Endogenously Tagged Rab Proteins: A Resource to Study Membrane Trafficking in Drosophila

Highlights

- A collection of YFP-tagged rab alleles allows functional in vivo studies

- FLYtRAB database links downloadable image data and annotated Rab localization terms

- Rab compartment polarity in epithelia is flexible and dynamic

- Similar intracellular Rab protein localization can indicate redundant Rab functions

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In Brief

Membrane trafficking is key to the cell biological mechanisms underlying development. As hypothesis-generating tools, Dunst et al. present a genetic resource for systematic visualization and manipulation of all membrane compartments controlled by Rab GTPases in Drosophila and a database summarizing Rab localization in different cell and tissue types.
Endogenously Tagged Rab Proteins: A Resource to Study Membrane Trafficking in Drosophila

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SUMMARY

Membrane trafficking is key to the cell biological mechanisms underlying development. Rab GTPases control specific membrane compartments, from core secretory and endocytic machinery to less-well-understood compartments. We tagged all 27 Drosophila Rabs with YFPMYC at their endogenous chromosomal loci, determined their expression and subcellular localization in six tissues comprising 23 cell types, and provide this data in an annotated, searchable image database. We demonstrate the utility of these lines for controlled knockdown and show that similar subcellular localization can predict redundant functions. We exploit this comprehensive resource to ask whether a common Rab compartment architecture underlies epithelial polarity. Strikingly, no single arrangement of Rabs characterizes the five epithelia we examine. Rather, epithelia flexibly polarize Rab distribution, producing membrane trafficking architectures that are tissue- and stage-specific. Thus, the core machinery responsible for epithelial polarization is unlikely to rely on polarized positioning of specific Rab compartments.

INTRODUCTION

Precise, flexible, and specialized regulation of membrane trafficking is key to tissue patterning and differentiation during the development of multicellular organisms. It underlies the development of cell and tissue polarity (Apodaca et al., 2012; Farr et al., 2009; Winter et al., 2012) and allows cells to specialize in the secretion or absorption of specific cargo in response to different signals or functional demands (Cao et al., 2012; Lu and Bilder, 2005). To understand developmental mechanisms, it is essential to be able to visualize and manipulate specific membrane trafficking pathways.

The Rab protein family provides a unique entry point to study membrane trafficking pathways and their function in development and differentiation. Rabs comprise a large family of lipid-modified GTPases that localize to specific subcellular membrane compartments. They cycle through GTP- and GDP-bound states, acting as molecular switches to recruit effector proteins that control compartment biogenesis (Seabra and Wasmieier, 2004; Zeigerer et al., 2012), functional properties (Liu and Storrie, 2012) and composition (Behnia and Munro, 2005; Zerial and McBride, 2001), and direct vesicle motility (Horgan and McCaffrey, 2011), tethering (Sinka et al., 2008), and fusion (Schimmöller et al., 1998).

A set of five Rabs (1, 5, 6, 7, 11), which we denote the core Rabs, has been maintained in almost all eukaryotes—from unicellular organisms to metazoans, fungi, and plants (Pereira-Leal, 2008; Pereira-Leal and Seabra, 2001). Functional studies in yeast and in tissue culture cells have revealed their key functions in regulating the core secretory and endocytic pathways common to all cells. Interestingly, recent genomic phylogeny studies have suggested that the putative last eukaryotic common ancestor (LECA) had a much larger repertoire—between 15–23 Rab proteins. This group includes the core Rabs, but also many others that are lost in different eukaryotic lineages. In contrast, the Rab family underwent a tremendous expansion correlated with the emergence of metazoans (Bock et al., 2001; Diekmann et al., 2011; Elias et al., 2012; Klöpper et al., 2012; Pereira-Leal and Seabra, 2001). This expansion has been proposed to reflect the greater complexity of membrane trafficking pathways required for cell communication, tissue patterning, and differentiated cellular functions. The functions of these Rab proteins are less well understood. Systematic analysis of the tissue specificity and subcellular localization of Rabs is an important first step in understanding how membrane trafficking pathways are organized in different cell types and how they are deployed during development and differentiation.

The ability to form epithelia underlies the organization of many different tissues. Epithelial cells adhere to each other to form sheets that separate different organismal compartments. They maintain distinct protein and lipid compositions on their apical and basolateral surfaces through targeted delivery, endocytosis, and recycling of specific cargo (Apodaca et al., 2012). Neurons also polarize trafficking of membrane and secreted proteins to organize the somatodendritic and axonal domains; these have been suggested to rely on sorting mechanisms similar to the basolateral and apical domains of epithelial cells, respectively (Dotti and Simons, 1990; Siegrist and Doe, 2007). Does the...
Rab machinery have a characteristic polarized architecture in epithelial cells and neurons? If so, are the Rabs that first appeared in metazoans more likely to be deployed in this way?

Much has been learned about the subcellular localization of Rab compartments by expressing tagged Rab proteins. However, unphysiological expression levels can alter membrane trafficking and distort the appearance of the relevant membrane compartments (Mottola et al., 2010). Tagging each Rab in its endogenous chromosomal locus would solve this problem. We therefore generated a toolkit for the systematic analysis of Rab protein expression and function in Drosophila melanogaster.

The Drosophila genome predicts 33 Rab proteins based on sequence similarity, and there is evidence that 27 are expressed (Chan et al., 2011; Zhang et al., 2007). Fourteen of these Rabs were already present in the LECA (between 2–3 billion years ago), and the other 13 first arose by duplication and divergence at the root of the metazoan lineage (~500 million years ago). While the genes encoding many Rab proteins have undergone further expansions in vertebrates, this is not the case in Drosophila (Diekmann et al., 2011; Elias et al., 2012; Klöpper et al., 2012). Each Drosophila Rab exists in only a single copy, facilitating analysis (http://flybase.org).

We used homologous recombination (Maggert et al., 2008; Rong and Golic, 2000) to fuse YFPMYC to the N terminus of each Drosophila Rab. This allows visualization of Rab distribution at endogenous expression levels. Furthermore, the YFPMYC tag provides a target for knockdown approaches, allowing specific and controllable reduction of Rab protein function (Brankatschk et al., 2014; Caussinus et al., 2012; Neumueller et al., 2012). We systematically analyze Rab protein tissue distribution and subcellular localization in six different tissues comprising 23 cell types. We construct an online image database FLYtRAB in which each Rab can be viewed in whole tissues at subcellular resolution and a searchable annotation database for the systematic analysis and comparison of Rab expression and localization (Tomancak et al., 2007). Analysis of these data provides insights into the evolution of membrane trafficking pathways and how they are deployed during cell polarization and tissue differentiation.

RESULTS

Construction and Verification of YFPMYC-Tagged rab Alleles

To generate a novel set of endogenous N-terminally tagged Rab proteins, we used homologous recombination to target the coding sequence for each Drosophila rab gene and replace the predicted start codon with DNA encoding YFPMYC (Figure 1A). We refer to these proteins as YRabs. Western blotting indicates that the YFP tag does not alter Rab4 protein levels (Figure S1A). Furthermore, tagging endogenous Rab1, Rab6, and Rab7 with YFP does not perturb Golgi morphology in salivary gland or fat body cells (Figures S1B–S1I). Finally, except for YrabX5, all new Yrab alleles produce viable homozygous flies, further confirming that their functions are not disturbed (Figure 1B).

Quantitative Profile of Rabs in Different Cell Types

To confirm the presence of YRab fusion proteins, and to examine their endogenous levels and proportions in different tissues, we performed semiquantitative western blotting against the YFPMYC tag in three larval tissues: the fat body (FB), wing disc (WD), and salivary gland (SG) (Figures 2A and 2B). We blotted equal amounts of tissue lysate from each homozygous YRab line and compared the signal strength of each YRab to a recombinant YFPMYC standard (Figures 2A and 2B).

YRabs generally exist as single proteins of the predicted size, with few isofoms. Several YRabs (3, 14, 32, 26, X4, X5) were not detected in any of the three tissues (Figures 2A and 2B). To confirm that these fusion proteins were produced at all, we blotted extracts of adult heads. This identified YRab3, YRab32, YRab26, YRabX4, and YRabX5, but failed to detect YRab14 (Figures S2A and S2A’).

Qualitatively, detectability of the different YRab proteins in WD, SG, and FB is consistent with mRNA expression data from modENCODE (Figure S2B). YRab14, YRab32, and YRabX4 are exceptions—we cannot detect these proteins in WD, FB, or SG although modENCODE (Chen et al., 2014) predicts moderate expression. Despite general qualitative agreement, YRab protein levels are not always proportional to reported mRNA levels in modENCODE (Figure 2B). This suggests that post-transcriptional mechanisms contribute significantly to regulating Rab protein levels.

It has been proposed that core Rabs that have been conserved since the LECA regulate pathways that are required in all cells, whereas Rabs that first arose in metazoans regulate tissue-specific pathways. Consistent with this, core Rabs (YRab1, YRab5, YRab6, YRab7, YRab11) are present in all three tissues examined by western blotting (Figures 2A and 2B). LECA-Rabs that are more often lost and those that arose in metazoans are more tissue-specific (Diekmann et al., 2011)—many of these are undetectable in some or all of the three tissues (Figures 2A and 2B).

Highly conserved LECA-Rabs tend to be present at the highest levels. We estimate that the most abundant YRabs (Figure 2B, red) are present at concentrations of at least 10 μM (for comparison, citrate synthase is present at 10 μM (Srere, 1967), while the least abundant detectable YRabs (Figure 2B, dark blue) are present at 20-fold lower concentrations. While Rab GEFs and GAPs can modulate Rab protein activity and flux through the corresponding compartment (Cabrera and Ungermann, 2013), Rab protein concentration may also help determine the capacity of the compartment. If so, then a large fraction of secreted and transmembrane proteins move through pathways controlled by core Rab proteins.

Finally, comparing YRab protein levels reveals unexpected heterogeneity in the proportions of even the highly conserved LECA-Rabs in different tissues. Conserved Rabs of the protein secretion machinery (YRab1, YRab2, YRab6, YRab8) are present in different proportions in WD, SG, and FB cells. Similarly, Rabs with conserved functions in endocytic trafficking and recycling (Yrab4, Yrab5, Yrab7, Yrab11) are also present in different proportions in different cell types. It will be interesting to see whether these differences are reflected by changes in trafficking through the corresponding compartments.

Systematic Analysis of Rab Protein Tissue Distribution and Subcellular Localization

To examine expression and subcellular localization in a broader range of cell types, we turned to immunofluorescence. For each
Figure 1. Generation of YFP\textsuperscript{MYC}-Tagged \textit{rab} Alleles

(A) Homologous recombination strategy for generating YFP\textsuperscript{MYC}-tagged \textit{Rab}s. \textit{Yrab} is shown as an example. 5' and 3' UTRs, gray; \textit{rab} coding regions, dark green; YFP, bright green; MYC, red. Scale bar, 1,000 bp.

(B) A table showing classical names and computed gene (CG) numbers of 27 \textit{Drosophila rab} genes and the viability of the corresponding YFP\textsuperscript{MYC}-tagged and loss-of-function alleles.

See also Figure S1 and Table S1.
Figure 2. YRab Protein Levels in Fat Body Cells, Salivary Glands, and Late Wing Discs

(A) Western blots of the indicated tissues dissected from homozygous YRab lines, along with indicated amounts of recombinant YFP-MYC (boxed in red), probed for MYC. YRabs are presented in gray-scale-coded squares that distinguish LECA Rabs according to how often they are lost in other eukaryotes. White squares indicate Rabs that arose in metazoans (see also legend in B). Purple boxes indicate oregonR controls.

(B) Heat map showing the amount of YRab (quantified from three western blots including those in A) present in 8 μg protein lysate from FBs, SGs, and late WDs. Legends explain gray scale and color codes.

(legend continued on next page)
YRab line, we imaged larval WD, SG, FB, and brains, as well as adult ovaries and testes (Janning, 1997). These tissues comprise over 23 different polarized and non-polarized cell types. We collected an array of 3D tiles from each tissue and stitched them (Preibisch et al., 2009) to form an image of the entire tissue at subcellular resolution. To analyze these data, we developed a hierarchical annotation scheme using a controlled vocabulary describing YRab expression and subcellular localization at the tissue and cell type level. The complete default annotation trees for each tissue can be found in the supplement. The terms “expressed”/“not expressed” describe the presence of a YRab in a tissue or cell type. To describe differential YRab expression in particular cell types within a tissue, we use the annotation term “elevated levels.” We use three independent terms to describe subcellular localization in general: cortical, intracellular punctate, and intracellular diffuse. A diffuse appearance could reflect either non-membrane-associated YRabs or small vesicles below the resolution of the light microscope. A particular YRab might exist in different pools within the cell and therefore be described by one or more of these terms. For polarized cell types, we add polarity annotation terms that separately describe the polarized distribution of each of the three possible pools (cortical, punctate, diffuse). For example, a particular YRab might have a diffuse intracellular pool that is not polarized, but a cortical pool that is polarized. Thus, each YRab in each tissue or cell type is characterized by multiple features. We annotated at total of 23 cell types in six tissues: three cell types in the CNS, ten in ovaries of different stages, four in testes, three in SG, one in the FB, and two cell types in both early and late third instar WD. To perform hierarchical clustering of YRabs according to specific chosen features, we constructed a binary matrix that displays all features of every YRab/tissue combination. All the YRab imaging data and annotations, including the binary matrix can be accessed through the Functional Yfp-Tagged RAB (FLYtRAB) database.

The FLYtRAB database is based on the scaffold described in Lécuyer et al. (2007) and Tomancak et al. (2007) and provides a link to Collaborative Annotation Toolkit for Massive Amounts of Image Data (CATMAID) (Saalfeld et al., 2009) to browse the original 3D data set for each YRab and tissue type. The open source software CATMAID (http://www.catmaid.org), originally used for EM data, allows online browsing of terabyte-scale image data. We extended the capabilities of the CATMAID software to allow visualization of multichannel immunofluorescence data. We also added tools that facilitate co-localization analysis, as well as cropping and downloading image data. This database is accessible at http://rablibrary.mpi-cbg.de.

Rabs and Tissue Specificity

Only the five core YRabs are expressed in all three tissues examined by western blotting. To ask whether this principle held over a broader range of cell types, we surveyed the FLYtRAB database. To help visualize these findings, we clustered YRabs according to their expression in different cell types (Figures 2C and S2C). The y

axis positions of the branch lines in the dendrogram indicate the similarity of cell-type expression between the two groups connected by the line. YRabs connected by a branch line with a similarity level of 1 are expressed in identical cell types. The set of YRabs that are expressed in all annotated cell types is highlighted in red. YRabs that separate at lower levels of similarity (orange and yellow branch lines) are increasingly dissimilar to the ubiquitous group (i.e., they are expressed in fewer cell types). YRabX5 and YRab32 form an out-group since they are not expressed in any annotated cell type (Figure 2C, gray).

The set of ubiquitously expressed YRabs contains all core YRabs except YRab5. YRab 5 is not detectable in migrating border cells, but is present in all other cell types. In contrast, LECA YRabs that are often lost during evolution (Figure 2B, group IV) tend to be expressed in fewer cell types, as do YRabs that arose in metazoans. The metazoan YRab26, YRab27, YRab3, and YRabX4 form an extremely tissue-specific group (Figure 2C, yellow). Together, these YRabs are expressed in only four annotated cell types and share the common feature of being expressed in neurons. YRab26 and YRab27 are expressed identically only in neurons, whereas YRab3 and YRabX4 are expressed in one and two additional cell types, respectively. These data generally support the idea that core Rabs organize the basic membrane trafficking machinery common to all cells, whereas other Rabs are more likely to have tissue-specific functions.

Rabs and Cell Polarity

To investigate the relationship between Rab protein localization and cell polarity, we examined the subcellular distribution of each Drosophila YRab in the neurons of the early second instar CNS and in three epithelial cell types with different functions (early third instar SG cells, ovarian follicle cells of different stages, along with early and late third instar WD cells).

Rab Compartment Organization in the CNS

Different cell types within the CNS can be easily identified by a combination of brain morphology and immunostaining. The CNS is surrounded by a layer of specialized glial cells that constitute a blood brain barrier (BBB). Neuronal cell bodies are located peripherally and extend axons and dendrites into more central regions, forming a neuropil. Cell bodies of primary neurons, which have already formed synaptic connections, are located closest to the neuropil. Secondary neurons are beginning to be born, and their cell bodies lie in more outer regions. Nuclei of both primary and secondary neurons stain with the post-mitotic marker Elav. Pluripotent neuroblasts and ganglion mother cells are mostly located in very peripheral regions, are large, and do not stain for Elav. Thus, we can easily compare the relative proportions of different YRabs in neuroblasts, cell bodies of primary and secondary neurons, and neuronal projections (Figure S3A).

Nine of the 22 YRabs detectable in neurons of the larval CNS are roughly equally distributed between cell bodies and the neuropil (Figures 3I–3Q and S3J, black). Six YRabs (1, 7, X1, 4, 6, 39)
are enriched in neuronal cell bodies (Figures 3C–3H and S3J, blue). Seven YRabs (3, 19, 23, 26, 27, 30, X4) are clearly enriched in the neuropil (Figures 3R–3X and S3J, magenta). Compared with YRabs that are uniformly distributed or enriched in cell bodies, YRabs that are enriched in the neuropil comprise predominantly metazoan YRabs. This group includes the highly CNS-specific YRab3, YRab26, YRab27, and YRabX4. YRab3 and YRab27 have well characterized roles in synaptic vesicle release (Mahoney et al., 2006; Südhof, 2013), and it would be interesting to examine whether other neuropil-enriched YRabs may have related functions.

mRNA localization to growth cones and to synaptic termini is thought to contribute to axon guidance and synaptic plasticity. Interestingly several localized mRNAs encode secreted and transmembrane proteins. These mRNAs appear to be locally translated, indicating that dendrites and axons have a functional ER/Golgi protein synthesis machinery (Jung et al., 2012; Swanger and Bassell, 2011). Do these outposts rely on the same Rab machinery as ER/Golgi assemblies in the cell body? Examining YRab protein distribution shows that canonical Golgi and ER Rabs (Rab1, Rab2, Rab6) are present in both cell bodies and the neuropil (Figures 3 and S3J). Thus, canonical Rab machinery likely regulates transport of secreted and membrane proteins in neuronal projections.

Does membrane trafficking change as cells differentiate? To examine this, we looked for YRabs with different expression levels in neuroblasts, neurons, and BBB glia. Neurons and glia both derive from neuroblasts. The expression of most YRabs (e.g., YRab6) does not differ between these cell types (Figures S3C and S3C′). However, neuroblasts express higher levels of some YRabs (4, 18, 21, 40) compared to neurons and BBB glia (Figures S3D–S3F and FLYtRAB). Conversely, some are enriched in differentiated neurons compared to neuroblasts. These include not only tissue-specific YRabs (X4, 3, 26, 27) but also YRab5 (Figures S3G–S3I and FLYtRAB). In contrast, YRab6 is undetectable in neurons and neuroblasts, but is expressed in BBB glia (Figures 3B and 3B′). Thus, the differentiation of glia and neurons may entail changes in membrane trafficking resulting from altered Rab protein expression.

Rab Compartment Organization in the Salivary Gland

To analyze Rab protein localization in an epithelium specialized for apical secretion, we turned to the third instar SG. The post-mitotic SG cells produce and release digestive enzymes and, later, glue proteins into the apical lumen. Each SG cell contains multiple large cup-shaped ER/Golgi compartments, which stain for the Golgi marker Lava Lamp. These occupy the medial and basal regions of each cell, but are excluded from the most apical regions (Szul et al., 2011). To help visualize the different YRab localization patterns in the SG we clustered them according to annotations features describing the polarized distribution of cortical, punctate, and diffuse pools (Figure 4G).

As expected, YRabs with known functions in ER-Golgi trafficking (YRab1, YRab2, YRab6) localize to large compartments in the basal/medial cytoplasm (Figures 4D–4E, S4N, S4O, and S4T). Several YRabs (19, 30, 11) are enriched in a diffuse apical pool (Figures 4B–4C′ and 4G, magenta, and S4L–S4K′). YRab11 does not cluster identically with YRab19 and YRab30 because it is also present in larger medial and basal structures like YRab39 and YRab7 (Figures 4G, green, and S4K–S4M). The only YRab that is basally polarized in the SG is YRab4 (Figures 4F, 4F′, 4G, blue, S4S, and S4S′). The apical group of Rabs (YRab11, YRab19, YRab30) are interesting candidates for mediating apical secretion in SG cells. Interestingly, YRab19 and YRab30 are also enriched in neuronal projections, which may indicate a common function in polarized secretion in these cells. Rab30 localizes to the Golgi in S2 cells—in contrast to its apical localization in SG cells. Its interaction partners in S2 cells include not only golgins, but also components of the exocyst—a complex that targets golgi-derived vesicles to the plasma membrane (Gillingham et al., 2014). If Rab30 mediates delivery of vesicle the golgi to the plasma membrane, then its different localization in the SG and S2 cells may simply reflect steady-state differences in its distribution.

Rab Compartment Organization in the Follicular Epithelium

To what extent is Rab compartment architecture a conserved feature of polarized epithelia? To address this, we examined other epithelia, beginning with the follicle epithelium (FE) of the ovary. When egg chambers emerge from the germarium, the FE is cuboidal and its apical surface contacts the germline cells. FE cells proliferate between stages 2 and 6, and their growth and patterning is controlled by apical signals from the germline cells (González-Reyes and St Johnston, 1998; López-Schier and St Johnston, 2001). At stage 6, FE cells stop dividing and begin to grow by becoming polyploid. At stage 8, FE cells contacting the oocyte start to elongate, becoming columnar over increasingly larger regions as the oocyte enlarges. At the same time, FE cells overlying the nurse cells flatten and become squamous.

To visualize intracellular YRab protein distribution features in early (stages 2–8) and late (stage 9) FE, we clustered them according to annotations describing the polarized distribution of cortical, punctate, and diffuse pools (Figures 5I and 5J). YRab proteins in the FE display two different architectures depending on the developmental stage: during early stages, the compartments organized by YRab4, YRab5, YRab7, YRab11, YRab19, YRab30, YRab39 and YRabX1 polarize apically toward the underlying germline (Figures 5B–5C′ and 5I, magenta). However, as the FE becomes columnar by stage 9, a new Rab architecture develops. Apical YRab polarization is lost (Figures 5F and 5F′), and YRab1, YRab2, YRab6, YRab7, YRab8, YRab10, and YRab18 now polarize toward the basal side of the cell (Figures 5G–5H and 5J, blue). Basal YRabs fall into two distinct groups.
in the dendrogram (Figure 5J, blue) because their compartment morphologies differ.

Comparing dendrograms depicting subcellular localization of YRabs in the SG, early FE, and late FE reveals no consistent pattern (Figure S5). Although the early FE and SG share the feature that YRab11, YRab19, and YRab30 localize apically, this feature disappears in the late FE. Neither does the SG exactly resemble an early FE. For example, many more YRabs localize apically in the early FE. Furthermore, YRab4 is basal in the SG and apical in the early FE.

**Rab Compartment Organization in the Wing Disc**

To analyze Rab compartment organization in a fourth epithelium, we turned to the larval WD, which later gives rise to the adult wing and thorax. The WD is a folded epithelial sac with an apical lumen and a basal side that is bathed in hemolymph. As the disc grows, it becomes pseudostratified on one side and squamous on the other—a feature common to many other developing epithelia such as the neural tube and retina.

We examined and annotated YRab localization in third instar WD before and after pseudostratification (Figure 6 and FLYRAB).

Before pseudostratification, few YRabs are distributed in a polarized fashion. YRab1 and YRabX1 are apically enriched, and YRab8 is basally enriched (FLYRAB and Figure 6F). YRab organization changes dramatically with pseudostratification. YRab1, YRab2, YRab4, YRab5, YRab7, YRab8, and YRabX1 become apically localized (Figures 6C, 6C', and 6G, magenta). YRab11 (Figures 6D, 6D', and 6G, magenta/blue) localizes to the extreme apical and basal ends of the cells.

Wing epithelial cells develop an additional interesting feature during pseudostratification. More than half of the YRabs in the WD are reproducibly represented in a characteristic structure found in the apical cytoplasm at the level of the junctions (Figures 6D–6F). We refer to this as the apical hub. It contains not only apically enriched Rabs like YRab1 (Figures 6C, 6C', and 6G, magenta) but also Rabs with an otherwise uniform distribution such as YRab6 (Figures 6E, 6E', and 6G, black). Strikingly, many Rabs in both the secretory (Rab1, Rab2, Rab6, Rab8) and endocytic pathways (Rab4, Rab5, Rab7, Rab11) are represented in this apical hub even when they also localize to other positions in the cell. Their localization is consistent with independent markers of ER and Golgi compartments (Figures 6H–6I). The dramatic Rab redeployment during pseudostratification is clearly seen by comparing the dendrograms shown in Figures 6F and 6G.

Taken together, systematic analysis of Rab localization in these epithelia reveals little similarity and provides no support for the idea that apical-basal epithelial polarization relies on a common Rab compartment architecture. To illustrate this dissimilarity, we clustered YRabs according to features describing...
Figure 5. Localization of YRabs in Ovaries

(A–H) Confocal sections of early (stages 2–8) (A–D) and late (stage 9) (E–H) ovaries from oregonR (A and E), Yrab4 (B and F), Yrab7 (C and G), and Yrab1 (D and H) probed for YFP (gray A–H and green A–H) and Phalloidin (magenta A–H). Scale bar, 10 μm.

(legend continued on next page)
polarity and compartment morphology, generating a dendrogram for each tissue (Figure S5). These clustering patterns highlight the differences between epithelial tissues and even between different developmental stages of the same tissue. Thus, epithelial cells deploy different subsets of Rab compartments in a polarized fashion depending on their specialized tasks.

**Rab Protein Divergence and the Evolution of Membrane Trafficking Routes**

To examine whether Rab tissue distribution and subcellular localization was related to Rab protein phylogeny, we performed clustering analysis based on annotation terms that describe compartment morphology and polarity in different cell types (Figure S6). This revealed several groups of Rabs with strong similarities in localization and cell type expression. Comparing this analysis with sequence-based phylogenetic trees shows that two of these groups (Rab19 and Rab30 and Rab3, Rab26, Rab27, RabX4) consist of Rabs that are evolutionarily related, but that diverged in the earliest metazoans. The fact that compartment morphology/polarity and tissue distribution of Rabs within these groups have evolved together for the last 500 million years suggests that their functions are interdependent. Other Rab pairs with similar subcellular and cell type distribution are not related phylogenetically (e.g., Rab23 and Rab35). Their shared features, however, suggest that it would be interesting to investigate whether they have similar functions.

**Redundant Functions for Cortically Localized Rab23 and Rab35 in Tissue Polarization**

YRab23 and YRab35 represent a phylogenetically unrelated Rab pair with similar subcellular localization patterns. These Rabs are both found at the cell cortex in all cell types where they are present (Figure S6, yellow box). We therefore wondered whether they might fulfill redundant functions in tissues where they are co-expressed. While YRab35 is generally expressed, YRab23 is mainly expressed in epithelial tissues such as the developing wing (FLYRAB).

Rab23 mutants disturb tissue polarity in the adult wing (Pataki et al., 2010). Normally, each wing cell produces a single, distally oriented hair. When Rab23 is lost, cells produce multiple hairs and hair orientation shifts away from the proximal-distal axis toward the wing margin (Figure 7B). No published classical allelic of rab35 exists. However, dominant-negative Rab35 expression and targeted Rab35 knockdown disturb actin organization in thoracic bristles (Zhang et al., 2009).

Anti-GFP RNAi and anti-GFP nanobody expression have been used to knock down endogenously GFP- and YFP-tagged proteins (Brankatsch et al., 2014; Caussinus et al., 2012; Neumüller et al., 2012). To ask whether these methods were generally effective at reducing levels of YFP-Rabs, we drove their expression in flies homozygous for Yrab23. Indeed, both methods strongly reduce levels of YRab23 (Figures S7A–S7C) and produce phenotype similar to rab23 mutants (Figures 7E, S7J, and S7K).

Although GFP RNAi efficiently reduces YRab35 levels (Figure S7E), its depletion causes only occasional subtle disturbances in hair morphology (Figure 7D and data not shown). To look for interactions between Rab23 and Rab35, we knocked down both Rabs in the developing wing blade of Yrab35;Yrab23 homozygous flies using nubbinGAL4. Simultaneous knockdown of YRab23 and YRab35 (Figure S7G) produces defects in hair polarity and morphology that would be expected from the sum of the individual knockdowns. The additional removal of YRab35 does not enhance hair polarity defects caused by loss of YRab23. However, knockdown of both Rabs produces a phenotype not seen in either knockdown alone: the formation of ectopic cross veins (Figures 7F and S7H). Thus, Rab23 and Rab35 function redundantly to organize the pattern of veins in the wing. This illustrates the power of subcellular localization screens to predict redundant Rab protein functions. Membrane trafficking is key to the regulation of many signaling systems that determine the wing vein pattern (Restrepo et al., 2014; Wang and Struhl, 2004). It will be interesting to further investigate how Rab35 and Rab23 influence signal transduction.

**DISCUSSION**

Here, we describe a genetic resource for visualization of endogenous Rab proteins. We provide a CATMAID-based annotated 3D image database (FLYRAB) that allows the user to browse Rab protein distribution with subcellular resolution in 6 different tissues comprising over 23 cell types. Dedicated search algorithms connect image data with the defined vocabulary in annotation trees and allow clustering of Rabs according to features of interest. All original recorded data are accessible and downloadable online—a feature that will allow users to investigate specific aspects of interest more deeply. Furthermore, hypotheses generated by searching this database can be easily tested with knockdown strategies that target the YFP tags. These will provide powerful tools to explore the different functions and specializations of membrane trafficking pathways in vivo. We exploited the comprehensive nature of this resource to address questions in cell differentiation, cell polarity, and Rab protein evolution.

**Rabs and Cell Differentiation**

Our analysis shows that cell differentiation is accompanied by quantitative and qualitative changes in Rab protein expression and localization, suggesting that rewiring of membrane trafficking pathways is a key feature of differentiation. For example, quantitative western blotting of FBs, SGs, and WDs revealed not only tissue-specific Rab protein expression, but also changes in the relative levels of Rabs that control basic cellular processes such as secretion, endocytosis, and recycling. Furthermore, neuronal differentiation is accompanied not only by expression of neuron-specific Rabs involved in synaptic vesicle release (Rab3, Rab26, Rab27), but also quantitative changes in levels of other Rab proteins. Rab21 levels drop during neuronal
differentiation, while Rab5 levels rise. Both Rabs are involved in early endocytic trafficking (Ali et al., 2014) and it would be interesting to know how changing their relative proportions influences the endocytic pathway in neurons. Rab4, Rab18, and Rab40 are more highly expressed in neuroblasts than in neurons. Interestingly, Rab18 mutations have been associated with Warburg micro syndrome, which is characterized by postnatal microcephaly (Bern et al., 2011; Cheng et al., 2014). It would be interesting to know whether neural stem cells function normally in patients with this syndrome. BBB glial cells also derive from neuroblasts (DeSalvo et al., 2011). Rab9 expression increases with differentiation of BBB glia, suggesting that it may have special functions there.

Finally, not only do Rab levels change as tissues develop—their subcellular localization can change as well. Rab proteins undergo a striking reorganization during development of both the ovarian follicular epithelium and the WD epithelium. Interestingly, although Rab proteins throughout the wing pouch re-localize as cells become pseudostratified, there appear to be few obvious differences in their expression levels or distribution within the wing pouch—despite the fact that this region is already being patterned by the activity of several different morphogens (Crozatier et al., 2004; Kicheva et al., 2012). The single exception is RabX6, which is expressed at elevated levels in the anterior compartment and in sensory organ precursor cells (FLYRAB).

Thus, morphogen spreading and signaling probably operates in a field of cells with relatively homogeneous membrane trafficking capabilities.

Rabs and Epithelial Polarity

Although we initially wondered whether epithelial polarity might depend on a standard polarized membrane trafficking architecture (Farr et al., 2009; Weisz and Rodriguez-Boulan, 2009), our observations do not support this idea. While many different Rabs can assume a polarized localization in specific epithelia at specific developmental stages, there is not a single Rab GTPase that is always apical or basal. Rab19 and Rab30 are the most consistently polarized—they are apical in SGs and early follicle cells and enriched in neuronal projections. However, they are unpolarized in other epithelia. Thus, different polarized distributions of Rab compartments may simply reflect differences in epithelial function. For example, one set of Rabs (4, 5, 7, 11, 19, 30, 39, X1) is apically localized in early follicle cells in the ovary, but not at later stages. Apical localization of these Rabs correlates with a time of intense communication between the germline and the overlying follicle cells (FLYRAB).

Thus, morphogen spreading and signaling probably operates in a field of cells with relatively homogeneous membrane trafficking capabilities.

**Functional Interdependence of Phylogenetically Related Rabs**

Duplicated genes with identical functions cannot be stably maintained during evolution—they generally rapidly diverge in
their expression patterns, acquire mutations, and may eventually adopt novel beneficial functions (neo-functionalization). Duplicates may also divide functions once present in a single ancestral protein (Taylor and Raes, 2004; Zhang, 2003). Our analysis has revealed two instances in which a group of phylogenetically related Rab proteins show extremely similar patterns of cell-type-specific expression and even subcellular localization. One group comprises Rab19 and Rab30 and the other Rab3, Rab26, Rab27, and RabX4. The members of both groups diverged from each other over 500 million years ago (Elias et al., 2012). This suggests that there has been selection to preserve their coordinate expression and subcellular localization, and their functions somehow depend on each other. One idea to account for this is that membrane trafficking pathways evolve by branching. When a Rab is duplicated, both Rabs are recruited to the same compartment by the same signals. One duplicate may acquire the ability to recruit a new effector. This could specify an alternative branch for cargo passing through the original compartment. Of course the Rab specifying the derivative branch might not function without the Rab that specified the original branch, explaining the requirement for coordinate expression. In the future, investigating this idea will depend on mapping the trafficking routes specified by these Rabs. The tools for Rab visualization and knockdown described here will facilitate these and other analyses.

**EXPERIMENTAL PROCEDURES**

**eYFP rab Alleles (Yrabs)**

YFP-tagged rab alleles were generated by ends-in homologous recombination and the initial genomic duplication was resolved using the I-Cre system as described by Rong and Golic (2000) and Maggert et al. (2008). Donor constructs for targeting rab genes are shown in the Supplemental Experimental Procedures. All rab donor constructs were verified by sequencing. Recombination events were verified by PCR.

**Fly Stocks**

Embryo collections and staging protocols were performed at 25°C; flies were raised on conventional cornmeal agar under a 12 hr light/12 hr dark cycle at 25°C. nubbin-Gal4 (#42699) and UAS-gfpRNAi (#41559 and #9331) are available from Bloomington Stock Center, rab235-SZ-3123 from DGRC (#125902). UAS-gfpNanobody flies were provided by the Affolter Lab and en(105)-Gal4 flies are from C. Dahmann.

**Immunohistochemistry**

Larval brains (44–48 hr after egg collecting [AEC]), salivary glands (72–80 hr AEC), early (88–72 hr AEC), and late (110–120 hr AEC) wing discs were
were dissected in ice-cold PBS (CNS, salivary glands) or Graces medium (wing disc), fixed with 4% PFA at room temperature and probed with one or more of the following: anti-GFP (Invitrogen), DAPI (Roche), anti-HRP-Cy5 (Dianova), anti-Elav (Developmental Studies Hybridoma Bank [DSHB]), anti-Dig (DSHB) or anti-Crb2.8 (gift from E. Knust). The larval fat bodies (72–80 hr AEC) were fixed with 4% PFA for 30 min at room temperature and stained with anti-GFP (Invitrogen), anti-Dig (DSHB), and DAPI (Roche). Anti-Lva (gift from E. Knust) and anti-KDEL (ENZO Life Science) were used to mark organelles. Aged (5–7 days) flies were fed for 1 day at room temperature (RT) with yeast paste; ovaries and testis were dissected and fixed with 4% PFA in PBS and stained for 3 days at room temperature with anti-GFP (Invitrogen), Phalloidin-555 (Roche), anti-Dig (DSHB), and DAPI (Roche). All samples were mounted in VectaShield mounting medium (Vector Labs) and photographs were acquired with an Olympus1000 confocal microscope and evaluated using FIJI imaging software.

**Annotation**

CATMAID image data sets were analyzed manually and annotated using a defined terminology to describe subcellular localization of individual YRabs (see Supplemental Experimental Procedures).

**Clustering**

Multiple annotation features that are represented in a binary matrix characterize the tissue distribution and subcellular localization of each YRab. We used this binary matrix to perform hierarchical clustering of YRabs according to specific annotation features using Past 3.01 (http://folk.uio.no/ohammer/ past). The Jaccard clustering metric was used to compute similarity coefficients. Rooted trees (dendrograms) were generated according to similarity coefficients based on Unweighted Pair Group Method with Arithmetic Mean (UPGMA) linkage (see Supplemental Experimental Procedures).

**Biochemistry**

Wing discs, fat bodies, and salivary glands from feeding 3rd instar larvae (110–120 hr AEC) were dissected in ice-cold Graces medium, homogenized with a plastic pestle in 1% TX-100 PBS lyses buffer, and pelleted at 20,000 × g for 5 min at 4°C. Protein content from recovered supernatants was measured using BCA (manufacturer protocol, Invitrogen) and 8 μg protein lysate \n lane were loaded on 12.5% SDS-PAGES. Blots were probed for MYC (CM-1, Gramsch). Additional blots (Figure S1) were probed for Rab4 (gift from M. Zerial) and α-Tubulin (gift from E. Knust).

**Calculation of Cellular YRab Concentration**

Comparing YRab signals to recombinant YFPMYC standards shows that there is more than 10 ng of the most abundant YRabs per lane, corresponding to ~0.2 pmol. Each lane contains four wing discs, and a wing disc has a volume of 0.005 μl. Thus the most abundant YRabs are present at more than 10 μM.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2015.03.022.

**AUTHOR CONTRIBUTIONS**

M.B. conceived and generated the YRab library. B.B. and A.M. verified the library. S.D., M.B., and S.E. collected the data. T.K., F.v.Z., and P.T. conceived and generated the YRab library. B.B. and A.M. verified the library. S.E. designed experiments, analyzed the data, and wrote the manuscript.

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Endogenously Tagged Rab Proteins: A Resource to Study Membrane Trafficking in *Drosophila*

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Figure S1, related to Figure1.

YFP tag does not disturb Rab function

(A) Western blots from wild type, and homozygous Yrab4 adult heads and larval tissues probed for Rab4 and alpha-Tubulin. Black and red asterisks indicate endogenous and YFP-tagged Rab4 bands, respectively.

(B-I'') Confocal images of salivary gland cells (B-E'') and fat body cells (F-I'') from wild type control animals, or the indicated Yrab recombinants, stained for Lava lamp (magenta). YRabs (green) are detected by endogenous YFP fluorescence. Phalloidin (grey) labels cell outlines. DAPI (blue) labels nuclei. Scale bar = 10µm.
### Table C: Cell Type by Yrabs

| Cell Type               | Yrabs |
|------------------------|-------|
| neuroepithelium        |       |
| neuron                  |       |
| neuroblast              |       |
| fat body cell           |       |
| cap cell                |       |
| female germinal stem cell & cytotelot |   |
| cystocyte               |       |
| follicle cell (germanum) |     |
| posterior nurse cell & octotelo |   |
| follicle cell (stage 1-6) |    |
| anterior pole follicle cell (stage 7-9) |   |
| posterior pole follicle cell (stage 10-12) | |
| nurse cell (stage 2-8)  |       |
| oocyte (stage 2-6)      |       |
| follicle cell (stage 8)  |       |
| posterior polar follicle cell (stage 9) | |
| nurse cell (stage 9)    |       |
| border follicle cell    |       |
| duct cell               |       |
| gland cell              |       |
| imaginal cell           |       |
| hub cell                |       |
| male germinal germ cell & spermatid | |
| spermatagonium          |       |
| spermatocyte            |       |
| ductal epithelium, proper (Type I) | |
| antipodal epithelium, proper (Type II) |     |
| ductal epithelium, proper (Type III) |   |
| antipodal epithelium, proper (Type IV) | |
| spermatid, proper (Type I) | |
| spermatid, proper (Type II) |   |
| spermatid, proper (Type III) |  |
| spermatid, proper (Type IV) |   |
**Figure S2, related to Figure2.**

**Expression of Rab proteins**

(A,A’) Western blots from Yrab9, white1118, Yrab3, Yrab26, Yrab27, YrabX4, Yrab14, Yrab32 and YrabX5 adult heads probed for MYC. (A) and (A’) show short and long exposures of the same membrane. Major detected YRab protein forms are labeled (red stars). Numbers indicate size in kDa.

(B) Published Rab-mRNA modENCODE data (Chen et al., 2014) from larval fat body (FB) cells, salivary glands (SG) and imaginal discs (ID) are summarized in a heat map (see also FlyBase). The legend (right side) shows the color code for rab mRNAs levels, which range from high (orange) to low (blue), undetected Rabs are labeled with ‘n.d.’ (black). YRabs are presented in greyscale-coded squares that distinguish LECA Rabs according to how often they are lost in other eukaryotes. White squares indicate Rabs that arose in metazoans. (C) List showing all the cell types for which YRab expression was annotated. Black squares indicate that the corresponding YRab is expressed; Greyscale coding of YRab boxes is described in Figure S2B.
Figure S3, related to Figure3.

YRab localization in larval brains

(A) Confocal section of a second instar larval brain at the level of the big brain commissure, colored to highlight annotated features: neurons (grey), BBB glia (red) and the neuropil (green).

(B-I') Confocal sections from 2\textsuperscript{nd} instar larval brain lobes from \textit{oregonR} (B,B'), \textit{Yrab6} (C,C'), \textit{Yrab4} (D,D'), \textit{Yrab18} (E,E'), \textit{Yrab40} (F,F'), \textit{Yrab5} (G,G'), \textit{YrabX4} (H,H') and \textit{Yrab3} (I,I') probed for YFP (B-I grey and B'-I' green), DAPI (B'-I' blue) and Elav (B'-I' red).

(J) Dendogram shows clustering of YRabs according to the relatedness of their polarized localization in neurons: undetectable YRabs (grey), uniformly localized YRabs (black), and YRabs enriched in neuronal cell bodies (blue) or in neuronal projections (magenta). Y-axis indicates level of YRab localization similarity (0=no similarity and 1=equal localization); Greyscale coding of YRab boxes is described in Figure S2B.
Figure S4, related to Figure4.

YRab localization in larval salivary glands

(A) An early 3rd instar larval salivary gland and adjacent fat body. Highlighting indicates fat body cells (brown), gland cells (green), imaginal ring cells (light green) and duct cells (yellow). The region photographed for each Yrab allele is boxed.

(B-U’) Confocal sections of salivary glands from indicated Yrab homozygotes stained for YFP. Scale bars = 20µm (B-U) or 5µm (H’-U’). YRabs are grouped according to selected localization features: (C-E, grey) indicates expression in only a subset of salivary gland cells; (F,G and T,T’,U,U’ in black) indicates uniform distribution in gland cells; (H-K and H’-K’, magenta) indicates apical localization; (L-R and L’-R’, green) indicates baso-medial localization; (S,S’ in blue) indicates very basal localization.
### Neuron

![Neuron Diagram](image)

### Gland cell

![Gland cell Diagram](image)

### Follicle cell (stage 2-8)

![Follicle cell (stage 2-8) Diagram](image)

### Follicle cell (stage 9)

![Follicle cell (stage 9) Diagram](image)

### Wing disc cell (before pseudostratification)

![Wing disc cell (before pseudostratification) Diagram](image)

### Wing disc cell (after pseudostratification)

![Wing disc cell (after pseudostratification) Diagram](image)
Figure S5, related to Figure 5.

YRab distribution in neurons and epithelia

(A-F) Dendrograms show YRabs clustered according to their similarity of subcellular localization in neurons (A) and different epithelia (B-F). Color code indicates polarized YRab distribution group: apically enriched/enriched in neuronal projections (magenta); medially enriched (brown); medially and basally enriched (green); basally enriched/enriched in neuronal cell bodies (blue); uniformly localized YRabs (black) and undetectable YRabs (grey). Dashed line indicates presence of YRabs in apical hub (wing discs). Dendogram-Y-axis indicates YRab localization similarities (0=no similarity and 1=equal localization); Greyscale coding of YRab boxes is described in Figure S2B.

(A'-F') Representative images of YRab localization (green) in the neurons and epithelia corresponding to the dendrograms in (A-F). The apico-basal axis is indicated by Crumbs (magenta), Phalloidin (magenta), or Discs large (grey) staining. DAPI (blue) labels nuclei. Scale bar = 10µm.
Figure S6, related to Figure6.

Two-dimensional clustering of YRab distribution

(A) Two dimensional clustering of annotation features describing intracellular YRab distribution. Dendrograms show similarity of YRab distribution (horizontal dendrogram) and similarity of annotation features (vertical dendrogram). Black squares at the intersection of each annotation term and YRab position indicate that the annotation term applies to that YRab. Greyscale coding of YRab boxes is described in Figure S2B. Main annotation groups are color-coded according to features describing YRab morphology and polarity. Detailed list of annotation features defining each branch can be found in supplementary methods. Yellow box indicates cortical cluster comprising YRabs 23 and 35.
Figure S7, related to Figure 7.

Verification of YRab23 and 35 knock down

(A-G) Wing discs of indicated genotypes stained for YFP.
(A) Control wing disc from Yraban23 homozygous larva.

(B,C) Wing discs from Yrab23 homozygous larvae in which anti-GFP RNAi (B) or anti-GFP degrading nanobody (C) is expressed in the posterior compartment under the control of engrailed-Gal4.

(D) Control wing disc from Yrab23;Yrab35 homozygous larva harboring a UAS:GFP RNAi construct but no GAL4 driver.

(E) Wing disc from Yrab35 homozygous larva that expresses anti-GFP RNAi under the control of nubbin-Gal4.

(F) Wing disc from Yrab23 homozygous larva that expresses anti-GFP RNAi under the control of nubbin-Gal4.

(G) Wing disc from Yrab23;Yrab35 homozygous larva that expresses anti-GFP RNAi under the control of nubbin-Gal4.

Scale bars = 100µm.

(H) Plot shows fraction of flies displaying ectopic wing veins in male and female flies of indicated genotypes. Time controlled knock-down of Yrabs 23 and 35 in the developing wing blade (nubbin-Gal4; tubulin-Gal80ts) at restrictive (left) and permissive (right, control) temperatures. Yrab23;Yrab35;nubbin>>gfpRNAi: n=13 (male), 9 (female); control: n=10 (male), 10 (female).

(I-K) Quantified hair polarity patterns of female wings of different genotypes corresponding to those shown in (I'-K').

(I'-K'') Magnifications of the boxed regions in (I'-K'). Arrowheads (J'',K'') indicate multiple wing hairs. Scale bars = 500µm.
| Name       | Donor construct 5\` from yfp tag | Donor construct 3\` from myc tag | CG number (FlyBase) |
|------------|----------------------------------|----------------------------------|---------------------|
| pYrab1     | 4098 bp                          | 4547 bp                          | CG3320              |
| pYrab2     | 4905 bp                          | 2981 bp                          | CG3269              |
| pYrab3     | 4508bp                           | 3287 bp                          | CG7576              |
| pYrab4     | 3510 bp                          | 3587 bp                          | CG 4921             |
| pYrab5     | 4396bp                           | 3436 bp                          | CG3664              |
| pYrab6     | 4097 bp                          | 3600 bp                          | CG6601              |
| pYrab7     | 4941 bp                          | 2947 bp                          | CG5915              |
| pYrab8     | 3986 bp                          | 3532 bp                          | CG8287              |
| pYrab9     | 4070 bp                          | 3338 bp                          | CG9994              |
| pYrab10    | 4455 bp                          | 3923 bp                          | CG17060             |
| pYrab11    | 3877 bp                          | 4214 bp                          | CG5771              |
| pYrab14    | 4007 bp                          | 3222 bp                          | CG4212              |
| pYrab18    | 4501 bp                          | 3007 bp                          | CG3129              |
| pYrab19    | 3794 bp                          | 3693 bp                          | CG7062              |
| pYrab21    | 4486 bp                          | 3679 bp                          | CG17515             |
| pYrab23    | 4045 bp                          | 3601 bp                          | CG2108              |
| pYrab26    | 5986 bp                          | 3280 bp                          | CG34410             |
| pYrab27   | 4369 bp | 4298 bp | CG14791 |
|----------|---------|---------|---------|
| pYrab30  | 4117 bp | 3373 bp | CG9100  |
| pYrab32  | 5492 bp | 1962 bp | CG8024  |
| pYrab35  | 5314 bp | 3518 bp | CG9575  |
| pYrab39  | 5445 bp | 2757 bp | CG12156 |
| pYrab40  | 4239 bp | 2630 bp | CG1900  |
| pYrabX1  | 5106 bp | 3624 bp | CG3870  |
| pYrabX4  | 5656 bp | 2710 bp | CG31118 |
| pYrabX5  | 5384 bp | 2093 bp | CG7980  |
| pYrabX6  | 5296 bp | 2963 bp | CG12015 |

**Table S1, related to Figure 1**

**Donor constructs for targeted recombination**

The table indicates donor plasmids, size of the homologous sequence 5’prime from yfp start codon, size of the homologous sequence 3’prime from myc tag DNA fragment and the computed gene number of the targeted rab locus.
Supplementary Experimental Procedures

Hair polarity algorithm
Hair polarity patterns in adult wings were quantified using an autocorrelation-based algorithm as described in (Merkel et al., 2014).

Annotation
CATMAID image datasets (catmaid.org) were analyzed manually and annotated using a defined terminology to describe tissue distribution and subcellular localization of individual YRabs. The default annotation trees for each tissue are as follows:

Salivary gland annotation

| not expressed | expressed |
|---------------|-----------|
|                | expressed|
|                | neuroepithelium elevated levels |
|                | neuroblast elevated levels |
|                | neuron elevated levels |
|                | intracellular punctate |
|                | medially enriched |
|                | basally enriched |
|                | intracellular diffuse |
|                | medially enriched |
|                | basally enriched |
|                | cortical |
|                | apically enriched |
|                | medially enriched |
|                | basally enriched |

Fat body annotation

| not expressed | expressed |
|---------------|-----------|
|                | fat body cell |
|                | intracellular punctate |
|                | intracellular diffuse |
|                | cortical |

CNS annotation

| not expressed | expressed |
|---------------|-----------|
|                | peripodial epithelium elevated levels |
|                | disc epithelium proper elevated levels |
|                | apical hub visible |
|                | intracellular punctate |
|                | medially enriched |
|                | basally enriched |
|                | intracellular diffuse |
|                | apically enriched |
|                | medially enriched |
|                | basally enriched |
cortical
apically enriched
basally enriched
medially enriched
late 3rd instar
peripodial epithelium
elevated levels
disc epithelium proper
elevated levels
apical hub visible
intracellular punctate
apically enriched
basally enriched
medially enriched
intracellular diffuse
apically enriched
basally enriched
medially enriched
cortical
apically enriched
basally enriched
medially enriched
Testis annotation
not expressed
expressed
hub cell
elevated levels
male germline stem cell and gonialblast
elevated levels
spermatogonium
elevated levels
intracellular punctate
apically enriched
medially enriched
basally enriched
intracellular diffuse
apically enriched
medially enriched
basally enriched
cortical
apically enriched
basally enriched
medially enriched
Ovary annotation
not expressed
expressed
germarium
cap cell
elevated levels
female germline stem cell and
cystoblast
elevated levels

intracellular punctate
cortical

intracellular diffuse
cortical
cystocyte
elevated levels
intracellular punctate
intracellular diffuse
cortical
cortical
elevated levels
intracellular punctate
intracellular diffuse
cortical

follicle cell
elevated levels
intracellular punctate
apically enriched
medially enriched
basally enriched
intracellular diffuse
apically enriched
medially enriched
basally enriched
cortical
apically enriched
medially enriched
basally enriched

presumptive nurse cell and oocyte
(stage 1)
elevated levels
intracellular punctate
intracellular diffuse
cortical

stage 2-8 egg chamber
follicle cell
elevated levels
intracellular punctate
apically enriched
medially enriched
basally enriched
intracellular diffuse
apically enriched
medially enriched
basally enriched
cortical
apically enriched
medially enriched
basally enriched

anterior polar follicle cell
elevated levels
posterior polar follicle cell
elevated levels
nurse cell
elevated levels
intracellular punctate
intracellular diffuse
cortical
oocyte
  elevated levels
  intracellular punctate
  intracellular diffuse
  cortical

stage 9 egg chamber
follicle cell
  elevated levels
  intracellular punctate
    apically enriched
    medially enriched
    basally enriched
  intracellular diffuse
    apically enriched
    medially enriched
    basally enriched
  cortical
    apically enriched
    medially enriched
    basally enriched

border follicle cell
  elevated levels

posterior polar follicle cell
  elevated levels

nurse cell
  elevated levels
  intracellular punctate
  intracellular diffuse
  cortical
Clustering

Multiple annotation features that are represented in a binary matrix characterize the tissue distribution and subcellular localization of each YRab. We used this binary matrix to perform hierarchical clustering of YRabs according to specific annotation features using Past 3.01 (http://folk.uio.no/ohammer/past/ and (Hammer et al. 2001)). The Jaccard clustering metric was used to compute similarity coefficients. Rooted trees (dendrograms) were generated according to similarity coefficients based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) linkage. The annotation features used for each hierarchical clustering are as follows:

Figure 2C
neuroepithelium
neuron
neuroblast
fat body cell
germarium,cap cell
germarium,female germline stem cell and cystoblast
germarium,cystocyte
germarium,follicle cell
germarium,presumptive nurse cell and oocyte (stage1)
stage 2-8 egg chamber,follicle cell
stage 2-8 egg chamber,anterior polar follicle cell
stage 2-8 egg chamber,posterior polar follicle cell
stage 2-8 egg chamber,nurse cell
stage 2-8 egg chamber,oocyte
stage 9 egg chamber,follicle cell
stage 9 egg chamber,posterior polar follicle cell
stage 9 egg chamber,nurse cell
stage 9 egg chamber,border follicle cell
duct cell
gland cell
imaginal cell
hub cell
male germline stem cell and gonialblast
spermatogonium
spermatocyte
early 3rd instar,disc epithelium proper
early 3rd instar,peripodial epithelium
late 3rd instar,disc epithelium proper
late 3rd instar, peripodial epithelium

Figure 4G
- gland cell, intracellular punctate, basally enriched
- gland cell, intracellular punctate, medially enriched
- gland cell, intracellular diffuse, apically enriched
- gland cell, cortical, apically enriched
- gland cell, cortical, medially enriched

Figure 5I-J
(I)
- stage 2-8 egg chamber, follicle cell, intracellular punctate, apically enriched
- stage 2-8 egg chamber, follicle cell, intracellular diffuse, apically enriched
- stage 2-8 egg chamber, follicle cell, cortical, medially enriched

(J)
- stage 9 egg chamber, follicle cell, intracellular punctate, basally enriched
- stage 9 egg chamber, follicle cell, intracellular diffuse, basally enriched

Figure 6F-G
(F)
- early 3rd instar, disc epithelium proper, intracellular punctate, apically enriched
- early 3rd instar, disc epithelium proper, intracellular diffuse, apically enriched
- early 3rd instar, disc epithelium proper, intracellular diffuse, basally enriched

(G)
- late 3rd instar, disc epithelium proper, intracellular punctate, apically enriched
- late 3rd instar, disc epithelium proper, intracellular punctate, basally enriched
- late 3rd instar, disc epithelium proper, intracellular diffuse, apically enriched
- late 3rd instar, disc epithelium proper, intracellular diffuse, basally enriched
- late 3rd instar, disc epithelium proper, cortical, basally enriched
- late 3rd instar, disc epithelium proper, apical hub visible

Figure S3J
- neuron, intracellular punctate, cell body enriched
- neuron, intracellular diffuse, cell body enriched
- neuron, intracellular diffuse, projection enriched

Figure S5A-F
(A)
- neuron, intracellular punctate, cell body enriched
- neuron, intracellular diffuse, cell body enriched
- neuron, intracellular diffuse, projection enriched

(B)
gland cell, intracellular punctate, basally enriched

gland cell, intracellular punctate, medially enriched

gland cell, intracellular diffuse, apically enriched

gland cell, cortical, apically enriched

gland cell, cortical, medially enriched

(C) stage 2-8 egg chamber, follicle cell, intracellular punctate, apically enriched

stage 2-8 egg chamber, follicle cell, intracellular diffuse, apically enriched

stage 2-8 egg chamber, follicle cell, cortical, medially enriched

(D) stage 9 egg chamber, follicle cell, intracellular punctate, basally enriched

stage 9 egg chamber, follicle cell, intracellular diffuse, basally enriched

(E) early 3rd instar, disc epithelium proper, intracellular punctate, apically enriched

early 3rd instar, disc epithelium proper, intracellular diffuse, apically enriched

early 3rd instar, disc epithelium proper, intracellular diffuse, basally enriched

(F) late 3rd instar, disc epithelium proper, intracellular punctate, apically enriched

late 3rd instar, disc epithelium proper, intracellular punctate, basally enriched

late 3rd instar, disc epithelium proper, intracellular diffuse, apically enriched

late 3rd instar, disc epithelium proper, intracellular diffuse, basally enriched

late 3rd instar, disc epithelium proper, cortical, basally enriched

late 3rd instar, disc epithelium proper, apical hub visible

Figure S6

neuron, cortical

neuron, intracellular punctate

neuron, intracellular diffuse

fat body cell, intracellular punctate

fat body cell, intracellular diffuse

fat body cell, cortical

germarium, female germline stem cell and cystoblast, intracellular punctate

germarium, female germline stem cell and cystoblast, intracellular diffuse

germarium, female germline stem cell and cystoblast, cortical

germarium, cystocyte, intracellular punctate

germarium, cystocyte, intracellular diffuse

germarium, cystocyte, cortical

germarium, follicle cell, intracellular punctate

germarium, follicle cell, intracellular diffuse

germarium, follicle cell, cortical

germarium, presumptive nurse cell and oocyte (stage 1), intracellular punctate

germarium, presumptive nurse cell and oocyte (stage 1), intracellular diffuse
germarium, presumptive nurse cell and oocyte (stage 1), cortical stage 2-8 egg chamber, follicle cell, intracellular punctate stage 2-8 egg chamber, follicle cell, intracellular diffuse stage 2-8 egg chamber, follicle cell, cortical stage 2-8 egg chamber, nurse cell, intracellular punctate stage 2-8 egg chamber, nurse cell, intracellular diffuse stage 2-8 egg chamber, nurse cell, cortical stage 2-8 egg chamber, oocyte, intracellular punctate stage 2-8 egg chamber, oocyte, intracellular diffuse stage 9 egg chamber, follicle cell, intracellular punctate stage 9 egg chamber, follicle cell, intracellular diffuse stage 9 egg chamber, nurse cell, intracellular punctate stage 9 egg chamber, nurse cell, intracellular diffuse stage 9 egg chamber, nurse cell, cortical gland cell, intracellular punctate gland cell, intracellular diffuse gland cell, cortical spermatogonium, intracellular punctate spermatogonium, intracellular diffuse spermatogonium, cortical spermatocyte, intracellular punctate spermatocyte, intracellular diffuse spermatocyte, cortical early 3rd instar, disc epithelium proper, intracellular punctate early 3rd instar, disc epithelium proper, intracellular diffuse early 3rd instar, disc epithelium proper, cortical late 3rd instar, disc epithelium proper, intracellular punctate late 3rd instar, disc epithelium proper, intracellular diffuse late 3rd instar, disc epithelium proper, cortical neuron, intracellular punctate, cell body enriched neuron, intracellular diffuse, cell body enriched neuron, intracellular diffuse, projection enriched germarium, follicle cell, intracellular punctate, apically enriched germarium, follicle cell, cortical, medially enriched stage 2-8 egg chamber, follicle cell, intracellular punctate, apically enriched stage 2-8 egg chamber, follicle cell, intracellular diffuse, apically enriched stage 2-8 egg chamber, follicle cell, cortical, medially enriched stage 9 egg chamber, follicle cell, intracellular punctate, basally enriched stage 9 egg chamber, follicle cell, intracellular diffuse, basally enriched gland cell, intracellular punctate, basally enriched gland cell, intracellular punctate, medially enriched gland cell, intracellular diffuse, apically enriched gland cell, cortical, apically enriched gland cell, cortical, medially enriched early 3rd instar, disc epithelium proper, intracellular punctate, apically enriched
early 3rd instar, disc epithelium proper, intracellular diffuse, apically enriched
early 3rd instar, disc epithelium proper, intracellular diffuse, basally enriched
late 3rd instar, disc epithelium proper, intracellular punctate, apically enriched
late 3rd instar, disc epithelium proper, intracellular punctate, basally enriched
late 3rd instar, disc epithelium proper, intracellular diffuse, apically enriched
late 3rd instar, disc epithelium proper, intracellular diffuse, basally enriched
late 3rd instar, disc epithelium proper, cortical, basally enriched
late 3rd instar, disc epithelium proper, apical hub visible
stage 2-8 egg chamber, nurse cell, cortical
stage 2-8 egg chamber, nurse cell, cytoplasmic diffuse
stage 2-8 egg chamber, nurse cell, cytoplasmic punctate
stage 2-8 egg chamber, oocyte, cortical
stage 2-8 egg chamber, oocyte, cytoplasmic diffuse
stage 2-8 egg chamber, oocyte, cytoplasmic punctate
stage 9 egg chamber, follicle cell, cortical
stage 9 egg chamber, follicle cell, cytoplasmic diffuse
stage 9 egg chamber, follicle cell, cytoplasmic diffuse, basally enriched
stage 9 egg chamber, follicle cell, cytoplasmic punctate
stage 9 egg chamber, follicle cell, cytoplasmic punctate, basally enriched
stage 9 egg chamber, nurse cell, cortical
stage 9 egg chamber, nurse cell, cytoplasmic diffuse
stage 9 egg chamber, nurse cell, cytoplasmic punctate

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