The two TRAPP complexes of metazoans have distinct roles and act on different Rab GTPases

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Originally identified in yeast, transport protein particle (TRAPP) complexes are Rab GTPase exchange factors that share a core set of subunits. TRAPPs were initially found to act on Ypt1, the yeast orthologue of Rab1, but recent studies have found that yeast TRAPP II can also activate the Rab11 orthologues Ypt31/32. Mammals have two TRAPP complexes, but their role is less clear, and they contain subunits that are not found in the yeast complexes but are essential for cell growth. To investigate TRAPP function in metazoans, we show that Drosophila melanogaster have two TRAPP complexes similar to those in mammals and that both activate Rab1, whereas one, TRAPP II, also activates Rab11. TRAPP II is not essential but becomes so in the absence of the gene parcas that encodes the Drosophila orthologue of the SH3BP5 family of Rab11 guanine nucleotide exchange factors (GEFs). Thus, in metazoans, Rab1 activation requires TRAPP subunits not found in yeast, and Rab11 activation is shared by TRAPP II and an unrelated GEF that is metazoan specific.

Introduction

Rab GTPases control many aspects of subcellular organization. They are typically active on only one particular organelle or vesicle class and so direct the subcellular localization of a wide range of proteins, including membrane trafficking machinery, molecular motors, and regulators of phosphoinositide levels or the activity of other GTPases (Stemmark, 2009; Hutagalung and Novick, 2011; Gillingham et al., 2014). This role in spatial organization of the cell requires specific guanine nucleotide exchange factors (GEFs) to activate each Rab in only the correct location (Barr, 2013; Ishida et al., 2016). GEFs for several Rabs have been identified, and among the best studied are the transport protein particle (TRAPP) complexes (Barrowman et al., 2010; Brunet and Sacher, 2014; Kim et al., 2016). The first TRAPP subunit was identified in yeast in a screen for mutations that interact with a mutation in a SNARE protein, and the corresponding protein was found to be part of a large protein complex that was termed TRAPP (Rossi et al., 1995; Sacher et al., 1998). Subsequent work reported the existence of three different TRAPP complexes in yeast (Barrowman et al., 2010; Choi et al., 2011; Brunet and Sacher, 2014; Kim et al., 2016). All three share a heptameric core of six proteins (Bet3 being present twice), with TRAPP I having no further subunits, TRAPP II having four additional subunits called Tca17, Trs65, Trs120, and Trs130, and TRAPP III having one additional subunit, Trs85. The shared TRAPP subunits are essential for membrane traffic through the Golgi apparatus, and consistent with this, TRAPP I was found to act as a GEF for Ypt1 (yeast Rab1), a GTPase essential for ER to Golgi and intra-Golgi traffic (Wang et al., 2000; Kim et al., 2006; Cai et al., 2008). TRAPP III was initially reported to have a more specific role in activating Ypt1 during autophagy, but recent work suggests that TRAPP I may not exist in vivo and that TRAPP III is responsible for the majority of Rab1 exchange activity in both secretion and autophagy (Meiling-Wesse et al., 2005; Lynch-Day et al., 2010; Thomas et al., 2017). In contrast, TRAPP II was proposed to act later in the Golgi as a GEF for the closely related GTPases Ypt31 and Ypt32, yeast orthologues of Rab11 (Jones et al., 2000; Morozova et al., 2006). This conclusion was initially questioned, but recent biochemical studies have shown both Rab1 and Rab11 GEF activity for TRAPP II from filamentous fungi and budding yeasts (Wang and Ferro-Novick, 2002; Pinar et al., 2015; Thomas and Fromme, 2016).

The shared core TRAPP subunits that are sufficient to act on Ypt1/Rab1 are very highly conserved in evolution and appear to be a universal feature of eukaryotic cells (Koumanadou et al., 2007). Mammals have orthologues of all of the yeast TRAPP subunits, including those specific to TRAPP II and TRAPP III. In addition, co-precipitation experiments have identified two further TRAPP subunits that are not present in yeast (Gavin et al., 2002; Scrivens et al., 2011). Examination of the proteins associated with each mammalian TRAPP subunit revealed that they form two complexes related to yeast TRAPP II and TRAPP III, with there being no evidence that mammals have a complex equivalent to TRAPP I, i.e., just the core subunits (Choi et al., 2011; Bassik et al., 2013; Borner et al., 2014). Mammalian TRAPP II contains seven core subunits and orthologues of Trs120 (TRAPPC9) and Trs130 (TRAPPC10). Mammalian TRAPP III contains the same seven core subunits and an orthologue of Trs85 (TRAPPC8) plus three further subunits:

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TRAPPC13 (an orthologue of yeast Trs65) and the two subunits not found in yeast (TRAPPC11 and TRAPPC12).

The precise roles of TRAPPII and TRAPPIII in mammals are not fully resolved. When assembled in vitro, the core subunits of the mammalian TRAP complexes have exchange activity on Rab1 (Kim et al., 2006), and mammalian TRAPPII has been reported to have the same activity when immunopurified from cells but to have no activity on Rab11 (Yamasaki et al., 2009). Moreover, there are also some striking differences to the yeast system. The most obvious is the existence of the two additional subunits in TRAPPII, TRAPPC11 and TRAPPC12, and these seem unlikely to have minor roles as at least TRAPPC11 is essential for secretion and cell viability (Wendler et al., 2010; Scrivens et al., 2011; Kim et al., 2016). In contrast, the TRAPPII subunits Trs120 and Trs130 are both essential for growth in yeast, and yet in mammals they do not appear to be required for cell viability even though Rab11 is an essential protein (Jankovics et al., 2001; Kim et al., 2016). Indeed, loss-of-function mutations in human TRAPPC9 are not lethal but cause mental retardation (Mir et al., 2009; Mochida et al., 2009; Philippe et al., 2009).

The TRAP complex subunits found in humans are well conserved across metazoans, and so we have used the tractable genetic system of Drosophila melanogaster to investigate TRAP in metazoans. We found that Drosophila contain two TRAP complexes that have the same composition as the human complexes, and we combined genetics and expression of recombinant TRAP complexes to investigate their function in vivo and their activity in vitro.

**Results**

**Purification of Drosophila TRAP complexes**

To investigate the composition of the TRAP complexes in Drosophila, we used a tandem affinity purification (TAP) approach to isolate them from the S2 Drosophila cell line (Fig. 1A). Stably transfected cell lines were generated expressing the TRAPPC3 subunit with a C-terminal TAP tag based on GFP and the streptavidin-binding peptide (SBP) tag. TRAPPC3 was chosen because its yeast orthologue, Bet3, is a core component of all TRAP complexes, and C-terminal tagging does not compromise its activity (Sacher et al., 1998). In parallel and as a control, we also generated cells lines expressing TAP-tagged forms of the Drosophila orthologue of Rgp1, a subunit of another complex proposed in other species to have GEF activity on a different Golgi Rab: in yeast, Rgp1 forms a complex with Ric1 that has exchange activity on Rab6, and both proteins are on a different Golgi Rab: in yeast, Rgp1 forms a complex with other complex proposed in other species to have GEF activity on Rab11 (Tong et al., 2011). Rgp1 that has exchange activity on Rab6, and both proteins are on a different Golgi Rab: in yeast, Rgp1 forms a complex with other complex proposed in other species to have GEF activity on Rab11 (Tong et al., 2011). Orthologue of Rgp1, a subunit of an-...
Composition and localization of TRAPP complexes in Drosophila tissues

These experiments rely on cultured cells and expressing the tagged TRAPP subunits in the context of the endogenous untagged protein. To instead investigate the behavior of tagged subunits in their native situation, we used CRISPR/Cas9-mediated homologous recombination to attach the SBP-GFP TAP tag to the C terminus of TRAPP subunits genes in the Drosophila genome. For this, we selected TRAPP3 as being in both complexes and TRAPP9, TRAPP11, and TRAPP12 as complex-specific subunits. All four tagged alleles produced viable flies when homozygous, and all four resulted in the expression of TAP-tagged proteins, which could be isolated from embryos and analyzed by...
Figure 2. The TRAPP complexes of Drosophila. (A) Mass spectrometry analysis of TRAPPC3, TRAPPC9, TRAPPC10, TRAPPC11, TRAPPC12, and TRAPPC13 tandem affinity purified from stably transfected cell lines. Total spectral counts for each protein are shown, with replicates shown in Fig. S1 B. Precipitations also contained lower levels of abundant chaperones and cytosolic enzymes. With the shared TRAPP subunit TRAPPC3, only three proteins apart from known TRAPP subunits were consistently present across all TAP purifications and several single-step purifications (catalase, ribosomal protein S26, and Gdi), and these were only present at trace levels in the TAP samples and so were not pursued further. (B) Immunoblot using the TRAPPC9 and TRAPPC12 antibodies of proteins associated with Rgp1, TRAPPC3, TRAPPC8, TRAPPC10, TRAPPC11, and TRAPPC13 after anti-GFP affinity purification from lysates of transiently transfected S2 cells. Molecular masses are given in kilodaltons. IP, immunoprecipitation. (C) Subunits present in the different the TRAPP complexes of yeast, flies, and humans. (D) Widefield micrographs of S2 cells transiently expressing GFP-tagged TRAPPC8, TRAPPC11, and TRAPPC13 and stained with antibodies against Golgin245 and GM130. Line scans of fluorescence intensity relative to the maximum are from representative Golgi stacks (dotted line on Golgin245 panels). The TRAPP subunits are enriched at the cis end of the stack. (E) Confocal micrographs of S2 cells labeled with antisera to endogenous TRAPC9 (TRAPPI), TRAPPC12 (TRAPPIII), and the Golgi proteins Golgin245 or GM130. Line scans are from representative Golgi stacks (dotted line on Golgi marker panels). Bars, 5 µm. Images in D and E are representative of at least two independent experiments, with at least three micrographs obtained from each.
mass spectrometry (Fig. 3, A and B). This revealed that as in the S2 cells, TRA PPC3 associated with all TRAPP subunits, whereas the specific subunits showed the same pattern of interaction seen in S2 cells, indicating that TRAPPII and TRAPPII exist in the native context (Fig. 3 B). This dichotomy into two complexes was also found in larvae and adults when the complexes were isolated and examined by immunoblotting (IB; Fig. 3 C).

Examination of the subcellular distribution of the tagged subunits in tissues revealed that it was possible to detect TRAPPC3 and the TRAPPII-specific subunits but not TRAPPII-specific TRAPPC9 in both wing discs and the salivary gland (Fig. 3 D). Interestingly, in salivary glands, TRAPPII showed a striking bimodal distribution in the Golgi stack with a clear concentration at both the cis and trans sides of the stack. The trans peak colocalized better with the clathrin adapter AP-1 than Golgin245 and thus appeared to correspond with the trans-Golgi network (Figs. 3 D and S1 D). This bimodal distribution was seen with both TRAPPC1 and TRAPPC12 as well as with TRAPPC3 and clearly indicates that TRAPPI is present on both ends of the Golgi stack in at least some tissues.

Generation of null alleles in TRAPPII- and TRAPPIII-specific subunits

Several chemical or transposon-based mutations in genes encoding TRAPP subunits have been isolated in screens for lethal genetic lesions or for defects in fertility (Fig. 4 A). This has shown that at least three of the core subunits are essential, along with TRAPPC8 and TRAPPC11 from TRAPPII. In some cases, the genetic lesion was not reported, and by sequencing the previously reported alleles, we were able to show that the lethal ethyl methane sulfonate (EMS) mutations in TRAPPC2, TRAPPC8, and TRAPPC11 were all caused by nonsense mutations (Fig. S2 B; see also the Characterization of potential TRAPPII complexes section of Materials and methods). In contrast, a nonsense mutation that truncates TRAPPC9 at residue 514 was reported to be viable but showed a temperature-sensitive defect in male fertility (Robinett et al., 2009). To investigate the role of the specific TRAPPI complex, we used the CRISPR/Cas9 system to generate mutations in the shared core subunit TRAPPC3 and in the complex-specific subunits TRAPPC9, TRAPPC10, and TRAPPC11 (Fig. S2, A–D). We were able to confirm the loss of TRAPPC10 protein in the relevant mutant by using TRAPPC9-GFP to precipitate the TRAPPII complex from the TRAPPC10 mutant flies (Fig. 4 B). Interestingly, in the absence of TRAPPC10, TRAPPC9 was associated with only the three core subunits, TRAPPC2, TRAPPC3, and TRAPPC5, that in mammals and yeast are present at one end of the core (Fig. 4 B; Kim et al., 2006; Cai et al., 2008). This is consistent with the finding in yeast that this part of the TRAP core can bind to either Tns85 (TRAPPC8) or Tns120 (TRAPPC9; Tan et al., 2013; Taussig et al., 2014), therefore suggesting that TRAPPC9 has the same location in TRAPPII as TRAPPC8 occupies in TRAPPIII and raising the possibility that TRAPPC10 either enables or stabilizes the assembly of the core complex in TRAPPII.

Examination of the phenotypes of the various mutations revealed that frameshift mutations in TRAPPC3 and TRAPPC11 were homozygous lethal, confirming that TRAPPII is essential for viability (Fig. 4 C). In contrast, a deletion of the entire TRAPPC9 coding region and a frameshift mutation in TRAPPC10 were both homozygous viable (Fig. 4 D). For both TRAPPC9 and TRAPPC10, the loss of the protein resulted in male sterility, indicating that both proteins contribute to the same activity of TRAPPII (Fig. 4 E). This is consistent with a previous study that found that a male sterile mutation corresponded to a 514-stop lesion in TRAPPC9 and indicates that this truncated form of the protein has little residual activity (Robinett et al., 2009). Collectively, these findings indicate that the TRAPPII complex is essential for viability, but the TRAPPIII complex appears only essential for normal male fertility, indicating that the two complexes have distinct roles in vivo.

In vitro assay of the exchange activity of TRAPPII and TRAPPIII

To compare the Rab exchange activity of TRAPPIII and TRAPPII, we established a method to prepare the two complexes in recombinant form. For each complex, the genes encoding all of the relevant subunits were coexpressed in insect cells using a multipromoter baculovirus vector. In both cases, cleavable tags were attached to one of the subunits, and by affinity purification, we were able to produce complete TRAPPII and TRAPPIII complexes (Fig. 5 A). The fact that each complex contains all of the subunits found in the specific TAP purifications suggests that these two complexes do indeed form in vivo and that no further subunits are required to hold them together.

When the purified TRAPPII and TRAPPIII complexes were tested with Drosophila Rab1, both were able to stimulate the exchange of bound nucleotides (Fig. 5 B). A previous study of the yeast TRAP complexes found that their exchange activity is enhanced if the relevant GTPase is attached to liposomes (Thomas and Fromme, 2016), and we also found increased nucleotide exchange with Rab1 when it was attached to the surface of liposomes. In contrast with these findings with Rab1, when we examined Rab11, we found that only TRAPPII had readily detectable exchange activity, with TRAPPIII having only minimal activity (Fig. 5 B). These activities appear specific, as neither TRAP complex stimulated exchange on the Golgi Rabs Rab2 and Rab18 or on the endosomal Rab, Rab5 (Fig. S3, A–C). Moreover, a similar Rab11 GEF activity was found for TRAPPIII when the assay was performed using a different purification procedure and a different assay for GTP exchange (Fig. S3, D and E).

Thus, it seems that as in plants and fungi, the presence of TRAPPC9 and TRAPPC10 allows the metazoan TRAPPII complex to act on Rab11. Interestingly, these two subunits form a stable complex when expressed without the shared subunits, adding further support to the notion that the two proteins act together to allow the TRAPP core complex to recognize Rab11 (Fig. 5 A).

TRAPPIII stimulates Rab1 recruitment to membranes in vivo

As described in the Generation of null alleles… section above, mutations in the TRAPPIII-specific subunit TRAPPC11 are lethal. However, we were able to use the FRT system in wing discs to generate clones of cells that lacked both copies of either TRAPPC3 or TRAPPC11. After 2.33 d, these clones were several cells in size, but after 3 d, clones were no longer detectable, suggesting that the cells eventually became defective and were removed (Fig. 6 A). To examine Rab1 in these clones, we generated them in a background where both alleles of Rab1 were tagged with YFP (Dunst et al., 2015). In clones of cells lacking TRAPPC3 or TRAPPC11, the levels of YFP-Rab1 on the Golgi were significantly reduced, whereas other Golgi markers were unaffected (Fig. 6, B–D). Thus, the levels of Rab1 on the
Figure 3. TRAPPII and TRAPPIII are present in embryos, larvae, and adults. (A) Silver-stained protein gels of TAPs of SBP-GFP–tagged TRAPPC11, TRAPPC9, TRAPPC3, and TRAPPC12 from embryos. (B) Mass spectrometry analysis of proteins associated with SBP-GFP–tagged TRAPPC11, TRAPPC9, TRAPPC3, and TRAPPC12 after TAP from embryos. Total spectral counts for each protein are shown. (C) Immunoblot analysis using the TRAPPC9 and TRAPPC12 antibodies of proteins associated with TRAPPC3, TRAPPC9, TRAPPC11, and TRAPPC12 after anti-GFP immunoprecipitation (IP) from lysates prepared from adults (left) or wandering third instar larvae (right) carrying SBP-GFP–tagged alleles. Arrows indicate GFP-tagged TRAP subunits; arrowheads indicate native TRAP subunits. Molecular masses are given in kilodaltons. (D) Confocal micrographs of SBP-GFP–tagged TRAPPC3, TRAPPC11, and TRAPPC12 in wing imaginal discs and salivary glands dissected from wandering third instar larvae and stained for GFP, GM130, and Golgin245. In each case, the tag was inserted at the genomic locus, and therefore, the tagged protein was expressed from its native promoter. Line scans are from representative Golgi stacks (dotted lines in GFP/Golgin245 panels). In the salivary gland, all three proteins were discretely located to both the cis and trans ends of the stack (arrows in insets), with a similar bimodal distribution also observed in follicle cells (not depicted). Bars: (main images) 5 µm; (insets) 1 µm. Images are representative of at least two independent experiments, with at least three micrographs obtained from each.
Golgi are reduced in the absence of TRAPPII, indicating that TRAPPII is a major activator of Rab1 in vivo.

TRAPPII is functionally redundant with Parcas, the Drosophila orthologue of the mammalian Rab11 GEF SH3BP5

These results indicate that TRAPPII can act as an exchange factor on Rab11, but it is known that Rab11 is essential in Drosophila (Jankovics et al., 2001), whereas TRAPPC9 and TRAPPC10, the specific subunits that define TRAPPII, are clearly not essential. This implies that TRAPPII is not the only Rab11 GEF in flies, and indeed, when we examined endogenous Rab11 in the TRAPPC9-null mutant, we could not detect any obvious change in its distribution in salivary glands or wing discs (not depicted). Recently, two closely related proteins in Caenorhabditis elegans, Rei-1 and Rei-2, were identified as putative Rab11 GEFs by their ability to bind to the GDP form of Rab11, and both Rei-1 and its mammalian orthologue SH3BP5
were confirmed to have Rab11 GEF activity in vitro (Sakaguchi et al., 2015). These proteins have no detectable similarity with TRAPP subunits or any other protein family. Drosophila have a single orthologue of Rei-1 and SH3BP5, which was initially isolated as a mutant with a defect in the formation of embryonic germ cells and named poirot (Sinka et al., 2002). This gene was subsequently shown to have other roles in development, including muscle formation, and was renamed parcas (pcs; Artero et al., 2003). The original pcs allele, pcs\(^{\text{gs}}\), is a null mutant, but nonetheless it is adult viable, albeit with reduced egg laying (Sinka et al., 2002). We thus tested the possibility that Parcas has Rab11 GEF activity that can explain why flies are viable in the absence of TRA PPII.

The Parcas protein is 477 residues long and it could be expressed and purified from Escherichia coli. This recombinant Parcas had GEF activity on Drosophila Rab11 but not Rab1 or Rab5, indicating that it behaves like its orthologues in C. elegans and humans (Fig. 7, A–C). The pcs gene is on the same chromosome as that encoding TRA PPC9, and so we combined the two mutations by recombination. Matings between flies carrying the single or double mutations confirmed that progeny homozygous for either of single mutations are viable; however, the double mutant stock produced no adult progeny homozygous for the chromosome carrying both mutations, indicating that loss of both genes is lethal (Fig. 7 D). These double mutant progeny completed embryogenesis but died in early larval stages. This striking genetic interaction is consistent with both TRAPPII and Pcs having Rab11 GEF activity and provides a simple explanation for why the individual loss of function mutations have a milder phenotype than loss of Rab11.

**Discussion**

The TRAPP complexes have emerged as fundamental components of membrane traffic machinery in eukaryotes. Since their discovery almost 20 years ago, most of the characterization of the complexes has been performed in yeast, and although recent work has shown that humans and other metazoans have two TRAP complexes (Bassik et al., 2013), these are not identical to their yeast counterparts. In this study, we have provided functional evidence from Drosophila to show what is shared between metazoans and yeast and also to resolve some of the apparent paradoxes that have emerged from comparing the yeast and mammalian systems. We find that as in yeast, both of the Drosophila TRAP complexes have GEF activity on Rab1, whereas TRAPPII also acts as a GEF on Rab11. In the only previous study in which mammalian TRAP complexes were tested on Rab11, TRAPPCII was immunoisolated from cells using antiserum to TRAPPC9, and activity was found on Rab1 but not Rab11 (Yamasaki et al., 2009). However, the subunit composition of the isolated complex was not determined, and it is also possible that the antibody or attached beads inhibited access to the Rab11 substrate. A recent study has suggested that mammalian TRAPPII can act on both Rab1 and Rab18 (Rab11 was not tested), although for reasons that are not clear, we...
were unable to detect this Rab18 activity within the Drosophila complex (Li et al., 2017).

Beyond these shared properties, there are some clear differences between the yeast and metazoan TRAP complexes. First, TRAP III contains two additional subunits, TRAPC11 and TRAPC12, that are absent from yeast. These two subunits are unlikely to have a metazoan-specific role as they are very widely conserved throughout eukaryotic phyla, including even filamentous fungi, and instead appear to have been lost in the budding yeast lineage (Fig. S4). TRAPC11 is distantly related to TRAPC10, and so it seems likely that in early eukaryotic evolution, there was an ur-TRAP complex that had two additional subunits, and then both duplicated to make on the one hand TRAPC8 and TRAPC9 and on the other TRAPC10 and TRAPC11, and hence two TRAP complexes. TRAPC11 is essential for the growth of both Drosophila and mammalian cultured cells, and therefore yeast must have found a means to bypass this requirement. Given that the core of shared subunits is sufficient for Rab1 GEF activity, the role of TRAPC11 seems most likely to be to direct the TRAP III Rab1 GEF activity to the correct location. It is possible that yeast have evolved an alternative mechanism to direct TRAP III to Golgi membranes via the core subunits (Sacher et al., 2001; Bassik et al., 2013). However, it is also possible that TRAPC11 allows the complex to have an additional activity that budding yeast no longer requires. Our finding that in some tissues, TRAPC11 is found on the TGN as well as on recycling endosomes (Emery et al., 2005; Satoh et al., 2005).

Our work has focused on TRAPC11 as the TRAP III subunit that is essential but absent from yeast. The TRAP III subunit that is shared with yeast, TRAPC8, has been reported
to be essential in Drosophila and in mammalian cultured cells, although its yeast orthologue Trs85 is not essential, perhaps for the same reasons suggested in the previous paragraph as to how yeast survive without TRA PPC11 (Cooper et al., 2010; Kim et al., 2016). Metazoans have two further TRA PPII subunits: TRA PPC13, a distant orthologue of the yeast protein Trs65 that has been suggested to contribute to TRA PP complex structure (Choi et al., 2011), and TRA PPC12, a protein that is absent from budding yeasts but present in a diverse range of eukaryotic phyla, suggesting it had been lost during budding yeast evolution (Fig. S4). The role of TRA PPC12 is unclear, and it is unrelated to other TRA PP subunits and has even been suggested to have additional roles outside of the TRA PP complex (Milev et al., 2015). TRA PPC12 has also been reported to bind to the Sec13/31 component of the COP II coat (Bassik et al., 2013; Zhao et al., 2017). However, although we found this interaction when we overexpressed tagged TRA PPC12, we did not recover Sec13/31 when we precipitated TRAPPII with other subunits (Fig. 2A). This suggests that if this interaction is physiological, then it may represent a distinct function of TRAPPC12 or possibly an intermediate in the assembly of TRAPPII.

A second striking difference between the yeast and metazoan TRAPPI complexes is that although TRAPPII can act on Rab11 in both classes of organism, in metazoans, it shares the role of activating Rab11 with the unrelated SH3BP5 protein family. There is clearly considerable redundancy between the two, as mutations in the SH3BP5 orthologues in Drosophila and C. elegans are viable as are mutations in TRAPPII in Drosophila and mammalian cultured cells (Sinka et al., 2002; Sakaguchi et al., 2015; Kim et al., 2016). Loss of one or the other Rab11 GEF does have consequences, with loss of TRAPPII from flies causing male infertility and loss of Parcas causing defects in several developmental processes, some of which have been linked to signaling by nonreceptor tyrosine kinases (Beckett and Baylies, 2006; Robinett et al., 2009; Hamada-Kawaguchi et al., 2015). In C. elegans, loss of the two SH3BP5 orthologues causes defects in embryonic cytokinesis (Sakaguchi et al., 2015). Thus, it seems likely that each of the two types of Rab11 GEF can provide much of the necessary activation of Rab11, but each also has unique specialized roles in particular cell types. Revealing this overlap in function between the two proteins should greatly assist dissecting the function of each class of GEF. It also seems likely that TRAPPII can contribute to the activation of Rab1 in vivo as both the yeast and Drosophila complexes have activity on Rab1, at least in vitro (Thomas and Fromme, 2016). This could provide a possible explanation for our observation that at least in S2 cells, the major pool of TRAPPII is found on the cis-Golgi. Indeed, a study of mammalian TRAPPC10 found an epitope-tagged version of the protein to be present on the early Golgi (Yamasaki et al., 2009). However, it is clear that the ability of TRAPPII to activate both Rab1 and Rab11 raises a conundrum because there is a growing consensus that Rab GEFs define the location of active Rabs within the cell (Barr, 2013; Blümer et al., 2013). However, in this case, the two Rabs are widely believed to act on different compartments and recruit very different effectors (Stenmark, 2009; Hutagalung and
and Rab11 (FBgn0015790). Trs20[1159]/TM6B, Tb[1], 23932 (w[1118]; Trs20[421] E(smoDN) Sb[1]; Wendler et al., 2010), 5058 (Trs20[1]/TM2), 23931 (w[1118]; FRT(w[hs])}2A), 25338 (w[1118]; Mi{ET1}gry[MB06920]/TM6C, Sb[1]), 26524 (w[1118]; Df(3L)BSC672, 62539 (w[1118]; TI{TI}Rab1[EYFP]; Dunst et al., 2015), 997 P{w[+mC]=His2Av-mRFP1}III.1 P{w[+mW.hs]=FRT(w[hs])}2A), 2520 (l(3)67BDl[1] kni-[ri-1] p[tg]/TM3, Sb[1]), 2509 (l(3)67BDp[1]- [ri-1] p[tg]/TM3, Sb[1]), 2489 (l(3)67BDd[1] kni-[ri-1] p[tg]/TM3, Sb[1]), 2509 (l(3)67BDd[1] kni-[ri-1] p[tg]/TM3, Sb[1]), 2488 (l(3)67BDc[2] kni-[ri-1] p[tg]/TM3, Sb[1]), 2493 (l(3)67BD[2] kni-[ri-1] p[tg]/TM3, Sb[1]), 2521 (l(3)67BDd[1] kni-[ri-1] p[tg]/TM3, Sb[1]), 2488 (l(3)67BD[2] kni-[ri-1] p[tg]/TM3, Sb[1]), 2493 (l(3)67BD[2] kni-[ri-1] p[tg]/TM3, Sb[1]), 7653 (w[1118]; Df(3R)Exel6174, P[w+]mC]=XP-U]Exel6174/TM6B, Tb[1]), 18399 (w[1118]; PBac[w+]mC]=WH]Trs33[f00985]/TM6B, Tb[1]).

### Construction of expression plasmids with SBF-GFP tag

The SBF-GFP tag consists of an SBF tag (Keefe et al., 2001), two tandem TEV protease recognition sites, and enhanced GFP (GenBank accession number AAB02574) with the A207K mutation to prevent dimerization. The sequence was codon optimized for expression in *Drosophila*, and two versions were synthesized (Integrated DNA Technologies) to tag proteins at either the N or C terminus and were then inserted into a pMT vector backbone for copper-inducible expression.

### cDNA clones

cDNA clones from the Berkeley Drosophila Genome Project were obtained from Drosophila Genomics Resource Center. These clones were: Rgp1 (clone RH52122), GenBank accession number BT012011, TRAPP3 (clone RE68712), GenBank accession number BT015980, TRAPP8 (clone FI18915), GenBank accession number BT131116, TRAPP9 (clone RE66325), GenBank accession number BT023878, TRAPP10 (clone LD45339), GenBank accession number YL18591, TRAPP11 (AT04056), GenBank accession number BT015311, TRAPP12 (RE19263), GenBank accession number YL089596, TRAPP13 (LD37668), GenBank accession number YL058665, and Sec3 (AT25839; GenBank accession number BT016143). RH52122 was corrected by inserting the sequence GA between nucleotides 282 and 283 of GenBank accession number BT012011. The coding sequence of this corrected clone encodes a longer version of Rgp1 corresponding with GenBank accession number NM_141259. The cDNA clone RE18773 (Berkeley Drosophila Genome Project) was corrected so that its coding sequence corresponded with GenBank accession number NM_139363. Open reading frames were amplified and inserted into corresponding expression vectors.

### Antibodies

Primary antibodies used were mouse anti-SBP (1:1,000; MAB10764; EMD Millipore), rabbit anti-tacin (1:3,000; 8227; Abcam), mouse anti–GFP (1:1,000; immunofluorescence [IF]; 1:2,000; 1181446001; Roche) mouse anti–V5 (1:2,000; R960-25; Invitrogen), rabbit anti–GM130 (1:500; ab30637; Abcam), goat anti–GMAP CRB614 and goat anti–Golgin245 CRB614 (1:2,000; Riedel et al., 2016), rabbit anti–Sec16 (1:2,000; Ivan et al., 2008), rabbit anti–TRA PPC9 AC1, rabbit anti–TRA PPC12 AC1 (IB, 1:1,000), rabbit anti–TRA PPC12 AC2 (IF, 1:1,000), and rabbit anti–Drosophila AP–1 γ (Hirst et al., 2009). Secondary antibodies were species-specific donkey anti–IgG, goat anti–IgG1, and goat anti–IgG2a antibodies coupled with Alexa Fluor 488, Alexa Fluor 555, or Alexa Fluor 647 (1:100; Thermo Fisher Scientific) and anti–IgG antibodies coupled with horseradish peroxidase (Santa Cruz Biotechnology, Inc.). GFP was also detected with the GFP booster Atto488 (1:400; gba488-100; ChromoTek).
Expression of tagged proteins in S2 cells for IF

*Drosophila* S2 cells (D.Mel-2; Invitrogen) were grown at 25°C in serum-free medium containing penicillin, streptomycin and l-glutamine (Express Five; 10486-025; Thermo Fisher Scientific). For transient expression cells were split in fresh medium and plated in 0.5-ml aliquots per well in a 24-well plate. Cells were allowed to settle for at least 60 min and then transfected with 0.4 µg of each expression plasmid using Fugene HD (Promega). Cells were incubated at 25°C for 24 h. The medium was replaced by fresh medium supplemented with copper sulfate to induce the expression of the tagged proteins. Cells were incubated for another 48 h, removed from the wells, and transferred to polytetrafluoroethylene multwell glass slides coated with poly-l-lysine (P4707; Sigma-Aldrich). Cells were allowed to settle for 60 min, washed three times with PBS, fixed for 10 min in 4% PFA in PBS, permeabilized for 10 min in PBS with 0.5% Triton X-100, blocked for 30 min in 20% fetal calf serum in PBS, and incubated for 60 min with primary antibodies diluted in 20% fetal calf serum in PBS. For the GFP-tagged proteins, the signal was typically boosted using an anti-GFP monoclonal. The cells were then washed three times for 20 min in PBS with 0.5% Tween-20, incubated for 60 min in species-specific secondary antibodies diluted in 20% fetal calf serum in PBS, washed three times for 20 min in PBS with 0.5% (vol/vol) Tween-20, and washed once for 20 min in PBS. Cells were mounted in Vectashield (H-1000; Vector Laboratories).

To generate stably transfected S2 cells lines, cells were split in fresh medium and plated in T25 flasks. Cells were allowed to settle for 60 min and then transfected with 2.5 µg expression plasmid and 0.25 µg pCooBlast using Fugene HD. Cells were incubated for 2 d, subjected to selection for 2 wk in medium with 25 µg/ml blasticidin, and maintained in medium with 5 µg/ml blasticidin. Cell sorting was then used to generated pooled populations of cells expressing the relevant tagged protein. Transfected S2 cells were split into fresh medium without out blasticidin in T75 flasks. 2 d before sorting, the medium was replaced with fresh medium containing 500 µM copper sulfate. Cells were removed from the flask, washed twice in fresh medium, filtered (0.04-0.2327; Celltrics; Partec) and sorted into groups of cells with low, intermediate, and high GFP intensity using a cell sorter. Cells were collected in medium with 5% fetal calf serum. Once cells started dividing, the medium was replaced with fresh medium (without fetal calf serum) with 5 µg/ml blasticidin.

**IF microscopy**

Salivary glands and wing discs were dissected from wandering third instar larvae in PBS, fixed for 30 min in 4% PFA in PBS, permeabilized three times for 20 min in PBS with 0.5% Triton X-100, blocked for 30 min in 20% fetal calf serum in PBS, incubated for 90 min with primary antibodies diluted in 20% fetal calf serum in PBS, washed three times for 20 min in PBS with 0.5% Tween-20, incubated for 60 min in species-specific fluorescently labeled secondary antibodies diluted in 20% fetal calf serum in PBS, washed three times for 2 min in PBS with 0.5% Tween-20, and washed once for 20 min in PBS. Tissues were mounted in ProLong Gold Antifade Mountant with or without DAPI (P36941 and P36934; Thermo Fisher Scientific). For comparing the distribution of different markers, each primary antibody was raised in a different species and then detected with species-specific antibodies labeled with different fluorochromes. To localize GFP-tagged proteins in tissues, the signal was boosted using Ato-488-labeled GFP booster (ChromoTek). Clones of TRPCC3 and TRPCC1 were generated in wing discs by heat-shocky yw hs-; FRT2A YFP-Rab1/His-RFP FRT2A YFP-Rab1 larvae and yw hs-; FRT2A YFP-Rab1/His-RFP FRT2A YFP-Rab1 larvae, respectively, at 37°C for 60 min, and wandering third instar larvae were dissected 56 or 72 h after the heat shock.

Widefield epifluorescence micrographs were obtained with an Axioplan microscope (ZEISS) equipped with a CoolSNAP HQ2 camera (Photometrics) using Micro-Manager software or a DM6 microscope equipped with a DFC365 FX camera using LAS X software (Leica Microsystems). Confocal images were taken with an SP8 microscope (Leica Microsystems) using LAS X software, and FIJI (ImageJ; National Institutes of Health) was used for subsequent analysis, including line scans of intensities across the Golgi stack (Schindelin et al., 2012). All images were acquired with a 63x 1.4 NA Plan Apochromat oil immersion lens except for whole discs (Fig. 6 A), which were with a 10x 0.4 NA Plan Apochromat dry lens. In all cases, the images shown are representative of at least two independent experiments with at least three micrographs obtained from each experiment.

**Western blot from lysates of adult flies**

10 adult male flies were collected on ice and homogenized with a motorized pestle in 100 µl lysis buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM EDTA, 1% Triton X-100, 5 mM β-mercaptoethanol, Complete EDTA-free protease inhibitors [18970600; Roche], and 1 mM PMSF). Lysates were cleared by centrifugation for 10 min at 20,000 g at 4°C. The protein concentration of the supernatants was determined with Bradford reagent (500-0006; Bio-Rad Laboratories). Lysates of parallel samples were adjusted to the same concentration and mixed with SDS sample buffer.

**Affinity purification of GFP-tagged proteins from transiently transfected cells and analysis by Western blotting**

Cells were plated in 2 ml per well in six-well plates and allowed to settle for at least 60 min before transfection with 2 µg of expression plasmid using Fugene HD (E2311). Cells were incubated at 25°C for 24 h, and the medium was then replaced with fresh medium supplemented with 100 µM copper sulfate to induce protein expression. Cells were incubated for another 48 h, collected in 1.5-ml tubes, washed in PBS, and lysed in 1 ml lysis buffer. Lysates were cleared by centrifugation for 10 min at 20,000 g at 4°C. Lysates were mixed with 5 µl equilibrated GFP-trap agarose slurry (gta-100; ChromoTek) and incubated for 60 min at 4°C with rotation. Beads were washed four times in 500 µl lysis buffer without PMSF. Proteins were eluted by incubation for 10 min at 90°C in 50 µl SDS sample buffer and analyzed by Western blotting.

**Affinity purification of GFP-tagged proteins from larvae and adult flies**

50 wandering third instar larva or fifty adult males were collected on ice and homogenized in 500 µl lysis buffer with a motorized pestle homogenizer. Lysates were cleared by centrifugation for 10 min at 20,000 g and 4°C. The protein concentration of the supernatants was determined with Bradford reagent. Lysates of parallel samples were adjusted to the same concentration and mixed with 20 µl equilibrated GFP-trap agarose slurry and incubated for 60 min at 4°C with rotation. Beads were washed four times in 500 µl lysis buffer without PMSF. Proteins were eluted by incubation for 10 min at 90°C in 50 µl SDS sample buffer and analyzed by Western blotting.

**Single-step purification and TAP from embryos and stable cell lines**

Embryos were collected for 24 h on 90-mm apple juice agar plates, with plates being kept for ≤2 d in the fridge after collecting. For a single purification, embryos from 15 plates were dounce homogenized in 10 ml lysis buffer (Jencons). For affinity chromatography from stably transfected cell lines, cells were grown in two T150 flasks (Sigma-Aldrich), induced with copper sulfate at least 2 d before harvest, and grown to a density of 10^6 to 10^7 cells per ml. Cells were pelleted at 200 g for 5 min, washed in PBS, and lysed in 2 ml lysis buffer. Lysates were cleared by...
centrifugation for 10 min at 20,000 g and 4°C. The protein concentration of the supernatants was determined with Bradford reagent. Lysates of parallel samples were adjusted to the same concentration and mixed with 20 μl equilibrated GFP-trap agarose and then incubated for 60 min at 4°C with rotation. Beads were transferred to Mobicol columns (M1003; Mobitec) and then washed four times with 500 μl lysis buffer without PMSF. Proteins were eluted from the beads for 30 min at 4°C in 150 μl TEV protease elution buffer (lysis buffer without PMSF; 1 mM DTT and 0.1 U/μl AcTEV; 12575-15; Invitrogen). The eluate was incubated with 20 μl slurry of high-capacity streptavidin agarose beads (20357; Thermo Fisher Scientific) for 1 h at 4°C with rotation. Beads were transferred to Mobicol columns and washed four times with 500 μl lysis buffer without PMSF. Proteins were eluted from the beads by rotation for 10 min at 4°C in 150 μl in lysis buffer without PMSF and with saturating concentrations of biotin. Proteins were precipitated with chloroform/methanol. The precipitate was dried in a concentrator and resuspended in 25 μl SDS buffer.

For protein identification by silver staining, proteins were fractionated by SDS-PAGE and visualized with the Pierce Silver Stain Kit (24612; Thermo Fisher Scientific). For protein identification by mass spectrometry, proteins were first fractionated by SDS-PAGE, and the gel was stained with Sypro Ruby protein gel stain (50562; Lonza). Gel lanes were cut into 1-mm slices and then into small cubes, transferred to a 96-well plate, and digested with trypsin. Tryptic peptides were identified by liquid chromatography coupled with tandem mass spectrometry by the Medical Research Council Laboratory of Molecular Biology biological mass spectrometry facility and then combined in Scaffold (Proteome Software). Identified proteins were filtered to match the criteria of having at least two exclusive unique peptides at an overall protein false discovery rate of <1%. The mass spectrometry data together with the complete Drosophila proteome from UniProt, fly genes from Flybase, and human genes from Ensembl as well as orthologous relationships from Ensembl were combined in a single custom-made relational database using Filemaker (Filemaker Inc.) in order to better distinguish background proteins from specific interactors.

Viability and fertility tests

To test viability, crosses were set up in triplicate by mating virgins heterozygous for the deficiency and a balancer with males heterozygous for the mutation and a balancer. Adults were removed after 5 d. To determine adult viability, each vial was cleared twice a day from the start of eclosion for 5 d. Adult progeny carrying either a balancer (heterozygous for the deficiency or the mutation) or no balancer (heterozygous for the deficiency and the mutation) were scored. To determine pupal viability, pupae carrying a balancer and pupae carrying no balancer were scored on a single day just before the start of eclosion. For TRAPPc11, we used the deficiency Df(3L)BSC672, for TRAPPc10, we used the deficiency Df(3R)BSC750, and for TRAPPc9, we used the deficiency Df(2L)Exel7078. To test male fertility, crosses were set up in triplicate by mating w[1118] virgins or w[+]; betaTub60D[2] Kr[If-1]/CyO virgins with males heterozygous for the mutation and the deficiency. Adult males were removed after 5 d. Pupal progenies were counted on a single day before eclosion.

Mutagenesis of TRAPP subunit-encoding genes by CRISPR/Cas9

For TRAPPc3, TRAPPc10, and TRAPPc11, we selected Cas9 target sites immediately downstream of the start codon to introduce indels. For TRAPPc9, we decided to remove the coding sequence from the genome by designing two pairs of Cas9 target sites. One target of each pair was upstream of the start codon, and the other target was downstream of the stop codon. Constructs expressing single guide RNAs were cloned into pCFD3, and constructs expressing two guide RNAs were cloned into pCFD4 (Port et al., 2014). Two guide RNA constructs were cloned for TRAPPc3, TRAPPc9, and TRAPPc11, and three guide RNA constructs were cloned for TRAPPc10.

Guide RNA–expressing constructs were injected into a stock expressing Cas9 in the germ line (Port et al., 2014). Stocks were generated from progeny of injected flies by crossing individual males heterozygous for a mutated chromosome to balancer females. For TRAPPc9, a total of 169 stocks were established from 44 injected flies; for TRAPPc3, a total of 90 stocks were established from 48 injected flies; for TRAPPc11, a total of 138 lines were established from 29 injected flies; and for TRAPPc10, a total of 200 stocks were established from 82 injected flies. Genomic DNA was prepared from single males taken from each of the established stocks, and the genomic DNA surrounding the Cas9 target site was amplified by PCR. The primers used were 5′-TCCAAACGTTAAACCGGGA-3′ and 5′-GGTGGCGCTGAAGTGTGTTT-3′ for TRAPPc3, 5′-TGCCAGGTCGATTACAT-3′ and 5′-ACTTGACCTGCTTTTCGTC-3′ for TRAPPc11, and 5′-GAAAGCACACTGTGAGCCG-3′ and 5′-GTCGTCCTGAGTGCAGTCC-3′ for TRAPPc10. The resulting amplicon was sequenced with one of the two primers used for PCR. Mixed trace files indicative of the presence of an indel in heterozygous flies were first analyzed by Poly Peak Parser (Hill et al., 2014). The mutant sequences were then aligned individually to the Drosophila genome using NCBI Blast (National Institutes of Health) to determine the mutation in each allele. To screen fly lines established from the TRAPPc9 mutagenesis experiment, we used the primers 5′-ACCCATGTGAGGATGTCGAG-3′ and 5′-ACCCATGTGAGGATGTCGAG-3′ that flank the coding sequence of TRAPPc9. This resulted in a PCR amplicon only for flies with a deletion of the TRAPPc9 coding sequence. This amplification was sequenced with one of the primers used for PCR and aligned against the Drosophila genome using the UCSC genome browser.

From the TRAPPc3 lines, a total of 24 lines showed an indel, representing 11 deletion alleles, two insertion alleles, and one allele with substitutions. All of the homozygous viable lines carried indels that did not shift the reading frame. Seven of the homozygous lethal alleles, lines 17, 19, 23, 44, 76, 77, and 68 (six deletions and one insertion), carried frameshift mutations. Six of these tested negative in a complementation test with Df(3L)AC1. Line 44 has not been included in this complementation test. For TRAPPc9, four fly lines representing two alleles were found to carry a deletion of the whole coding sequence. None of these lines was lethal over the deficiency Df(2L)Exel7078, and lines 105 and 141 were kept for further studies. For TRAPPc10, a total of 29 lines with indels representing 15 alleles were isolated. None of these lines was lethal over Df(3R)BSC750. Of the 138 lines that were established for TRAPPc11, four failed to complement TRAPPc11[MB06920]. Three of the four lines represent one insertion allele and one deletion allele. For the fourth line, we were unable to determine the type of the mutation.

Genomic tagging of TRAPP subunits

TRAPPc3, TRAPPc9, TRAPPc12, and TRAPPc11 were targeted at the C terminus as for expression in S2 cells. Cas9 target sequences were selected close to the stop codon, which were 5′-GGTTGCTTCCA GAGGTTGGCTTGGG-3′ for TRAPPc11, 5′-TGGAGGAAACA TGAGCGATGGG-3′ for TRAPPc9, 5′-ACTTGAACTCAAAACTC CAAAGG-3′ for TRAPPc12, and 5′-GGAGGTTCATCAGCAGCTTG CGAGG-3′ for TRAPPc3, and the corresponding guide RNAs were cloned into pCFD3. Donor DNA constructs were cloned with a central region encoding the tag, which was flanked by homology arms, each corresponding with 800–1,000 bp of genomic DNA. The protospacer-adjacent motif in each of these constructs was mutated to prevent Cas9 from cutting the donor DNA. Both the donor DNA and the guide RNA were injected into a stock expressing Cas9 in the germ line.
For tagging of TRAPPC3, 90 stocks from 17 injected flies; for TRAPPC9, 148 stocks from 14 injected flies; for TRAPPC11, 130 stocks from 18 injected flies; and for TRAPPC12, 92 stocks from 29 injected flies were set up. These lines were screened by PCR with two primer pairs. One primer of each pair bound to the DNA encoding the tag and the other bound in the flanking genomic DNA of the donor plasmid. Positive lines were subjected to a second PCR, with primers flanking the donor DNA to confirm that the entire donor DNA integrated into the correct place. The following primers were used: 5′-GCCAATTTGTGCAGCTTTG-3′ and 5′-ACCGCCCGGGAATCTAAATC-3′ for TRAPPC3, 5′-TACCGGATCATCTGCGAAA-3′ and 5′-ACCGCCGCGAAATTGCTGTG-3′ for TRAPPC9, 5′-CCGCGAGCAATGAGAG-3′ and 5′-GAGCTGGCAACATACGC-3′ for TRAPPC11, and 5′-AACACTAATCACCACGGGTT-3′ and 5′-TCCGACACCCACTAAACGAG-3′ for TRAPPC12. The amplicon of at least two lines per gene was sequenced. All lines for TRAPPC9, TRAPPC11, and TRAPPC12 were homozygous viable and were kept as stable stocks without a balancer. All lines for TRAPPC3 were homozygous viable but sterile.

Characterization of potential TRAP complex alleles

The EMS alleles l(3)63B[2], l(3)63B[c][1], l(3)63B[b][5], and l(3)63B[h][1] were found with Flybase Cytosearch. They were previously mapped to the cytogenetic region, which also contains TRAPPC11 (Wohwill and Bonner, 1991). Of these five mutations, l(3)63B[2] failed to complement TRAPPC11[MB06920] and TRAPPC11[7]. We therefore changed the name of this allele to TRAPPC11[63B2]. Flies heterozygous for TRAPPC11[63B2] and the deficiency Df(3L)BSC672 do not develop into adulthood, but in rare cases, they pupariate. Furthermore, flies heterozygous for the CRISPR alleles TRAPPC11[19] and TRAPPC11[63B2] in rare cases develop into adults.

The alleles l(3)67B[2][1], l(3)67B[c][1], l(3)67B[d][2], l(3)67B[e][2], l(3)67B[f][1], l(3)67B[h][1], l(3)67B[i][1], l(3)67B[j][1], l(3)67B[k][1], l(3)67B[l][1], l(3)67B[m][1], l(3)67B[n][1], l(3)67B[o][2], l(3)67B[p][2], l(3)67B[q][2], l(3)67B[r][2], and l(3)67B[s][2] were found with Flybase Cytosearch. They were previously mapped to the same cytogenetic region as TRAPPC3 (Leicht and Bonner, 1988), but all of these alleles complemented the TRAPPC3 alleles 76, 77, and 84, indicating that none is an allele of TRAPPC3. EMS alleles of TRAPPC11[63B2], TRAPPC11[12], TRAPPC11[59], TRAPPC12[2], and TRAPPC12[421] were sequenced from heterozygous flies. Natural variations to the reference genome of Drosophila were identified from the Drosophila Genes Reference Panel via the UCSC genome browser and the Drosophila Population Genome Project via Ensembl. All above-mentioned EMS alleles had a nonsense mutation in the corresponding gene. We also obtained a fly line with a piggyBac insertion in the coding sequence of TRAPPC6. Mutants heterozygous for this allele, called TRAPPC6[j00985], and the deficiency Df(3R)6174 are lethal, suggesting that TRAPPC6 in flies is essential in contrast with yeast Trs33 and mammalian TRAPPC6A and TRAPPC6B (Kim et al., 2016).

Generation of antibodies to TRAPPC9 and TRAPPC12

Full-length TRAPPC12 corresponding with GenBank accession number AAL90334 was fused at its N terminus to GST by cloning the coding sequence of cDNA clone RE19263 into the expression vector pGEX–6P-1. The N terminus of TRAPPC9 corresponding with amino acids 20–417 of GenBank accession number ABU18182 was fused at its N terminus to GST by cloning the corresponding region of the coding sequence of cDNA clone RE66325 into the expression vector pGEX–6P-1. The expression of the GST-fusion proteins was induced overnight at 16°C in E. coli BL21 (DE3; Agilent Technologies) with 0.2 µM IPTG. Cells were harvested and resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 150 mM NaCl, 10 mM DTT, and complete protease inhibitors). The lysate was sonicated and cleared by centrifugation at 50,000 g for 15 min at 4°C. GST proteins were purified from the supernatant by affinity chromatography using glutathione Sepharose 4B (GE Healthcare). Antibodies were raised in rabbits by Cambridge Research Biochemicals.

To purify TRAPPC12 antisera, GST and TRAPPC12-GST were coupled to CNBr-activated Sepharose 4B beads (GE Healthcare). GST antibodies were first removed using a GST column, and TRAPPC12 antibodies were purified with a TRAPPC12-GST column. Antibodies were eluted with 0.1 M glycine, pH 2.5, and fractions were neutralized with 1 M Tris, pH 8.8. Protein concentration was determined with Bradford reagent. Peak fractions were pooled, dialyzed in PBS at 4°C overnight, and stored at −80°C. Two sera were purified separately (TRAPPC12 AC1 and TRAPPC12 AC2). To purify TRAPPC9 antisera, the N terminus of TRAPPC9 corresponding with amino acids 23–514 of GenBank accession number ABX18182 was fused at its N terminus to His-MBP by cloning the corresponding region of the coding sequence of cDNA clone RE66325 into the expression vector pETM-41. The expression of this protein was induced overnight at 16°C in E. coli BL21 (DE3) with 0.2 mM IPTG and 0.2% glucose. Cells were harvested and resuspended in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.1 mM EDTA, 10 mM DTT, and complete protease inhibitors). Cells were lysed by sonication. The lysate was cleared at 50,000 g for 15 min. The TRAPPC9 (aa23–514)-6×His-MBP fusion protein was purified from the cleared lysate with amylose resin (EBS218; New England Biolabs, Inc.), and coupled with CNBr-activated Sepharose 4B beads. TRAPPC9 antibodies were bound to the beads and then eluted with 0.1 M glycine, pH 2.5, and fractions were neutralized with 1 M Tris, pH 8.8. Protein concentration was determined with Bradford reagent. Peak fractions were pooled, dialyzed in PBS at 4°C overnight, and stored at −80°C.

Recombinant Rabs and TRAP complexes

Rabs were expressed in the E. coli strain BL21-DE3 (Agilent Technologies). The cDNA of Drosophila Rab1 (NP_732610), Rab2 (NP_477090), Rab11 (NP_599137), Rab18 (NP_524744), and Rab5 (NP_722795) were amplified and cloned into pET28a in frame with a C-terminal His6 tag. The Rab C-terminal cysteine residues were replaced by His tags to allow membrane anchoring. Cell growth and protein expression were as described by Galindo et al. (2016). Cell pellets were resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl2, and 1 mM DTT) with 20 mM imidazole and complete protease inhibitors. After sonication, the lysate was cleared by centrifugation at 32,000 g for 30 min at 4°C. Cleared lysates were mixed with Ni-NTA beads and incubated for 1 h at 4°C. Resin was washed with 10 bed volumes of buffer A plus 40 mM imidazole and a final wash with 10 bed volumes of buffer A plus 200 mM imidazole. Eluted fractions were analyzed by SDS-PAGE, and imidazole was removed using a PD10 desalting column. Proteins were concentrated to ~1 mg/ml, snap frozen in liquid N2, and stored at −80°C. Purified Rabs were loaded with mnt-GDP (Invitrogen) as previously described by Thomas and Fromme (2016). Parcas (CG7761; NP_611010; Poirot) cDNA was synthesized (Epoch Life Science) and cloned into pGEX-6P N-terminally GST-tagged protein was expressed in BL21-DE3 growing at 37°C and purified as described previously for the C. elegans protein (Sakaguchi et al., 2015) but eluted by incubating the glutathione Sepharose slurry overnight with PreScission protease (~10 U/ml) at 4°C.

TRAP complex tubes were expressed in Sf9 cells using the Multibac system (Nie et al., 2014). The TRAP subunit genes were synthesized (Epoch Life Science). C1 (NP_651778), C2 (NP_648841), C3
GEF assays were performed in HKM buffer (20 mM Hepes-KOH, pH 7.4, 150 mM KOAc, 2 mM MgCl₂, and 1 mM DTT). All reagents, Rabs, and C2L (NP_610662) were inserted sequentially into pACEBac1, with each expression cassette separated by 30-bp linkers to generate the pACEBac1-C1-C6 vector. The TRAPPII-specific genes C9 (NP_610044) and C10 (NP_650431) were cloned into pIDC, and the TRAPPIII-specific subunits C8 (NP_649135) and C11 (NP_647785) were cloned into pIDC; meanwhile, the other two TRAPPIII subunits C12 (NP_649255) and C13 (NP_609365) were inserted into pIDC. Cre recombinase (New England Biolabs, Inc.) was used to fuse the appropriate pID vectors with pACEBac1-C1-C6 to generate a plasmids for the coexpression of all TRAPPII and TRAPPIII subunits. In these plasmids, the C3 subunits were tagged with Strep-TagII, and either C11 (TRAPPIII) or C10 (TRAPPIII) was FLAG tagged. The linker sequence between each subunit and the tag included a site for PreScission protease (GST–human rhinovirus [HRV]-3C protease). Bacmids using the EM BacY baculovirus genome and virus production were made as described previously by Nie et al. (2014).

500-ml cell suspensions (2 × 10⁶ cells/ml) were infected with 5 ml of P2 baculovirus and incubated at 27°C and 124 rpm. Cells coexpressing TRAP core subunits, all TRAPPII subunits, or all TRAPPIII subunits were harvested after 66 h (75–80% viability) by centrifugation at 2,250 g for 10 min at 4°C. Pellets were washed once with PBS, centrifuged again, and stored at −80°C. Frozen pellets were vortexed in buffer B (50 mM Hepes-KOH, pH 7.44, 110 mM NaCl, 1 mM DTT, and 0.1% Igepal CA-630) with inhibitors (1 mM PMSF, cOmplete protease inhibitors, 0.4 µM pepstatin, 0.24 µM leupeptin, and 5 µM MG132) using 30 ml per 500 ml of initial culture; Sigma-Aldrich) and incubated on a rotation wheel for 1 h at 4°C. Beads were washed three times with 10 bead volumes of buffer B plus 0.05% Igepal CA-630. Bound material was eluted by washing the beads with five volumes of buffer B with 100 µg/ml of FLAG peptide or 2.5 mM desthiobiotin as appropriate. Eluted fractions were analyzed by SDS-PAGE, concentrated, and buffer exchanged. Alternatively, the bound complexes were eluted by incubating the slurry with PreScission protease (1.50 µU/ml overnight at 4°C, and the eluates then were bound to glutathione-Sepharose to remove the PreScission protease. The TRAP core complex was purified further by gel filtration using a Superose 16/30 column (GE Healthcare) equilibrated in buffer B plus 0.05% Igepal CA-630.

GEF activity assays

GEF assays were performed in HKM buffer (20 mM Hepes-KOH, pH 7.4, 150 mM KOAc, 2 mM MgCl₂, and 1 mM DTT). All reagents, Rabs, and TRAP complexes were buffer exchanged into HKM. The activity of His-tagged Rabs was confirmed by the exchange of nonfluorescent GTP for mant-GDP using a PHERASTAR plate reader. Reactions containing 250 nM mant-GDP–labeled Rab alone, Rab and 200 µM GTP, or adding 10 mM EDTA to the former mix were set up in 96-well black-bottomed plates (Corning), and fluorescence decay was measured at 30°C. Drosophila Rab5 and the Vps9 domain of Rabex5 were used as a control. More rapid exchange rates were measured using an LS55 fluorimeter (PerkinElmer) under multiple turnover conditions. The mant-GDP→GTP exchange was performed by adding 250 nM mant-GDP Rab to the solution, warming the solution to 30°C for 150 s, adding 200 µM, and waiting 120 s before adding 50 nM of the corresponding TRAP complex. Fluorescence measurements (360 nm excitation and 448 nm emission) were recorded for 20 min. Assays in the presence of liposomes were performed by preincubating 300 µM liposomes with the mant-GDP Rab before adding the GTP. All GEF assays were performed with n ≥ 3. To determine exchange rates, curves of fluorescence versus time were fitted to a single exponential curve with an additional linear drift term using the Prism７ software (GraphPad Software) as described previously by Richardson and Fromme (2015). The resulting rate constants were divided by the concentration of GEF to give the exchange rate.

Synthetic liposomes were prepared using an “insect Golgi” mixture based on previous lipidomics studies (Drin et al., 2008; Guan et al., 2013) with Ni²⁺−DOGS included when needed to anchor His-tagged Rabs for GEF assays. Thus, phosphatidylcholine (14 mol%), phosphatidyethanolamine (62%), phosphatidylserine (3%), phosphatidic acid (1%), phosphatidylinositol (10%), phosphatidylinositol-4-phosphate (1%), diacglycerol (1%), ceramide (5%), cholesterol (3%), and Ni²⁺−DOGS (5%) were extruded through 400- and 100-nm filters (Whatman) as previously described (Christis and Munro, 2012).

Online supplemental material

Fig. S1 shows SBP-GEF–tagged TRAP complex subunits in Drosophila S2 cells. Fig. S2 shows sequence details of EMS alleles in various TRAP complex subunits. Fig. S3 shows Rab GEF assays with recombinant TRAP complex subunits. Fig. S4 shows the evolutionary conservation of TRAP complex subunits and Parcas/SH3BP5. Fig. S5 shows Localization of YFP-Rab4 in WT and TRAPC9 mutant larval tissues.

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