RESEARCH PAPER

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Interaction with the effector dynamin-related protein 1 (Drp1) is an ancient function of Rab32 subfamily proteins

Carolina G Ortiz-Sandoval[1], Sarah C Hughes[1,2], Joel B Dacks[1], and Thomas Simmen[1,*]

[1]Faculty of Medicine and Dentistry; Department of Cell Biology; University of Alberta; Edmonton, Alberta, Canada; [2]Faculty of Medicine and Dentistry; Department of Medical Genetics; University of Alberta; Edmonton, Alberta, Canada

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The mitochondria-associated membrane (MAM) is an endoplasmic reticulum (ER) domain that forms contacts with mitochondria and accommodates Ca^{2+} transfer between the two organelles. The GTPase Rab32 regulates this function of the MAM via determining the localization of the Ca^{2+} regulatory transmembrane protein calnexin to the MAM. Another function of the MAM is the regulation of mitochondrial dynamics mediated by GTPases such as dynamin-related protein 1 (Drp1). Consistent with the importance of the MAM for mitochondrial dynamics and the role of Rab32 in MAM enrichment, the inactivation of Rab32 leads to mitochondrial collapse around the nucleus. However, Rab32 and related Rabs also perform intracellular functions at locations other than the MAM including melanosomal trafficking, autophagosome formation and maturation, and retrograde trafficking to the trans-Golgi network (TGN). This plethora of functions raises questions concerning the original cellular role of Rab32 in the last common ancestor of animals and its possible role in the last eukaryotic common ancestor (LECA). Our results now shed light on this conundrum and identify a role in Drp1-mediated mitochondrial dynamics as one common denominator of this group of Rabs, which includes the paralogues Rab32A and Rab32B, as well as the more recently derived Rab29 and Rab38 proteins. Moreover, we provide evidence that this mitochondrial function is dictated by the extent of ER-association of Rab32 family proteins.

Introduction

Contacts between the endoplasmic reticulum (ER) and mitochondria are called the mitochondria-associated membrane (MAM)[1,2] and require the formation of proteinaceous tethers.[3] Whereas such a function can be fulfilled by the ER-mitochondria encounter structure (ERMES) in fungi and some other lineages,[4,5] or by the recently discovered ER membrane protein complex (EMC),[6] mammalian cells rely on additional tethering structures. Of these, the mitofusin-2 complex is one of the best-characterized.[7] However, mitofusin-2 (Mfn2) is not only a MAM tether, but also a GTPase, which together with mitofusin-1 (Mfn1) promotes mitochondria fusion.[8-10] This finding highlights another function of the MAM, which is the regulation of mitochondrial dynamics, a term referring to the balance of fusion and fission determining structure and distribution of mitochondria. This functional connection between MAM-forming or MAM-regulating proteins and mitochondrial structure is well established, but often not mechanistically understood. For instance, the knockdown of the MAM enrichment determinant phosphofurin acidic cluster sorting protein 2 (PACS-2) leads not only to the disruption of the MAM, but also to the fragmentation of mitochondria.[11]

Among the enzymes that regulate mitochondrial dynamics is dynamin-related protein 1 (Drp1), a GTPase that, in its GTP-bound form, cleaves mitochondria.[12] Ca^{2+} released from the MAM activates Drp1 via calcineurin and Ca^{2+}/calmodulin-dependent protein kinase I (CaMKI), thus causing mitochondria fission.[13] Conversely, Drp1 is inhibited by cAMP-dependent protein kinase (PKA) phosphorylation on Ser656 that is regulated by PKA-anchoring proteins (AKAPs).[14,15] Interestingly, active Drp1 localizes to tubules in the proximity of ER-mitochondria contacts, where it can oligomerize and interact with mitochondria-associated cardiolipin,[16,17] suggesting that mitochondrial division occurs at the MAM.[18] Oligomerized Drp1 progressively restricts and ultimately cleaves a mitochondrial tube.[19–21] Our lab has recently identified Rab32, a Ras-related GTPase, as a determinant of Drp1 activity.[22] However, exactly how Rab32 performs this function has remained unclear.

© Carolina G Ortiz-Sandoval, Sarah C Hughes, Joel B Dacks, and Thomas Simmen
*Correspondence to: Thomas Simmen; Email: Thomas.Simmen@ualberta.ca
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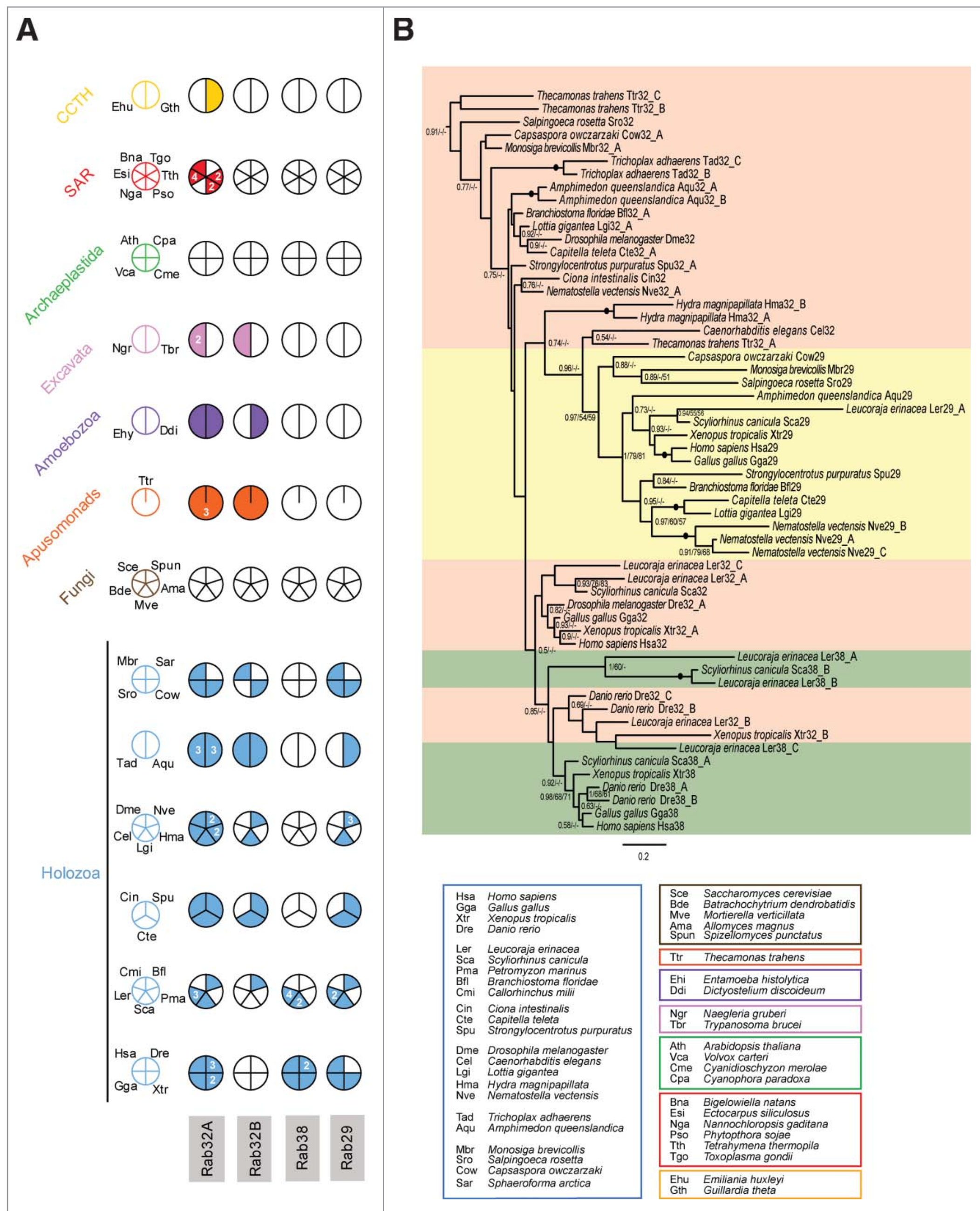

Figure 1. Distribution of the Rab32 family proteins across eukaryotic taxa. (A). Coulson plot representing the distribution of the Rab32 family proteins across different organisms of the 6 major eukaryotic supergroups. Colored or blank pieces of the pie chart indicate presence or absence of Rabs in each organism, respectively. Numbers indicate when there was more than one homolog found in each specific organism. The non-abbreviated name for each organism is included in their corresponding color boxes. (**B).** Phylogenetic evolution of Rab32 family proteins. Clades containing sequences of each specific family member are color coded (Rab32A, orange; Rab29, yellow; Rab38, green). Numerical values represent Bayesian posterior probabilities and maximum likelihood bootstrap values (RAxML and PhyML); black circles indicate 1.00/95/95 support values for MrBayes, RAxML, and PhyML, respectively.

Rab proteins are part of the Ras-superfamily of GTPases that regulate diverse aspects of membrane trafficking.[23] They are known to have already diversified extensively by the point, at which the Last Eukaryotic Common Ancestor (LECA) gave rise to the existing lineages present on earth today.[24,25] Past work has demonstrated that the LECA had between 19–23 Rab subfamilies, including 2 paralogues of Rab32 designated Rab32A and Rab32B.[26]

Rab32 is part of the group of ER-localized Rab proteins, which determine diverse ER-associated functions, including the formation of autophagosomes, peroxisome precursors and lipid droplets.[27] Many ER-localized Rabs are implicated in the regulation of ER morphology. For instance, Rab10 marks the position of ER tubule growth.[28] In the case of Rab32, this function is reflected in its ability to promote translocation of MAM proteins such as calnexin to the cellular periphery.[22] Other Rab32-related proteins include Rab29 and Rab38. These have both originated more recently in the single-celled ancestor of animals and within the vertebrates, respectively.[24,26] Rab29 has been implicated in roles at the trans-Golgi network (TGN),[29] while Rab38 has been shown to act at melanosomes.[30] This diverse set of intracellular locations for Rab32-related proteins raises the question of what these proteins might have as an ancestral cellular role in animals, and what general function they might play in other eukaryotic cells.

Our study confirms that Rab32, Rab38 and Rab29 form an evolutionarily conserved Rab subfamily and shows that all members of the Rab32 family in mammalian cells can interfere with mitochondrial dynamics when inactive. Under that condition, they sequester their effector Drp1 to block

mitochondria fission dependent on their own extent of localization to the ER and the MAM.

Results

Rab38 and Rab29 are non-redundant descendants of the evolutionarily ancient Rab32A

A grouping of Rab proteins according to their sequence similarity led to the discovery that Rab29, Rab38 and Rab32A/B form a subfamily among the Rabs.[26] We first examined when the Rab32 ancestor diversified, via homology searching and phylogenetics. Our study included at least 2 representative organisms from each of the 6 major eukaryotic supergroups, thus improving the taxonomic breadth compared to previous studies.[24-26]. As shown in **Figure 1A**, Rab32A and Rab32B are the only members of the family that are present in all supergroups, except Archaeplastida, whereas both Rab38 and Rab29 are Holozoa-specific. Rab32 showed an ancient split very early in evolution, which led to Rab32A and Rab32B (Supplemental **Fig. 1**), consistent with previous findings.[26] Interestingly, we detected that the presence of Rab32B always coincides with the presence of Rab32A, but that Rab32B is lost in Metazoa concurrently with the appearance of Rab38. This raises the possibility that, in this lineage, Rab38 could have taken over redundant functions from Rab32B allowing for loss of the latter. Moreover, Rab29 is never found alone, but always in the presence of at least one of the other family members (Rab32A, Rab38 or Rab32B). Therefore, Rab29 and Rab38 may perform non-redundant functions that have either been added during evolution to the Rab32 repertoire or that have been lost from the ancient Rab32A/B.

Next, we performed phylogenetic analyses using 2 maximum likelihood methods (RAxML and PhyML), and a Bayesian inference method (MrBayes). These analyses indeed demonstrated that Rab29 diverged earlier than Rab38, as its split appeared closer to the root of the tree in Filazoa (**Fig. 1B**), while Rab38 appears to have arisen as early as cartilaginous fish, although the latter is based exclusively on reciprocal BLAST results as the phylogeny was unresolved (**Fig. 1B**). Together, our results suggest that both Rab38 and Rab29 are a result of Holozoan expansion of Rab32A. Also, we predict that the functions performed by Rab29 and Rab38 are likely non-redundant to Rab32A, since all 3 paralogues are retained in the Holozoa and vertebrates respectively.

Rab32 family proteins associate with mitochondria and the ER

Given the set of diverse, non-overlapping, intracellular locations and functions attributed to Rab32A (referred to as Rab32 from here on) and its relatives, we attempted to identify their shared cellular role by testing their extent of association with the ER, mitochondria and lysosomes by immunofluorescence microscopy and subcellular fractionation. We first identified and validated antibodies for each Rab using Western blot (data not shown) and immunofluorescence protocols (Supplemental **Fig. 3**). Via confocal immunofluorescence images, we determined overlaps with marker proteins by quantifying Manders coefficients. As shown in **Figures 2A-C**, we were indeed able to detect some overlap of endogenous staining with markers of the ER, mitochondria and lysosomes for all 3 Rabs in HeLa cells. However, the extents of this overlap varied between the Rabs and the respective organelle we were probing for. While the highest extent of overlap was detected between lysosomal LAMP-1 and Rab38, we determined that the overlap of the ER marker ERp57 was highest with Rab32 (**Figs. 2 A, C, and D**). Conversely, Rab32 showed little overlap with LAMP-1 and Rab29 overlapped the least with the ER. However, all 3 Rabs showed a comparable apposition to mitotracker staining (**Figs. 2 B and D**). To further determine the subcellular localization of the Rab32 family proteins to mitochondrial membrane contact sites, we used biochemical fractionation with a Percoll protocol that can isolate ER-mitochondria contact sites from mitochondria and a microsomal fraction which contains ER, endosomes and lysosomes, each identified by their markers ERp57, syntaxin-7, Rab7 and LAMP-1, respectively.[31] We noticed in agreement with our immunofluorescence micrographs, that Rab29, Rab32 and Rab38 were all found in purified mitochondria. In contrast, Rab32 and Rab38, but not Rab29 showed significant overlap with the MAM (**Fig. 2E**). The combination of our immunofluorescence and fractionation data indicated that all Rab32 family proteins show association with mitochondria, but that there is a gradient of ER-association from Rab32 to Rab38 and to Rab29, which shows the lowest association with the ER.

Rab32 family proteins interact with their effector Drp1

The common association of Rab32 family proteins with mitochondria together with their gradient-style association with the ER led us to propose the hypothesis that a role in mitochondrial dynamics could be shared by all Rab32 family proteins, but might be determined by their association with the ER. If this were the case, Rab32 family proteins could interact with an effector protein involved in mitochondrial dynamics that requires the ER to function. Three candidate proteins could fulfill such a role: *i.* Mitofusin-2 is a MAM tether, but also promotes mitochondria fusion.[7] However, we were unable to detect significant amounts of active Rab32 family proteins co-immunoprecipitating with mitofusin-2 (data not shown). *ii.* Fis1 forms a complex with MAM-localized BAP31. However, the formation of this complex is restricted to induction of apoptosis.[32] *iii.* Drp1 is a GTPase that uses ER tubules to mediate mitochondria constriction.[16] Given the common, partial localization to the ER, Drp1 is a candidate Rab32 effector protein.

Thus, we tested the extent of interaction between endogenous Drp1 and FLAG-tagged Rab32 family proteins via co-immunoprecipitation in HEK293T cells. We found that all Rab32 family proteins can interact with Drp1. This interaction is strongest for Rab32 and weaker for Rab38 and Rab29 (**Fig. 3A**). In the case of Rab interactors, it is critical to determine whether they interact with active or inactive Rabs. This quality can indicate whether a Rab interactor is, for instance, an effector. Effectors are expected to localize to membranes where active Rabs are found and should interact preferentially with GTP-bound Rabs.[33] We had noticed

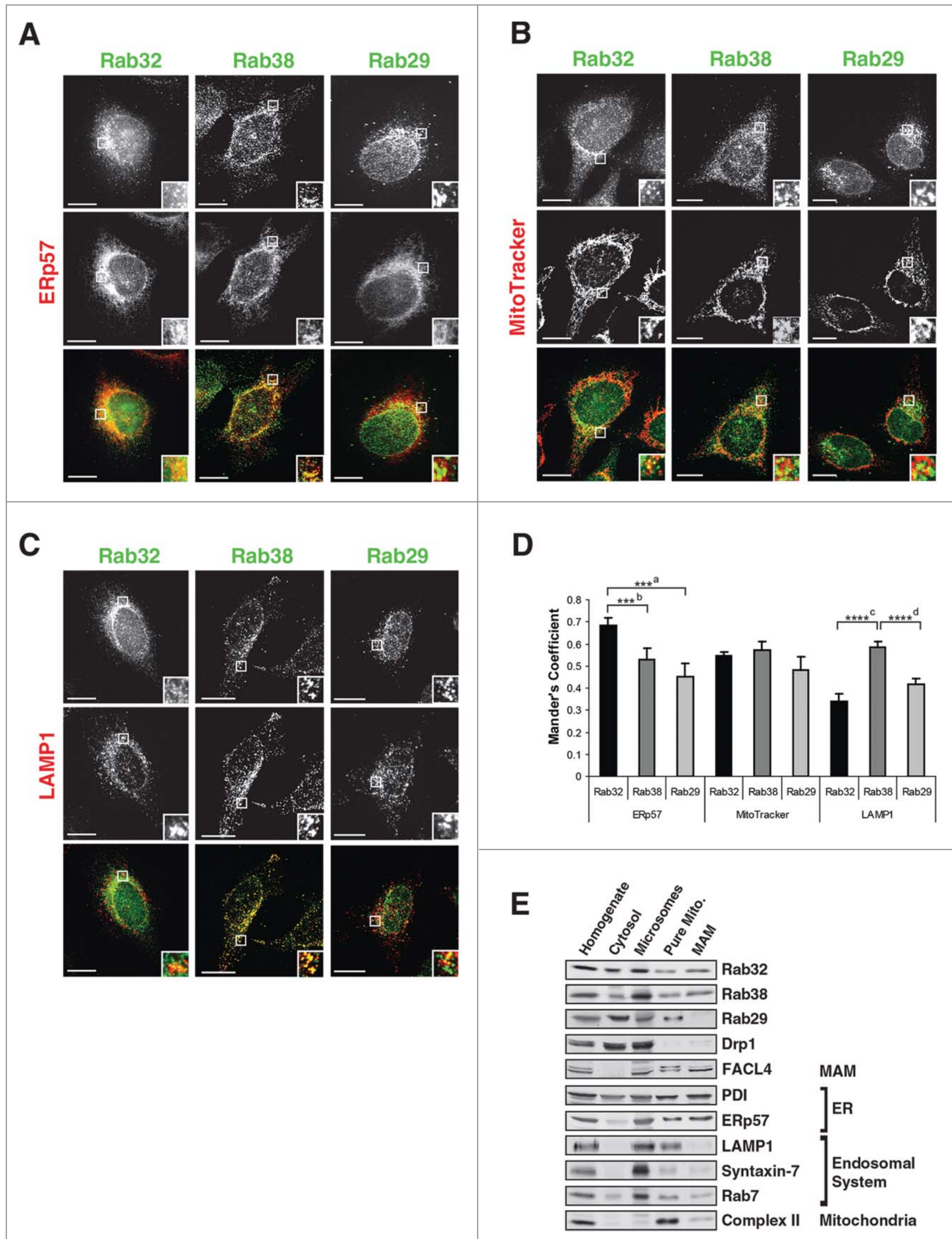

Figure 2. Localization of Rab32 Family Proteins to the ER, Mitochondria and Lysosomes. A-C. HeLa cells were processed for confocal immunofluorescence microscopy for Rab32 family proteins and the ER (detected with ERp57, (**A**), mitochondria (visualized with Mitotracker, (**B**) and lysosomes (stained with LAMP-1, (**C**). Yellow color in the overlap indicates apposition of the Rab and ER or mitochondria staining. Pictures correspond to extended focus images. Scale bar = 25 μm. Representative images are shown. (**D**). Co-localization was quantified using the Manders coefficient for the localization of Rabs with the ER, mitochondria and lysosomes, as shown by representative images in panels A-C. $P_a = 0.0011$, $P_b = 0.0064$, $P_c = 0.0001$, $P_d = 0.0004$. (Standard deviation is shown, n = 6) **(E).** Rab32 family distribution between microsomes (containing ER, endosomes and lysosomes), mitochondria and the MAM. HeLa homogenates were fractionated via the Percoll fractionation protocol into cytosol, microsomes, pure mitochondria, and MAM, as indicated on the right side. Loading was as follows: Homogenate 0.6% of total, cytosol, microsomes, pure mitochondria, MAM (all 2.4% of total).

previously that Drp1 indeed is found on light ER membranes like active Rab32.[22] Next, we repeated our co-immunoprecipitation protocol with GDP-bound Rab32 T39N (inactive) and GTP-bound Rab32 Q85L (active). For this purpose, we used the Drp1 S656A mutant that is active and cannot be phosphorylated by PKA. This showed that inactive Rab32 showed only about one fourth of the interaction detected with active Rab32 (**Fig. 3B**). Consistent with their reduced binding to Drp1, the distinct increase of binding between active Rab29 and Rab38 to Drp1 was less pronounced than in the case of Rab32 (**Fig. 3C**). Moreover, an overlap in protein localization between all dominant-active Rab32 family proteins and Drp1, as well as mitochondria and the ER could be detected (Supplemental Figs. 4–6). Our findings therefore demonstrate that Drp1 is an effector protein for Rab32 family proteins, and that Rab32 is the most efficient interactor of the 3 members.

The extent of the association of Rab32 family with the ER and their effector Drp1 determines their ability to modulate mitochondrial dynamics

If our identification of Drp1 as a common effector for Rab32 family proteins is correct, then

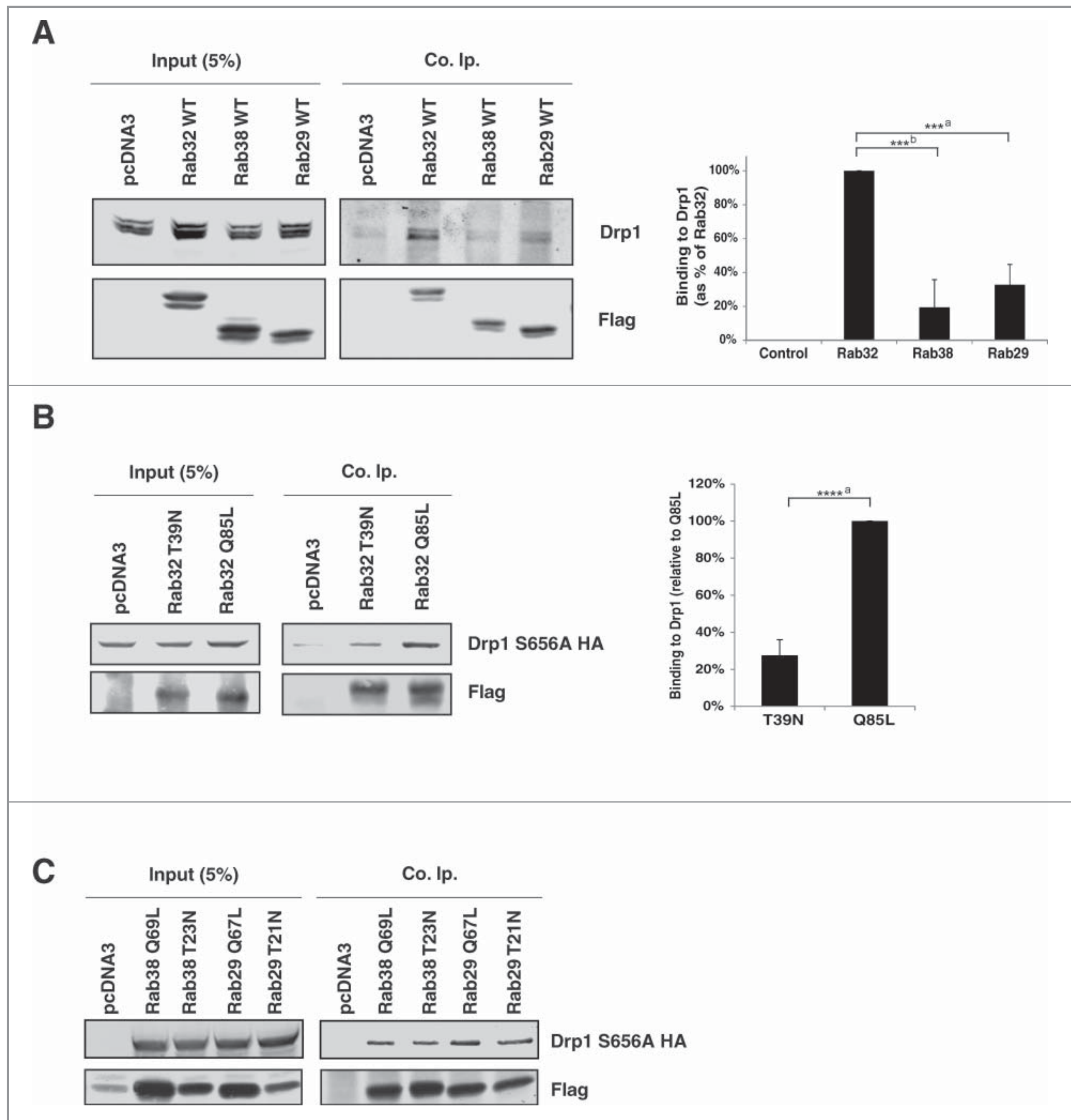

Figure 3. Interaction of Rab32 Family Proteins with their Effector Drp1. (A). Co-immunoprecipitation of Rab32 family proteins with endogenous Drp1. HeLa cells were transfected with FLAG-tagged Rab32 family wild type constructs, followed by lysis and incubation with anti-FLAG antibodies. Immunoprecipitates were analyzed for anti-FLAG (Rabs) and co-immunoprecipitating endogenous Drp1. Amounts of Drp1 immunoprecipitated were determined following the normalization to the amounts of Rab proteins immunoprecipitated. $P_a = 0.0027$, $P_b = 0.0014$. (Standard deviation is shown, $n = 3$) (**B).** Co-immunoprecipitation of dominant-active Rab32 Q85L with dominant-active HA-tagged Drp1 S656A. HeLa cells were transfected with HA-tagged Drp1 S656A and FLAG-tagged Rab32 Q85L and Rab32 T39N constructs, followed by lysis and incubation with anti-FLAG antibodies. Immunoprecipitates were analyzed for anti-FLAG (Rab32 Q85L) and co-immunoprecipitating HA-tagged Drp1. $P_a = 0.0001$. (Standard deviation is shown, $n = 3$) Amounts of Drp1 immunoprecipitated were determined following the normalization to the amounts of Rab proteins immunoprecipitated. (**C).** Co-immunoprecipitation of dominant-active and dominant-inactive Rab38 and Rab29 with dominant-active HA-tagged Drp1 S656A. HeLa cells were transfected with HA-tagged Drp1 S656A and FLAG-tagged Rab38 and Rab29 constructs as indicated, followed by lysis and incubation with anti-FLAG antibodies. Immunoprecipitates were analyzed for anti-FLAG (Rabs) and co-immunoprecipitating HA-tagged Drp1.

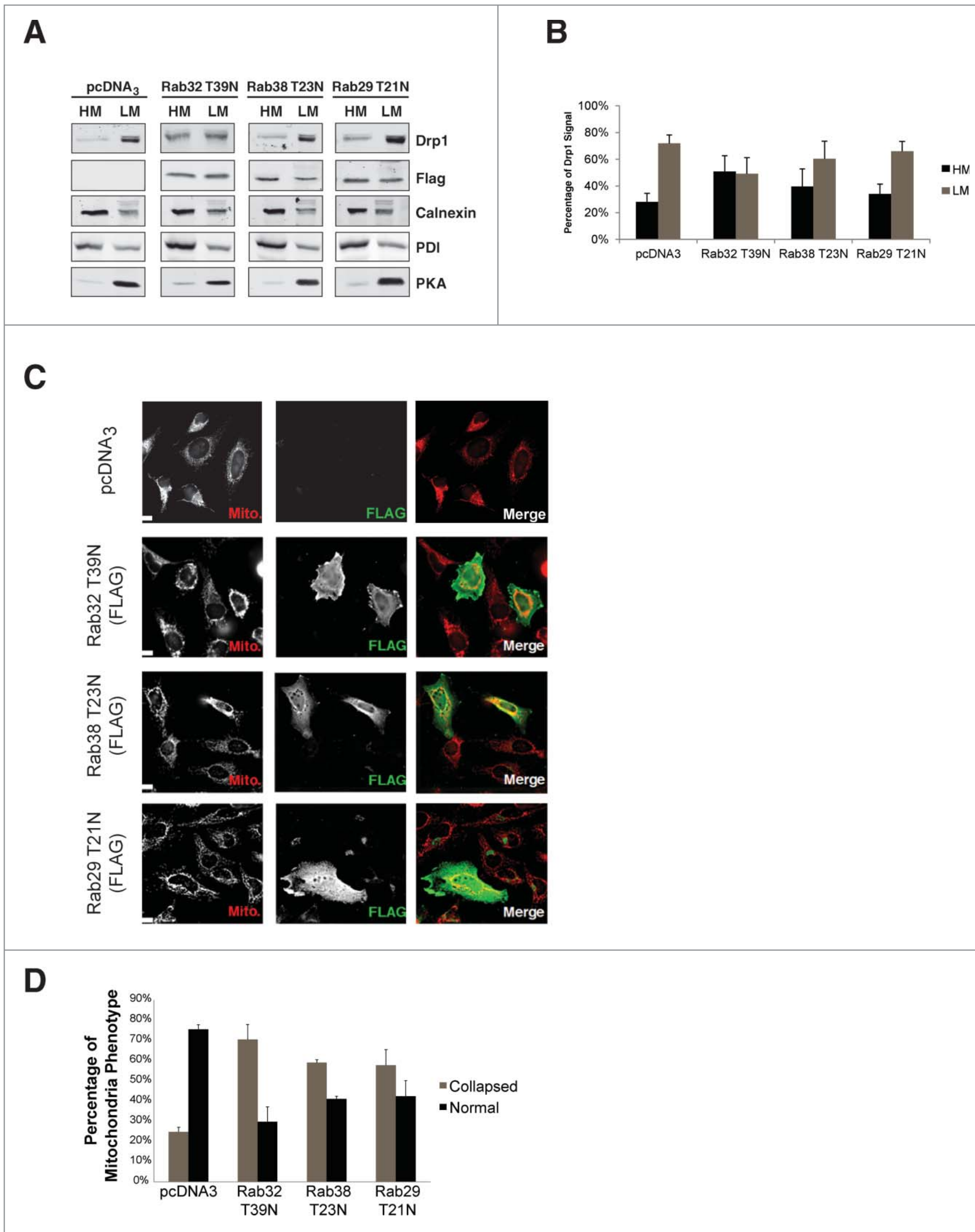

Figure 4. Rab32 Family Proteins Determine Drp1 Localization and Mitochondrial Dynamics. (A). HeLa cells were transfected with the indicated plasmids or dominant-negative Rab32 family protein cDNA constructs. Homogenized HeLa cell lysates were separated into heavy and light membranes (HM and LM, respectively). Membrane fractions were analyzed as indicated by SDS-PAGE and western blotting for Drp1, Calnexin (MAM marker), PDI (general ER marker), PKA-RII and FLAG (Rab32 family constructs). (**B**)**.** Quantification of the membrane fractionation in the presence of Rab32 family inactive proteins. The graph shows the Drp1 amounts in the heavy and light membrane fractions. (Standard deviation is shown, n = 3) (**C**)**.** Rab32 family proteins influence mitochondria membrane dynamics. Mitotracker-loaded HeLa cells were transfected with the pcDNA3 empty plasmid as well as FLAG-tagged constructs encoding Rab32 T39N, Rab38 T23N and Rab29 T21N. Transfected cells were identified by their positive FLAG signal. Scale bar = 25 μm. (**D**)**.** Quantification of the effects of dominant-negative Rab32 family proteins on mitochondrial dynamics. 100 transfected cells from 3 independent experiments were evaluated for the presence of collapsed mitochondria with the standard deviation shown.

we should detect that the function of Drp1 is linked to the activity of Rab32 family proteins. We first analyzed the ability of dominant-inactive Rab32 family proteins to alter the subcellular distribution of Drp1 in HeLa cells. This showed that Rab32 removed Drp1 from light ER membranes (**Fig. 4A**) and led to a two-fold increase of Drp1 on heavy membranes that contain mitochondria and the MAM. This effect was seen to a lesser extent with Rab29 and Rab38 (**Fig. 4B**).

As a second test for our hypothesis, we determined the extent by which dominant-inactive Rab32 family proteins lead to a collapse of the mitochondrial network around the nucleus, a phenotype that resembles Drp1 knockout cells.[22,34] Consistent with our previous results, we detected that all Rab32 family proteins have this ability when comparing to non-expressing cells (**Fig. 4C**). Whereas Rab32 T39N roughly tripled this amount, Rab38 T23N and Rab29 T21N only led to a more modest, roughly twofold increase in the collapsed mitochondria phenotype (**Fig. 4D**). This effect was not tied to PKA, which is used by Rab32 to promote Drp1 phosphorylation.[22] As published,[35] this ability is lost in Rab29 and Rab38, since they are not A-kinase anchor proteins (AKAPs) and are therefore not able

to bind or mislocalize PKA (**Fig. 4A**). Moreover, we were unable to detect altered Drp1 phosphorylation with any of these mutants (data not shown). Therefore, our results showed that the extent of the Drp1-Rab32 family protein interaction determines their control of mitochondrial dynamics.

Discussion

Our results identify Drp1 as a common effector protein for the Rab32 protein family. Rab32 is one of the primordial Rabs thought to have been present in the LECA,[26] and thus is presumed to have performed a fundamental function. However, current information on Rab29, Rab32, and Rab38 do not provide a clear conclusion as to what this ancestral function might have been. Our findings now indicate that one common function of the 3 Rabs is their interaction with the mitochondria fission factor Drp1, dictated by their association with the ER. Since dominant-negative Rab32 family proteins lead to the accumulation of Drp1 on heavy membranes, we speculate that Rab32 family activity is needed to localize Drp1 to lighter, peripheral portions of the ER. This is also consistent with the inability of dominant-active Rab32 to affect the subcellular fractionation of Drp1.[22]

The Drp1 GTPase had originally been discovered on the ER, where it can determine the distribution and morphology of the ER.[36,37] However, Drp1 fulfills a more clear-cut role in mitochondrial dynamics by forming oligomers in the cytosol before interacting with mitochondria-localized proteins such as Fis1, mitochondrial fission factor (Mff) and the 2 mitochondrial dynamics proteins of 49 and 51 kDa (MiD49, MiD51).[38] This interaction is preceded by the apposition of ER tubules and the actin cytoskeleton with mitochondria that then constrict.[16,39] Analogous to this, Drp1 also promotes the constriction of ER-derived peroxisomes.[40] Our findings raise the question how this ER-associated function is related to the known functions of the Rab32 family proteins on other organelles and how these potentially novel functions have originated.

Some insight might be gained from the Rab32 family protein tissue distribution and the effects observed upon Rab32 family protein mutation in whole animals. However, all Rab32 family proteins show wide tissue distributions. Rab38 is present in lung, liver, kidney, stomach and skin.[41] While both Rab32 and Rab38 have been tied to melanosome maturation and trafficking toward this lysosome-related organelle, published evidence suggests that it is Rab38 that has a predominant role in this function: Not only do mutations in Rab38 lead to the chocolate fur phenotype in mice, Rab38 is also a melanocyte differentiation antigen and is required for the stability of immature melanosomes.[42–44] Rab29's tissue distribution closely resembles the one of the other family members: it is found predominantly in heart, liver, pancreas and kidney, where it appears to function in TGN-endosome recycling.[29,45] A screen for factors required for typhoid toxin export has identified Rab29 as necessary for the transport of bacteria-containing vacuoles to the plasma membrane.[46] In the case of Rab32, highest expression has been detected in liver, but significant amounts of the Rab32 mRNA also appear in kidney, spleen, lung and heart tissue of humans and mice.[35,47,48] Interestingly, Rab32 appears to regulate a lysosome-directed pathway that can deliver a Salmonella typhi-targeting toxin.[49] Moreover, Rab32 is required for the formation of autophagosomes in both humans and *D. melanogaster*.[50,51] Some or all of these functions may be tied to the function of Rab32 at the MAM, since these membranes have recently been proposed as a point of origin for autophagosome formation.[52] Together with its localization to both the ER and lysosome-related organelles, these findings implicate Rab32 in the regulation of membranous contacts between the ER and other organelles, especially mitochondria[53] and lysosomes.[54]

We propose that ER-associated modulation of mitochondrial dynamics were part of the original repertoire of Rab32 functions. The LECA clearly possessed ER, endolysosomes and mitochondria [55]. It has further been demonstrated that a Drp1 homolog was present in the LECA and proposed as being involved in mitochondrial dynamics as its primordial function.[56,57] With Rab 32A and B being present in the LECA and Rabs 29 and 38 arising later in the holozoan lineage, we can conclude that Rab38 and Rab29 could perform non-overlapping functions with Rab32, but that ancient functions must be shared by all family members. Moreover, many eukaryotic taxa possess one or more Rab32 paralogues that we expect to all regulate mitochondrial dynamics. Our study, however, not only provides starting hypotheses for the functional validation in those organisms, it also raises questions about the regulation of mitochondrial dynamics in organisms such as plants or fungi that do not have Rab32 family proteins.

Interestingly, the sole *D. melanogaster* Rab32 regulates adaptor protein 3 (AP-3)-independent vesicular trafficking that is critical for pigment granule biogenesis.[58] Together with the cooperative activity of Rab32 and 38 with AP-1 and 3, as well as with the biogenesis of lysosome-related organelle complexes 2 and 3 (BLOC-2/3),[59,60] all of which are ancient protein machinery,[55,61] a role for lysosome-related organelles could also have been part of the original repertoire of Rab32 functions. A similar dichotomy is observed for PACS-2, another MAM-regulatory protein, that also regulates endosomal trafficking.[73] Therefore, a collection of ER-, mitochondria- and lysosome-related functions of this family are not as inconsistent as it might seem, as the membranes of all 3 organelles have functional and physical ties among each other.[62] Furthermore, trafficking pathways exist between both the ER and mitochondria and lysosomes.[63,64] The extent to which these pathways exist in all eukaryotic cells and the role of Rabs for these represent an exciting avenue of future investigation.

Materials and Methods

Antibodies and reagents

All chemicals were from Sigma (Oakville, ON). Protein A-Sepharose was from Repligen (Needham, MA). The antibodies were used at 1:1000 for Western blots and 1:100 for immunofluorescence. They have been purchased as follows: rabbit anti-Rab29, rabbit anti-Rab32 (Sigma Prestige Antibodies, St. Louis,

MO), rabbit anti-Rab38 (abcam, Cambridge UK ab155956, used for IF), mouse anti-Rab38 (Abnova, Taipei, Taiwan, used for Western blot), rabbit anti-PKA RII, rabbit anti-LAMP1 (Santa Cruz Biotechnology, Dallas, TX), mouse anti-ERp57 (StressMarq, Victoria, BC), rabbit anti-Drp1 (Cell Signaling, Danvers, MA), rabbit anti-Syntaxin 7 (ProteinTech, Chicago, IL), mouse and rabbit anti-FLAG (Rockland, Gilbertsville, PA; Sigma, Oakville, ON), goat anti-FACL4, rabbit anti-Rab7, mouse anti-complex 2 (abcam, Cambridge, UK), mouse anti-PDI, (Thermo-Pierce, Rockford, IL), rabbit anti-calnexin antibody (our lab[65]). Mitotracker and goat anti-mouse/rabbit secondary fluorescent antibodies were from Life Technologies (Carlsbad, CA). Plasmids for N-terminally FLAG-tagged Rab38 and Rab29 have been constructed by overlap extension using oligos whose sequence are available upon request. All other plasmids used in this study have been described previously.[22]

Homology searching and Phylogenetic analysis

Candidate Rab32 family protein sequences were identified by BLAST using databases of the National Center for Biotechnology Information (NCBI), the Joint Genomes Institute (JGI), Origins of Multicellularity of the Broad Institute, and individual genome projects. Protein sequences for *Schyliorhinus canicula* were translated manually using the software Sequencher v4.9. Sequences identified with an E-value <0.05 were considered candidate homologous sequences, and were validated by a variation of the RBH method described in.[66] The accession numbers of all sequences identified in this analysis is found in Supplemental **Figure 2**. When the homology search was finished, all the positive and negative hits were visualized using the Coulson Plot Generator.

After collecting and classifying all the homologous protein sequences throughout the eukaryotic supergroups, they were first aligned using MUSCLE v3.6[67] and then manually adjusting the alignment using MacClade v4.08. All alignments are available from the authors upon request. Trees were built using 2 maximum likelihood methods, RAxML v2.2.3[68] and PhyML v2.4.4,[69] and a Bayesian inference method, MrBayes v3.1.2.[70] For MrBayes trees, 30 000 000 Markov Chain Monte Carlo generations were used with a stop rule of <0.01 average standard deviation of split frequencies; the burn-in value was determined in a graph manner using Excel, removing usually the first 25% of the trees preceding the plateau. Maximum-likelihood bootstrap values were obtained using PhyML and Phylip 3.66 for RAxML trees. Trees were visualized using FigTree v1.2.

Immunofluorescence microscopy and quantification of mitochondria collapse

To determine Rab32 family protein overlap and mitochondrial collapse, 250,000 cells were seeded on glass coverslips in 6-well plates and incubated overnight at 37°C. The next day, 2 μL of MitoTracker (Life Technologies, Carlsbad, CA) was added, followed by further incubation for 20–30 min at 37°C. After 3 washes with PBS++ (PBS containing 0.1 g/L $CaCl_2$ and 0.1 g/L $MgCl_2$), cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Cells were next permeabilized with 2 mL IF washing solution (PBS++, 0.2% BSA, 0.1% Triton X-100) for 1–2 minutes, washed twice with PBS ++, and incubated with IF blocking solution (1× DPBS, 2% BSA, 0.5% Saponin). Primary and secondary antibodies were incubated with inverted coverslips in a wet chamber on parafilm for 1 hour each. Coverslips were next washed with PBS++ and transferred from H_2O onto a drop of ProLong Antifade Gold (Life Technologies, Carlsbad, CA). Control and mitochondria phenotype images were obtained with an Axiocam on an Axioobserver microscope (Carl Zeiss, Jena, Germany) using a 100× plan-Apochromat lens. Co-localization images were acquired on an Axioobserver Z1 Confocal Microscope (Zeiss, Jena, Germany) using an Ultraview ERS Spinning Disc Confocal system (Perkin Elmer, Waltham MA) and a Hamamatsu 9100 EMCCD camera. Images were then processed (after manual delineation of cells) using ImageJ Coloc 2 (Fiji) to determine the Mander's coefficients, used to determine the extent of co-localization.[71] To quantify alteration of the mitochondria phenotype, an ImageJ algorithm previously published by us was used.[72] Briefly, cells were manually delineated, and the center of the nucleus of each cell was manually indicated. Next, the total fluorescence intensity was concentrically measured by the algorithm (quantifying fluorescence intensity per pixel). After establishing a baseline, cells were grouped into normal and altered mitochondrial distance from the nucleus.

Percoll gradient fractionation, heavy and light membrane separation

Heavy and light membrane separation as well as Percoll gradient fractionation of MAM and mitochondria was described previously.[22]

Co-immunoprecipitation

HEK 293 T cells were transfected with the plasmids indicated (Metafectene, Biontex, Martinsried, Germany) and grown for 48 h. On the day of the experiment, cells were washed twice with PBS and then incubated with 2 mM DSP (Thermo, Rockford, IL) in PBS++ for 30 min. After triple washing with 10 mM NH_4Cl in PBS++, cells were harvested in 150 μL lysis buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40). Cell lysates were vortexed for 10 seconds and spun down at 4°C at 800 rpm for 5 min. The post-nuclear supernatant was precleared and incubated overnight with 5 μL of anti-Flag antibodies. 25 μL of PAS beads were used to precipitate Rabs and their associated proteins.

Author Contributions

COS conducted the experiments; TS designed most of the experiments; SCH contributed ideas and reagents; JBD designed the bioinformatics experiments; SCH and JBD edited the manuscript; and TS and COS wrote the manuscript.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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