, 39, 44).

In *Drosophila melanogaster*, downstream of the Ras effectors, a PI3K pathway is involved in cell growth (6, 35, 70) and a mitogen-activated protein kinase pathway is involved in cell determination (22, 37), cell survival (4, 33), and cell proliferation (29, 46). It has been shown that the $Ras^{G12V\ E37G}$ allele acts in synergy with the mitogen-activated protein kinase pathway to induce cell hyperplasia (29). The Rap1 GTPase has been involved in morphogenesis where Ras and Rap seem to function via distinct pathways (2). *Drosophila* Ral has been suggested to regulate cell shape changes through the JNK pathway (54). A connection between the Rap and Ras pathways and the Ral pathway has not been established yet.

We have revisited the Ras/Rap-Ral connections with an in vivo model, *D. melanogaster.* A two-hybrid approach with these three GTPases as baits and iterative screens with prey as baits

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was followed by a genetic analysis. We report the following: (i) the conservation of the protein-protein interaction network from Ras/Rap to components of the endocytosis machinery, via RAL and RLIP, and to the exocyst, as in mammals, with the fly harboring a simplified version of the Ral transduction pathway; (ii) the molecular and phenotypic characterization of RGL, a *Drosophila* orthologue of mammalian RalGEFs; (iii) that the Ral pathway interacts genetically with the Ras pathway, although our results do not favor the existence of a linear Ras-RalGEF-Ral pathway, as documented in mammalian cell lines; and (iv) genetic interactions that are consistent with a linear activation pathway from Rap to Ral. These results shed a different light on the paradigmatic relationships of Rap and Ras with Ral. They support the idea that the same set of signaling proteins can be used to build different networks, probably in a tissue-specific manner.

MATERIALS AND METHODS

Molecular biology and plasmid handling. Standard techniques in molecular biology were used for cloning, PCR, sequencing, and Northern blotting (51). When PCR was used for cloning, all of the constructs were sequenced. The backbone plasmid used for transgene experiments was pUAST (8). When targeting expressed proteins to membranes was desirable, we used a modified pUAST where the 3′ side of the polylinker was modified by inserting a double-stranded oligonucleotide encoding the last 18 amino acids of K-Ras, mainly consisting of a polybasic region followed by a CAAX motif that allows prenylation.

Two-hybrid screens. Two-hybrid screens and assays were carried out with a LexA-based two-hybrid system (64) and yeast strains L40ΔGAL4 (a gift of P. Legrain and M. Fromont-Racine) and AMR70 (a gift of R. Sternglanz) or Y187 (Clontech). A 0-to-24-h *Drosophila* embryo cDNA library was a generous gift of S. Elledge. Standard techniques in yeast handling were used.

Drosophila stocks. Flies carrying *UAS-Rgl1FL*, *UAS-Rgl1CAAX*, *UAS-Rgl2FL*, *UAS-Rgl2CAAX*, *UAS-Ral*^{wt}, *UAS-Ral*^{S25N}, and *UAS-Ral*^{G20V} were generated by standard methods of P-element transformation. Flies carrying transgenes harboring various Rap and Ras alleles under an upstream activation sequence (UAS) promoter were generous gift from several colleagues: *UAS-Rap1*^{V12} and *UAS-Ras1*^{V12} were generously provided by I. Hariharan and D. Montell, respectively. The effector loop mutants *UAS-Ras1*$^{V12\ S35}$, *UAS-Ras1*$^{V12\ G37}$, and *UAS-Ras1*$^{V12\ C40}$ are from G. Rubin's laboratory (29).

For overexpression studies, Rgl and Ral transgenes were used in combination with the Gal4 drivers *da-Gal4* (ubiquitous expression in embryo, FlyBase data), *Gal4*^{MS1096} (12), *GMR-Gal4* (52, 53), and *sca-Gal4* (40). The *sca-Gal4* driver was also used for interaction crosses. *sca-Gal4* drives expression in proneural clusters; its expression initiates in the second larval instar and progresses to the late pupal stages (15). Gal4 driver strains were generously provided by E. Hafen (*GMR-Gal4*), B. Limpourg-Bouchon (*da-Gal4* and *Gal4*^{MS1096}), and F. Schweisguth (*sca-Gal4*). Fly crosses were performed at 25°C unless noted otherwise.

Deletions in the Rgl locus (ΔRgl) were generated by excision of the P elements in the l(3)02840 line (16) and in the EP3365 line (48). Molecular, genetic, and phenotypic characterization of these alleles of Rgl will be described elsewhere.

Flies were prepared for scanning electron microscopy as described previously (30). The mounted samples were ion coated and observed with a scanning electron microscopte (Hitachi Instruments, Inc.). Preparation of flies for Nomarski optics was performed in Hoyer's solution following standard fixation procedures in glycerine-acetic acid (1:4) (74).

Gene and protein references. The genes and proteins referred to herein are identified by the following GenInfo (gi) numbering: human RLIP76, gi 974142; mouse REPS1, gi 2677842; human POB1, gi 18598813; human POB1, gi 2895090; mouse RGL, gi 8394179; mouse RalGDS, gi 193572; mouse Rlf, gi 1354500; *Drosophila* RLIP, gi 4104637; *Drosophila* RGL1, gi 12001829; *Drosophila* RGL2, gi 6652993; and *Drosophila* REPS, CG6192.

RESULTS

Protein networks are conserved between flies and mammals. Protein-protein interactions are more strictly conserved than the sequences of the involved proteins. The fly Ras1, Rap1, and Ral GTPases were used as baits in a first round of two-hybrid screens of a *Drosophila* embryo cDNA library. Subsequently, a second round was performed in order to identify partners of proteins identified as Ral, Rap1, or Ras1 partners in the first round.

(i) A protein interaction network is conserved downstream of the RAL GTPase in flies and mammals. A screen with fly Ral as a bait led to the identification of an orthologue of human RLIP76, an effector of the human RalA and RalB GTPases. We named this protein RLIP (28). RLIP and RLIP76 proteins share 38% identity and 63% similarity, most of which is concentrated in the central region that contains a RhoGAP region and the Ral-binding domain (RalBD) (Fig. 1). The regions that are N-terminal to the GAP domain and C-terminal to the RalBD share no significant homology between humans and flies. Even if sequences with low levels of compositional complexity are not filtered (60), the levels of homology only reach 28 and 17%, respectively (Fig. 1). This lack of significant conservation in the primary structure contrasts with the ability of both human (amino acids [aa] 1 to 209) and fly (aa 1 to 206) N-terminal regions to interact with the fly and human μ2 medium chain of adaptin AP2 (28).

A two-hybrid screen performed to identify partners of the C-terminal region of *Drosophila* RLIP (last 144 aa) identified a protein encoded by the predicted gene CG6192. The predicted protein harbors an EH domain (positions 259 to 354), a proline-rich region containing putative SH3-binding sites (aa 534 to 665), and a putative PDZ class 1 binding motif at its C terminus (18, 36, 79). Our two-hybrid data show that it displays the functional ability to bind to RLIP, encapsulated within its last 140 aa and corresponding to the shortest cDNA fragment identified in our screen. The presence and topology of these features make this protein the most probable unique fly orthologue of the mammalian proteins REPS1 and REPS2/POB1, a conclusion supported by taxonomic proximity alignment (71). Thus, we named this protein REPS. Human POB1 is a partner of mammalian RLIP76/RalBP1 and is involved in its endocytotic function (26, 41). The global identity between mouse and fly REPS is only 21%, and most of it is accounted for by the EH domains (51%), whereas the RLIP binding regions share only 19% identity: interaction between the RLIP and REPS proteins involves regions of low homology between species in both proteins (Fig. 1). Evolution seems to have allowed large changes in the primary protein sequences of the C termini of REPS and RLIP, as long as one crucial characteristic is maintained: the ability for RLIP and REPS within each species to interact. This hypothesis is consistent with the absence of interspecies interaction between RLIP and REPS (data not shown).

Finally, a recently identified effector of human Ral is the human orthologue of yeast Sec5, a protein involved in processes using the exocyst machinery. Via SEC5, RAL participates in the correct basolateral targeting of membrane proteins in polarized epithelial cells as well as in secretion (39). RalA and RalB bind to the first 120 aa of SEC5, a region absent in yeast Sec5. The same region of fly SEC5 as well as full-length fly SEC5 binds to activated alleles of human and fly Ral, and these interactions are sensitive to effector loop mutations (data not shown). In flies, as in humans, the exocyst seems to be a RAL effector.

Taken together, these results show that, at the molecular

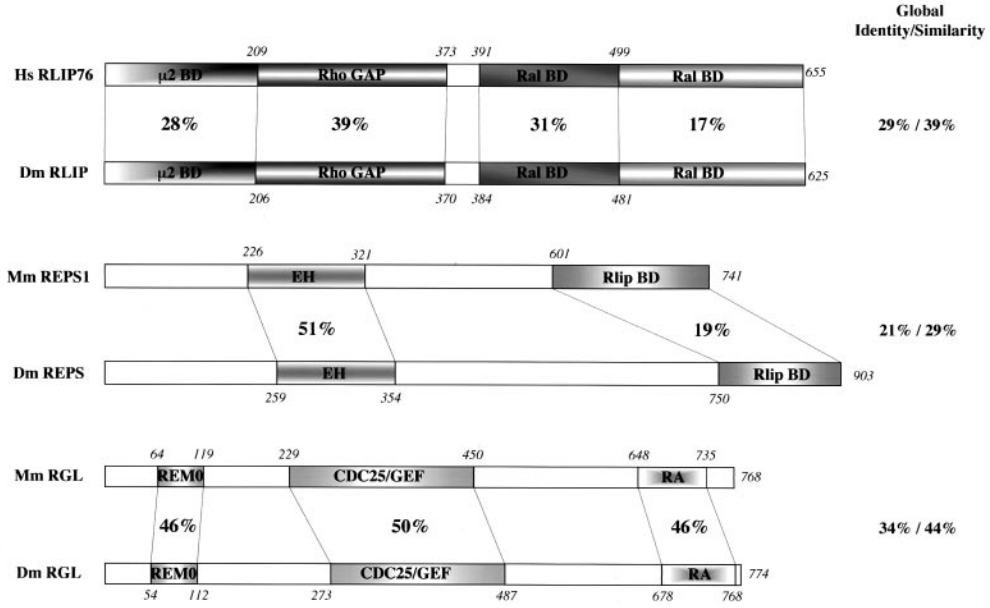

FIG. 1. Alignment of primary structures of RGL, RLIP, and REPS in humans (Hs) (RLIP76), mice (Mm) (RGL and REPS1), and *Drosophila* (Dm). The REM0, CDC25/GEF, RA, RhoGAP, and EH domains were identified by using the SMART domain search engine (http://smart.embl-heidelberg.de/). The RalBD and μ2 binding domain (BD) were identified as minimal interacting regions in two-hybrid assays (26). The REPS BD in RLIP proteins and the RLIP BD in REPS proteins are defined in this work and in published data (24). Local and global identities (as percentages) were determined by using ClustalX alignment and MacBoxShade. Notice that the REM0, CDC25/GEF, and RA domains of *D. melanogaster* RGL share 46.4, 44.7, and 40.2% identity with the equivalent domains of mouse RalGDS, respectively. *D. melanogaster* RGL is thus globally and locally closer to RGL, hence its name. For each couple, the upper scheme represents the mammalian protein, and the lower scheme represents the *Drosophila* protein.

level, the pathway downstream of Ral is similar in flies and in mammals at least to the second level of neighboring. Such conservation of protein-protein interactions in species so distant in evolution also suggests that these two-hybrid interactions are biologically significant, pointing most probably to similar functions (Fig. 2).

(ii) Two-hybrid screens with *Drosophila* RAS1 and RAP1 as baits identify a *Drosophila* RalGEF, RGL. A two-hybrid screen with the same *Drosophila* embryo library and *Drosophila* wild-type RAS1, an orthologue of human Ras, and RAP1, an orthologue of human Rap1, identified several cDNAs of various lengths encoding the same protein 24 and 64 times, respectively (Table 1).

From a plasmid-based fly embryo cDNA library (9), we cloned a 3,862-bp-long cDNA composed of a 593-bp 5′ untranslated region (UTR), a 944-bp 3′ UTR, and an open reading frame of 2,322 bp encoding 774 amino acids. The mRNA might be longer by 84 nucleotides on its 5′ side since bp 1 of our cDNA corresponds to bp 85 of a recently identified expressed sequence tag (EST) (RE62655). In this latter EST as well as in our cDNA, all frames are closed upstream of the proposed ATG and major positions of the fly Kozak consensus sequence (−6, −3, and −1) are found (ACUAUAAUG). The open reading frame encodes a predicted protein containing an REM0/LTE1 domain, a CDC25/GEF domain, and a Ras association (RA) domain (Fig. 1). Such an organization is found in the mammalian RalGEFs RalGDS, RGL, and Rlf, suggesting that we had identified a *Drosophila* exchange factor for Ral. Globally, this putative fly RalGEF is 34% identical to mouse RGL and 28% identical to mouse RalGDS. Rlf shows less

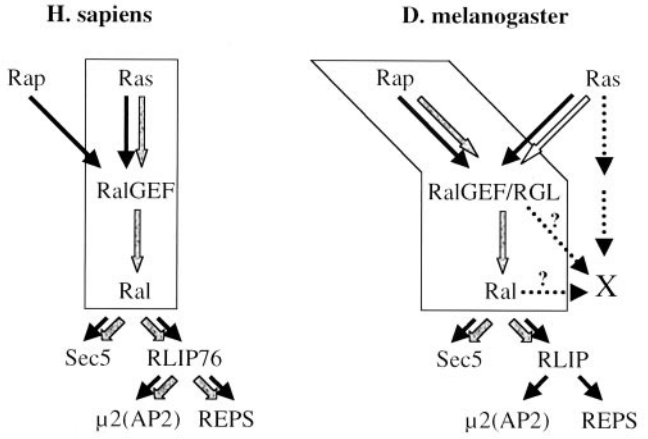

FIG. 2. Conservation of the Ral-centered signaling network in mammals and flies. Black arrows reflect protein-protein interactions as deciphered mainly by two-hybrid assays in the present study and elsewhere (see text for references). Gray arrows reflect functional data obtained in various mammalian cell lines and genetic interactions in *Drosophila*. In flies, the white arrow between Ras and RGL reflects a genetic interaction that does not fit with a linear functional path from one to the other. Preliminary data suggest a genetic interaction between fly Ral and RLIP. In mammals, a linear pathway goes from Ras to Ral, with no intervention of Rap, whereas in flies, Rap seems to activate a Ral pathway that interacts with Ras. In flies, dotted arrows reflect that the observed Ras-Rgl/Ral genetic interaction might be accounted for by the convergence of the Ras and the Ral pathways, with Ras using an effector that does not discriminate between the three effector loop mutations used in this work.

TABLE 1. Two-hybrid screens[a]

Bait protein[b]	No. of screened colonies	No. of positive clones (His^+ $LacZ^+$)	No. of colonies of protein as prey/screen[c]			
			Rgl	Ras1	Ras2	Rap1
Ras1	15×10^6	53	24	0	0	0
Rap1	8×10^6	144	64	0	0	0
Rg1	12×10^6	125	0	2	3	24

[a] Two-hybrid screens were performed with the listed fly proteins and a 0-to-24 h *D. melanogaster* embryo cDNA library.

[b] Wild-type Ras1, wild-type Rap1, and the C-terminal region of fly RGL (starting at aa 488, just after the GEF domain) were used as bait in the two-hybrid screens.

[c] The number of times the indicated proteins were identified as independent prey in positive clones are given.

homology. The three conserved regions also present a closer identity to their counterparts in RGL than in RalGDS (Fig. 1). We named this fly protein RGL. In silico searches with BLAST homology for other potential RalGEFs in the *Drosophila* genome database with fly RGL, the RGL CDC25/GEF domain, and the SOS CDC25/GEF domain showed that Rgl is the only gene in the fly genome that encodes a RalGEF of the RalGDS/RGL/Rlf family, i.e., containing both a RalGEF domain and an RA domain. Flies have one other potential exchange factor for RAL that belongs to the RalGPS/RalGEF2 family, which is devoid of RA domains but harbors a PH domain, SH3-binding motifs, and a CDC25/GEF domain. In mammals, RalGPS proteins have been shown to act as RAL activators (17, 47). Fly RalGPS is encoded by the predicted CG5522 gene.

Reciprocally, a two-hybrid screen performed with the C-terminal region of fly RGL (aa 488 to 774, which includes the RA domain) identified RAP1, RAS1, and RAS2 as partners (RAS2 is a fly orthologue of mammalian R-Ras and/or TC21) (Table 1). This region of *Drosophila* RGL does not interact with fly RAL, CDC42, RAC1, or RHO1 (data not shown), exhibiting a specificity restricted to a few GTPases of the Ras family. Activated Ras1 and Rap1 G12V mutants, as well as dominant-negative S17N mutants, were tested for interaction with the RA domain of RGL: only the G12V mutants bind (data not shown). Thus, fly RGL behaves as an effector of Rap1 and Ras1 (and probably of Ras2).

In summary, the set of interactions obtained by several two-hybrid screens with fly proteins forms a protein interaction network where orthologous proteins from *Homo sapiens* and *D. melanogaster* constitute isomorphic protein linkage maps (Fig. 2). The proteins involved are RAS1, RAS2, RAP1, and RGL, and its targets are RAL, RLIP, REPS, and the medium chain of AP2 and SEC5. This orthology of networks justifies *D. melanogaster* as a suitable model organism to study Ral signaling. Conservation of protein-protein interactions throughout evolution reinforces their biological relevance.

Genomics of *Drosophila* Rgl. (i) Rgl encodes two isoforms of the RGL protein. The Rgl gene was localized by in situ polyethylene chromosome hybridization (Christian Biémont, personal communication) and by filter hybridization to P1 phages (Genome Systems, Inc.) (data not shown) on the left arm of the third chromosome in the region 70C. This localization was confirmed by the sequence of the *D. melanogaster* genome (1). The full-length cDNA of RGL (gi 12001829) was used to map the intron-exon structure of the gene (Fig. 3).

Analysis of the genomic sequence of *Drosophila* Rgl revealed that the second intronic sequence of Rgl is partially contained in an EST (LD16082). Sequencing the whole EST showed that there is an alternatively transcribed form of Rgl, apparently due to a second transcription initiation. We named the putative encoded protein RGL2 (gi 6652993). It corresponds to five additional ESTs found in a normalized embryo library (http://www.fruitfly.org/blast/index.html) (50). RGL2 corresponds to the predicted gene CG8865. The exon-intron structure of the RGL2 mRNA shows that RGL1 and RGL2 share the last five exons, but they differ from each other at their two first exons (Fig. 3). As a consequence, RGL1 and RGL2 proteins share the same LTE1/REM0, CDC25/GEF, and RA domains and differ at their N termini. The first 9 aa of RGL1 are replaced by 189 aa in RGL2; this latter extension bears no homology with any known domain or protein.

(ii) Rgl is ubiquitously expressed during embryonic development. Using a probe common to Rgl1 and Rgl2, Northern blot analysis showed a unique ~4 kb transcript expressed throughout *Drosophila* development, in embryos, larvae, pupae, and adults (Fig. 4). The same mRNA was detected with an Rgl1-specific probe. RNA in situ hybridization of whole-mount

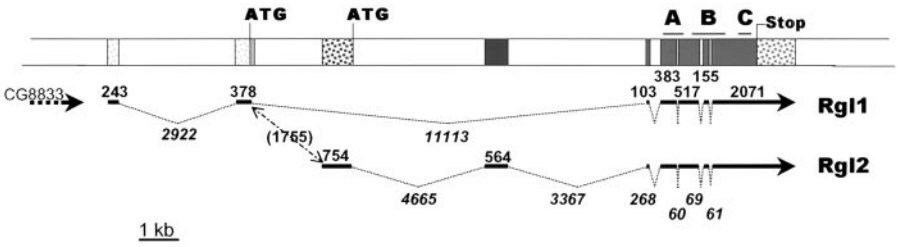

FIG. 3. Schematic representation of the exon-intron structure of the Rgl gene. Rgl1 mRNA is made of a first noncoding exon, a second essentially noncoding exon which contributes the ATG translation initiation codon and eight codons, four coding exons, and a final seventh exon which contains 1,144 coding base pairs and a 3′ UTR. Rgl2 mRNA is made of seven exons, with the last five exons held in common with Rgl1. The seven exons of Rgl1 are represented in light and dark gray, and the Rgl2 exons are represented in black and dark gray. Dark gray represents exons common to Rgl1 and Rgl2. Shading represents coding regions; spotted boxes represent noncoding regions. A, B, and C give the approximate localizations of the regions encoding the LTE1/REM0 domain, the CDC25/RasGEF domain, and the RA domain, respectively. The specific ATG codons and the common Stop codon are approximately represented. CG8833 is a predicted gene upstream of and on the same strand as Rgl. It is transcribed; corresponding ESTs can be found at http://www.fruitfly.org/blast/index.html. Intron sizes are indicated in italic type; exon sizes are indicated in roman type.

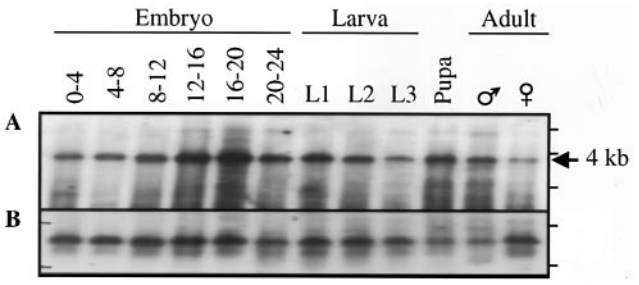

FIG. 4. Expression of Rgl mRNA during *Drosophila* developmental stages. RNAs were extracted from embryos of the indicated ages (in hours), from larvae of the 1st (L1), 2nd (L2), and 3rd (L3) instar stages, from pupae, and from male (♂) and female (♀) adults. The cDNA of Rgl1 was used as a probe (A); a probe corresponding to ribosomal protein RP49 was used as a loading control (B).

embryos with a full-length Rgl1 probe confirmed early expression, even at the preblastoderm stage, suggesting a maternal contribution for the Rgl transcript. At later stages, Rgl appeared to be expressed ubiquitously in the whole embryo (data not shown). Using an Rgl2-specific probe, no Rgl2 transcript could be detected either by Northern blot or by in situ hybridization. Thus, RGL2 seems to be expressed at a very low level and/or according to a very restricted spatiotemporal pattern. This could be consistent with the observation that overexpression of Rgl2 has dramatic developmental consequences (see below). It is also consistent with the fact that only one Rgl2 EST appeared in EST databases generated with nonnormalized embryo libraries—only normalized libraries allowed the five additional ESTs to be identified.

Genetics of the Ral-Rgl pathway. As opposed to what has been shown in studies with mammalian cell lines, in *Drosophila*, the Ral pathway interacts with both the Rap and Ras pathways. To gain insight into the functions of Rgl and Ral, and to decipher the functional network(s) where they act in vivo, a genetic approach was used. Transgenic strains were established in which expression of Rgl1, Rgl2, Ral, the dominant-negative allele Ral^{S25N}, and the constitutively active Ral^{G20V} is under the control of UASs. Similarly we generated transgenic flies expressing constitutively activated versions of Rgl1 and Rgl2 (Rgl1-CAAX and Rgl2-CAAX, respectively). Constitutive activation was achieved by expressing RGL proteins fused to a membrane localization sequence consisting of a polybasic motif followed by a CAAX box, allowing the post-translational farnesylation of proteins. Such fusions leading to membrane localization have been shown to mimic the activation by Ras of many Ras effectors, including mammalian RalGEFs (38, 77).

We employed the GAL4-UAS transcription system in which GAL4 produced in a particular pattern acts in *trans* to activate the expression of UAS transgenes in a corresponding pattern (8). We characterized phenotypes by using four readouts with four GAL4 drivers. Ubiquitous expression was obtained with the *da-GAL4* driver. *GMR-GAL4* drives expression in the eye (24). Expression in the notum and wing imaginal disk (drivers *sca-GAL4* and $Gal4^{MS1096}$) (12, 40) allowed monitoring of the patterns and morphology of notum bristles and wing structures.

(i) Phenotypes due to alleles of Ral and of Rgl. Is Rgl more than a RalGEF? Ubiquitous overexpression of wild-type Ral or wild-type Rgl1 gave no phenotype, whereas Rgl2 caused lethality at the embryonic and larval stages. Constitutively activated Rgl1 or Rgl2 caused embryonic and larval lethality, as did expression of the constitutively active Ral^{G20V} (data not shown) (see reference 54). In contrast, ubiquitous expression of dominant-negative Ral^{S25N} gave no lethal effect and flies demonstrated a characteristic Ral^{S25N} loss-of-bristle phenotype (see below and reference 54).

Flies expressing wild-type Ral and Ral^{S25N} in the eyes displayed no phenotype. The expression of activated Ral^{G20V} led to a weak rough eye phenotype, reminiscent of the phenotype described when human Ral^{G23V} is expressed under the same conditions (55).

The expression of wild-type Rgl1 yielded no phenotype while Rgl2 gave a few fusions of ommatidia and weakly rough eyes, a phenotype also seen with activated Rgl1 (Rgl1-CAAX). Activated Rgl2 (Rgl2-CAAX) caused a more-profound disturbance of eye patterning, the degeneration of multiple ommatidia, and a reduction in eye size, as documented by scanning electron microscopy (Fig. 5).

The $Gal4^{MS1096}$ driver allows the expression of UAS transgenes in the dorsal mesothoracic disk, which gives two principal derivatives, the wing and the thorax. Under this driver, none of the Ral alleles gave any wing phenotype. Wild-type Rgl1 and Rgl2 gave no phenotype either. Flies expressing the activated Rgl1 or Rgl2 (Rgl1/2-CAAX) exhibited a similar phenotype with an almost complete absence of wing or wings with an almost complete absence of veins and an overall reduction of wing size. The penetrance was stronger with activated Rgl2 than with activated Rgl1 (data not shown). The thorax phenotypes were similar to the ones seen with the more-restricted *sca-GAL4* driver that are described here below (data not shown).

Thus in both eyes and wings, the difference between the stronger phenotype due to activated Rgl and the weaker phenotype due to activated Ral, suggests that Ral does not carry all of the functions supported by its exchange factors Rgl1 and Rgl2, which therefore might also act via a Ral-independent pathway(s).

Under the control of *sca-Gal4*, the expression of wild-type forms of Ral, Rgl1, or Rgl2 had no effect on the pattern of sensory bristles. Flies expressing the constitutively active allele Ral^{G20V} or constitutively active Rgl1 and Rgl2 exhibited missing macrochaetae (1 to 3 for nota and/or head). Many of the remaining macrochaetae on the nota and head exhibit shaft morphology modifications (data not shown). Activated Rgl alleles gave macrochaetae that are hooked and/or had actin bundles at their ends while macrochaetae in Ral^{G20V} flies were short, burned, and misplaced apparently coming out of non-socket cells (data not shown). The expression of dominant-negative Ral^{S25N} led to the disappearance of the bristle shafts of most microchaetae and macrochaetae on the notum and the head. Only bristle sockets remained. Nota displayed bold cuticular surfaces with a wild-type socket pattern (Fig. 6B). This phenotype was dose- and temperature-sensitive and is similar to the one found in flies expressing Ral^{S25N} under the ubiquitous *da-GAL4* driver. It is similar to the Ral^{S25N} phenotype previously described (54). Such a phenotype (absence of the

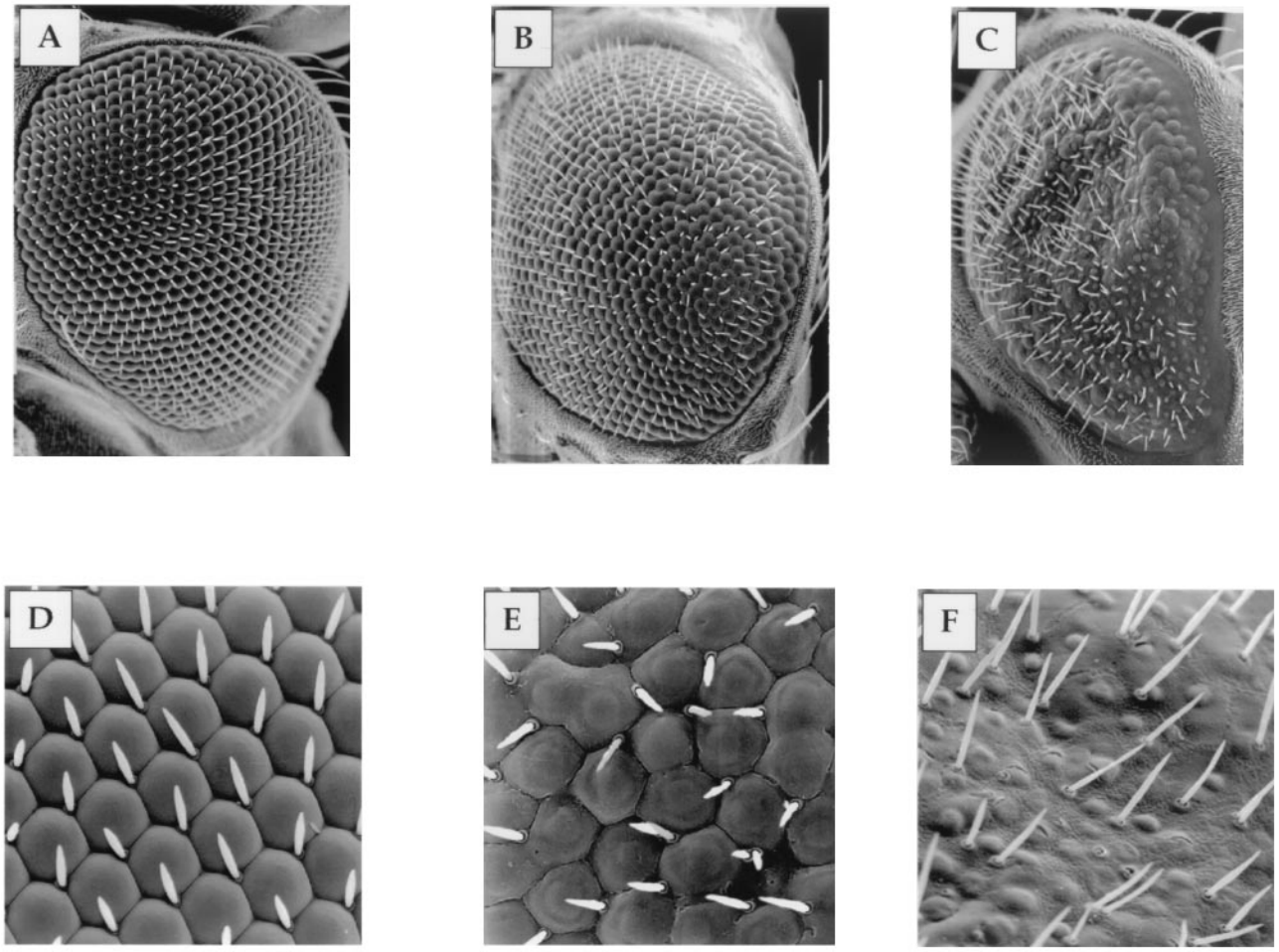

FIG. 5. Effects of RAL exchange factor RGL on eye development. Adult compound eyes from *GMR-Gal4*/+ (A and D), *GMR-Gal4*/+ *UAS-Rgl1-CAAX*/+ (B and E), and *GMR-Gal4*/+-*UAS-Rgl2-CAAX*/+ (C and F) flies were examined by scanning electron microscopy. Flies carrying *GMR-Gal4*/+ display a wild-type eye phenotype (A and D). Whole-eye views (A to C) and high magnification of a small part (D to F) are shown.

shafts of multiple sensory bristles on the nota affecting both macrochaetae and microchaetae) reflects a disturbance in the development of sensory organ progenitor cells. Since sensory organ progenitor cells seem exquisitely sensitive to alleles of the Ral pathway, they were chosen for further investigations.

(ii) Completing the network: *Drosophila* RGL, which looks like a RalGEF, behaves as a RalGEF in vivo. When biochemistry fails, genetics speak. Based on sequence homology and topology of domains, as well as on interaction data, we have predicted that RGL is a *Drosophila* exchange factor for RAL (see above). Unfortunately, we were unable to obtain any soluble RGL protein, precluding a biochemical investigation of our prediction. An alternative approach to this question consists of using genetics. Phenotypes generated by dominant-negative alleles of GTPases are usually suppressed by overexpression of the corresponding exchange factor (see, for instance, reference 45). This is consistent with the molecular mechanism underlying the phenotype: a dominant-negative GTPase interacts in a nonproductive way with its exchange factor and the overall result is a titration of the exchange factors that can no longer activate the wild-type endogenous GTPase.

Expression of Ral^{S25N} under the control of *sca-GAL4* leads to bristle disorganization with a loss of microchaetae and macrochaetae (Fig. 6B) (54). Expression of Rgl1 under *sca-GAL4* gives no bristle phenotype (Fig. 6A). When Rgl1 and Ral^{S25N} are coexpressed under *sca-GAL4*, the loss-of-bristle phenotype of Ral^{S25N} is suppressed and the wild-type pattern of macrochaetae and microchaetae is restored (Fig. 6C). The bristles of flies coexpressing Rgll and Ral^{S25N} were indistinguishable from those of wild-type flies: their length, morphology, and orientation were normal. This was also true when only the N-terminal REM0/RalGEF region (aa 54 to 487) (Fig. 1) of Rgl1 was used or when Rgl2 was used (data not shown). These data functionally support the hypothesis that RGL1 and RGL2 are exchange factors for RAL.

(iii) The activation of RAL required for the differentiation of sensory bristles might be independent of RAS1 signaling. In mammalian cells, Ral proteins are activated in response to Ras activation. Ras-GTP binds and activates RalGEFs that subsequently activate Ral. We tested this model, established with mammalian cell lines, in an in vivo system of bristle development in *Drosophila*.

Scabrous-Gal4-driven expression of an activated form of

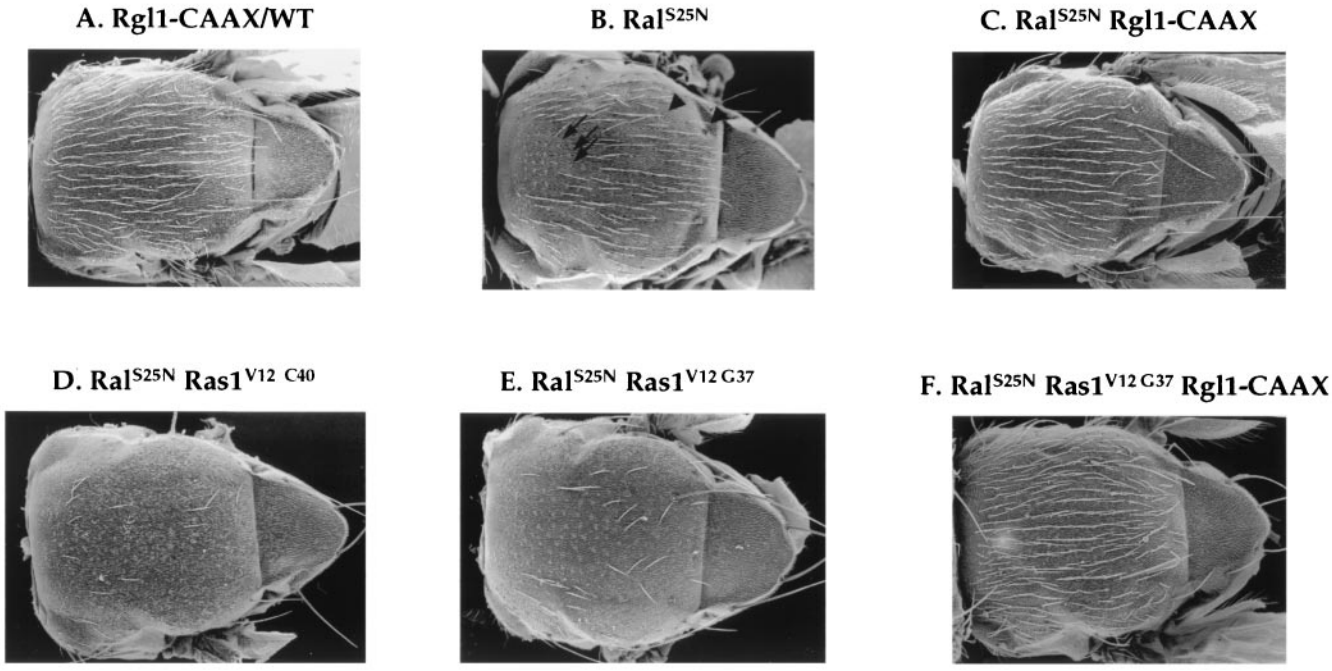

FIG. 6. Genetic interactions between Ras1, Ral, and Rgl1 alleles on the notum. Adult nota of flies harboring combinations of the transgenes mentioned above each picture and expressed in the proneural cluster under a *sca-Gal4* driver were examined by scanning electron (I) and/or Nomarski (II) microscopy. Notice that microchaeta organization on the nota in flies expressing Rgl1-CAAX is normal and serves as a wild-type reference (A). (B) Most of the microchaetae expressing Ral^{S25N} contained one socket but no shaft. Arrowheads indicate macrochaeta sockets without shaft; arrows indicate microchaeta sockets without shaft. (C) The socket without shaft phenotype resulting from Ral^{S25N} expression was rescued by coexpression of the activated form of Rgl1, and wild-type bristle patterning was restored. (D and E) The socket without shaft phenotype for both macrochaetae and microchaetae was largely enhanced by coexpression of Ral^{S25N} and Ras1$^{V12\ C40}$ or Ras1$^{V12\ G37}$. The remaining bristles have wild-type morphology and orientation. (F) Coexpression of the Rgl1-CAAX transgene suppressed these enhanced phenotypes (Ral^{S25N}, Ras1$^{V12\ G37}$, and Rgl1-CAAX). (G) Nota of flies in which Ras1$^{V12\ S35}$ is expressed display ectopic macrochaetae near existent ones (see boxed region). (H) Flies coexpressing Ral^{S25N} Ras1 $^{V12\ S35}$ display clusters of macrochaeta sockets (see boxed region) and microchaeta sockets arranged as in Ras1$^{V12\ S35}$ flies, but these sockets have no shaft. Reciprocally, the Ral^{S25N} microchaeta phenotype was largely enhanced by Ras1$^{V12\ S35}$ coexpression (compare with panel B). An example of the notum of a dissected late pupa is shown since expression of Ral^{S25N} together with Ras1$^{V12\ S35}$ gives a high level of lethality at the late pupal stage.

Drosophila Ras1 (Ras1^{V12}) caused lethality which was not rescued by the coexpression of Ral^{S25N}. Three effector loop mutants of activated Ras1 (Ras1$^{V12\ S35}$, Ras1$^{V12\ G37}$, and Ras1$^{V12\ C40}$) (29) were used to avoid this embryonic lethality. These effector loop mutations correspond to mammalian Ras mutants (T35S, E37G, and Y40C) which interact with a subset of Ras effectors, RAF, RalGEFs, and PI3K, respectively, but not exclusively, and which have been allowed to distinguish between different effector pathways downstream of Ras. Similar to the situation with mammalian Ras mutants, in two-hybrid assays, *Drosophila* Ras1$^{V12\ G37}$ interacted with RGL while Ras1$^{V12\ C40}$ and Ras1$^{V12\ S35}$ did not (data not shown). In vivo in *Drosophila*, expression of these mutants gave different phenotypes in both eyes and wings, and genetic data are consistent with the idea that these effector loop mutants use different pathways (22, 29).

We reasoned that if Ras1$^{V12\ G37}$, but neither Ras1$^{V12\ C40}$ nor Ras1$^{V12\ S35}$, acts on Rgl, a dominant-negative Ral^{S25N} which blocks Rgl (see "Completing the network: *Drosophila* RGL, which looks like a RalGEF, behaves as a RalGEF in vivo" above) might functionally interact with Ras1$^{V12\ G37}$, but neither with Ras1$^{V12\ C40}$ nor with Ras1$^{V12\ S35}$. Reciprocally, Ras1$^{V12\ G37}$, but not the other two alleles, might suppress Ral^{S25N} phenotypes by hyperactivating Rgl.

We could not see any effect on bristle patterning of the ectopic expression of Ras1$^{V12\ G37}$ and Ras1$^{V12\ C40}$ under *sca-GAL4*: the distribution and morphology of both type of bristles (macrochaetae and microchaetae) are normal (data not shown). Ras1$^{V12\ S35}$ promotes the development of extra macrochaetae in the vicinity of the existing ones on the notum and scutellum. Macrochaetae, but not microchaetae, were affected (Fig. 6G). Oversignaling by epidermal growth factor receptor and by Ras1^{V12} in proneural clusters produced similar bristle phenotypes; these phenotypes are mediated by the Ras/Raf signaling pathway (15).

The coexpression of Ras$^{V12\ G37}$ as well as Ras$^{V12\ C40}$ with Ral^{S25N} dramatically enhanced the Ral^{S25N}-induced loss-of-bristle phenotype. Both macrochaeta and microchaeta patterns were affected, leading to large bold regions on the notum (Fig. 6D and E). Ras$^{V12\ C40}$ produced a more-dramatic effect than

II.

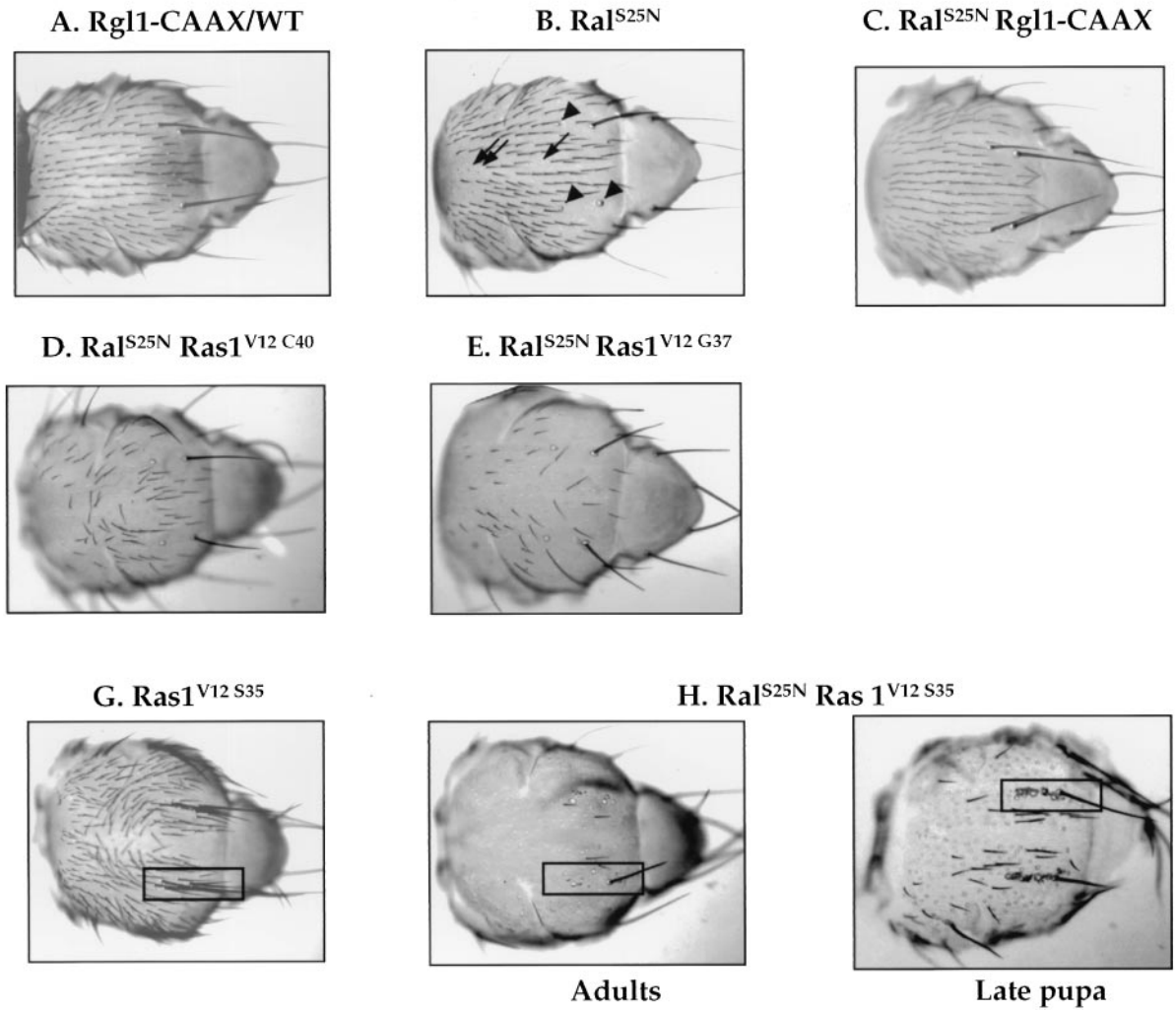

FIG. 6—*Continued*.

Ras$^{V12\ G37}$: 70% of flies coexpressing Ras$^{V12\ C40}$ and Ral^{S25N} had lost most of their bristles while this percentage was less than 25% for flies coexpressing Ras$^{V12\ G37}$ and Ral^{S25N}.

This enhanced loss-of-bristle phenotype was completely restored (for Ral^{S25N} and Ras$^{V12\ C40}$) or largely rescued (for Ral^{S25N} and Ras$^{V12\ G37}$) by adding the activated form of Rgl1: coexpression of the three transgenes Ral^{S25N}, Rgl1-CAAX, and Ras$^{V12\ G37/C40}$ produced flies with a wild-type notum (Fig. 6F).

The expression of Ral^{S25N} together with Ras1$^{V12\ S35}$ under *sca-GAL4* leads to a high level of lethality in late pupa. The observation of phenotypes was thus performed on dissected late pupa in addition to rare adults. Flies coexpressing Ras$^{V12\ S35}$ and Ral^{S25N} displayed the same pattern of extra macrochaeta sockets as Ras$^{V12\ S35}$ flies but these sockets had no shaft. Similarly, the pattern of microchaetae was normal, as in Ras$^{V12\ S35}$ flies, but here again, the cuticular surface was bold (Fig. 6H). Thus, the loss-of-bristle and the extra macrochaeta phenotypes due to Ral^{S25N} and Ras$^{V12\ S35}$, respectively, do not seem to be affected by each other. The Ral^{S25N} bold phenotype, however, is more pronounced in a Ras$^{V12\ S35}$ context.

Altogether, contrary to our predictions, in the development of sensory organs on the notum, we observed an unexpected enhanced expressiveness of the Ral^{S25N} phenotype, which displayed no Ras allele specificity. These results argue against a model where Ras1$^{V12\ G37}$ or any of the other mutants activate RGL and the Ral pathway in the notum. They suggest that, in *Drosophila*, the Ral and the Ras pathways are rather independent, although they intersect in the development of sensory organs.

(iv) Genetic interactions argue that RAP1 acts via an RGL-RAL signaling pathway. In mammalian cell lines, the Rap and the Ral pathways have no documented functional connection, although Rap and RalGEFs are capable of physical interactions. We have revisited this issue in vivo.

The *sca-Gal4*-driven expression of an activated form of *Drosophila* Rap1 (Rap1^{V12}) caused 100% lethality at the embryonic stage and no *sca-Gal4 UAS-Rap1^{V12}* larvae were recovered, probably because the Rap1-GDP/Rap1-GTP cycle must

be regulated during morphogenesis (2). Flies expressing Ral^{S25N} are viable (0% lethality). Coexpression of Ral^{S25N} partially rescued the lethal effect of $Rap1^{V12}$, from 0% survival to a 10 to 13% survival rate. In all surviving flies, a wild-type pattern of macrochaeta and microchaeta bristles was restored on the thorax. Therefore, the coexpression of dominant-negative Ral suppressed the lethal effect of $Rap1^{V12}$ throughout embryonic development, and on the other hand, activation of the Rap1 signaling pathway rescued the Ral^{S25N} loss-of-bristle phenotype. Coexpression of either the activated Ral^{G20V} or an activated Rgl1 (Rgl1-CAAX) did not rescue the $Rap1^{V12}$ lethal phenotype.

The simplest explanation is that Rap1 acts via the RalGEF protein RGL to activate Ral. During embryogenesis, the overactivation of a Rap1/Rgl signaling pathway by $Rap1^{V12}$ can be decreased by the coexpression of Ral^{S25N} which sequesters RGL (RalGEF) proteins. Reciprocally, throughout mesothoracic bristle development, the additional activation of endogenous RGL1 and consequently of its effector RAL results in reestablishment of the wild-type bristle patterning affected by Ral^{S25N}.

The present data are consistent with a model where the Rgl-Ral signaling pathway would function as an effector of the Rap1 GTPase. Alternatively, a functional Ral pathway might be required for effective Rap signaling, but the interaction between RGL and Rap-GTP favors the former hypothesis.

(v) Genetic interactions between Ras, Rap, Rgl, and Ral alleles in eye development. Since in many mammalian cell lines Ras signaling is via the Ral pathway and we couldn't see this pathway at work in bristle development, we wondered whether such a cascade could be effective in other tissues. We used eye development, where Ras function is well documented, as a readout. Similarly, we tested whether the Rgl-Ral signaling pathway acts as an effector of Rap1 in eye development, as it appears in the notum.

Under the eye-specific driver *GMR-GAL4*, expression of $Ras1^{V12\,C40}$ or of $Ras1^{V12\,G37}$ gives a rough eye phenotype. We couldn't see any effect of the expression of Ral^{S25N} or Rgl1 on the rough eye phenotype of flies expressing $Ras1^{V12\,C40}$ (data not shown). The mild rough eye of flies expressing $Ras1^{V12\,G37}$ (22, 29) (Fig. 7A) is enhanced when signaling by the Rgl-Ral pathway is decreased either by coexpression of Ral^{S25N} or in flies homozygous for a deletion in the Rgl locus (ΔRgl). Two other deletions in Rgl give the same results. The $Ras1^{V12\,G37}$ phenotype is insensitive to overexpression of Rgl1 (Fig. 7A). These data suggest that the rough eye phenotype of the $Ras1^{V12\,G37}$ allele is not due to the activation of a RalGEF-Ral pathway, although they do not rule out some contribution of the Ral pathway to Ras signaling.

Flies expressing activated $Rap1^{V12}$ under *GMR-GAL4* display a rough eye phenotype (Fig. 7B). Coexpression of the dominant-negative allele Ral^{S25N} with $Rap1^{V12}$ partially suppresses this latter phenotype, consistent with the interaction seen under *sca-GAL4*, where dominant-negative Ral rescues partially lethality due to $Rap1^{V12}$. Similarly, when $Rap1^{V12}$ is expressed in the background of a homozygous hypomorphic allele of the Rgl locus (ΔRgl), the rough eye phenotype is partially suppressed. Two other hypomorphic alleles of Rgl give a similar suppression. Reciprocally, coexpression of wild-type Rgl1 with $Rap1^{V12}$ enhances the rough eye phenotype (Fig. 7B). So in eyes as in nota, the Rap GTPase seems to signal through the RalGEF RGL and the Ral GTPase or to require an effective Ral pathway to signal.

DISCUSSION

Ral proteins appeared in evolution with multicellular eukaryotes, where they seem to be expressed ubiquitously even though modulation of their expression might be involved in development (80).

The functions of Ral proteins remain unclear. They are not oncogenic per se, but they facilitate Ras transformation, participate in cell motility, and are required for metastatic evolution of Ras-transformed cells as well as for Ras-induced stimulation of cyclin D1 expression (20, 23, 25, 34, 59, 63, 69). They are involved in phospholipase D activation, endocytosis, and exocytosis (10, 19, 28, 39, 41, 58, 65). There is not yet a unifying theory that relates these latter functions to the former cancer-connected phenotypes.

In mammalian cell lines, Ral proteins were shown to be involved in not only H-Ras and K-Ras but also TC21 signaling via a family of Ras effectors, the RalGEFs (42, 49). Once Ras is bound to GTP, it binds and activates these RalGEFs, which in turn activate Ral proteins. There are also Ras-independent pathways that activate Ral (17, 43, 47, 68, 78).

Rap proteins are GTPases once described as antagonistic to Ras oncoproteins. Their function remains elusive. They were reported to be functionally connected to integrin signaling, and they are able to bind RalGDS, one of the mammalian RalGEFs, with a higher affinity than Ras, yet this interaction does not lead to the activation of Ral in cell lines (13, 31, 57, 75).

We wanted (i) to address the question of the contribution of the Ral pathway to cellular functioning by using approaches with different methodological biases; (ii) to be within the frame of a whole organism, where cells have to communicate with neighboring cells of different types and integrate various signals; (iii) to have several readouts, assuming that signaling pathways might be using signaling modules following different architectures in different situations; and (iv) to use the power of genetics to establish signaling cascades as well as functional interactions between distinct signaling pathways. *D. melanogaster* can fulfill these requirements.

First, we show that an exchange factor for Ral of the RalGEF family, which is an orthologue of mammalian RGL, exists in *Drosophila*. In fact, flies express two orthologues, RGL1 and RGL2, probably generated by the use of two promoters and alternative splicing. RGL1 and RGL2 share the same RalGEF domain as well as the C-terminal domain that binds Ras and Rap, but they differ in their N termini. Combined data from several two-hybrid screens, including the present one, suggest that the Ras/Rap-Ral network is very similar in mammals and in *Drosophila* (Fig. 2). Physical interactions connect RGL to RAS1 (Ras in humans), RAS2 (R-Ras and/or TC21 in humans), and RAS3 (Rap1 in humans) as well as RAL to RLIP (RLIP76 in humans) and SEC5 (the same in humans). RLIP is connected to the orthologous μ2 chains of the AP2 complexes as well as to REPS (the same in humans). The conservation of such a large network confirms that *Drosophila* is a suitable model to study the Ral pathway in a physiological context. It is noteworthy that in *Caenorhabditis elegans*, all the proteins of

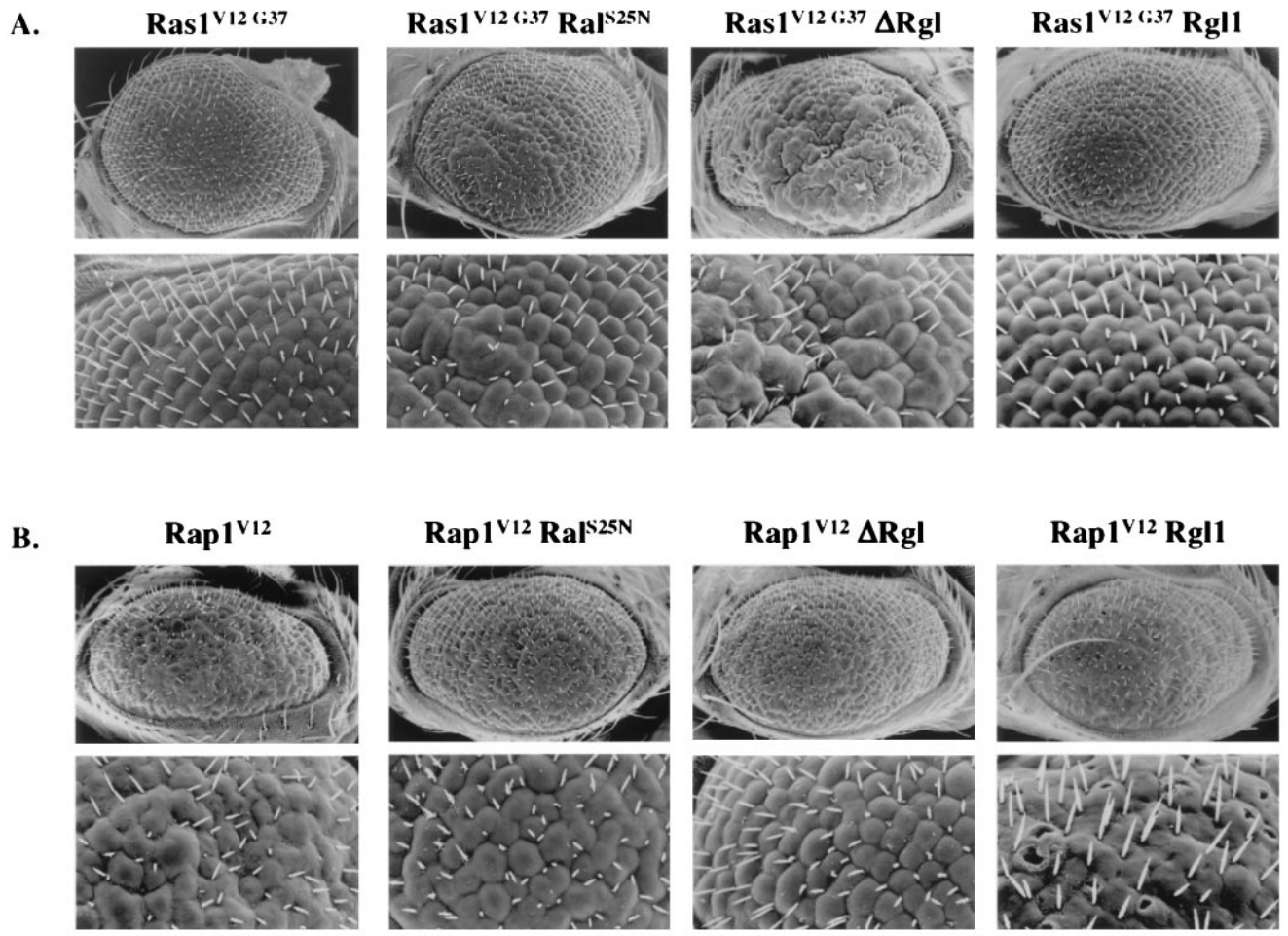

FIG. 7. Genetic interactions between Rap1, Ras1, Ral and Rgl1 alleles in the eyes. Eyes of flies harboring combinations of the transgenes mentioned above each picture and expressed under a *GMR-GAL4* driver were examined by scanning electron microscopy. Whole-eye views and high magnifications of a small part are shown. Notice that the eye organization of flies expressing wild-type Rgl1 under *GMR-GAL4* or homozygous for a deletion in the Rgl locus (ΔRgl) is normal (data not shown) (for an example, see Fig. 5). (A) Genetic combinations with $Ras1^{V12\,G37}$, an activated allele of Ras1 harboring a mutation in the effector loop (see text). Flies expressing $Ras1^{V12\,G37}$ display a mild rough eye phenotype. Coexpression of Ral^{S25N} enhances this phenotype. The rough eye phenotype is also enhanced in flies expressing $Ras1^{V12\,G37}$, which are homozygous for a deletion of the Rgl locus (ΔRgl). This phenotype is barely influenced by the coexpression of a wild-type allele of Rgl1. (B) Genetic combinations with $Rap1^{V12}$, an activated allele of Rap1. $Rap1^{V12}$ expressed under *GMR-GAL4* yields a rough eye phenotype that is partially suppressed by the coexpression of Ral^{S25N} or in flies homozygous for a deletion of the Rgl locus (ΔRgl). This phenotype is enhanced by the coexpression of a wild-type allele of Rgl1.

this network exist and certain interactions have been shown (67), as opposed to what is seen in *S. cerevisiae*, suggesting that they are important for metazoans.

We have generated several lines of transgenic flies to decipher the functional relationships between the different actors. Phenotypes of the transgenic flies suggest that, like in mammals, the function of Rgl is not totally accounted for by the activation of Ral, since an activated allele of Ral does not mimic the activated alleles of Rgl. Could activated Rgl phenotypes be due to the titration of endogenous RAS1 or RAP1 by the RA domain of the RGL transgenes? If so, coexpression of activated RGL with either wild-type Ras1 or wild-type Rap1 should attenuate the Rgl phenotypes. This is not the case. Flies coexpressing activated RGL and RAS1 display some new phenotypes which are not seen when each transgene is individually expressed (extra veins under *en-GAL4*; heterogeneity of ommatidia size under *GMR-GAL4*) or keep displaying the Rgl phenotype (bristle morphology under *sca-GAL4*). Flies coexpressing activated RGL and RAP1 even display an enhanced Rgl phenotype (in eyes and on wings) or keep displaying the bristle morphology phenotype due to activated RGL (data not shown). Ral-independent functions of RGL might be mediated by protein-protein interactions with domains other than the Ras/Rap and Ral interacting domains, and recently, mammalian RalGDS was shown to interact with β-arrestin (5). However we cannot rule out totally the titration hypothesis. An alternative explanation might be that Ral has to cycle between a GDP state and a GTP state, which would be accelerated by activated RalGEF and not mimicked by activated Ral that is blocked in a GTP-bound state. Although the existence of Ral-

independent functions of RalGEFs is suggested both in mammals (for examples, see references 23 and 73) and in flies (our results), the clarification of this question requires further investigation.

But is RGL an actual exchange factor for RAL? The effects in bristle development of a dominant-negative Ral are suppressed by the increased expression of Rgl. The simplest explanation is that RGL is a bona fide exchange factor for Ral.

We investigated interactions between the Ral pathway and two of its interlocutors, the Ras and Rap GTPases. In mammalian cell lines, the $Ras^{G12V\ E37G}$, $Ras^{G12V\ Y40C}$, and $Ras^{G12V\ T35S}$ alleles activate the Ral pathway via interaction with RalGEFs, the PI3K pathway, and the Raf pathway, respectively, although things might be more complicated since $Ras^{G12V\ Y40C}$ might be acting together with $Ras^{G12V\ E37G}$ to activate RalGEFs upon epidermal growth factor stimulation (61). $Ras^{G12V\ E37G}$ does not activate the Raf nor the PI3K pathway. In *Drosophila* also, these different Ras alleles drive different pathways (22, 29); however, nothing is known about the connection between the Ras and Ral pathways.

If a Ras-Ral pathway exists, a dominant-negative allele of Ral should attenuate effects due to $Ras^{G12V\ E37G}$ but not phenotypes due to $Ras^{G12V\ Y40C}$ or $Ras^{G12V\ T35S}$. Indeed, in HeLa cells, dominant-negative alleles of Ral do block a $Ras^{G12V\ E37G}$ phenotype (25). Reciprocally, $Ras^{G12V\ E37G}$, but not the two other alleles, might attenuate a Ral dominant-negative phenotype. Our two-hybrid results show that, as in mammals, fly RGL behaves as an effector of fly RAS1, and this interaction is mediated by the RA domain of RGL (data not shown). When searching for genetic interactions between Ras1 and Ral, we found that all three Ras alleles enhanced the Ral^{S25N} loss-of-bristle phenotype. These results show an actual genetic interaction between the Ras and Ral pathways but do not support the classical model of a linear pathway from Ras to Ral, a conclusion strengthened by the absence of the Ras allele specificity of the observed interactions. An alternative model would be an intersection of the Ral and Ras pathways and would involve a yet undefined Ras effector whose interaction with Ras would not be selective for the three effector loop mutations tested here (Fig. 2).

Rap1 is another GTPase of the Ras family that can interact with most Ras effectors, including RalGEFs. No functional Rap1-RalGEF, Rap1-PI3K, or Rap1-Raf interactions have been documented, except for an isoform of B-Raf, described as activated by Rap1 (56, 66). The originally suggested antagonism between Ras and Rap (32) remains a murky issue, and Rap1 and Ras seem to participate in rather independent pathways, although recent data challenge this idea, at least in vesicle trafficking at synapses (81). In *Drosophila*, where Rap1 is required for morphogenesis, Ras1 and Rap1 act in distinct pathways (2). No functional effector of Rap has been identified. Our two-hybrid data show that *Drosophila* RGL behaves as a Rap1 effector. Our genetic data support the idea that this interaction is functional: a dominant-negative allele of Ral is able to rescue lethality caused by an activated allele of Rap1, and reciprocally, in the surviving flies, activated Rap1 rescues the bristle development phenotype of a dominant-negative Ral. Similarly, in eyes, Rgl and Ral seem to act downstream of Rap1. Although we cannot rule out an alternative model where Rap and Ral signals converge towards a common downstream target, our results rather argue in favor of a linear Rap-Rgl-Ral pathway (Fig. 2). Consistent with this model, preliminary data with an *engrailed-GAL4* driver show that the phenotype displayed by Ral^{S25N} in wings mimics the one obtained by overexpression of a negative regulator of Rap, RapGAP (R. Fehon, personal communication). Thus, titrating RGL proteins by the expression of a dominant-negative Ral mimics the inactivation of Rap1 by an excess of its GAP.

Taken together, our genetic data from *Drosophila* shed a different light on signaling networks as they were established in mammalian cell lines. In both developmental systems used here (eye and notum), Ras1 and Ral do not seem to be linearly connected. In contrast, we show that Rap1 and Ral act as if they were participating in a common transduction pathway. These data do not rule out that in some other tissues, a Ras-Ral pathway might indeed exist, but they suggest that a molecular Lego might assemble signaling modules following various architectures in different tissues. We speculate that this should also be the case in mammals. An alternative model would be that we cannot reveal a Ras-Ral pathway in our experimental system, just as the Rap-Ral pathway couldn't be revealed in mammalian cell lines, and that, in the same tissue, Ras-Ral and Rap-Ral pathways are functional.

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G.M. and M.B. contributed equally to this work.

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REFERENCES

1. **Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne, P. G. Amanatides, S. E. Scherer, P. W. Li, R. A. Hoskins, R. F. Galle, R. A. George, S. E. Lewis, S. Richards, M. Ashburner, S. N. Henderson, G. G. Sutton, J. R. Wortman, M. D. Yandell, Q. Zhang, L. X. Chen, R. C. Brandon, Y. H. Rogers, R. G. Blazej, M. Champe, B. D. Pfeiffer, K. H. Wan, C. Doyle, E. G. Baxter, G. Helt, C. R. Nelson, G. L. Gabor Miklos, J. F. Abril, A. Agbayani, H. J. An, C. Andrews-Pfannkoch, D. Baldwin, R. M. Ballew, A. Basu, J. Baxendale, L. Bayraktaroglu, E. M. Beasley, K. Y. Beeson, P. V. Benos, B. P. Berman, D. Bhandari, S. Bolshakov, D. Borkova, M. R. Botchan, J. Bouck, P. Brokstein, P. Brottier, K. C. Burtis, D. A. Busam, H. Butler, E. Cadieu, A. Center, I. Chandra, J. M. Cherry, S. Cawley, C. Dahlke, L. B. Davenport, P. Davies, B. de Pablos, A. Delcher, Z. Deng, A. D. Mays, I. Dew, S. M. Dietz, K. Dodson, L. E. Doup, M. Downes, S. Dugan-Rocha, B. C. Dunkov, P. Dunn, K. J. Durbin, C. C. Evangelista, C. Ferraz, S. Ferriera, W. Fleischmann, C. Fosler, A. E. Gabrielian, N. S. Garg, W. M. Gelbart, K. Glasser, A. Glodek, F. Gong, J. H. Gorrell, Z. Gu, P. Guan, M. Harris, N. L. Harris, D. Harvey, T. J. Heiman, J. R. Hernandez, J. Houck, D. Hostin, K. A. Houston, T. J. Howland, M. H. Wei, C. Ibegwam, et al.** 2000. The genome sequence of Drosophila melanogaster. Science **287:**2185–2195.
2. **Asha, H., N. D. de Ruiter, M. G. Wang, and I. K. Hariharan.** 1999. The Rap1 GTPase functions as a regulator of morphogenesis in vivo. EMBO J. **18:** 605–615.
3. **Barbacid, M.** 1987. ras genes. Annu. Rev. Biochem. **56:**779–827.

4. **Bergmann, A., J. Agapite, K. McCall, and H. Steller.** 1998. The Drosophila gene hid is a direct molecular target of Ras-dependent survival signaling. Cell **95:**331–341.
5. **Bhattacharya, M., P. H. Anborgh, A. V. Babwah, L. B. Dale, T. Dobransky, J. L. Benovic, R. D. Feldman, J. M. Verdi, R. J. Rylett, and S. S. G. Ferguson.** 2002. β-Arrestins regulate a Ral-GDS–Ral effector pathway that mediates cytoskeletal reorganization. Nat. Cell Biol. **4:**547–555.
6. **Bohni, R., J. Riesgo-Escovar, S. Oldham, W. Brogiolo, H. Stocker, B. F. Andruss, K. Beckingham, and E. Hafen.** 1999. Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1–4. Cell **97:**865–875.
7. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1990. The GTPase superfamily: a conserved switch for diverse cell functions. Nature **348:**125–132.
8. **Brand, A. H., and N. Perrimon.** 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development **118:**401–415.
9. **Brown, N. H., and F. C. Kafatos.** 1988. Functional cDNA libraries from Drosophila embryos. J. Mol. Biol **203:**425–437.
10. **Brymora, A., V. A. Valova, M. R. Larsen, B. D. Roufogalis, and P. J. Robinson.** 2001. The brain exocyst complex interacts with RalA in a GTP-dependent manner: identification of a novel mammalian Sec3 gene and a second Sec15 gene. J. Biol. Chem. **276:**29792–29797.
11. **Cantor, S. B., T. Urano, and L. A. Feig.** 1995. Identification and characterization of Ral-binding protein 1, a potential downstream target of Ral GTPases. Mol. Cell. Biol. **15:**4578–4584.
12. **Capdevila, J. A., and I. Guerrero.** 1994. Targeted expression of the signaling molecule Decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. EMBO J. **13:**4459–4468.
13. **Caron, E., A. J. Self, and A. Hall.** 2000. The GTPase Rap1 controls functional activation of macrophage integrin alphaMbeta2 by LPS and other inflammatory mediators. Curr. Biol. **10:**974–978.
14. **Chardin, P., and A. Tavitian.** 1986. The ral gene: a new related gene isolated by the use of a synthetic probe. EMBO J. **5:**2203–2208.
15. **Culi, J., E. Martin-Blanco, and J. Modolell.** 2001. The EGF receptor and N signalling pathways act antagonistically in *Drosophila* mesothorax bristle patterning. Development **128:**299–308.
16. **Deak, P., M. M. Omar, R. D. Saunders, M. Pal, O. Komonyi, J. Szidonya, P. Maroy, Y. Zhang, M. Ashburner, P. Benos, C. Savakis, I. Siden-Kiamos, C. Louis, V. N. Bolshakov, F. C. Kafatos, E. Madueno, J. Modolell, and D. M. Glover.** 1997. P-element insertion alleles of essential genes on the third chromosome of Drosophila melanogaster: correlation of physical and cytogenetic maps in chromosomal region 86E-87F. Genetics **147:**1697–1722.
17. **de Bruyn, K. M. T., J. de Rooij, R. M. F. Wolthuis, H. Rehmann, J. Wesenbeek, R. H. Cool, A. H. Wittinghofer, and J. L. Bos.** 2000. RalGEF2, a pleckstrin homology domain containing guanine nucleotide exchange factor for Ral. J. Biol. Chem. **275:**29761–29766.
18. **Falquet, L., M. Pagni, P. Bucher, N. Hulo, C. J. Sigrist, K. Hofmann, and A. Bairoch.** 2002. The PROSITE database, its status in 2002. Nucleic Acids Res. **30:**235–238.
19. **Frankel, P., M. Ramos, J. Flom, S. Bychenok, T. Joseph, E. Kerkhoff, U. R. Rapp, L. A. Feig, and D. A. Foster.** 1999. Ral and Rho-dependent activation of phospholipase D in v-Raf-transformed cells. Biochem. Biophys. Res. Commun. **255:**502–507.
20. **Gildea, J. J., M. A. Harding, M. J. Seraj, K. M. Gulding, and D. Theodorescu.** 2002. The role of Ral A in epidermal growth factor receptor-regulated cell motility. Cancer Res. **62:**982–985.
21. **Goi, T., G. Rusanescu, T. Urano, and L. A. Feig.** 1999. Ral-specific guanine nucleotide exchange factor activity opposes other Ras effectors in PC12 cells by inhibiting neurite outgrowth. Mol. Cell. Biol. **19:**1731–1741.
22. **Halfar, K., C. Rommel, H. Stocker, and E. Hafen.** 2001. Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity. Development **128:**1687–1696.
23. **Hamad, N. M., J. H. Elconin, A. E. Karnoub, W. Bai, J. N. Rich, R. T. Abraham, C. J. Der, and C. M. Counter.** 2002. Distinct requirements for Ras oncogenesis in human versus mouse cells. Genes Dev. **16:**2045–2047.
24. **Hay, B. A., R. Maile, and G. M. Rubin.** 1997. P element insertion-dependent gene activation in the Drosophila eye. Proc. Natl. Acad. Sci. USA **94:**5195–5200.
25. **Henry, D. O., S. A. Moskalenko, K. J. Kaur, M. Fu, R. G. Pestell, J. H. Camonis, and M. A. White.** 2000. Ral GTPases contribute to regulation of cyclin D1 through activation of NF-kappaB. Mol. Cell. Biol. **20:**8084–8092.
26. **Ikeda, M., O. Ishida, T. Hinoi, S. Kishida, and A. Kikuchi.** 1998. Identification and characterization of a novel protein interacting with Ral-binding protein 1, a putative effector protein of Ral. J. Biol. Chem. **273:**814–821.
27. **Jullien-Flores, V., O. Dorseuil, F. Romero, F. Letourneur, S. Saragosti, R. Berger, A. Tavitian, G. Gacon, and J. Camonis.** 1995. Bridging the Ral GTPase to Rho pathways: RLIP76, a Ral effector with CDC42/Rac GTPase activating protein (GAP) activity. J. Biol. Chem. **270:**22473–22477.
28. **Jullien-Flores, V., Y. Mahe, G. Mirey, C. Leprince, B. Meunier-Bisceuil, A. Sorkin, and J. H. Camonis.** 2000. RLIP76, an effector of the GTPase Ral, interacts with the AP2 complex: involvement of the Ral pathway in receptor endocytosis. J. Cell Sci. **113:**2837–2844.
29. **Karim, F. D., and G. M. Rubin.** 1998. Ectopic expression of activated Ras1 induces hyperplastic growth and increased cell death in *Drosophila* imaginal tissues. Development **125:**1–9.
30. **Kimmel, B. E., U. Heberlein, and G. M. Rubin.** 1990. The homeo domain protein rough is expressed in a subset of cells in the developing Drosophila eye where it can specify photoreceptor cell subtype. Genes Dev. **4:**712–727.
31. **Kishida, S., S. Koyama, K. Matsubara, M. Kishida, Y. Matsuura, and A. Kikuchi.** 1997. Colocalization of Ras and Ral on the membrane is required for Ras-dependent Ral activation through Ral GDP dissociation stimulator. Oncogene **15:**2899–2907.
32. **Kitayama, H., Y. Sugimoto, Y. Matsuzaki, Y. Ikawa, and M. Noda.** 1989. A ras-related gene with transformation suppressor activity. Cell **56:**77–84.
33. **Kurada, P., and K. White.** 1998. Ras promotes cell survival in Drosophila by downregulating hid expression. Cell **95:**319–329.
34. **Lee, T., L. Feig, and D. J. Montell.** 1996. Two distinct roles for Ras in a developmentally regulated cell migration. Development **122:**409–418.
35. **Leevers, S. J., D. Weinkove, L. K. MacDougall, E. Hafen, and M. D. Waterfield.** 1996. The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. EMBO J. **15:**6584–6594.
36. **Letunic, I., L. Goodstadt, N. J. Dickens, T. Doerks, J. Schultz, R. Mott, F. Ciccarelli, R. R. Copley, C. P. Ponting, and P. Bork.** 2002. Recent improvements to the SMART domain-based sequence annotation resource. Nucleic Acids Res. **30:**242–244.
37. **Lu, X., T. B. Chou, N. G. Williams, T. Roberts, and N. Perrimon.** 1993. Control of cell fate determination by p21ras/Ras1, an essential component of torso signaling in Drosophila. Genes Dev. **7:**621–632.
38. **Matsubara, K., S. Kishida, Y. Matsuura, H. Kitayama, M. Noda, and A. Kikuchi.** 1999. Plasma membrane recruitment of RalGDS is critical for Ras-dependent Ral activation. Oncogene **18:**1303–1312.
39. **Moskalenko, S., D. O. Henry, C. Rosse, G. Mirey, J. H. Camonis, and M. A. White.** 2002. The exocyst is a Ral effector complex. Nat. Cell Biol. **4:**66–72.
40. **Nakao, K., and J. A. Campos-Ortega.** 1996. Persistent expression of genes of the *Enhancer of Split* complex suppresses neural development in Drosophila. Neuron **16:**275–286.
41. **Nakashima, S., K. Morinaka, S. Koyama, M. Ikeda, M. Kishida, K. Okawa, A. Iwamatsu, S. Kishida, and A. Kikuchi.** 1999. Small G protein Ral and its downstream molecules regulate endocytosis of EGF and insulin receptors. EMBO J. **18:**3629–3642.
42. **Osada, M., T. Tolkacheva, W. Li, T. O. Chan, P. N. Tsichlis, R. Saez, A. C. Kimmelman, and A. M. Chan.** 1999. Differential roles of Akt, Rac, and Ral in R-Ras-mediated cellular transformation, adhesion, and survival. Mol. Cell. Biol. **19:**6333–6344.
43. **Park, J. B.** 2001. Regulation of GTP-binding state in RalA through Ca2+ and calmodulin. Exp. Mol. Med. **33:**54–58.
44. **Park, S.-H., and R. A. Weinberg.** 1996. A putative effector of Ral has homology to Rho/Rac GTPase activating proteins. Oncogene **11:**2349–2355.
45. **Powers, S. K., K. O'Neill, and M. Wigler.** 1989. Dominant yeast and mammalian RAS mutants that interfere with the CDC25-dependent activation of wild-type RAS in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**390–395.
46. **Prober, D. A., and B. A. Edgar.** 2000. Ras1 promotes cellular growth in the Drosophila wing. Cell **100:**435–446.
47. **Rebhun, J. F., H. Chen, and L. A. Quilliam.** 2000. Identification and characterization of a new family of guanine nucleotide exchange factors for the Ras-related GTPase Ral. J. Biol. Chem. **275:**13406–13410.
48. **Rorth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm, G. M. Rubin, K. Weigmann, M. Milan, V. Benes, W. Ansorge, and S. M. Cohen.** 1998. Systematic gain-of-function genetics in Drosophila. Development **125:**1049–1057.
49. **Rosario, M., H. F. Paterson, and C. J. Marshall.** 2001. Activation of the Ral and phosphatidylinositol 3′ kinase signaling pathways by the Ras-related protein TC21. Mol. Cell. Biol. **21:**3750–3762.
50. **Rubin, G. M., L. Hong, P. Brokstein, M. Evans-Holm, E. Frise, M. Stapleton, and D. A. Harvey.** 2000. A *Drosophila* complementary DNA resource. Science **287:**2222–2224.
51. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
52. **Sauer, F., D. A. Wassarman, G. M. Rubin, and R. Tjian.** 1996. TAF(II)s mediate activation of transcription in the *Drosophila* embryo. Cell **87:**1271–1284.
53. **Sawado, T., F. Hirose, Y. Takahashi, T. Sasaki, T. Shinomiya, K. Sakagushi, A. Matsukage, and M. Yamaguchi.** 1998. The DNA replication-related element (DRE):DRE-binding factor system is a transcriptional regulator of the *Drosophila* E2F gene. J. Biol. Chem. **273:**26042–26051.
54. **Sawamoto, K., P. Winge, S. Koyama, Y. Hirota, C. Yamada, S. Miyao, S. Yoshikawa, M. H. Jin, A. Kikuchi, and H. Okano.** 1999. The Drosophila Ral GTPase regulates developmental cell shape changes through the Jun NH(2)-terminal kinase pathway. J. Cell. Biol. **146:**361–372.
55. **Sawamoto, K., C. Yamada, S. Kishida, Y. Hirota, A. Tagushi, A. Kikuchi, and H. Okano.** 1999. Ectopic expression of constitutively activated Ral GTPase inhibits cell shape changes during Drosophila eye development. Oncogene **18:**1967–1974.
56. **Schmitt, J. M., and P. J. Stork.** 2000. Beta 2-adrenergic receptor activates

extracellular signal-regulated kinases (ERKs) via the small G protein rap1 and the serine/threonine kinase B-Raf. J. Biol. Chem. **275:**25342–25350.
57. **Sebzda, E., M. Bracke, T. Tugal, N. Hogg, and D. A. Cantrell.** 2002. Rap1A positively regulates T cells via integrin activation rather than inhibiting lymphocyte signaling. Nat. Immunol. **3:**251–258.
58. **Sugihara, K., S. Asano, K. Tanaka, A. Iwamatsu, K. Okawa, and Y. Ohta.** 2001. The exocyst complex binds the small GTPase RalA to mediate filopodia formation. Nat. Cell Biol. **17:**17.
59. **Suzuki, J., Y. Yamazaki, G. Li, Y. Kaziro, and H. Koide.** 2000. Involvement of Ras and Ral in chemotactic migration of skeletal myoblasts. Mol. Cell. Biol. **20:**4658–4665.
60. **Tatusova, T. A., and T. L. Madden.** 1999. BLAST 2 sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol. Lett. **174:** 247–250.
61. **Tian, X., G. Rusanescu, W. Hou, B. Schaffhausen, and L. A. Feig.** 2002. PDK1 mediates growth factor-induced Ral-GEF activation by a kinase-independent mechanism. EMBO J. **21:**1327–1338.
62. **Toda, T., I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Broek, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler.** 1985. In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell **40:**27–36.
63. **Urano, T., R. Emkey, and L. A. Feig.** 1996. Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. EMBO J. **15:**810–816.
64. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
65. **Voss, M., P. A. O. Weernink, S. Haupenthal, U. Moller, R. H. Cool, B. Bauer, J. H. Camonis, K. H. Jakobs, and M. Schmidt.** 1999. Phospholipase D stimulation by receptor tyrosine kinases mediated by protein kinase C and a Ras/Ral signaling cascade. J. Biol. Chem. **274:**34691–34698.
66. **Vossler, M. R., H. Yao, R. D. York, M. G. Pan, C. S. Rim, and P. J. Stork.** 1997. cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell **89:**73–82.
67. **Walhout, A. J. M., R. Sordella, X. Lu, J. L. Hartley, G. F. Temple, M. A. Brasch, N. Thierry-Mieg, and M. Vidal.** 2000. Protein interaction mapping in C. elegans using proteins involved in vulval development. Science **287:**116–122.
68. **Wang, K. L., and B. D. Roufogalis.** 1999. Ca2+/calmodulin stimulates GTP binding to the ras-related protein ral-A. J. Biol. Chem. **274:**14525–14528.
69. **Ward, Y., W. Wang, E. Woodhouse, I. Linnoila, L. Liotta, and K. Kelly.** 2001. Signal pathways which promote invasion and metastasis: critical and distinct contributions of extracellular signal-regulated kinase and ral-specific guanine exchange factor pathways. Mol. Cell. Biol. **21:**5958–5969.
70. **Weinkove, D., T. P. Neufeld, T. Twardzik, M. D. Waterfield, and S. J. Leevers.** 1999. Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor. Curr. Biol. **9:**1019–1029.
71. **Wheeler, D. L., D. M. Church, A. E. Lash, D. D. Leipe, T. L. Madden, J. U. Pontius, G. D. Schuler, L. M. Schriml, T. A. Tatusova, L. Wagner, and B. A. Rapp.** 2002. Database resources of the National Center for Biotechnology Information: 2002 update. Nucleic Acids Res. **30:**13–16.
72. **White, M. A., C. Nicolette, A. Minden, A. Polverino, L. Van Aelst, M. Karin, and M. H. Wigler.** 1995. Multiple Ras functions can contribute to mammalian cell transformation. Cell **80:**533–541.
73. **White, M. A., T. Vale, J. H. Camonis, E. Schaefer, and M. H. Wigler.** 1996. A role for Ral guanine nucleotide dissociation stimulator in mediating Ras-induced transformation. J. Biol. Chem. **271:**16439–16442.
74. **Wieschaus, E., and C. Nüsslein-Volhard.** 1998. Looking at embryos, p. 179–214. *In* D. B. Roberts (ed.), Drosophila, a practical approach, 2nd ed., vol. 191. IRL Press, Oxford, United Kingdom.
75. **Wittinghofer, A., and C. Herrmann.** 1995. Ras-effector interactions, the problem of specificity. FEBS Lett. **369:**52–56.
76. **Wolthuis, R. M., and J. L. Bos.** 1999. Ras caught in another affair: the exchange factors for Ral. Curr. Opin. Genet. Dev. **9:**112–117.
77. **Wolthuis, R. M., N. D. de Ruiter, R. H. Cool, and J. L. Bos.** 1997. Stimulation of gene induction and cell growth by the Ras effector Rlf. EMBO J. **16:**6748–6761.
78. **Wolthuis, R. M., B. Franke, M. van Triest, B. Bauer, R. H. Cool, J. H. Camonis, J. W. Akkerman, and J. L. Bos.** 1998. Activation of the small GTPase Ral in platelets. Mol. Cell. Biol. **18:**2486–2491.
79. **Yaffe, M. B., G. G. Leparc, J. Lai, T. Obata, S. Volinia, and L. C. Cantley.** 2001. A motif-based profile scanning approach for genome-wide prediction of signaling pathways. Nat. Biotechnol. **19:**348–353.
80. **Zhao, Z., and S. A. Rivkees.** 2000. Tissue-specific expression of GTPas RalA and RalB during embryogenesis and regulation by epithelial-mesenchymal interaction. Mech. Dev. **97:**201–204.
81. **Zhu, J., Y. Qin, M. Zhao, L. Van Aelst, and R. Malinow.** 2002. Ras and Rap control AMPA receptor trafficking during synaptic plasticity. Cell **110:**443–455.
82. **Zwartkruis, F. J., R. M. Wolthuis, N. M. Nabben, B. Franke, and J. L. Bos.** 1998. Extracellular signal-regulated activation of Rap1 fails to interfere in Ras effector signalling. EMBO J. **17:**5905–5912.