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Thirty-One Flavors of Drosophila Rab Proteins

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ABSTRACT

Rab proteins are small GTPases that play important roles in transport of vesicle cargo and recruitment, association of motor and other proteins with vesicles, and docking and fusion of vesicles at defined locations. In vertebrates, >75 *Rab* genes have been identified, some of which have been intensively studied for their roles in endosome and synaptic vesicle trafficking. Recent studies of the functions of certain Rab proteins have revealed specific roles in mediating developmental signal transduction. We have begun a systematic genetic study of the 33 *Rab* genes in Drosophila. Most of the fly proteins are clearly related to specific vertebrate proteins. We report here the creation of a set of transgenic fly lines that allow spatially and temporally regulated expression of Drosophila Rab proteins. We generated fluorescent protein-tagged wild-type, dominant-negative, and constitutively active forms of 31 Drosophila Rab proteins. We describe Drosophila *Rab* expression patterns during embryogenesis, the subcellular localization of some Rab proteins, and comparisons of the localization of wild-type, dominant-negative, and constitutively active forms of selected Rab proteins. The high evolutionary conservation and low redundancy of Drosophila Rab proteins make these transgenic lines a useful tool kit for investigating Rab functions *in vivo*.

THE process of intracellular transport is important for almost every aspect of cellular function and for proper organism development. In highly compartmentalized eukaryotic cells, a large group of monomeric small GTPases, termed Rab proteins, orchestrate vesicle trafficking among distinct cellular membrane compartments, including cargo selection, vesicle budding, moving, tethering, docking, and targeting (Pfeffer 2001; Pfeffer and Aivazian 2004; Ali and Seabra 2005; Jordens *et al.* 2005; Pfeffer 2005). Rab proteins are members of the larger family of Ras-like GTPases, which regulate vesicle trafficking, transmembrane signal transduction, and cytoskeletal rearrangements, among other functions (Satoh *et al.* 1992a,b; Hernandez-Alcoceba *et al.* 2000).

Like most other small GTPases, Rab proteins undergo two alternate conformational transitions upon binding to either GDP or GTP. In response to signal stimuli, guanine nucleotide exchange factors interact with Rab GTPases, trigger their binding to GTP, and enable their interactions with various targets and effector proteins. GTPase-activating proteins work in the opposite direction, accelerating GTP hydrolysis and leaving GDP-bound Rab proteins inactive. In the GTP-bound active form, each Rab can interact with a different complex of proteins (effectors) to facilitate the delivery of transport vesicles to different acceptor membranes (Molendijk *et al.* 2004; Pfeffer and Aivazian 2004).

Mutations in *Rab* genes can affect cell growth, motility, and other biological processes. The first member of the Rab subfamily GTPases to be studied, Sec4p, was identified in yeast as an essential protein required for secretory vesicle exocytosis (Salminen and Novick 1987). Mammalian relatives of this yeast protein were identified and formally designated Rab (*ra*s-like genes in rat *b*rain) proteins. Different Rab proteins are found to be specifically associated with distinct subcellular membrane compartments and some have become standard markers for these compartments. Rab1 is present in the endoplasmic reticulum, Rab6 in the Golgi, Rab3 in synaptic vesicles, Rab5 in early endosomes, Rab7 and Rab9 in late endosomes, and Rab11 in the recycling endosome (Pfeffer 2001; Pfeffer and Aivazian 2004; Ali and Seabra 2005; Jordens *et al.* 2005; Pfeffer 2005).

Mutations affecting Rab GTPases and their regulatory proteins and effectors have been identified in multiple

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developmental disorders and malignancies. These include Griscelli syndrome, an autosomal recessive disorder caused by a mutation in *Rab27a* and characterized by pigment dilution in the hair and uncontrolled T-cell activation; choroideremia, an X-linked form of retinal degeneration with slow onset and progression caused by a mutation in Rab escort protein-1; and Hermansky–Pudlak syndrome, an autosomal recessive disorder caused by a mutation in Rab geranylgeranyl transferase and characterized by partial albinism and a tendency to bleed (Pereira-Leal *et al.* 2001a; Seabra *et al.* 2002). Mounting evidence also shows that Rab proteins may influence the progression of some cancers (Lanzetti *et al.* 2000; Cheng *et al.* 2004; Amillet *et al.* 2006). For example, Rab32, which is an A-kinase-anchoring protein, has recently been shown to be hypermethylated and inactivated by epigenetic silencing in colorectal and other cancers (Mori *et al.* 2004).

Most prior studies of Rab proteins have been carried out in extracts, yeast cells, or cultured mammalian cells. Although the different Rab proteins have similar sequences and share GTP/GDP recycling mechanisms, their upstream triggers, binding proteins, and downstream effects vary greatly. Much remains to be learned about how Rab proteins coordinate the control of vesicle movement/targeting with other key players and how proper cellular signaling is transduced by Rab-regulated vesicle trafficking.

Recent studies led to the appreciation that Rab proteins modulate signal transduction in development. Early embryonic cell fates are regulated by secreted signaling proteins such as Hedgehog (Hh), Wnt (int-1 in the mouse and wingless in Drosophila), and TGF-β/Dpp (Decapentaplegic). The spatial and temporal control of signal concentration is critical for normal development, and Rab-regulated intracellular trafficking regulates signal gradients and transduction. The signaling range of Dpp, a secreted protein that controls anterior–posterior patterning during Drosophila wing development, depends on the activity of Rab5, which controls early endocytic trafficking. Rab5 modulates Wnt signaling by targeting the Wnt protein to early endosomes (Seto and Bellen 2006). Constitutively active Rab7 causes increased destruction of Dpp signal and shortens its range of action (Entchev *et al.* 2000; Entchev and Gonzalez-Gaitan 2002). In the Hh pathway, Smoothened (Smo), a transmembrane protein that transduces Hh signals, translocates to the plasma membrane upon Hh stimulation. This relocalization affects its activity level, which can be blocked *in vivo* by inhibiting endocytosis with constitutively active Rab7. In contrast, dominant-negative Rab5 stabilizes Smo in the plasma membrane (Zhu *et al.* 2003). These results suggest that Rab proteins modulate Smo localization by regulating endocytosis and perhaps also exocytosis. Mouse Rab23 is a negative regulator of Hh signaling in the developing neural tube (Eggenschwiler *et al.* 2001, 2006; Evans *et al.* 2003, 2005; Guo *et al.* 2006; Wang *et al.* 2006). These studies clearly indicate that Rab proteins are important for controlling developmental signals to ensure proper morphogenesis and organismal growth.

We have chosen to create a tool kit for Drosophila Rab proteins to take advantage of three key opportunities. First, there are fewer Rab proteins in Drosophila than in vertebrates. Hence, there is less likelihood of redundant gene functions that may confound genetic analyses. Second, Drosophila genetics will be useful in identifying interacting genes and proteins. Third, most developmental signaling pathways are evolutionarily conserved from Drosophila to humans and are easily studied in the fly, offering opportunities to understand the roles of Rab proteins in developmental signal transduction. Characterization of Rab functions in flies is therefore likely to improve our understanding of the normal cellular functions of Rab proteins and the molecular nature of Rab-related diseases.

Most Drosophila Rab protein sequences can be clearly related to one or a few of the >75 vertebrate *Rab* genes. We identified 33 fly *Rab* genes and isolated cDNA clones representing 31 of them. We generated transgenic flies that can be stimulated to produce yellow fluorescent protein (YFP)-tagged wild-type, dominant-negative (DN; a T/S → N change that is GTP binding defective) and constitutively active (CA; a Q → L change that is GTPase defective) forms of each of the 31 Drosophila Rab proteins. Here we describe the generation of fluorescently tagged Drosophila Rab proteins and the transgenic animals, as well as the verification and initial characterization of the subcellular localizations of some of the tagged Drosophila Rab proteins both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Bioinformatics studies: To construct the tree of Rab protein sequence relationships, first the whole set of Drosophila Rab protein sequences were aligned using ClustalW 1.83. Second, pairwise distances of all Rab protein sequences were calculated using Blosum62. A neighbor-joining tree of Rab proteins was constructed on the basis of the distance matrix using MEGA3.1. The confidence in branch structure was ascertained using 1000 bootstrap samples from the original alignment, each of which was used to construct a Neighbor-Joining tree. The number shown at each branchpoint indicates the percentage of time that a particular branch appeared in these 1000 trees. The length of each branch indicates the distance calculated based on Blosum62 between any pair of proteins.

Cloning, construction, germline transformation, and crosses: cDNAs of 31 Drosophila *Rab* genes were amplified from Drosophila embryo total RNA and inserted into pDONR201 (Invitrogen, San Diego) to generate pENTR-Rab constructs according to the manufacturer's instructions. No PCR products were obtained using primers for the remaining two Rab genes predicted from the genome sequence. Site-directed mutagenesis was performed to generate the DN and CA versions of each Rab. A T/S → N change was designed to obtain the DN form while a Q → L change was designed to obtain the CA form of each Rab protein (with some exceptions

as indicated on supplemental Table 2 at http://www.genetics.org/supplemental/). An N-terminal YFP- or dsRed2-tagged pUASp and pUAST construct was fused with the Gateway (Hartley *et al.* 2000; Walhout *et al.* 2000) cassette fragment and cloned into the destination UAS construct. LR recombination assays were performed with each version of pENTR-Rab (now served at the entry vector) and the destination vector (pUAS-YFP-ccd/pUAS-dsRed2-ccd) to generate the final N-terminal YFP- or dsRed2-tagged *Rab* transgene. YFP and dsRed2 tags were purchased from CLONTECH (Palo Alto, CA).

Purified DNA containing each construct was injected to establish transgenic Drosophila lines. Multiple lines of flies that carried each *P* element were recovered and analyzed.

All fly crosses described here were performed on standard media at 22°–25°.

DNA isolation/inverse PCR: Genomic DNA isolation and determination of the flanking sequence of the insertions by inverse PCR were performed as described previously (Bellen *et al.* 2004). The procedures and primers used were as for the EY collection (*P{EPgy2}* insertions) described in Bellen *et al.* (2004).

***In situ* hybridization:** Whole-mount wild-type Drosophila embryos collected 0–18 hr after egg laying were fixed according to the standard protocol (Zhu *et al.* 2003). *In situ* hybridization was carried out using DIG-labeled riboprobes. Sense and antisense riboprobes were generated by *in vitro* transcription using a linearized plasmid of pCR4 containing the full-length coding sequence of each *Rab* gene.

Cell culture, transfection, and antibody staining Drosophila S2R+ cells (a line derived from embryos) were cultured essentially as described previously (Yanagawa *et al.* 1998). A total of 2×10^5 cells were seeded in a 24-well plate 1 day prior to transfection. Cells were cotransfected with the pUAS construct and pActin5c-GAL4 using Effectene (QIAGEN, Valencia, CA). A total of 200 ng of DNA was used in total for each well. Forty-eight hours after transfection, cells were fixed in 4% paraformaldehyde for 20 min at room temperature and examined with confocal microscopy. Mammalian HeLa cells were cultured in DMEM (Life Technologies) medium supplemented with 10% fetal bovine serum. Cells were fixed and images were taken between 12 and 24 hr on a Leica TCS-SP5 confocal microscope. Primary antibody against Myc (Santa Cruz Biotechnology) was used at a dilution of 1:500. Anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes, Eugene, OR) was used at a dilution of 1:1000. DAPI was used to stain the nuclei in all the cell cultures.

Tissue dissection and antibody staining: Tissue collection, fixation, and staining were performed using standard procedures (Zhu*et al.* 2003). Antibody dilutions were used for primary antibodies raised against Chaoptin (mAb 24B10), 1:50 (Van Vactor *et al.* 1988); Drosophila Rab5, 1:50 (Wucherpfennig *et al.* 2003); and mouse Rab11, 1:250 (BD Biosciences). Secondary antibodies conjugated to Cy3 or Cy5 (Jackson ImmunoResearch, West Grove, PA) were used at 1:250. All antibody incubations were performed at 4° overnight in the presence of 5% normal goat serum. All fluorescent images were taken on a Zeiss LSM510 confocal microscope.

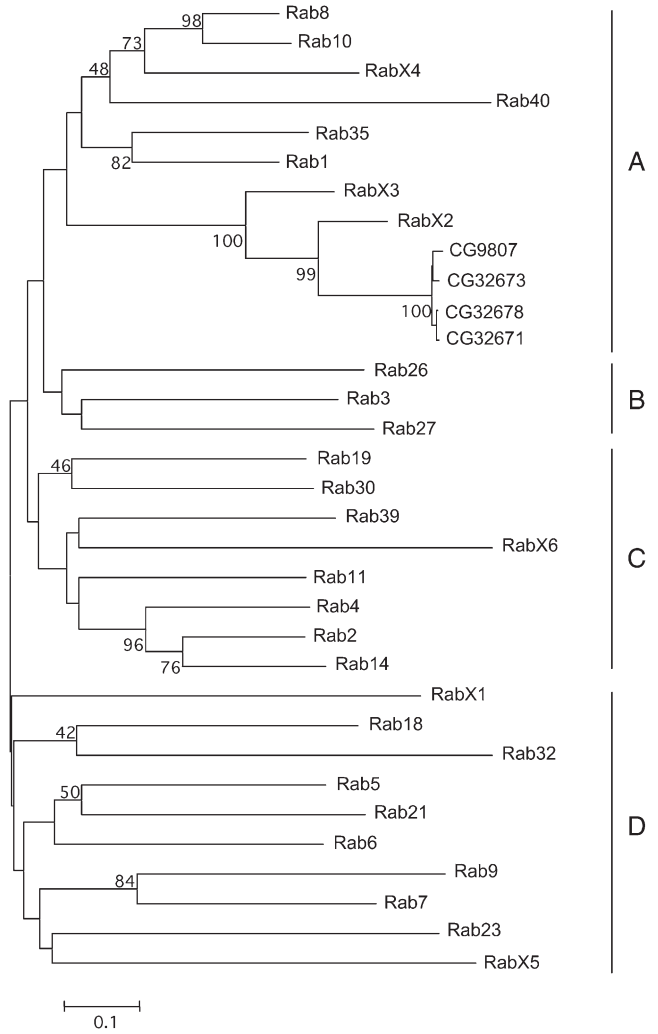

Figure 1.—Phylogenetic tree of 33 predicted Drosophila Rab proteins. The number shown between each pair of branches is the bootstrap value that measures how consistent the data are. The value is calculated from a new data set (a pseudosample) by randomly copying one character from the original data matrix. It represents the percentage of 1000 bootstrap pseudosamples with replacement supporting that branch. Only bootstrap values >40% are shown. The length of the unit represents the divergence of proteins.

RESULTS

Identification of all members of the Drosophila *Rab* gene family: By searching the *Drosophila melanogaster* genome sequence (Release 4.3), we found that the fly *Rab* gene family consists of 33 members. To identify the *Rab* genes, we took advantage of the high evolutionary conservation of Rab protein sequences. These sequences have features common to the GTPases of the Ras superfamily, as well as Rab-specific motifs that cluster in and around the "switch" regions. Switch sequences are involved in the transition between the GDP- and GTP-bound conformations (Pereira-Leal and Seabra 2001).

The Drosophila Rab proteins were aligned using CLUSTALW 1.8 Multiple Sequence Alignment (Jeanmougin *et al.* 1998). The alignment is shown in supplemental Figure 1 at http://www.genetics.org/supplemental/. A neighbor-joining tree was constructed using BLOSUM matrix and other default parameters (Figure 1). The neighbor-joining algorithm is an effective method for reconstructing phylogenies. It is capable of clustering sequences that have substantially variable rates of

change during evolution (Saitou and Nei 1987). Rab proteins were classified into four major "branches" (A–D in Figure 1). The proteins within branches A–C are more closely related to each other than to proteins in the D group.

Compared to a previously published study (Pereira-Leal and Seabra 2001), we have identified four "new" Drosophila *Rab* genes in release 4.3: CG9807, CG32671, CG32673, and CG32678. The sequences of the four predicted Drosophila Rab proteins are 98% similar. The genes are located in a cluster on the X chromosome at cytological location 9D-F. Two previously identified Drosophila *Rab* genes, *RabX2* and *RabX3*, are located nearby at 9C1 and 9F13. The six proteins are in branch A of the phylogenetic tree (Figure 1A). Their sequence similarity and proximity on the X chromosome suggest that they evolved relatively recently. The same cluster is also observed in genomes of other Drosophila species (http://rana.lbl.gov/drosophila/) but not in mouse and human genomes (http://genome.ucsc.edu). An interesting feature of these six genes is that they have only one protein-coding exon while other Drosophila *Rab* genes have multiple coding exons. The six genes may be derived from duplication and rearrangement events (Presgraves 2005).

The expression patterns of *Rab* genes in the embryo: Many vertebrate *Rab* genes are widely or ubiquitously expressed, but some are transcribed in tissue- or organ-specific patterns (Ayala *et al.* 1989; Nagata *et al.* 1990; Bao *et al.* 1998). Tissue-specific expression may provide clues about the biological functions of Rab proteins. If a tissue has a special secretory role, then a Rab expressed only in that tissue may control a specific type of secretion. For example, mammalian Rab27A protein is produced specifically in melanocytes and cytotoxic T lymphocytes. In keeping with its expression pattern, this Rab controls melanosome transport in melanocytes (Chen *et al.* 1997) and lytic granule exocytosis in cytotoxic T lymphocytes (Stinchcombe *et al.* 2001). Mutation of the *Rab27A* gene causes the human diseases Griscelli syndrome, Hermansky–Pudlak syndrome, and choroideremia. These diseases are characterized by pigment dilution in the hair and uncontrolled T-cell activation, reflecting the gene's specific functions in two cell types (Stinchcombe *et al.* 2001).

To explore when and where Drosophila *Rab* genes are transcribed during embryonic development, whole-mount *in situ* hybridizations were performed. Twenty-one of the Drosophila *Rab* genes are ubiquitously expressed, although in some instances with higher levels in certain tissues (supplemental Table 1 at http://www.genetics.org/supplemental/). Examples of *in situ* hybridization patterns for *Rab* genes are shown in Figure 2. In embryos (Figure 2A), Drosophila *Rab5* mRNA is ubiquitous but much more abundant in the garland cells, a group of cells that may function as nephrocytes and that have a rapid rate of fluid-phase endocytosis (Koenig and Ikeda 1990). A similar staining pattern is observed in third instar larvae: *Rab5* RNA is enriched in the garland cells that surround the esophagus (Figure 2A′). Drosophila *Rab3*, *Rab2*, *Rab26*, and *RabX4* (Figure 2, B, C, D, and E, respectively) are expressed mostly in the nervous system, whereas Drosophila *Rab32* (Figure 2F) is expressed in the Malpighian tubules, which have kidney-like functions. Finally, the expression pattern of *Rab30* represents the majority of *Rab* genes; it is expressed in multiple tissues throughout embryogenesis (Figure 2G; data not shown). These data suggest that certain tissues and organs may use a distinctive set of trafficking or signaling proteins for their development or functions.

Generating a set of YFP-tagged Drosophila Rab proteins for determining Rab functions: Of the 33 Drosophila *Rab* genes found in the genome, we succeeded in isolating 31 using RT–PCR to amplify mRNA sequences from total embryo RNA. CG32671 and CG32678 were not recovered. They may be expressed at low levels or not at all in embryos. They are among the four newly identified Drosophila *Rab* genes that are highly similar to each other and were not investigated further. For the other 31 genes, the cDNAs were cloned into vectors to create fusion proteins with a YFP tag at the N terminus of the Drosophila Rab protein in an arrangement suitable for *P*-element-mediated transformation of flies. The transformation vectors are either pUAST (Brand and Perrimon 1993; Brand *et al.* 1994) or pUASp (Rorth 1998) so that the inserted fusion genes can be expressed under the control of the yeast GAL4 transcription factor, allowing spatio-temporal control of expression with a large number of available Drosophila GAL4-driver strains (Brand *et al.* 1994). Multiple transgenic flies were generated and the insertion sites were mapped by inverse PCR (information on the lines contributed to the Bloomington Stock Center is in Table 1 and supplemental Table 3 at http://www.genetics.org/supplemental/).

To test whether YFP-tagged proteins function as wild-type proteins, we overexpressed *UAS-YFP-Rab11WT* in a ubiquitous manner in flies homozygous for a previously isolated *Rab11* null mutation (Dollar *et al.* 2002). A single copy of the transgene rescued the zygotic lethality to adulthood (data not shown). Therefore at least this YFP-tagged Rab protein is capable of replacing the endogenous gene. Nonetheless, anyone employing the lines that we have made is advised to check the level of wild-type activity carefully in their particular assay.

To create transgenes encoding DN forms of different Drosophila Rab proteins, GTP-binding-defective proteins were generated by mutating the T/S amino acids in the GTP-binding domain to N. To obtain CA forms of Drosophila Rab proteins, GTPase-defective Q → L changes were created. Although other amino acid changes have been used to produce DN and CA Rab proteins, these two types of mutation have been used

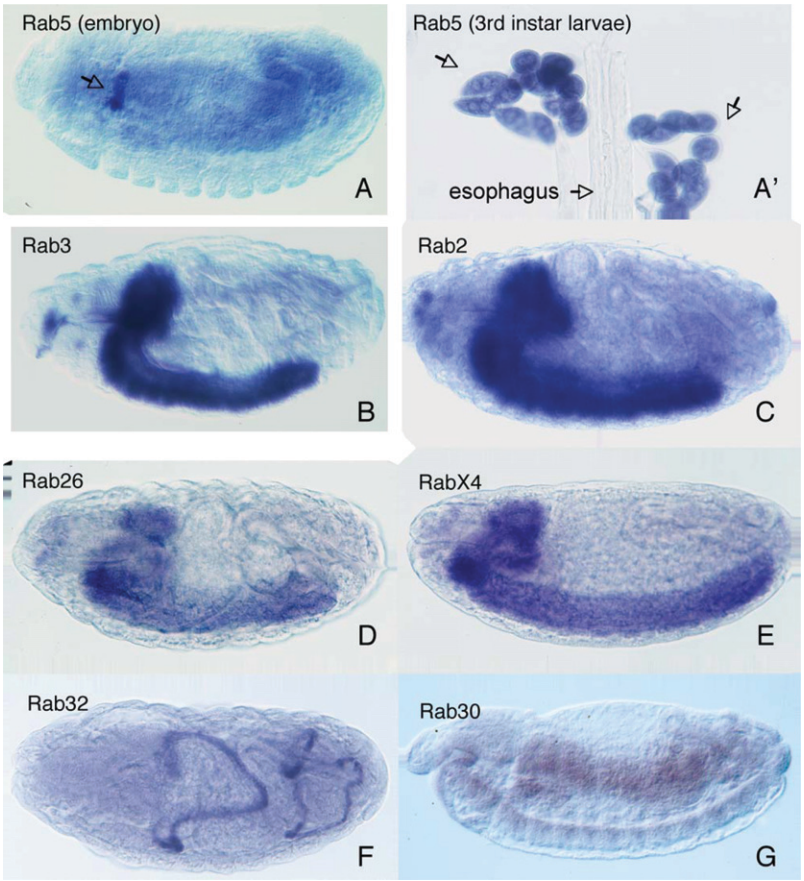

Figure 2.—*In situ* hybridization of Drosophila *Rab* gene probes to localize transcripts in Drosophila whole-mount embryos. For each *Rab* gene, one stained preparation of a particular embryonic stage is shown. The stage that has the most representative staining pattern is shown. (A and A′) *Rab5* mRNA signals in embryos (A) and third instar larvae (A′). (B–G) *In situ* patterns of Rab3, Rab2, Rab26, RabX4, Rab32, and Rab30.

extensively in other laboratories and demonstrated to be effective in many tested Rab proteins (Feng *et al.* 1995; Press *et al.* 1998; Dinneen and Ceresa 2004a,b; Pasqualato *et al.* 2004). Some of the Rab proteins, including Rab18, Rab40, RabX2, RabX3, RabX6, CG9807, and CG32673, do not have the conserved T/S or Q amino acid in the GTP- or GDP-binding domain. In these cases we mutated the amino acid in the position corresponding to N or L (supplemental Figure 1 and supplemental Table 2 at http://www.genetics.org/supplemental/). These mutated Rab proteins may or may not act like typical DN or CA Rab proteins.

Colocalization of corresponding Mus and Drosophila Rab proteins: The sequence similarity between fly and mammalian Rab proteins raises the question of whether corresponding proteins are also functionally conserved and located in the same intracellular compartments. We first compared the localization of putative fly and mouse orthologs by cotransfecting mouse and fly *Rab* genes into mammalian cells and observing the proteins with double labeling. It is not entirely clear which intracellular compartments in fly and mammalian cells are analogous, so differences in localization could reflect nonconserved protein functions or changes in the nature of cellular compartments. Rab protein localization may help in resolving the remaining questions about which compartments are functionally comparable between species.

We used three well-characterized mouse *Rab* genes (5, 7, and 11), each one encoding an N-terminal YFP tag, for transfection studies. Transgenes encoding presumed mammalian and dsRed2-tagged Drosophila Rab orthologs were cotransfected into mammalian HeLa cells. Each corresponding pair of mammalian and Drosophila versions of Rab5, Rab7, and Rab11 colocalized, suggesting that the targeting systems in mammalian cells are able to recognize and properly transport the fly Rab proteins (Figure 3, A–C). The localization pattern of each pair of Rab proteins seems different from the others. To test this, mammalian *Rab5* and Drosophila *Rab7* were coexpressed and the proteins were observed to be in distinct membrane compartments (Figure 3D). This rules out the possibility that overexpression, and consequent clogging of cell transport systems, leads to accumulation of proteins in the same intracellular compartment.

Comparing the localization of YFP-tagged Rab proteins to endogenous proteins: Functional fluorescent Rab chimeras using GFP, YFP, CFP, and RFP attached to the Rab NH2 terminus have been widely used in cell biology to observe Rab intracellular movements. In mammalian cultured cells, the presence of fluorescent proteins at the N terminus of Rab9 causes less efficient membrane association, probably due to differences in prenylation efficiency and/or differences in recruiting downstream effectors (Barbero *et al.* 2002).

TABLE 1

Characterization of selected transgenic UAS-YFP-Rab Drosophila strains

Rab	Annotation symbol	WT construct/line information	DN construct/line information	CA construct/line information
Rab1	3320	UAST/01(80C1)	UAST/01(87B8); 04(58B9)	UASp/12a(68C13); 12c(1E4)
Rab2	3269	UAST/02(86E18)	UAST/01(42C7); 05(85E1)	UASp/02(61C8); 25(46E6)
Rab3	7576	UASp/02(55B9); 05b(95E1)	UASp/04L(25C6)	UASp/11(76D1); 29a(59E3)
Rab4	4921	UASp/9(28B1); 32(100A7)	UASp/37(32D2); 46b(72D7)	UASp/13(33C1); 28(76A1)
Rab5	3664	UASp/02(94A14); 08b(59F5)	UASp/01(44B8); 02(75B2)	UASp/01a(65A9); 24(24C5)
Rab6	6601	UAST/01(57F6)	UAST/01(23F3); 03(92B3)	UASp/05(61C9); 23(3B6)
Rab7	5915	UASp/18(96C1); 21(21B2)	UASp/06(79A2); 19(10E6)	UASp/14(100D2); 19(22A1)
Rab8	8287	UASp/09(68C2); 45(42E7)	UASp/09(42E1); 12(83B4)	UASp/05(27F4); 10(63F5)
Rab9	9994	UASp/13(21B7); 22(79E4)	UASp/04(70D7); 11(33A2)	UASp/10(31F4); 20(61C9)
Rab10	17060	UASp/13(34C4); 21(65A9)	UASp/15(23C5); 25a(97D2)	UASp/01(35D1); 27a(86C5)
Rab11	5771	UASp/32(89B7)	UASp/06(88F1); 35(59A3)	UASp/24(43C5); 31(61C8)
Rab14	4212	UASp/5L(22E1); 12(100D1)	UASp/01(36B2); 02(99F6)	UASp/02(34D1); 07(67C1)
Rab18/RP4	3129	UAST/01(82C5)	UAST/02(30B5) 03(87B8)	UASp/02(99A11); 12(37C1)
Rab19/RP3	7062	UAST/02(39B4)	UAST/04(70E2); 05(53D14)	UASp/06(62A6); 15(51E2)
Rab21	17515/40304	UAST/04(25B1)	UAST/01(99A5); 03(58A3)	UASp/02(53F8); 09(4F2)
Rab23	2108	UASp/01(93D4); 02(21C2)	UASp/01(86E11); 02(58E1)	UASp/04(66D12)
Rab26	7605	UAST/01(73D5); 05(35D1)	UAST/02(48A3); 03(74D2)	UAST/01(83C4); 04(54E2)
Rab27	14791	UASp/01(35D1); 14(86E11)	UASp/02(89A5)	UASp/05(23A3); 07(3C1)
Rab30	9100	UASp/10(53C9)	UASp/07(98A7)	UASp/11(39E3)
Rab32/RP1	8024	UASp/03(27F1); 11(76D5)	UASp/04(42C8)	UASp/03(85A5); 08(47F8)
Rab35	9575	UASp/15(51A2)	UAST/01(23C4); 06(68B1)	UASp/01(95F1); 11(37B13)
Rab39	12156	UASp/13(58D4)	UAST/04(37B1); 05(76F1)	UASp/02(5C2); 14(92B3)
Rab40	1900	UAST/03(61C1); 05(60F5)	UAST/01(91F4); 04(26D1)	UASp/07(22D1); 13(61B3)
RabX1	3870	UASp/10(84F6); 12(27D3)	UAST/01(44B3); 14(62B1)	UASp/02(92B3); 10(25C6)
RabX2	2885	UASp/08(92C1)	UASp/02(24A2); 05(67B11)	UASp/10(22B1); 21(84F6)
RabX3	32670/2532	UASp/24(94A1); 38(50B6)	UAST/01(47A7); 03(70C10)	UASp/19(98A7)
RabX4	31118/13638	UASp/19(94A1)	UAST/02(91F11); 03(23F3)	UASp/09(44B8); 13a(66D10)
RabX5	7980	UASp/13b(27D3); 22b(77B4)	UAST/01(47C1)	UASp/10(6C8)
RabX6	12015	UASp/03(79A2); 04(39B4)	UAST/01(31F4); 02(82C3)	UASp/13(60A3); 27(73B5)
CG9807	9807	UASp/21(chr.2); 25(75E2)	UAST/01(49F11); 03(82D5)	UASp/04(56F11); 14b(96E6)
CG32673	32673	UASp/35(97D7); 42(28C4)	UAST/01(57E1); 02(87A2)	UASp/05(80A4); 17(26B2)
CG32671	32671			
Rab9D	32678			

Only one line from each chromosome (X, II, or III) and a maximal two lines for each gene were selected to send to the stock center. The detailed information is in this table and in supplemental Table 3 at http://www.genetics.org/supplemental/. Columns indicate Drosophila Rab names, CG numbers, the construct that was used to generate the transgenic lines, and the insertion sites identified by inverse PCR. CG32671, CG32673, CG9807, and CG32678 are very similar and our PCR identified CG9807 and CG32673 products.

To explore this issue in flies, we compared YFP-Rab5 and YFP-Rab11 protein localization with endogenous Drosophila Rab5 and Rab11 in wild-type photoreceptor cells. To characterize the YFP signal precisely with respect to photoreceptor cell boundaries, we costained the photoreceptors (PR) with an antibody that labels PRs, mAb 24B10 (Zipursky 1982).

Endogenous Drosophila Rab5 has a localization pattern that closely mimics the YFP-Rab5WT (Figure 4, A and B), including the punctae present in the apical domain (arrowheads in Figure 4, A′and B′). We also compared the localization of endogenous Rab11 protein and overproduced YFP-Rab11WT in photoreceptors. Rab11, which is required for the trafficking of rhodopsin to photoreceptor rhabdomeres, accumulates in a distinct ring-like pattern at the base of photoreceptor rhabdomeres (Satoh *et al.* 2005). YFP-Rab11WT produced in photoreceptors exhibited a very similar distinct ring-like localization pattern (compare arrows in Figure 4, C and D).

To further compare the localization of endogenous Rab5 and tagged Drosophila Rab5, we produced YFP-tagged Rab5WT in S2R+ cells and stained the cell with anti-Rab5 antibody. This experiment has an inherent limitation, since the antibody will detect both endogenous protein and the YFP-tagged protein. The Rab5 antibody signal colocalized with the YFP-Rab5WT signal, detected by fluorescence (Figure 4E). The interpretation is that the endogenous protein is not in any location that the YFP-tagged protein fails to reach. Similarly, Rab11 detected by antibody, and YFP-Rab11 detected by fluorescence, colocalized in S2R+ cells (Figure 4F). In summary, these data show that there is no detectable cellular location where only the endogenous Rab

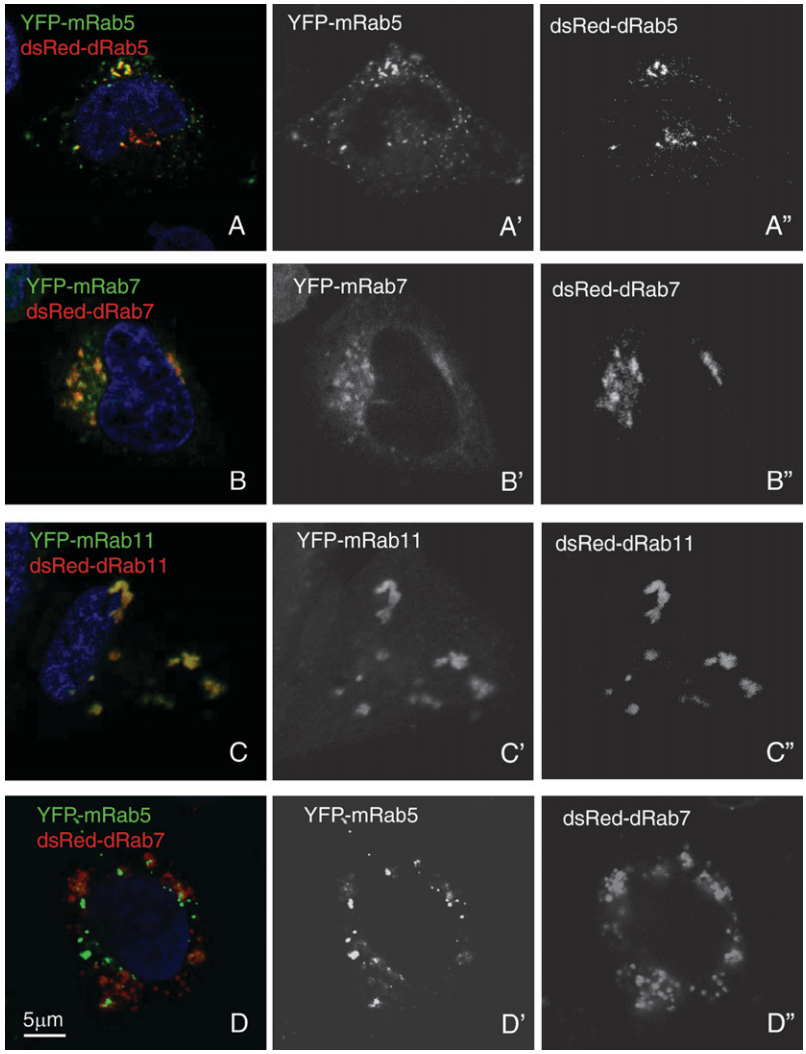

FIGURE 3.—Conserved subcellular localization of mammalian and fly Rab proteins. The mammalian versions of well-characterized Rab5, Rab7, and Rab11 were cloned with a N-terminal YFP tag (shown in green and gray). Each pair of mammalian and dsRed-tagged Drosophila Rab proteins 9 (shown in red and gray) was cotransfected into HeLa cells and visualized with a Leica confocal microscope. Drosophila Rab5, Rab7, and Rab11 are localized in a punctate pattern and colocalized with mammalian Rab5 (A–A′′), Rab7 (B–B′′), and Rab11 (C–C′′), respectively. As a control, mammalian Rab5 and Drosophila Rab7 are not colocalized (D–D′′).

protein is found, although we cannot determine whether the YFP-tagged protein is in abnormal locations. To test this possibility, we introduced a myc-2xFYVE transgene, an endosome marker (WUCHERPFENNIG *et al.* 2003), together with YFP-Rab5WT (Figure 6C). YFP-Rab5WT was present mainly in FYVE-marked early endosomes. Such tests for an individual YFP-Rab protein in comparison to independent markers are recommended in the context of any particular tissue and developmental stage.

Intracellular Drosophila Rab protein locations *in vivo*: Mammalian Rab proteins serve as useful markers of different intracellular compartments, reflecting their highly specialized roles in controlling trafficking events between particular compartments. The limited knowledge of compartments and trafficking in Drosophila cells makes Rab proteins potentially valuable as markers for different compartments. As a first step, we applied this approach to larval eye–brain complexes, where highly stereotyped architecture makes subcellular localization particularly interesting. Drosophila photoreceptor neurons are highly polarized cells that allow us to assess YFP-Rab protein localization in brain cell bodies and synapses and relate these observations to known Drosophila Rab protein localization and functions (Figure 5). For each assay we tested at least two individual transgenic lines to minimize the effects of different expression levels that may be caused by transgenic lines. We examined the location of all 31 Drosophila Rab proteins for each version—wild type (WT), DN, and CA—and observed obvious differences between the localization of different Drosophila Rab proteins. Here we report the subcellular localization of WT, DN, and CA forms of some of the better-characterized Rab proteins: Drosophila Rab3, Rab4, Rab5, Rab7, and Rab11. The locations of the WT versions of these proteins have been reported previously and provide a reference for their localization.

The WT version of YFP-Rab3 (Figure 5, C and D), a known synaptic vesicle protein, is localized almost exclusively to synaptic terminals in the brain (Figure 5D; DIANTONIO *et al.* 1993; GEPPERT *et al.* 1997). The DN and CA versions of YFP-Rab3 are more abundant in cell bodies and less abundant in synapses when compared to the wild-type protein (Figure 5, A and E *vs.* C; Figure 5, B

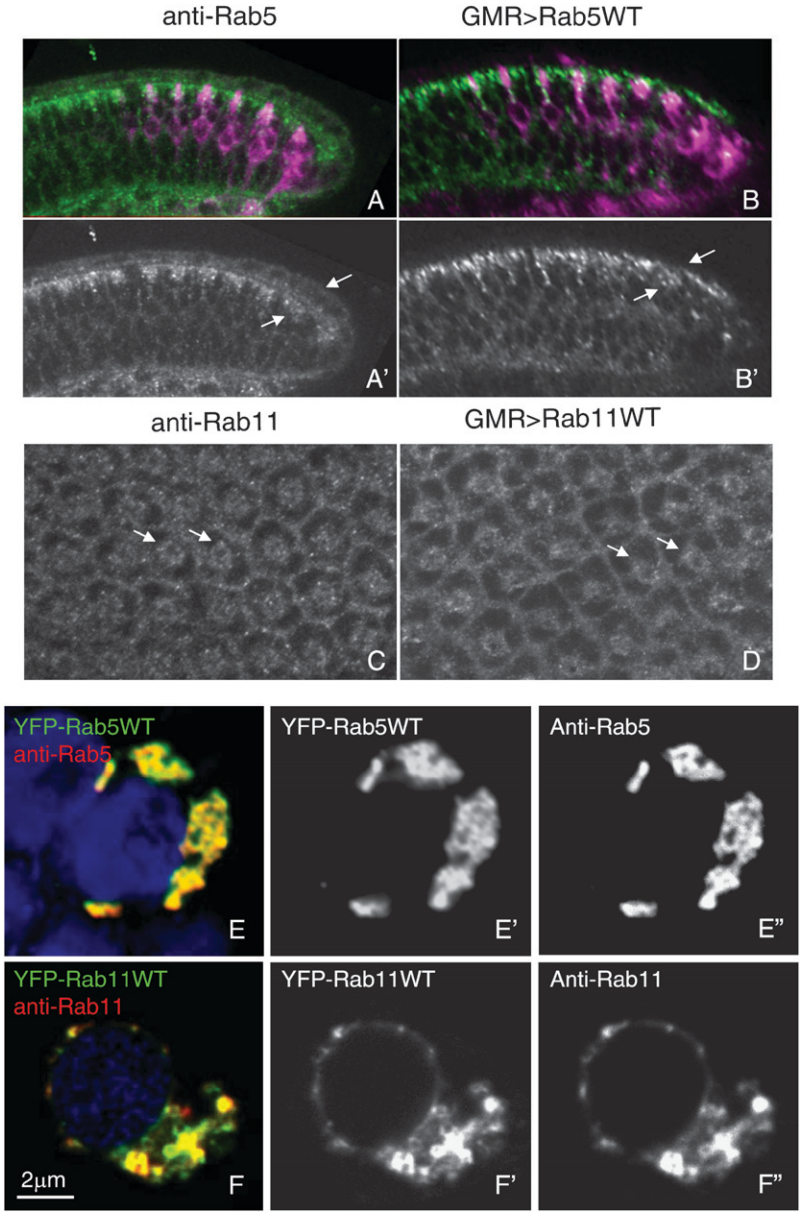

FIGURE 4.—Colocalization of YFP-Rab5WT and YFP-Rab11WT with endogenous Rab5 and Rab11 proteins. (A and B) Longitudinal sections of third instar larval eye discs. Photoreceptor stainings with mAb 24B10 are in magenta. A and A′ show Rab5 antibody staining in green and gray, respectively, revealing a punctate localization that is enriched distally (between arrows). B and B′ show YFP-Rab5WT driven in photoreceptors with GMR-GAL4. The overexpressed fusion protein exhibits the same localization pattern as the endogenous protein. (C and D) Anti-Rab 11 staining (C) and photoreceptor-driven YFP-Rab11WT (D) in third instar eye imaginal discs. The cross sections reveal very similar localization patterns. Note the ring-like structure of Rab11-positive vesicles around the rhabdomeres (arrows). (E and E′′) Colocalization of endogenous Rab5 and exogenous tagged Rab5 proteins in S2R+ cells. Cells were transfected with YFP-Rab5WT and then fixed and stained with anti-Rab5 antibody (red and gray). (F and F′′) Colocalization of endogenous Rab11 and exogenous tagged Rab11 proteins in S2R+ cells. Cells were transfected with YFP-Rab11WT and then fixed and stained with anti-Rab11 antibody (red and gray).

and F *vs.* D), suggesting a defect in protein trafficking or organelle distribution (or both) in loss- and gain-of-function mutants.

Mammalian Rab4 has been reported to accumulate at the cytosolic surface of endosomes in Chinese hamster ovary cultured cells (VAN DER SLUIJS *et al.* 1991, 1992). We find that YFP-Rab4WT exhibits a punctate localization pattern in the cytoplasm of cell bodies (Figure 5I), with little protein at synaptic terminals (Figure 5J). DN and CA versions of YFP-Rab4 are present at higher levels in axons than is YFP-Rab4WT (Figure 5, H and L *vs.* J). Furthermore, both the CA and the DN version lack the characteristic punctate cell body distribution (Figure 5, G and K *vs.* I). In mammalian cells, the production of WT or CA versions of Rab4 changes the morphology of the transferrin compartment and causes the formation of membrane tubules, while production of Rab4DN significantly reduces vesicle recycling and degradation (MCCAFFREY *et al.* 2001). Our data also suggest that there is aberrant formation and/or distribution of the cellular compartment with which Rab4 is normally associated. Alternatively, the mutant proteins are unable to reach their normal location.

Rab5 is an endosome protein that is critical for endosome fusion along the endocytic pathway. The native Rab5 protein is present in endosomes at synaptic terminals as well as in cell bodies (WUCHERPFENNIG *et al.* 2003). We observed the YFP-Rab5WT fusion protein in a punctate pattern in cell bodies and in nerve terminals (Figure 5, O and P) in agreement with previously published data (WUCHERPFENNIG *et al.* 2003). The mutations in YFP-Rab5DN and YFP-Rab5CA affect Rab5

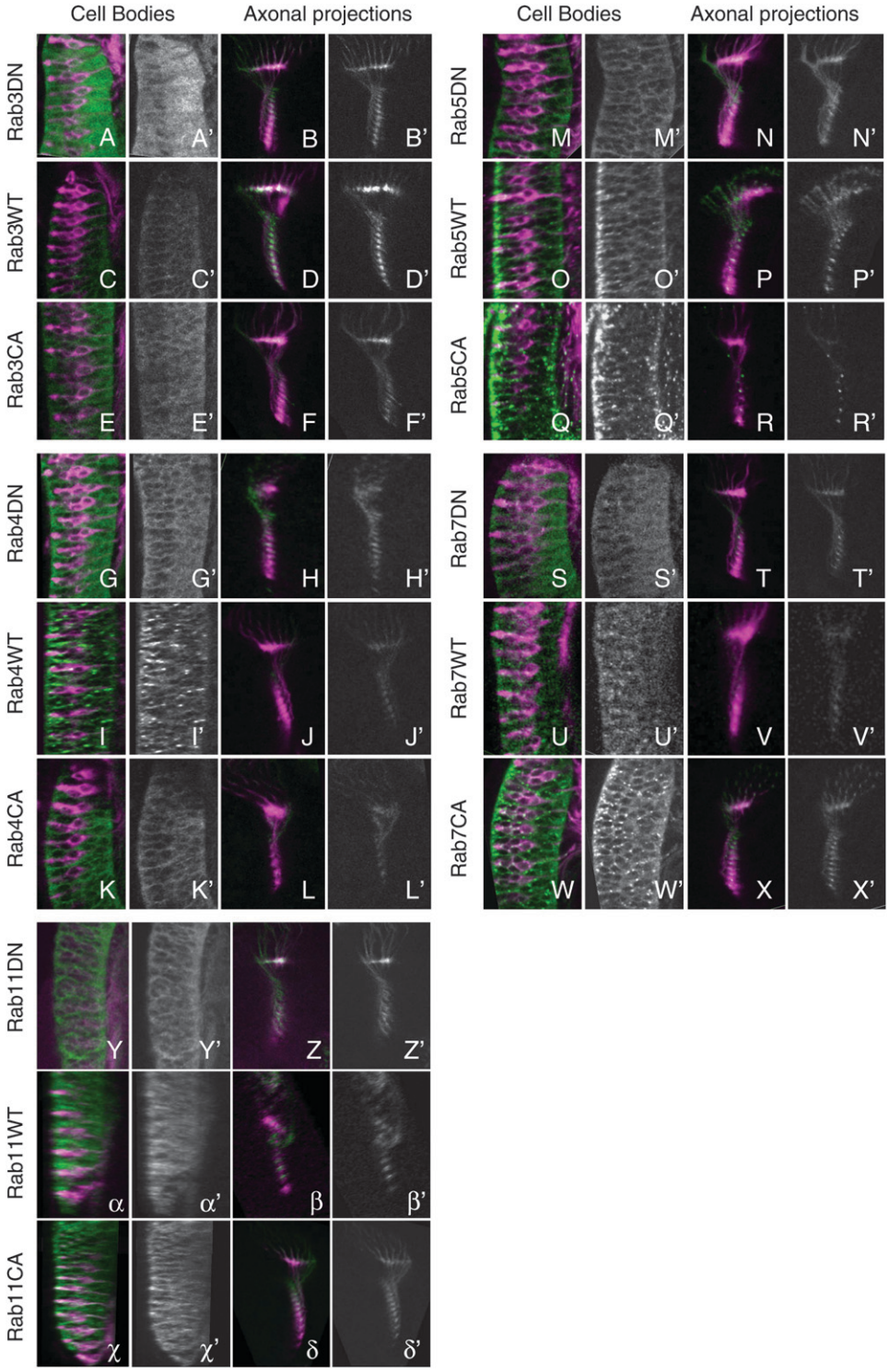

FIGURE 5.—Comparison of tagged protein localization of WT, DN, and CA versions of Drosophila Rab proteins. YFP-Rab variants were expressed specifically in photoreceptors of transgenic flies using GMR-GAL4. Images of third instar eye-disc sections and terminal axonal projections of photoreceptors in the optic lobe are shown for Rab3, Rab4, Rab5, Rab7, and Rab11. Tissues were stained with the photoreceptor-specific antibody mAb 24B10 (magenta). YFP-Rab proteins are shown in green and gray.

protein localization, as corresponding mutations did for Rab4. YFP-Rab5DN has a less punctate pattern in the cell body and nerve terminals (Figure 5, M and N) than the WT (Figure 5, O and P). YFP-Rab5CA, on the other hand, has increased intensity in the punctae and they are larger in size than wild type Rab5, especially at the apical end of cell bodies where most early endosomes are concentrated (Figure 5, Q and R *vs.* O and P). To further investigate whether Rab5WT retains its normal distribution, we coexpressed dsRed-Rab5WT with YFP-Rab5DN or YFP-Rab5CA in S2R+ cells. dsRed-Rab5WT and YFP-Rab5CA were mainly colocalized (Figure 6B), suggesting that both WT and CA forms are localized to early endosomes, the correct subcellular compartments. YFP-Rab5DN and dsRed-Rab5WT, in contrast, are not colocalized (Figure 6A). Rab5DN protein was

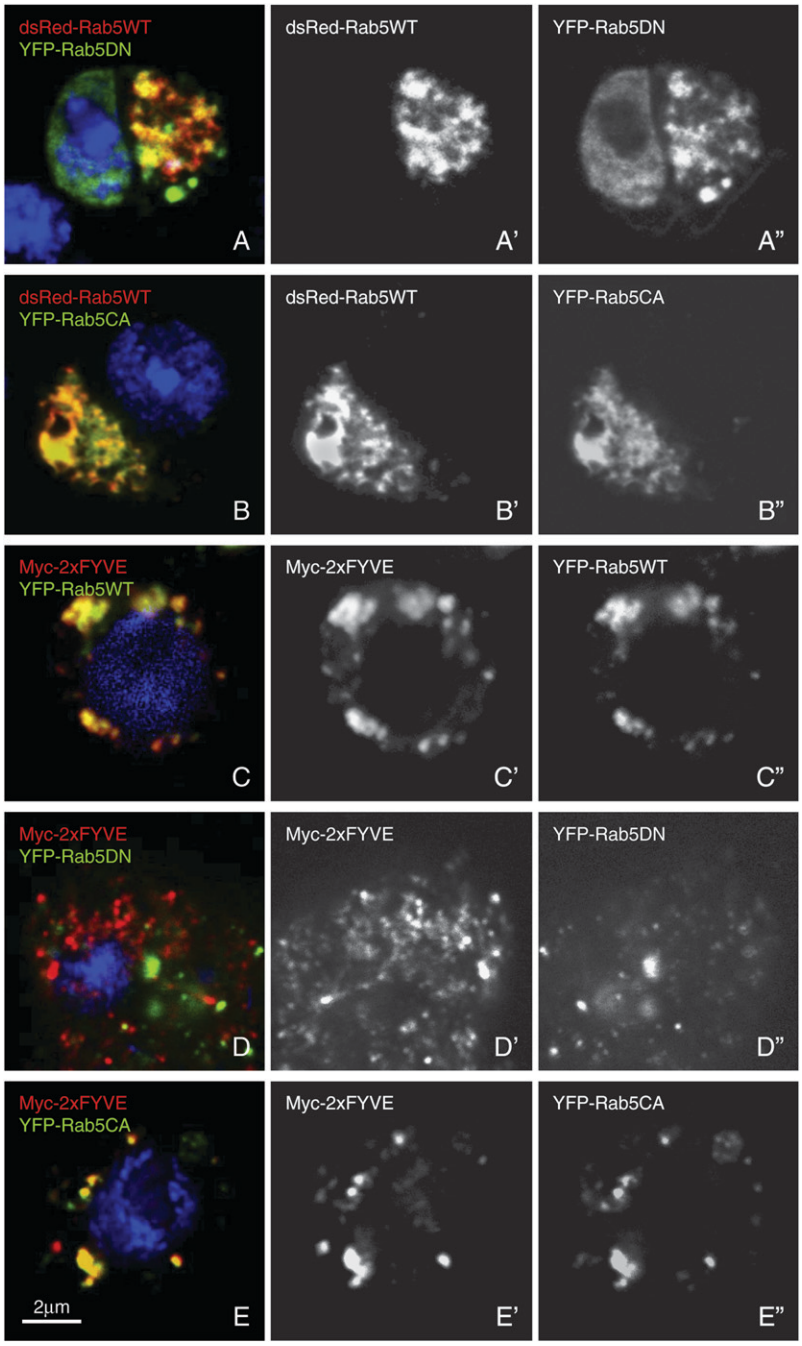

FIGURE 6.—Reduction of YFP-Rab5DN endosomal localization. (A and B) dsRed-Rab5WT (red and gray) and YFP-Rab5DN (A; green and gray) or YFP-Rab5CA (B; green and gray) were coexpressed in cultured Drosophila S2R+ cells. dsRed-Rab5WT and YFP-Rab5CA are mainly colocalized while dsRed-Rab5WT and YFP-Rab5DN are not. (C–E) A pUAST-myc-2xFYVE (red and gray) construct that labels early endosomes was cotransfected with pUAST-YFP-Rab5WT (C), pUAST-YFP-Rab5DN (D), or pUAST-YFP-Rab5CA (E). YFP-Rab5WT and YFP-Rab5CA are colocalized mainly with Myc-2xFYVE whereas YFP-Rab5DN is not.

more dispersed (Figure 6A′′), suggesting that the GDP-bound Rab5DN shifts to a different location or that the cells making it have altered compartmentalization.

The late endosome marker YFP-Rab7WT exhibits a punctate localization pattern (Figure 5U) similar to that of Rab5. Again, the CA form of Rab7 displays a much more pronounced punctate localization pattern (Figure 5W), with stronger staining intensity, than the WT form. As in the case of Rab5, YFP-Rab7DN protein exhibits a more dispersed localization pattern and YFP-Rab7CA protein aggregates in more and larger punctae (Figure 5S).

We investigated in detail the localization of the WT (Figure 5, α and β), DN (Figure 5, Y and Z), and CA (Figure 5, χ and δ) fusion proteins of the well-characterized recycling endosome protein Rab11. While the WT (Figure 5, α and β) and CA (Figure 5, χ and δ) forms of YFP-Rab11 were located in cell bodies and synapses, DN YFP-Rab11 caused a loss of photoreceptor structure in the eye disc (Figure 5Y; data not shown). Adults making YFP-Rab11DN in photoreceptor cells exhibited developmental defects. Low levels of Rab11DN gave mild developmental defects (supplemental Figure 2B at http://www.genetics.org/supplemental/) compared to control flies (supplemental Figure 2A at http://www.genetics.org/supplemental/) and high levels caused massively reduced eyes or even lethality (data not shown),

confirming a previously reported cell lethality of dominant-negative Rab11 (Emery *et al.* 2005; Jafar-Nejad *et al.* 2005).

Our data show that DN and CA versions of Drosophila Rab proteins often have distributions that differ from WT. Either the mutant proteins affect the structure of organelles with which they typically associate or the proteins become mis-localized. To determine whether the mutant forms alter targeting or the morphology of the compartments themselves, we used Rab5 fusion proteins (Figure 6, C–E). We coexpressed the myc-2xFYVE transgene with YFP-Rab5WT, YFP-Rab5DN, or YFP-Rab5CA. Compared to Rab5WT, Rab5DN displayed a more dispersed pattern and was not found where myc-2xFYVE labeled early endosomes. Rab5WT and Rab5CA were mainly present in FYVE-marked early endosomes (Figure 6, C and E, respectively). The data indicate that, at least for Rab5, the DN proteins are mislocalized. It is possible that GDP-bound Rab5 is trapped in the cytosol by RabGDIs (Feng *et al.* 1995; Press *et al.* 1998; Dinneen and Ceresa 2004a,b; Pasqualato *et al.* 2004).

In summary, our *in vivo* localization data indicate that YFP-Rab5 fusion proteins faithfully recapitulate endogenous protein localization and that CA and/or DN versions of Rab proteins may accumulate in aberrant compartments. Together, the data show a high level of specificity in protein localization, but this should be examined in detail with independent markers for any particular tissue type.

Identifying intracellular locations of the novel RabX proteins: Twenty-three of the Drosophila Rab proteins are at least 80% similar to their mammalian counterparts, while 6 Drosophila Rab proteins have <40% sequence similarity to any Rab protein in other species. We refer to the latter as Drosophila RabX proteins. The distant relationship of the 6 RabX proteins to other Rab proteins makes it difficult to predict the functions or locations of the proteins. We have carried out a preliminary characterization of YFP-RabX protein localization to initiate the study of their biology.

Overproducing YFP-tagged wild-type RabX proteins in either Drosophila or mammalian cells produced a punctate pattern for RabX1, RabX4, and RabX5 (data not shown). These patterns are likely to report the locations of the untagged proteins, since other YFP-tagged WT Rab proteins localize to their proper cellular compartments (Figures 4 and 6 and data not shown). To localize RabX4, we cotransfected Drosophila S2R+ cells with several dsRed-tagged *Rab* genes and YFP-tagged RabX4. RabX4 colocalized mainly with Drosophila Rab5 (Figure 7A), which is involved in early endosome trafficking, suggesting that Drosophila RabX4 acts in the same compartment as Rab5. RabX4 is highly expressed in the nervous system and less expressed elsewhere (Figure 2E). The RabX4 protein did not colocalize with YFP-tagged Rab7, Rab9, or Rab11 (Figure 7, B–D). *RabX1* transcripts were barely detectable by *in situ* hybridization to embryos, and RabX5 showed signals in a very limited region (supplemental Table 1 at http://www.genetics.org/supplemental/). Their YFP-tagged proteins have a distinctive localization in S2R+ cells that does not overlap with YFP-tagged Rab5, Rab7, Rab9, or Rab11 (data not shown). We conclude that the novel RabX4 is likely to function in the endosomes of neural cells, while the other RabX proteins are in different, not easily identified, compartments.

DISCUSSION

Rab proteins are crucial in the control of targeted vesicle trafficking, movements of organelles, and assembly of subcellular compartments and cytoskeletal elements. Understanding how vesicle trafficking is regulated is important in answering many basic questions about intracellular events. Our understanding of Rab functions to date is based on studies of only a few Rab proteins, mostly in yeast and cultured mammalian cells. In this work, we have endeavored to create a powerful new tool set that will help researchers explore the fascinating biology of Rab proteins in a systematic way.

The new transgenic Rab fly strains will allow researchers to screen for loss- and gain-of-function phenotypes associated with altered Drosophila Rab functions in chosen tissues and at defined stages. These tools will be useful in investigating how vesicle trafficking affects developmental signaling and other cellular functions, as we have demonstrated with the initial characterizations of Rab3, Rab4, Rab5, Rab7, and Rab11 transgenic insertions in this study. Furthermore, as we have shown in Figures 3–7, most Rab proteins are located in a punctate pattern and define a compartment of the cell in flies. Therefore, our YFP-tagged Rab proteins can serve as a set of intracellular markers to label different steps of the endocytic and exocytic pathways for diverse studies. Our data show that most of the YFP-tagged Rab5 is accurately localized to endosomes, where it resides together with the endogenous protein (Figures 4 and 6). Similarly, YFP-Rab11 exhibits the same localization as endogenous Rab11 (Figure 4). Therefore, these lines can serve as cellular markers for different compartments. We will deposit these strains in the Bloomington Stock Center for public use.

"New" Drosophila *Rab* genes: We report the identification of four previously unknown Drosophila *Rab* genes: CG9807, CG32671, CG32673, and CG32678. These genes are located in a cluster on the X chromosome at cytological location 9D-F with two other *Rab* genes, *RabX2* and *RabX3*. On the basis of a previous study that investigated X-linked small GTPases, the cluster of Drosophila *Rab* genes in the middle of the *D. melanogaster* X chromosome, along with nearby genes encoding oxidative phosphorylation proteins, are candidates for the genetic basis of hybrid inviability. Hybrid

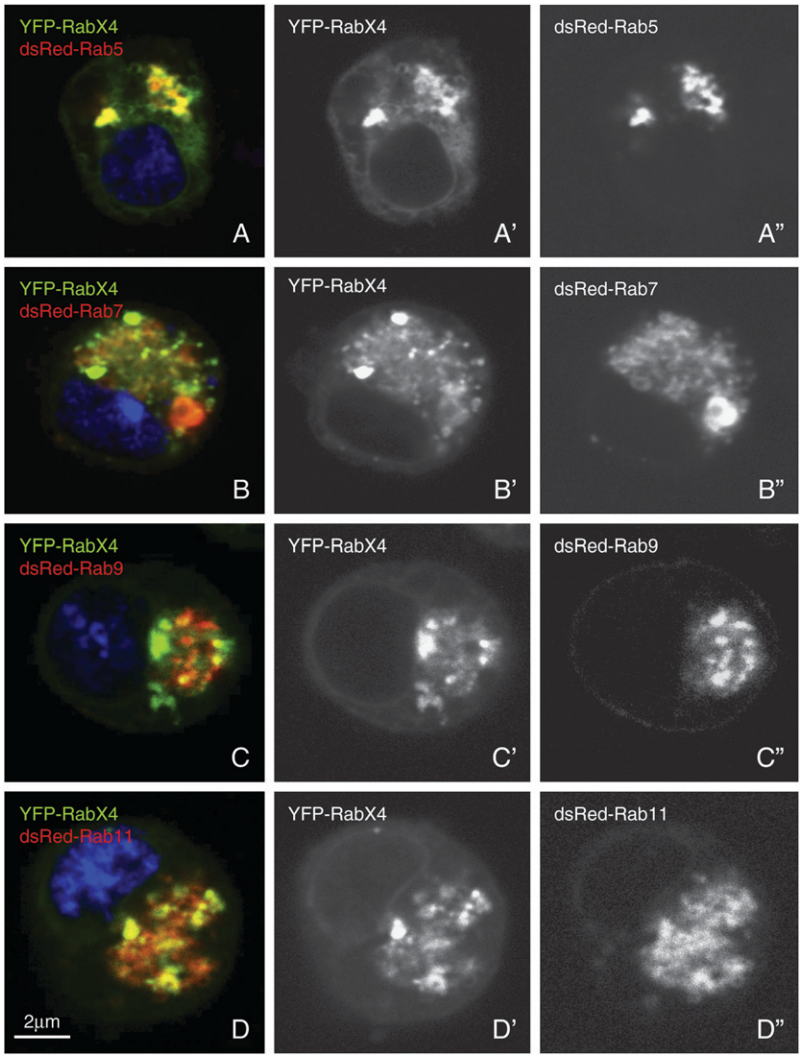

FIGURE 7.—The subcellular localization of a newly studied type of Drosophila Rab, RabX4. (A) dsRed-tagged Rab5 (red and gray) and YFP-tagged RabX4 (green and grays) were cotransfected into Drosophila S2R+ cells. The majority of the RabX4 protein colocalized with Rab5, a protein reported to be located in the early endosome. (B–D) dsRed-tagged Rab7 (B), Rab9 (C), or Rab11(D) and YFP-tagged RabX4 were cotransfected into Drosophila S2R+ cells, respectively. No colocalization was observed between each pair of proteins.

inviability is a form of reproductive isolation in which occasional mating events between two species give rise to a hybrid that is fertile but nevertheless does not leave any offspring. The RabX genes may be responsible for hybrid inviability among Drosophila species due to their potential functions in mitosis (HUTTER 2002).

Implications of expression patterns of Drosophila *Rab* genes for ascertaining function: A *Rab* gene that is detectably transcribed only in certain tissues probably functions mainly in those tissues. Our *in situ* hybridizations with probes for different Drosophila *Rab* genes show that transcripts from 21 of the genes are ubiquitous throughout the embryonic stages. Seven *Rab* genes are preferentially transcribed in specific tissues or organs. Ubiquitously expressed *Rab* genes presumably function in cellular vesicle trafficking, a process that is highly conserved from yeast to mammals and required in all eukaryotic cell types.

Some mammalian *Rab* genes are expressed in specific tissues to perform highly specialized functions. *Rab27a* is required in melanocytes to control melanosome trafficking (IZUMI *et al.* 2003), and *Rab3* is required in neurons to regulate synaptic vesicle trafficking (SCHLUTER *et al.* 2002). Thus even seemingly fundamental trafficking events required in all cells may be altered by a dedicated set of Rab proteins to refine the events for a specific task. The seven Drosophila *Rab* genes that are differentially expressed are members of all four evolutionary branches (Figure 1). *Rab2*, *Rab3*, *Rab26*, and *RabX4* transcripts are enriched mainly in neural cells (Figure 2). The expression patterns of *Rab3* and *Rab26* are consistent with a previous study that shows high expression of mammalian *Rab3A* and *Rab26* in brain tissues (GURKAN *et al.* 2005).

During the analysis of *Rab* gene expression patterns we identified a previously uncharacterized gene, *RabX4*, which is specifically transcribed in the nervous system. RabX4 is a member of the A branch of proteins (Figure 1). No clear mammalian ortholog of RabX4 exists. Wild-type YFP-RabX4 colocalizes with Rab5 within cells (Figure 7A), so RabX4 may function in endocytosis specifically in neurons.

Some fly *Rab* gene expression patterns do not correlate well with their mammalian counterparts. For example, Drosophila *Rab32* is specifically transcribed in the Malpighian tubules, which serve a function similar to that of a kidney, while mammalian *Rab32* is highly expressed in lymph, trachea, uterus, ovary, and liver, but not in the kidney (Gurkan *et al.* 2005). The discrepancy between fly and mammalian expression patterns of *Rab32* suggests that the two genes have functionally diverged. Since the molecular functions of Rab proteins are likely to be conserved, dependent as they are on precise protein structures, the evolutionary difference may lie in which tissues employ that particular Rab function.

Functional tests of dominant-negative constructs: Several types of tests can be applied to ascertain whether the dominant-negative Drosophila transgenes are in fact succeeding in specifically reducing the function of the corresponding endogenous proteins.

First, the phenotype caused by the dominant-negative construct can be compared to the phenotype of a conventional mutation in the same gene. Mutations have been made only in Drosophila *Rab5*, *Rab6*, and *Rab11*. Many other uncharacterized *P*-element insertion strains that potentially affect some other *Rab* genes are also available (Bellen *et al.* 2004). Our experiments reveal that the properties of at least one dominant-negative Rab protein, Rab11DN, are in agreement with known properties of its loss-of-function phenotype, despite the possibly incomplete inactivation that is obtained with a dominant-negative Rab11. Both Rab11DN and the *Rab11* mutant have external sensory organ development problems that cause a loss-of-bristle phenotype (supplemental Figure 2D at http://www.genetics.org/supplemental/; Emery *et al.* 2005; Jafar-Nejad *et al.* 2005) and both cause cell lethality in the eyes (supplemental Figure 2B at http://www.genetics.org/supplemental/; Satoh *et al.* 2005). The similar phenotypes indicate that Rab11DN specifically interferes with the function of the corresponding endogenous wild-type protein.

The Rab5DN and Rab7DN flies were generated by mutating the same amino acids that were mutated for other studies (Feng *et al.* 1995; Press *et al.* 1998; Dinneen and Ceresa 2004a,b; Pasqualato *et al.* 2004). An experiment with Rab5 that has been reported previously (Zhu *et al.* 2003) was repeated and confirmed with our lines (data not shown) and indicates that the DN flies are functioning equivalently to other lines available in the fly community. Hence, we used these same amino acid changes in all other DN/CA Rab lines that we developed. The Rab proteins that do not have the particular conserved T/S or Q in the GTP/GDP-binding domain may not have the expected DN or CA effects.

The phenotype caused by a dominant-negative protein can be compared to the phenotype of the corresponding loss-of-function mammalian mutants to see whether similar defects are observed. Not many mammalian Rab proteins have been studied *in vivo* due to the difficulty in making mutants when the genes have potential or known redundant functions. Mice carrying Rab mutations are available only for *Rab3* (Schluter *et al.* 2004), *Rab23* (Eggenschwiler *et al.* 2001), and *Rab27* (Wilson *al.* 2000). The phenotypes obtained with fly dominant-negative proteins can be compared to the phenotype of the corresponding loss-of-function mammalian mutants, particularly at the molecular and cellular level. Our initial studies of DN versions of fly Rab23 and -27 have not revealed many defects (data not shown), so the endogenous proteins may be too abundant to be inhibited or may be redundant with other proteins. Work on Rab3DN is still in progress.

We must emphasize two issues. First, the DN constructs may not specifically affect one protein, especially in the cases of RabX2, RabX3, and the four "new" Rab proteins where the Rab proteins are very similar. Second, some dominant-negative Rab proteins do not always function properly due to protein instability. For example, it has been reported that the dominant-negative Rab27 protein is rapidly degraded *in vivo*, which precludes the use of transgenic mouse models to study Rab27 function (Ramalho *et al.* 2002). Using dominant-negative forms of Rab proteins to reveal the functions of the normal protein requires caution with respect to specificity, level of inactivation, redundancy among proteins, and protein stability.

Relationship between Drosophila Rab protein localization and function: Protein localization data provide clues about two connected issues. One is whether a particular cellular compartment is formed under the influence of the Drosophila Rab protein, in which case the compartment may be absent if the protein is not functioning. A second possibility is that a mutant form of the Rab protein may not prevent the formation of a certain intracellular compartment, *e.g.*, endosomes, but the amino acid change may prevent the protein from accumulating in the proper compartment. These two possibilities can be distinguished by using other markers that define the presence or absence of a compartment in the presence of various forms of Rab proteins and by looking at the ultrastructure of each candidate compartment with electron microscopy.

We compared the subcellular localization of WT, DN, and CA versions of the whole set of Drosophila Rab proteins *in vivo*. In most cases, the DN versions of the proteins were distributed differently within cells compared to the wild-type protein. Data from photoreceptor neurons in the developing third instar larval eye discs show these differences quite dramatically. YFP-Rab3WT, for example, is almost exclusively located at synaptic terminals in the brain. This is consistent with the function of Rab3 as a synaptic vesicle regulator. In contrast, the YFP-Rab3DN and YFP-Rab3CA versions of the protein are localized mostly to the cell bodies. This mislocalization of Rab3 has been observed with the

dominant-negative version of mammalian Rab3D. In pancreatic acini, dominant-negative Rab3D does not accumulate in the secretory granules where most wild-type Rab3D is located (Chen *et al.* 2003). There are no reports about the localization of Rab3CA. Our data show that both loss- and gain-of-function mutations can cause defects in protein localization.

The three versions of Rab5 have distinct patterns of intracellular staining. The YFP-Rab5WT fusion protein is present in a punctate pattern both in the cell bodies and in the nerve terminals of the photoreceptor neurons, which is consistent with previously published data that Drosophila Rab5 is associated with early endosomes in the presynaptic terminal of the neuromuscular junction (Wucherpfennig *et al.* 2003). Strikingly, the CA and DN mutations of Drosophila *Rab5* cause opposite effects on the protein localization pattern. The YFP-Rab5DN version of the protein loses the punctate pattern in the cell body and nerve terminals while the YFP-Rab5CA version of the protein displays increased intensity in larger punctae, especially at the apex of cell bodies. The same dispersed pattern of Rab5DN is observed in the neuromuscular junction (Wucherpfennig *et al.* 2003). A previous study using mammalian cells shows that loss of Rab5 causes loss of the endosome compartment, and gain of function leads to an enlarged early endosome compartment (Bucci *et al.* 1992). Therefore, our observations of loss of punctate staining with Rab5DN and enhanced punctate staining with Rab5CA are consistent with previous observations.

Some DN and CA Drosophila Rab proteins, such as Rab4DN and Rab4CA, do not show the stereotypical punctate pattern. Rab4 is an endosome protein (van der Sluijs *et al.* 1991, 1992) and this redistribution of DN and CA forms may reflect the malfunction of endosome assembly or trafficking. Although no obvious loss- or gain-of-function phenotypes have been observed with overexpression of Rab4DN in our assays (data not shown), further detailed studies need to be performed to look for subtle changes in vesicle trafficking. On the other hand, the effects of Rab11CA, Rab11WT, and Rab11DN are in full agreement with previous reports about Rab11 function, especially the cell lethality associated with loss of Rab11 function (Emery *et al.* 2005; Jafar-Nejad *et al.* 2005) . The dynamic localization of endosome Rab proteins over time was observed in a study that compared Rab4, Rab5, and Rab11 localization (Sonnichsen *et al.* 2000). Three major endosome populations were identified. The first group contains only Rab5, a second contains Rab4 and Rab5, and a third contains Rab4 and Rab11. The possibly overlapping functions of Rab4 with Rab5 and with Rab11 may explain the lack of obvious defects caused by Rab4DN, while Rab5 and Rab11, in most cases, play major roles in endosome trafficking.

The late endosome protein YFP-Rab7WT exhibits a punctate localization pattern similar to that seen with Rab5. The CA form of Rab7 displays a pronounced punctate localization pattern with stronger staining intensity than its WT variant, while the DN form has more dispersed and lesser staining intensity compared to WT. This pattern is similar to what we observed with some Rab dominant-negative proteins, such as Rab5DN, suggesting that the DN forms of Rab proteins may abolish binding to modulators and prevent recruitment of downstream effectors. We infer that the GDP-bound Rab proteins are mislocalized in the cytosol, while the GTP-bound CA forms may have increased activity since the proteins are enriched in specifically targeted vesicles or other intracellular compartments.

Differences in pUAST and pUASp expression vectors: We used the well-established UAS/GAL4 regulatory system (Brand and Perrimon 1993) to generate *Rab* transgenic lines. The GAL4 system has proven to be an extremely useful tool for spatial and temporal control of Drosophila gene expression. We began using the pUASp vector, since pUASp drives expression in germline cells and is suitable for detecting defects at relatively early embryonic stages. Unfortunately, the level of protein production that we observed using pUASp is often lower than that with another vector, pUAST (Brand and Perrimon 1993). The difference between pUASp and pUAST is that the basal promoter and the 3′-UTR in the GAL4-responsive expression vector are changed for the purpose of driving germline expression (Rorth 1998). pUASp contains a *P* transposase promoter and a *fs(1)K10* 3′ terminator, while pUAST has a *hsp70* promoter and a SV40 3′ terminator. Although it is difficult to compare transgenic lines since expression levels are determined by multiple factors, we did observe generally weaker phenotypes, even in zygotic tissue, with pUASp in cases in which both pUASp and pUAST lines are available (data not shown). In addition, we found that we were unable to enhance the expression level significantly in lines using pUASp by keeping the flies at 29° where *GAL4* is more active.

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