Rab8a regulates the exocyst-mediated kiss-and-run discharge of the Dictyostelium contractile vacuole

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Abstract

Water expulsion by the contractile vacuole in Dictyostelium is carried out by a giant kiss-and-run focal exocytic event during which the two membranes are only transiently connected but do not completely merge. We present a molecular dissection of the GTPase Rab8a and the exocyst complex in tethering of the contractile vacuole to the plasma membrane, fusion and final detachment. Right before discharge, the contractile vacuole bladder sequentially recruits Drainin, a Rab11a-effector, Rab8a, the exocyst complex and LvsA, a protein of the Chediak-Higashi family. Rab8a recruitment precedes the nucleotide-dependent arrival of the exocyst to the bladder by a few seconds. A dominant-negative mutant of Rab8a strongly binds to the exocyst and prevents recruitment to the bladder suggesting that a Rab8a GEF activity is associated with the complex. Absence of Drainin leads to over-tethering and blocks fusion, while expression of constitutively active Rab8a allows fusion but blocks vacuole detachment from the plasma membrane, inducing complete fragmentation of tethered vacuoles. An indistinguishable phenotype is generated in cells [...]
Rab8a regulates the exocyst-mediated kiss-and-run discharge of the Dictyostelium contractile vacuole

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ABSTRACT Water expulsion by the contractile vacuole (CV) in Dictyostelium is carried out by a giant kiss-and-run focal exocytic event during which the two membranes are only transiently connected but do not completely merge. We present a molecular dissection of the GTPase Rab8a and the exocyst complex in tethering of the contractile vacuole to the plasma membrane, fusion, and final detachment. Right before discharge, the contractile vacuole bladder sequentially recruits Drainin, a Rab11a effector, Rab8a, the exocyst complex, and LvsA, a protein of the Chédiak–Higashi family. Rab8a recruitment precedes the nucleotide-dependent arrival of the exocyst to the bladder by a few seconds. A dominant-negative mutant of Rab8a strongly binds to the exocyst and prevents recruitment to the bladder, suggesting that a Rab8a guanine nucleotide exchange factor activity is associated with the complex. Absence of Drainin leads to overtethering and blocks fusion, whereas expression of constitutively active Rab8a allows fusion but blocks vacuole detachment from the plasma membrane, inducing complete fragmentation of tethered vacuoles. An indistinguishable phenotype is generated in cells lacking LvsA, implicating this protein in postfusion detethering. Of interest, overexpression of a constitutively active Rab8a mutant reverses the lvsA-null CV phenotype.

INTRODUCTION Focal delivery of exocytic vesicles to specific domains of the plasma membrane (PM) is called focal exocytosis and is crucial for cell polarity, function, and fate. This process is used either for addition of bulk membrane or local delivery of specific cargo. It contributes to epithelial cell organization in apical and basolateral domains (Mellman and Nelson, 2008), to addition of membrane at the base of the phagocytic cup necessary for uptake of large particles (Groves et al., 2008), or at the cell front for efficient cell motility (Spiczka and Yeaman, 2008). Focal exocytosis can occur either by full fusion, when the vesicle collapses into the PM, or by a kiss-and-run mechanism (Rizzoli and Jahn, 2007), when the vesicle forms a transient exocytic pore at the PM without membrane mixing (Tse et al., 1993; Zimmerberg et al., 1994). The exact mechanisms restricting fusion are unclear (Harata et al., 2006).

Each step, from vesicle delivery to exocytosis and final pore closure, is highly regulated. The first contact between two membranes is defined as tethering that facilitates soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) engagement and pore formation. Tethering is mediated by multisubunit protein complexes (Sztul and Lupashin, 2009), among which the exocyst complex is one of the best studied. The exocyst is an octameric complex of the subunits Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84. It was first discovered in yeast (TerBush and Novick, 1995), where it tethers post-Golgi secretory vesicles to the PM to provide additional membrane material for cell wall biogenesis and bud growth. In mammalian cells, the exocyst is involved in focal exocytic processes, including neurite outgrowth, cytokinesis, various vesicular trafficking steps in polarized epithelial cells, and invasive cell motility (Hazuka et al., 1999; Inoue et al., 2003; Murthy et al., 2003; Yeaman et al., 2004; Spiczka
In plants, the exocyst is involved in polarized secretion, pollen tube formation (Hala et al., 2008), and cytokinesis (Fendrych et al., 2010). In fungi, it has similar functions in hypha growth (Taheri-Talesh et al., 2008).

Research on the exocyst has concentrated on its assembly and targeting to the PM. In yeast, delivery of the complex to the PM depends on the interaction of Sec15p with the small GTPase Sec4p (Guo et al., 1999). Essential for targeting at the PM are Sec3p and Exo70p via interaction with the small GTPases Cdc42p, Rho1p, and Rho3p, as well as with phosphatidylinositol 4,5-biphosphate (PI(4,5)P2; He et al., 2007; Zhang et al., 2008). Interference with any of the eight subunits blocks secretion and arrests growth. In mammalian cells, targeting works similarly. Exo70 interacts with PI(4,5)P2 at the PM (Liu et al., 2007). In contrast to yeast, however, mammalian Exo70 does not interact with Rho3 but does interact with TC10 (Inoue et al., 2003). No major role has been proposed for Sec3c, as it lacks a Rho-binding domain (Matern et al., 2001). Sec15 interacts with Rab11-GTP on recycling endosomes (Zhang et al., 2004) but not with the Sec4 homologue Rab8. Final assembly of the complex seems to take place at the PM. In yeast, transport of all subunits, except Sec3p and Exo70p, depend on the actin cytoskeleton (Boyd et al., 2004). Studies in mammalian cells show two subcomplexes. RaIA, a small GTPase that does not exist in yeast, regulates their assembly (Moskalenko et al., 2003) into a rod-like structure (Hsu et al., 1998).

Mechanisms of exocyst targeting and its involvement in focal exocytosis have been investigated in detail, but little is known about its possible role in kiss-and-run exocytosis and even less about mechanisms of tether dissociation or “detethering.” In the present study, we used Dictyostelium as a model organism to address these questions. Membrane trafficking in Dictyostelium is similar to that in animal cells in both the secretory and endocytic pathways (Duohon and Cardelli, 2002; Neuhaus et al., 2002). Dictyostelium possesses a further major organelle—the contractile vacuole (CV) system, a specialized osmoregulatory organelle found in freshwater protozoa and amoeba. In Dictyostelium, it consists of a dynamic network of interconnected membrane tubules and bladders. Under hypo-osmotic conditions, as water enters into the cytoplasm, H₂O⁺ and HCO₃⁻ ions are pumped into the CV lumen. Following the osmotic gradient, water streams into the CV. When the CV bladder reaches its maximal diameter of 2–4 μm, it discharges its content through a pore at the PM (Heuser et al., 1993) in a giant kiss-and-run process. Subsequently, CV and PM separate, and the empty CV bladder tubulates, fragments, and finally is reincorporated into the CV network. Cortical transport of the bladder and anchoring of the CV membrane in the cortical cytoskeleton are mediated by the class V myosin MyoJ (Jung et al., 2009). The force that drives discharge and tubulation likely depends on the curvature-regulating function of MEGAP1, a Rab-GAP family protein with an F-BAR domain (Heath and Insall, 2008). A temperature-sensitive mutant of secA, the Sec1 orthologue, blocks CV discharge (Zanchi et al., 2010), suggesting a parallel with exocyst-mediated exocytosis in yeast and mammalian cells.

The CV cycle is controlled by Rab proteins and their regulators. Dictyostelium has 52 Rab proteins (Weeks et al., 2005). For the vast majority of them, a human orthologue is clearly identified. Rab8α, 11a, 11c, and 14 have been localized to the CV (Bush et al., 1994, 1996; Harris et al., 2001; Du et al., 2008). Of note, Rab8 and 11 regulate targeting of the exocyst to the PM in other systems. Disgorgin, a Dictyostelium homologue of the mouse Rab27a GTPase-activating protein (GAP; Reczek and Bretscher, 2001), was identified as a Rab8α GAP and functions in the CV cycle (Du et al., 2008). Exposure of Disgorgin-knockout cells to low osmotic conditions results in strongly enlarged bladders that rapidly disappear after discharge (Du et al., 2008), likely by fragmentation into numerous smaller structures that are prevented from reassembling in a bladder. Hydrolysis of GTP by Rab8α was suggested to be necessary for CV–PM fusion, and Rab8α was claimed to be an essential protein (Du et al., 2008).

Drainin is a further conserved Rab-GAP like protein (with no measurable GAP activity) that regulates CV discharge (Gersch et al., 2002; Bos et al., 2007; Du et al., 2008). Drainin is recruited to the bladder in a Rab11a-GTP dependent manner shortly before discharge and remains during the whole process (Du et al., 2008). Under hypo-osmotic conditions, the CV bladders of drainin-null cells become >10-fold larger than in wild-type cells. Instead of discharging, the bladder expands in close apposition to the PM. In these areas the membranes are tightly connected by a palisade-like structure closely resembling the “tethers” observed by Heuser (Figure 3 in Heuser, 2006). As a result of overinflation, abnormal CV discharge occurs explosively via rupture of the apposed membranes. Drainin might function as a volume sensor that signals to the palisade of spacers to prepare for pore formation and fusion (Becker et al., 1999).

A further class of regulators of small GTPases associated with the CV function is made up of Rho-Gdis, as knockout of Rho-GDI1 or Rho-GDI2 leads to inflated CV bladders (Rivero et al., 2002). Rho-GDI1 binds to Rac1a/b/c, RacB, RacC, and RacE, but none of them has been linked to the CV cycle. Besides small GTPases and their regulators, LvsA, a member of the family of Chédiak–Higashi syndrome (CHS) proteins, functions in CV discharge (Gerald et al., 2002). CHS is a human autosomal disorder characterized by the presence of enlarged endolysosomal compartments. In certain cell types, this is associated with defects in the secretion of lysosomal hydrolases (Page et al., 1998). The Dictyostelium homologues are called Lvs (large volume sphere) A–F (Kwak et al., 1999). Green fluorescent protein (GFP)–LvsA labels the CV bladder when it reaches its maximal diameter and remains associated throughout the discharge phase until it concentrates in a very bright patch at the PM (Gerald et al., 2002). No CV activity was detectable in lvsA-null cells. The CV network was fragmented into small punctae near the nucleus and at the cell periphery (Wu et al., 2004). LvsA is proposed to preserve the structure of the CV during discharge and to play a role in pore closure (Du et al., 2008) but its precise molecular function is unclear.

Although scattered morphological and molecular information about the process of CV discharge has been reported, the data suffer from a lack of integration and mechanistic understanding. In this study, we took advantage of the inducible, easily observable process of CV discharge to study the function of the exocyst and its regulators in kiss-and-run focal exocytosis. We describe a molecular cascade that drives the entire process from targeting the bladder to the PM to pore closure and bladder detachment from the PM. Our major finding is that the exocyst complex, together with the essential protein Rab8α, plays a role in focal exocytosis by the kiss-and-run mechanism. We present evidence that CV-associated Rab8α is activated by a guanine nucleotide exchange factor (GEF) activity associated with the exocyst complex that acts at the time of its recruitment onto the CV bladder. In addition, we propose a new role for a CHS protein in “detethering” a vacuole from its target membrane after fusion, shedding more light on this mysterious protein family.

RESULTS
Interfering with Rab8α function alters CV morphology and its discharge cycle
As described earlier, ablation of the Disgorgin gene encoding a Rab8α GAP resulted in a prominent CV discharge defect, but the
forms of Rab8a in a strictly regulated manner by adapting to Dictyostelium a co/posttranslational degradation system (Banaszynski et al., 2006). In brief, Rab8a is fused at the C-terminus of the destabilizing domain (DD). On translation, the DD domain causes rapid degradation of the DD fusion protein, but addition of the compound Shield-1 rapidly and reversibly stabilizes it (Supplemental Figure S1 and Supplemental Movie S1), allowing us to observe direct and acute effects of Rab8a mutant expression. Fusion of DD-GFP or monomeric red fluorescent protein (mRFP; Supplemental Figure S2) to the N-terminus of Rab8A did not alter its localization to the CV (Du et al., 2008). Both colocalized with the CV markers VatA (Neuhaus et al., 1998), a subunit of the vacuolar H⁺-ATPase (V-ATPase; Supplemental Figure S2, A and B, arrowheads) and calmodulin (CaM, Supplemental Figure S2C, arrowhead; Zhu and Clarke, 1992) on large CV bladders in close proximity to the PM. Some CV bladders deeper inside the cell were Rab8a negative but VatA positive (Supplemental Figure S2A, arrow) and CaM positive (Supplemental Figure S2C, arrow), indicating transient association of Rab8a with the CV. The remaining structures are endosomes, known to be VatA positive but CaM negative (see, e.g., Figure 2A, asterisk, later in the paper). Live-cell imaging (Supplemental Movies S2 and S3) confirmed CV localization and showed that mRFP/GFP-Rab8a and DD-GFP-Rab8a (Figure 1A, arrow) also localized to the juxtanuclear region. This area corresponds to pericentriolar recycling endosomes (Charette et al., 2006) and Golgi, possibly indicative of CV-independent functions of Rab8a. Recruitment of DD-GFP-Rab8a wild type (WT) to the CV was temporally regulated. It appeared at the CV bladder shortly before it reached its maximal diameter and stayed on the contracting bladder during the entire discharge process, finally remaining as a patch at the PM (Figure 1A, arrowheads). Because DD-GFP tagging did not affect the specific recruitment of Rab8a WT, we used that system to express Rab8a mutants that are predicted to interfere with its functional cycle, by locking them in their GTP-bound, constitutively active (Q74L = CA) or nucleotide-free, dominant negative (N128I = DN) form (Powell and Temesvari, 2004). Similar to Rab8a WT, DD-GFP-Rab8a CA localized to small vesicles at PM and tubules and vesicles that move bidirectionally between PM and cell center (arrowhead). Bottom, magnification of areas in top (Supplemental Movie S4, right image; Supplemental Movie S5, left image). (C) DD-GFP-Rab8a DN appeared cytosolic. Arrowheads point to exocytic-like events. The discharge process was slowed down (Supplemental Movies S6 and S7, 80 s). Scale bars, 5 μm.

FIGURE 1: Mutations of Rab8a have an effect on the functional CV cycle. For live-cell imaging by widefield microscopy, cells were incubated for 30 min with HL5c medium diluted 1:2 with water and imaged. (A) DD-GFP-Rab8a WT was recruited to CV bladder shortly before discharge and remained until completion (arrowhead); series 1, 60 s (Supplemental Movie S2), and series 2, 28 s (Supplemental Movie S3). Rab8a WT was also in a juxtanuclear region (arrow). (B) DD-GFP-Rab8a CA localized to small vesicles at PM and tubules and vesicles that move bidirectionally between PM and cell center (arrowhead). Bottom, magnification of areas in top (Supplemental Movie S4, right image; Supplemental Movie S5, left image). (C) DD-GFP-Rab8a DN appeared cytosolic. Arrowheads point to exocytic-like events. The discharge process was slowed down (Supplemental Movies S6 and S7, 80 s). Scale bars, 5 μm.

cells were viable (Du et al., 2008). The authors reported their failure at generating a Rab8a knockout, indicating a role for Rab8a not restricted to the CV cycle. Therefore we expressed dominant mutant
fixed cells, these DD-GFP-Rab8a CA-positive structures colocalized at the cell periphery with VatA (Figure 2A) and CaM (Figure 2B), establishing their identity as part of the CV system. Serial x, y sections at various positions through the cell clearly showed that the collapsed crescent shape seen at bottom and mid-height was formed by the accretion of small individual vesicles at the PM (Figure 2A, arrowheads), as best observed in tangential sections at the top of the cell. These "mini-CV bladders" were nevertheless functional, as discharge events were observed (Figure 1, left, and Supplemental Movies S4 and S5). In contrast to the WT and CA forms, DD-GFP-Rab8a DN appeared entirely cytosolic (Figures 1C and 2, C and D). Nevertheless, live imaging revealed exocytic events strongly reminiscent of CV bladder discharge (Supplemental Movies S6 and S7), even though the average duration of the events was twofold longer than in wild-type cells or cells expressing DD-GFP-Rab8a WT (compare the time stamps in Figure 1, A and C). The CV function appears to be not completely abrogated, likely because of basal expression of endogenous Rab8a. Labeling for VatA and CaM showed that cells expressing DD-GFP-Rab8a DN possessed an apparently normal CV system with inflated CV bladders (Figure 2C) and CV network (Figure 2D). To assess more quantitatively the effect of DD-GFP-Rab8a DN overexpression, we counted the number of CV bladders per cell and measured their size. To this purpose, Ax2, DD-GFP-Rab8a WT, and DD-GFP-Rab8a DN cells were stained for CaM and analyzed with ImageJ. About 70% of DD-GFP-Rab8a DN cells had two or more CV bladders, whereas this was only the case for <30% of Ax2 and DD-GFP-Rab8a WT cells. Greater than 70% of Ax2 and DD-GFP-Rab8a WT cells had one or no CV bladder (Supplemental Figure S1D). The maximal diameter of CV bladders was also affected (Supplemental Figure S1E). Compared to wild-type cells, the diameter of CV bladders in most

**FIGURE 2:** Mutations in Rab8a induce changes in the morphology of the CV system. Cells overexpressing DD-GFP-Rab8a CA (A, B) or DD-GFP-Rab8a DN (C, D) were treated as in...
DD-GFP-Rab8a DN–expressing cells was significantly larger. In cells with one bladder it resulted in a doubling of bladder volume. Expression of DD-GFP-Rab8a WT affected CV size, but only significantly in cells with three or more bladders. These data suggest that expression of DD-GFP-Rab8a DN severely prolongs the CV cycle, resulting in cells with more and larger CV bladders. We conclude that Rab8a CA induces CV structures that are fragmented and tightly associated with the PM, whereas Rab8a DN induces larger CV bladders because of an inefficient discharge process. The opposite phenotypes of Rab8a CA and DN suggested a role for Rab8a in regulating the efficiency of membrane docking, tethering, and/or fusion. We hypothesized that Rab8a regulates CV–PM tethering via the exocyst complex, a known effector of Sec4 (the yeast Rab8a orthologue).

The exocyst complex localizes to Rab8a-positive CV bladders during discharge

Live-cell imaging showed that the exocyst subunit GFP-Sec15 was partly cytosolic and was recruited to preexisting large mRFP-Rab8a–positive vacuoles. Supplemental Movie S8 shows multiple examples of discharge events that are visible either in an “en face” manner and others that occur sideways. The former became visible as ring structures (Figure 3D, top, and Supplemental Movie S8) and the latter as rings, patches, (and line scan) taken every 2.5 s. White arrowheads indicate the time of first appearance of the corresponding protein over background. (C) Quantification of the relative fluorescence signals of GFP-Sec15 and mRFP-Rab8a during six discharge events of similar size and duration. (D) Galleries showing two discharge events from an “en face” (upper) or sideways (lower) view. Asterisks indicate first time of recruitment of the corresponding proteins. Interval between two frames is 2.5 s. Each frame is 5 μm wide. (E–G) Cells expressing GFP-Sec8/mRFP-Sec15 (E; Supplemental Movie S9), GFP-Sec15/mRFP-Rab8a (F; Supplemental Movie S10), or GFP-Sec15/mRFP-Rab11a (G; Supplemental Movie S11) were imaged by TIRFM/IRCM. The darkest areas of the IRCM image define places of discharge, where GFP-Sec8/mRFP-Sec15 (E) and GFP-Sec15/mRFP-Rab8a (F) colocalized (arrowhead). (G) mRFP-Rab11a was evenly distributed over the network, whereas GFP-Sec15 localized (arrowhead) to dark areas of the IRCM image. (H) Zooms on examples of discharge sites where the center of the fluorescent area is darker and corresponds to a brighter zone in IRCM. Scale bars, 5 μm.
and dots (Figure 3D, bottom, and Supplemental Movie S8). Rab8a was recruited before Sec15, as indicated by comparing their time of appearance around vacuoles (Figure 3D) and by monitoring their respective intensities along a line scan as a function of time (Figure 3B, kymographs). Quantification of six discharge events of their respective intensities along a line scan as a function of time revealed a lag of up to 10 s, followed by a series of events shown in Supplemental Movie S8 and quantitated in Figure 3, A–D. In some sections across the site where the discharging CV contacted the PM, the staining appeared as patches or doublet of dots (Figure 4, B, E, and F). In a few striking cases, the exocytic site was observed in an “en face” view, revealing details of the exocyst arrangement. When the section is tangential to the plane of contact between the CV bladder and the PM, GFP-Sec15 localizes to two concentric rings (Figure 4C). We propose that the outer circle represents the projection of the outline of the VatA-positive CV bladder membrane, and the inner circle represents the outline of the pore. We conclude that the different localizations shown in Figure 4 are snapshots from the temporal series of events shown in Supplemental Movie S8 and quantitated in Figure 3, A–D.

We suggest that the structures observed by Heuser (see Figure 3 in Heuser, 2006) and Becker and colleagues (see Figure 3 in Becker et al., 1999) at the site of contact between CV and PM correspond to the exocyst. In support of this claim, the length measured for this palisade or picket fence was estimated between 15 and 30 nm, in perfect agreement with the dimension of the exocyst complex estimated to between 10 and 30 nm, in perfect agreement with the dimension of the exocyst complex (Hsu et al., 1998; Munson and Novick, 2006). Taken together, these results not only confirm our hypothesis that the exocyst is involved in CV discharge, but they also suggest that the complex assemblies and interacts directly with Rab8a.
Identification of novel exocyst interaction partners acting in CV discharge

To further dissect the function of the exocyst, we made several (unsuccessful) attempts to knock out the sec15 gene, strongly indicating that, like Rab8a, the exocyst is essential. As an alternative, we chose to identify exocyst interaction partners using Y2H screens with full-length Sec15 (Supplemental Table S1A) and with the C-terminal half of Exo70 (Exo70-C; Supplemental Table S1B), as well as using anti-GFP IPs (Supplemental Table S1C). For proteomic analyses, the samples from two separate IP experiments were separated by SDS–PAGE and either stained with colloidal Coomassie (Figure 6D) or silver (Supplemental Figure S3), cut into slices, and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

The results of the Y2H and IP/MS screens can be separated into three categories. The most relevant proteins with highly significant Hybrigenics or Mascot scores are listed in Supplemental Table S1, A–C.

First, we identified a series of interacting partners from a subset of membrane-trafficking pathways that are briefly mentioned in the Discussion. We believe that involvement of the exocyst in tethering events besides its involvement in CV discharge accounts for its essentiality.

Second, we obtained confirmations for some direct/indirect interactions between the exocyst subunits themselves. First, because Sec8 and Sec15 do not interact directly in the octameric complex, assembly of tagged subunits was verified by coIP performed with a cell line overexpressing GFP-Sec8 and mRFP-Sec15. Then IPs from GFP-Sec8– or GFP-Sec15–expressing cells confirmed coassembly of tagged and endogenous subunits and suggested recruitment of Sec15 to the CV bladder membrane depends on Rab8a in its GTP-bound form, but Western blot of anti-GFP immunoprecipitates (IPs) revealed that mRFP-Sec15 interacted with all three forms of Rab8a (Figure 5D). When normalized to the amount of immunoprecipitated GFP-Rab8a, about five times more mRFP-Sec15 was pulled down with DD-GFP-Rab8a DN than with DD-GFP-Rab8a WT or CA. Trapping of the exocyst complex by the DN form of Rab8a scavenges and blocks the exocyst-associated GEF and thus hinders its CV recruitment.

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First, we identified a series of interacting partners from a subset of membrane-trafficking pathways that are briefly mentioned in the Discussion. We believe that involvement of the exocyst in tethering events besides its involvement in CV discharge accounts for its essentiality.

Second, we obtained confirmations for some direct/indirect interactions between the exocyst subunits themselves. First, because Sec8 and Sec15 do not interact directly in the octameric complex, assembly of tagged subunits was verified by coIP performed with a cell line overexpressing GFP-Sec8 and mRFP-Sec15. Then IPs from GFP-Sec8– or GFP-Sec15–expressing cells confirmed coassembly of tagged and endogenous subunits and suggested recruitment of Sec15 to the CV bladder membrane depends on Rab8a in its GTP-bound form, but Western blot of anti-GFP immunoprecipitates (IPs) revealed that mRFP-Sec15 interacted with all three forms of Rab8a (Figure 5D). When normalized to the amount of immunoprecipitated GFP-Rab8a, about five times more mRFP-Sec15 was pulled down with DD-GFP-Rab8a DN than with DD-GFP-Rab8a WT or CA. Trapping of the exocyst complex by the DN form of Rab8a scavenges and blocks the exocyst-associated GEF and thus hinders its CV recruitment.
mRFP-Rab8a also identified five of the V-ATPase subunits—VatA, B, C, D, and M (Supplemental Table S1C). Absence of other abundant CV proteins from the IP/MS, such as Rhesus 50 (Benghezal et al., 2001) and CaM (Figure 6D), excludes unspecific interaction with the very abundant V-ATPase. Interaction of the exocyst with the V-ATPase might be of great mechanistic importance to specify the recruitment of the activated complex to the CV before discharge. Of interest, VatA was found in complex with Disgorgin (Du et al., 2008).

Huntingtin was also identified as a high-confidence Y2H interactor of both Sec15 and Exo70, possibly offering a mechanistic background to the recent observation that Huntingtin-null Dictyostelium cells appear to be extremely osmosensitive (Myre et al., 2011). However, the most interesting Y2H hit with Sec15 was LvsA, a Chédiak–Higashi–related protein of the BEACH family already involved in CV bladder discharge (Du et al., 2008). Most important, anti-GFP IPs

that in Dictyostelium the exocyst might be subdivided into two subcomplexes. Indeed, five of the eight exocyst subunits—Sec6, Sec8, Sec10, Sec15, and Exo70—were identified by IP/MS and likely belong to one subcomplex. Sec3, despite being identified in the Exo70-C Y2H screen, was absent from all the IPs (Figure 6D) and might thus belong to a second subcomplex. Moreover, GFP-Sec15 coprecipitated with mRFP-Rab8a (Figure 6B), and anti-Rab8a Western blot identified endogenous Rab8a in every IP from Sec15-overexpressing cells (Figure 6D), confirming the interaction of the Rab8a with Sec15.

Third and most important, we identified novel partners for the exocyst that are likely linked to its CV-tethering functions. In the Y2H screen with Sec15 and Exo70-C, we identified subunits of the V-ATPase, VatA, and VatE, respectively (Supplemental Table S1, A and B). Confirming these hits, IP/MS from stable cell lines expressing combinations of GFP-Sec8 or GFP-Sec15 with mRFP-Sec15 or mRFP-Rab8a also identified five of the V-ATPase subunits—VatA, B, C, D, and M (Supplemental Table S1C). Absence of other abundant CV proteins from the IP/MS, such as Rhesus 50 (Benghezal et al., 2001) and CaM (Figure 6D), excludes unspecific interaction with the very abundant V-ATPase. Interaction of the exocyst with the V-ATPase might be of great mechanistic importance to specify the recruitment of the activated complex to the CV before discharge. Of interest, VatA was found in complex with Disgorgin (Du et al., 2008).

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from stable cell lines expressing GFP-LvsA revealed association with endogenous Sec8 and Sec15 (Supplemental Table S1C), confirming the Y2H results and strengthening the possibility that they might act together in the CV cycle. We also included a GFP-Drainin–expressing cell line because of its reported role in CV bladder discharge (Becker et al., 1999; Du et al., 2008). It is remarkable that anti-GFP IPs from cells expressing GFP-Drainin and mRFP-Sec15 followed by Western blot revealed that Drainin and the exocyst interact in vivo (Figure 6C). To assess whether genetically interfering with CV-discharge affects the spectrum of Sec15-binding partners, we generated lvsA-null and drainin-null cell lines overexpressing Sec15. In these cases, anti-GFP IP/MS failed to detect the exocyst interactors identified in functional conditions.

Finally, Rac1a was also detected by both Y2H (Supplemental Table S1B) and IP/MS (Supplemental Table S1C) approaches and might be one of the small GTPases that regulate exocyst anchoring at the PM. This hypothesis is strengthened by identification of DGAP1, a known Rac1a-interacting protein and regulator, in some of the exocyst IPs (Figure 6D and Supplemental Table S1C).

**Drainin regulates the commitment to pore formation for CV discharge**
GFP-Drainin and mRFP-Sec15 were both found on the CV bladders in contact with the PM, but, whereas GFP-Drainin was enriched at the back of the bladder, mRFP-Sec15 concentrated in dots and small patches at the bladder-PM contact sites (Figure 7, A and B). Of interest, the CV marker VatA was also partially excluded from the contact sites, possibly as a consequence of a safeguard mechanism that prevents diffusional spilling at the PM after

**FIGURE 7:** Drainin is essential for correct localization of the exocyst, and Rab8a for CV discharge. Cells were treated as in Figure 1, fixed, and labeled with indicated antibodies. (A, B) GFP-Drainin and mRFP-Sec15 colocalized, but GFP-Drainin localized more prominently to the back of discharging CV bladder (arrow), whereas mRFP-Sec15 concentrated in lips at places of CV–PM contact (arrowheads). (C–F) Drainin-null cells exhibited large, VatA-positive CV bladders, tightly connected to PM. Rab8a localized to CV–PM contact zones in large patches (C, D, arrowhead). GFP-Sec15 strongly accumulated at enlarged CV–PM contact zones (E, F, arrowhead). Scale bars, 1 μm.
fusion. We suggest that this exclusion zone around the fusion pore is the place of maximal tethering of the CV bladder to the PM. In drainin-null cells, this precise spatial arrangement was specifically altered. When exposed to hypo-osmotic medium they exhibit large, overinflated CV bladders tightly apposed to the PM, and both Rab8a and Sec15 localized to this broad area of contact (Figure 7, C–F, arrowheads). In extreme cases, Sec15 accumulated in an intensely stained and flat patch between the adjacent membranes (Figure 7, E and F), without forming “lips” around a fusion pore as was visible in Figures 3B and 4, E and F. We propose that Drainin is involved in restricting the activated Rab8a–exocyst complex to the CV bladder–PM contact zone and in the ensuing commitment to fusion pore formation.

**LvsA promotes detethering of the CV bladder from the PM after discharge**

GFP-LvsA localizes exclusively to the CV bladders in closest proximity to the PM (Figure 8A, arrowheads), and its arrival is coincidental with visible CV bladder contraction (Gerald et al., 2002). The other bladders were often negative (Figure 8A, arrows). Exposure of lvsA-null cells to hypo-osmotic medium resulted in the disappearance of the CV reticulum. Instead, we observed CaM-positive CV structures collapsed against the PM, forming a hemispherical cap structure (Figure 8B) strongly reminiscent of the DD-GFP-Rab8a CA–induced phenotype. In both cases, numerous small CV-derived vesicles were docked beneath the PM (compare Figure 2, A and B, to Figure 8, B–E), indicative of postdischarge fragmentation of the CV, most probably by misfunctioning of the detethering and retrieval process from the PM. Despite this aberrant morphology, Rab8a and GFP-Sec15 colocalized with VatA at the collapsed CV vesicles cell. All the CV bladders were VatA positive (arrow), but only those close to PM were additionally GFP-LvsA-positive (arrowhead). (B) In lvsA-null cells CV bladders were fragmented and collapsed against PM, forming a VatA-positive crescent in tangential sections (arrowheads). Transverse sections showed that these crescents were built out of numerous small vesicles (arrow). (C, D) Rab8a colocalized with VatA at the collapsed CV bladder (arrowhead). (E) GFP-Sec15 and VatA localized to the same collapsed CV bladder (arrowhead), but GFP-Sec15 localized more peripherally than the VatA-positive CV bladder, likely because it is localized between PM and CV (arrows in magnification). All scale bars, 1 μm, except B, 5 μm.

**FIGURE 8:** LvsA is involved in detethering of CV bladders after discharge. Cells were treated as in Figure 1, fixed, and labeled for VatA. (A) The x, y confocal sections from bottom to top of the
phenotype, we propose that joint action of LvsA and hydrolysis of GTP by Rab8a, possibly via the action of the Disgorgin GAP, is necessary for disassembly of the exocyst and release of the CV bladder.

In a previous report, a genetic interaction was identified between LvsA and Disgorgin (Du et al., 2008). Therefore we tested the consequences of overexpressing various forms of Rab8a in lvsA-null cells. As shown in Figure 9, DD-GFP-Rab8a WT perfectly colocalized with VatA at collapsed and fragmented CV structures (Figure 9A), whereas DD-GFP-Rab8a DN remained cytosolic and did not affect the formation of the CV crescent (Figure 9C). In sharp contrast, expression of DD-GFP-Rab8a CA almost completely reversed the formation of the hemispherical CV cap induced by low osmolarity in lvsA-null cells (Figure 9B). Whereas in wild-type cells, both DD-GFP-Rab8a WT and CA localized to the CV (Supplemental Figure S1 and Figures 2 and 5), in lvsA-null cells, the CV localization of DD-GFP-Rab8a CA was greatly reduced but not abolished. In the few cells that showed some degree of collapsed CV, localization was visible (Figure 9E). In both cell types, DD-GFP-Rab8a WT is CV associated (Figure 9D), and DD-GFP-Rab8a DN did not localize to the CV (Figure 9F). These results suggest that an active CV discharge cycle is rescued by overexpression of DD-GFP-Rab8a CA in lvsA-null cells.

**DISCUSSION**

Exocytosis is a highly coordinated event of central importance in the homeostasis of cellular membranes. In this study, we report that the small GTPase Rab8a, together with the exocyst tethering complex, regulates the kiss-and-run exocytosis-like event of CV bladder discharge. We propose a detailed functional model (Figure 10) integrating our data with previous work in the field.

Before detailing the steps of our model, it is worth briefly mentioning that additional roles of the exocyst in *Dictyostelium* are likely because our unbiased Y2H screens revealed high-confidence binding partners involved in vesicle coat assembly, such as CopA (α-COP orthologue of the COP complex) and adaptor-related protein complex 1B (β-adaptin orthologue AP1B), members of the family of small GTPases as well as Ras and ArfA (Arf1 orthologue), and some of their regulatory GEFs and GAPs. A potential role of the exocyst in post-Golgi traffic, as known for other organisms, was strengthened by the identification of the subunit CopB by IP/MS. Y2H revealed a large number of hits corresponding to a proteasome subunit, PsmD7. IP/MS confirmed the potential importance of this finding by identifying six other proteasome subunits (Supplemental Table S1C), as well as the ubiquitin UbcQ fusion
Coming back to the model, we emphasize that the completion of a CV discharge cycle follows a precise sequence of events (Figure 10):

Step 1. In yeast, the Rab11 homologue, Ypt32p recruits Sec2, the Sec4-GEF, to the exocytic vesicle (Ortiz et al., 2002). In Dictyostelium, Rab11a was proposed to be activated by a threshold of hypo-osmotic stress (Du et al., 2008), and expression of Rab11a-DN induces enlarged CV bladders and renders cells osmosensitive (Harris et al., 2001). We propose that Rab11a-GTP first recruits Drainin onto CV bladders (Du et al., 2008), which, when the CV bladder reaches its maximal volume, in turn indirectly recruits Rab8a-GTP.

Step 2. Shortly afterward, the cytosolic exocyst complex is recruited to the CV concomitant with activation of Rab8a. Our data indicate that the GEF activity might be exerted by an unidentified exocyst-associated Rab8a-GEF or directly by one of the exocyst subunits, in analogy to the function of the Vsp39 subunit of the HOPS tethering complex, which directly activates Rab7 upon recruitment (Wurmser et al., 2000). Disgorgin, a Rab8a-GAP, reaches the CV bladder at the same time by an independent mechanism (Du et al., 2008).

Step 3. The CV bladder is translocated through and anchored at the actin cortex by the Dictyostelium type V myosin MyoJ for subsequent tethering. MyoJ-null cells show a strong accumulation of CV bladders in the cell center (Jung et al., 2009).

In yeast, the mechanisms dictating the choice of the tethering place at the PM have been subjects of intense research. We propose the existence of two subcomplexes in Dictyostelium. One contains Sec3 and maybe Exo84, and the other one contains Sec15 plus Sec6, Sec8, Sec10, Exo70, and maybe Sec5. In analogy to the mammalian system, where Exo70 is targeted at the PM by the small GTPase TC10 (Inoue et al., 2003), our data suggest a similar role for Rac1a in Dictyostelium. Rac1a is important for organization of the Dictyostelium actin cytoskeleton (Dumontier et al., 2000), and knockout mutants of the Rac1a binding partner Rho-GDI1 show enlarged CV bladders (Rivero et al., 2002). We speculate that Rac1a might have a Cdc42-like exocyst–PM targeting role (Adamo et al., 2001; Zhang et al., 2001) in the various exocytic pathways of Dictyostelium, but further functional studies might be hampered by the fact that Rac1a is functionally redundant with Rac1b and c (Chung et al., 2000; Dumontier et al., 2000; Palmieri et al., 2000).

Steps 4–9. After exocyst tethering of the CV bladder at the PM, Drainin stimulates Disgorgin action, leading to dissociation of the complex.
Rab8a-GDP exocyst complex from the CV bladder membrane (Step 4). Because Drainin is absent from the CV bladder–PM contact zone, the Rab8a-GTP–exocyst tethering complex remains and even concentrates (Step 5) at this interface. We observed this concentration during tethering of the CV bladder to the PM and pore formation as a progressive change in the localization of Rab8a and of the exocyst from a ring, to lips, to a dot structure (Steps 5–8). Analyses of drainin-null cells support this hypothesis. The CV bladders of drainin-null cells are broadly spread beneath the PM, and the two membranes are connected by a palisade-like structure (Becker et al., 1999). We extend and strengthen this observation by localizing GFP-Sec15 in drainin-null cells, in which it is highly enriched in a large area between the CV bladder and the PM, suggesting that absence of Drainin-mediated enhancement of Disgorgin activity leads to uncontrolled accumulation of the exocyst tethers and a block in discharge. We suggest that these zones are the places of SNARE engagement (Step 5) and that Disgorgin-mediated concentration of Rab8a-GTP and the exocyst (Steps 3–5) is a prerequisite for pore formation (Step 6). Of interest, overexpression of Rab8a or Disgorgin rescues the drainin-null phenotype (Du et al., 2008), showing that excess of Disgorgin might overcome the lack of activation by Drainin and allow pore formation. Following pore formation, the CV bladder undergoes discharge (Step 7). Despite the fact that in DD-GFP-Rab8a DN cells Rab8a cannot hydrolyze GTP, the cells are apparently still able to undergo CV discharge, even though the whole cycle is slowed down. We suggest that the Rab8a GDP/GTP cycle dictates the efficiency and timing of the entire process via the recruitment and regulation of the exocyst.

Finally, the collapsed CV bladder is detethered from the PM and reintegrated into the tubulovesicular CV system to start a new cycle of refill and discharge. Detethering seems to depend on the concerted action of Disgorgin and LvsA (Step 8). We propose that Sec15 recruits LvsA to the CV bladder as it reaches the PM. One function of LvsA might be to prevent homotypic fusion of CV bladders when preparing for pore formation and discharge. In the absence of LvsA in normal osmotic conditions, enlarged bladders are generated, in analogy to the lvsB-null phenotype characterized by enlarged lysosomes that might be caused by increased fusion (Harris et al., 2002). A further possible function of LvsA could be in the detethering of the CV after discharge, because in lvsA-null cells under hypo-osmotic conditions, the collapsed vacuoles tethered to the PM fragment vanish, reminiscent of the disgorgin-null cell phenotype (Du et al., 2008). In cells expressing DD-GFP-Rab8a CA, numerous small CV-derived vesicles also accumulate beneath the PM, but this phenotype is even more prominent than in lvsA-null and disgorgin-null cells because hydrolysis of GTP by Rab8a is completely blocked, whereas in the other mutants it likely happens with reduced uncatalyzed efficiency. We propose that hydrolysis of Rab8a-bound GTP by Disgorgin, together with a still-to-be-discovered biochemical activity of LvsA, is necessary for detethering from the PM (Steps 8 and 9).

Finally, our finding that expression of Rab8a CA reverses the collapsed and fragmented CV phenotype of lvsA-null cells is in perfect agreement with previously reported results (Du et al., 2008). Indeed, it is logical that expression of Rab8a CA phenocopies the knockout of Disgorgin, the Rab8a GAF, which CV phenotype was shown to be rescued by further knockout of the LvsA gene. The Rab8a (Disgorgin)–LvsA genetic interaction corroborates the physical interaction reported here. How this complex behavior is performed at the biochemical level will be the topic of further studies and will shed light on the function of the still mysterious proteins of the Chédiak-Higashi family.

**MATERIALS AND METHODS**

**Dictyostelium cell culture**

The wild-type Dictyostelium discoides strain Ax2 was cultivated axenically at 22°C in HL5c medium (ForMedium, Hunstanton, United Kingdom) supplemented with 10 U/ml penicillin and 10 μg/ml streptomycin (Life Technologies, Carlsbad, CA). The mutant strains were cultivated in the richer HL5 medium (14.3 g/l bactopeptone [L37; Oxoid, Basingstoke, United Kingdom], 7.15 g/l yeast extract, 18.0 g/l maltose monohydrate, 1.29 g/l Na2HPO4 × 12H2O, 0.49 g/l KH2PO4). The GFP and mRFP fusion protein constructs were expressed in the Ax2 background, selected, and maintained in 10 μg/ml G418 or 10 μg/ml blasticidin (Life Technologies) in HL5c, respectively. The drainin-null strain and the GFP-Drainin fusion construct were kind gifts of G. Gerisch (Max Planck Institute of Biochemistry, Martinsried, Germany; Becker et al., 1999). The lvsA-null strain and the GFP-LvsA–expressing strain were kindly provided by A. DeLozanne (University of Texas, Austin, TX; Kwak et al., 1999; Gerald et al., 2002). The Q74L and N128I Rab8a mutant cDNAs were a kind gift of L. Temesvari (Clemson University, Clemson, SC; Powell and Temesvari, 2004). The Rab8a Q74L mutant is unable to hydrolyze GTP and therefore is constitutively active (CA). The Rab8α N128I mutant mimics the nucleotide-free form and therefore is dominant negative (DN).

**Antibodies**

The following antibodies were used: a mouse monoclonal antibody against the A subunit of the V-ATPase complex (VatA; monoclonal antibody, 221-35-2; Neuhaus et al., 1998) and rabbit polyclonal antibodies against Dgap1 (Faix and Dittrich, 1996), Rhesus50 (Benghezal et al., 2001), and calmodulin (Ulbricht and Soldati, 1999). The antibodies against mRFP, Sec3, and Rab8α were obtained during this study by immunization of rabbits with purified mRFP protein, purified GST-Sec3 N-terminus (amin acids [aa] 1–188), and a synthetic Rab8α peptide (aa 172–186: NH2-H2-DIKKRRMIDTPNQ-CONH2). Commercial anti-GFP antibodies were used for immunoprecipitation (11 814 460 001, Roche, Indianapolis, IN; and GFP-Trap-A, ChromoTek ( Martinsried, Germany ), immunofluorescence, and immunoblot (598; MBL, Woburn, MA). As secondary antibodies for immunofluorescence, goat anti–mouse or goat anti–rabbit immunoglobulin G (IgG) coupled to Alexa 488 or Alexa 594 (Molecular Probes, Invitrogen, Carlsbad, CA) was used at a 1:2000 dilution. For immunoblots, goat anti–rabbit IgGs or goat anti–mouse IgGs conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) were used at dilutions between 1:2000 and 1:10,000.

**Immunofluorescence**

For immunofluorescence, cells on coverslips (grade 0; Hecht KG, Sondheim, Germany) were fixed by rapid freezing as described (Neuhaus et al., 1998; Hagedorn et al., 2006). The cells were plated on these coverslips the day before, exposed for 30 min to medium diluted 1:2 with water to enhance the activity of the contractile vacuole, and plunge-frozen. Then coverslips were transferred to phosphate-buffered saline (PBS) at room temperature and blocked by incubation in PBS containing 2% fetal bovine serum (FBS) for 10 min. PBS with 2% FBS was used for the antibody dilutions and washing steps. Cells were incubated with primary antibodies for 60 min, washed three times, and incubated with fluorescently labeled secondary antibodies and 4′,6-diamidino-2-phenylindole (1 μg/ml) for 60 min. After three washes, the slides were mounted in ProLong Antifade (Molecular Probes). Immunofluorescence samples were documented with a Leica SP2 confocal microscope (Leica, Wetzlar, Germany) using a 100×, 1.4 numerical aperture, oil immersion.
objective. Recording parameters for fields of 1024 × 1024 pixels with appropriate electronic zoom (2–8x) were 4x line averaging and 0.1- to 0.32-μm vertical steps. Images and stacks were processed with ImageJ (National Institutes of Health, Bethesda, MD).

Live-cell imaging
For live-cell imaging, cells were transferred to 35-mm, optically clear plastic μ-Dish (Ibidi, Munich, Germany) with phosphate buffer (15 mM KH2PO4, 2 mM Na2HPO4, pH 6.0) and imaged immediately with a Leica AF6000 LX widefield microscope (frames were taken every second). To monitor the effect of the compound Shield-1 on the cells, they were imaged directly in the 12-well Petri dishes by phase-contrast or fluorescence microscopy using a Zeiss Axioptot 2 microscope with a 100x Achroplan water immersion objective (Carl Zeiss, Jena, Germany), and images were recorded with a charged-coupled device (CCD) camera (Imago Sensicam; PCO AG, Kelheim, Germany) and processed with ImageJ. For imaging with a spinning disk confocal microscope (Marianas SDC; Intelligent Imaging Innovations, Denver, CO; mounted on a Leica DMIRE2 inverted microscope), cells were plated on 35-mm μ-Dish at 30% confluence and incubated overnight in HL5c. At 1 h before imaging, the medium was replaced with low-fluorescence medium (LoFlo; ForMedium). Just before recording, LoFlo was replaced with LoFlo diluted 1:2 with water, and the cells were overlaid with a thin agar sheet (2% in water) as described (Yumura et al., 1984). For TIRFM and IRCM, cells were placed onto glass coverslips (22 mm diameter, n = 1.5255, D263M; Menzel, Braunschweig, Germany) mounted in an Attofluor cell chamber (Invitrogen) and overlaid with ~1.5 ml of HL5c medium. The coverslip was precoated with 0.1% poly-l-lysine solution (Sigma-Aldrich, St. Louis, MO) for 5 min. For imaging, the chamber was mounted on a custom-built TIRFM based on an Olympus IX-70 body (Olympus, Hamburg, Germany) coupled with a Zeiss (100x, 1.45 numerical aperture) objective. Details of a similar TIRFM setup have been published (Merrifield et al., 2005). The setup was further modified for IRCM by introducing widefield illumination from an amber (594 nm, Luxeon K2; Philips Lumileds, San Jose, CA) light-emitting diode introduced into the light path via an 8.92-part-silvered pellicle (BP145B1; Thorlabs, Munich, Germany). The 488/568 DBX dichroic mirror had sufficient residual reflection at ~594 nm to allow IRCM. Custom timing electronics were fabricated to control the illumination mode using transistor–transistor logic (Radio Spares, Mörfelden-Walldor, Germany) such that alternate TIRFM and IRCM images could be acquired. For simultaneous imaging of red and green fluorescence, an image splitter (Multiplex Micro-Imager; Optical Insights, Santa Fe, NM) divided the red and green components of the images with a 565-nm dichroic mirror (QS65; Chroma, Brattleboro, VT) and passed the red component through a 585-nm long-pass filter (585 nm AELP; Omega Optical, Brattleboro, VT) and the green component through a 525- to 575-nm bandpass filter (HQ525/50; Chroma). The images were then projected side by side onto a back-illuminated CCD camera (Cascade 512B; Roper Scientific, Ottobrunn, Germany). Each cell was imaged using MetaMorph (Molecular Devices, Sunnyvale, CA) for up to 18 min with 50- to 150-ms exposures at 1 Hz with TIRFM and IRCM alternated. For each acquisition session, an alignment image was acquired consisting of sparsely scattered 0.2-μm fluorescence isothiocyanate–conjugated beads (Molecular Probes). There was sufficient bleedthrough from green to red channels to image the beads in both the green and red channels, which thus provided fiducial markers in the x, y plane.

Immunoprecipitation
Two different protocols were applied. In protocol A, the anti-GFP antibody and protein G–Sepharose beads were added subsequently. In protocol B, a beads-coupled llama antibody was used.

Protocol A. A total of 2 × 10⁷ cells were exposed for 30 min to medium diluted 1:2 with water, lysed in 600 μl lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 50 mM sucrose, 5 mM EDTA, 0.3% Triton X-100, complete protease inhibitors, 5 mM ATP, 1 mM GTPγS, 1 mM dithiothreitol), incubated on a rotating wheel at 4°C for 15 min, and centrifuged. For preclearing, 10 μl of a slurry of prewashed G Sepharose beads (G Sepharose 4B fast flow beads [Sigma-Aldrich]) were added to the lysate and incubated 30 min at 4°C on a rotating wheel and centrifuged. The supernatant was mixed with 6 μl of anti-GFP antibody and incubated 3 h on a rotating wheel at 4°C. Afterward, 30 μl of a slurry of G Sepharose beads were added, followed by a 5-h incubation on a rotating wheel at 4°C. Beads were washed twice with 500 μl of lysis buffer with Triton X-100 and three times with 500 μl of lysis buffer without Triton X-100 and taken up in 60 μl of SDS sample buffer for analysis on SDS–PAGE. As a negative control either a cell line expressing solely GFP or no GFP was used.

Protocol B. A total of 1 × 10⁷ cells were exposed for 30 min to medium diluted 1:2 with water, lysed in 600 μl of lysis buffer (see earlier discussion), incubated on a rotating wheel at 4°C for 15 min, and centrifuged. The lysate was incubated with 25 μl of GFP-Trap-A (ChromoTek) for 2 h on a rotating wheel at 4°C. Beads were washed as described and taken up in 60 μl of SDS sample buffer for analysis on SDS–PAGE.

Silver stain and protein analysis by mass spectrometry analysis
Gel silver staining and protein analysis by mass spectrometry were performed exactly as described (Gotthardt et al., 2006).

Analysis of mass spectrometry data
To analyze the raw mass spectrometry data, the following steps were undertaken. First, all the proteins that were identified in any lane, as well as in the negative control lanes, were ignored. Second, we used a stringent cutoff for identification and ignored all proteins falling under a Mascot score of 100, with the exception of proteins identified in multiple lanes and/or in the two screens, as, for example, ubqC or proteins that belong to complexes (exocyst, proteasome, and V-ATPase) and at least one subunit was above the threshold. These hits were, in screen 1, ap3b-1, nek2, nek3, exoc5, exoc7, vatD, vatM, vatE, psmD1, psmC4, psmC6, rac1A, ubqC, and dymB, and, in screen 2, psmC2, psmC4, psmC5, vatB, vatC, nek3, copB, and ubqC.

Immunoblots
Immunoblots were performed exactly as described (Gotthardt et al., 2006).

Vector construction
For construction of the GFP and mRFP fusion proteins the full-length cDNAs were cloned into the vector pGEM T easy, sequenced, and then subcloned in the respective vectors, pDXA-GFP (Diekmann et al., 2010) or mRFPmCas (Fischer et al., 2004). The DD domain is a protein destabilization domain originally from the FK506- and rapamycin-binding protein (FKBP12). Fused to a protein of interest, it leads to immediate degradation of the fusion during or after translation. By addition of the compound Shield-1 the conformation of the DD domain is stabilized and protected from degradation (Banaszynski et al., 2006). For construction of the DD-GFP-Rab8a vectors, the DD
domain was amplified by PCR from a plasmid (Herm-Gotz et al., 2007) and cloned 5’ of the GFP sequence into pDXA-GFP. Then the sequences of Rab8a WT, CA, and DN (kindly provided by L. Temesvari, Clemson University; Powell and Temesvari, 2004) were inserted in-frame into the plasmid pDXA-DD-GFP.

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