Regulation of contractile vacuole formation and activity in *Dictyostelium*

Fei Du¹, Kimberly Edwards^{1,3},
Zhouxin Shen¹, Binggang Sun^{1,4},
Arturo De Lozanne², Steven Briggs¹
and Richard A Firtel^{1,*}

¹Section of Cell and Developmental Biology, Division of Biological Sciences, University of California San Diego, La Jolla, CA, USA and

²Section of Molecular Cell & Developmental Biology, Institute for Cellular and Molecular Biology, University of Texas at Austin, Austin, TX, USA

The contractile vacuole (CV) system is the osmoregulatory organelle required for survival for many free-living cells under hypotonic conditions. We identified a new CV regulator, Disgorgin, a TBC-domain-containing protein, which translocates to the CV membrane at the late stage of CV charging and regulates CV–plasma membrane fusion and discharging. *disgorgin*[−] cells produce large CVs due to impaired CV–plasma membrane fusion. Disgorgin is a specific GAP for Rab8A-GTP, which also localizes to the CV and whose hydrolysis is required for discharging. We demonstrate that Drainin, a previously identified TBC-domain-containing protein, lies upstream from Disgorgin in this pathway. Unlike Disgorgin, Drainin lacks GAP activity but functions as a Rab11A effector. The BEACH family proteins LvsA and LvsD were identified in a suppressor/enhancer screen of the *disgorgin*[−] large CV phenotype and demonstrated to have distinct functions in regulating CV formation. Our studies help define the pathways controlling CV function.

The EMBO Journal (2008) 27, 2064–2076. doi:10.1038/emboj.2008.131; Published online 17 July 2008

Subject Categories: membranes & transport; cell & tissue architecture

Keywords: BEACH proteins; contractile vacuole; *Dictyostelium*; Rab; RabGAP

Introduction

The contractile vacuole (CV) complex is an osmoregulatory organelle of free-living amoebae and protozoa, which controls the intracellular water balance by accumulating and expelling excess water out of the cell, allowing cells to survive under hypotonic stress as in pond water. In the absence of a functional CV complex, cells cannot expel

water, become highly swollen, and lyse. Significant work has been carried out to characterize the properties and function of the CV system. In *Dictyostelium*, the CV system consists of tubules and vacuoles (or bladders) that are interconvertible (Gerisch *et al*, 2002). The tubular structures function as collecting ducts to accumulate excess water, whereas the bladders fuse with the plasma membrane enabling the bladder's contents to be released into the extracellular medium thus expelling water from the cell body (Heuser *et al*, 1993). When cells are in isotonic medium, the CV system exhibits limited activity, but when cells are placed in hypotonic medium, the CV system is rapidly activated: the bladders fill with water and then fuse to the plasma membrane, discharging their contents (Heuser *et al*, 1993; Gabriel *et al*, 1999). Heuser (2006) has suggested that the CV does not disappear during the discharge stage but, instead, collapses, and flattens against the plasma membrane, maintaining its distinct membrane components.

Although several putative regulatory components have been demonstrated to associate with the *Dictyostelium* CV system, including vacuolar-H⁺-ATPase (V-ATPase), LvsA, Drainin, Rab11A, and Rab14, little is known about the regulation of the CV system and the pathways that control CV discharging (Heuser *et al*, 1993; Bush *et al*, 1996; Becker *et al*, 1999; Harris *et al*, 2001; Gerald *et al*, 2002; Wu *et al*, 2004). V-ATPase is a conserved enzyme in all eukaryotic cells that transforms the energy generated from ATP hydrolysis to active transport H⁺. This transmembrane electrochemical potential of H⁺ is then used to transport other ions or macromolecules (Beyenbach and Wiczeorek, 2006). Heuser *et al* (1993) has proposed that the *Dictyostelium* V-ATPase establishes a proton gradient to transport other ions into CVs, resulting in an inward flux of water. The BEACH protein LvsA is required for cytokinesis and phagocytosis and for proper water homeostasis (De Lozanne, 2003). LvsA translocates to the CV membrane after the vacuole reaches its maximum diameter and has been proposed to regulate CV biogenesis and discharge (De Lozanne, 2003). Lastly, Becker *et al* (1999) demonstrated that Drainin, a TBC domain (RabGAP-domain)-containing protein, is required for proper CV discharge and localizes to CV bladders. *drainin*[−] cells exhibit enlarged CV bladders and are partially sensitive to low osmotic stress. Although Drainin has been proposed to be a potential RabGAP (Gerisch *et al*, 2002), no Rab has been linked to the function of Drainin.

Rab GTPases belong to the Ras superfamily of small GTPase proteins, having central functions in regulating membrane trafficking, including vesicle transport, formation, movement, tethering, and fusion with target membranes (Jordens *et al*, 2005; Grosshans *et al*, 2006b). In total, 16 of the 54 *Dictyostelium* Rabs have been examined, and 2, Rab11A and Rab14, have been linked to the CV (Weeks *et al*, 2005). Rab11A localizes to both CV bladder and tubular structures and cells expressing dominant-negative Rab11A exhibit abnormal, enlarged CV bladders in hypotonic buffer and are sensitive to low osmolarity stress (Harris *et al*, 2001).

*Corresponding author. Section of Cell and Developmental Biology, Division of Biological Sciences, University of California San Diego, Natural Sciences Building Room 6316, 9500 Gilman Drive, La Jolla, CA 92093-0380, USA. Tel.: +1 858 534 2788; Fax: +1 858 822 5900; E-mail: rafirtel@ucsd.edu

³Present address: Department of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA

⁴Present address: Johnson & Johnson Pharmaceutical Research and Development, San Diego, CA 92121, USA

Received: 24 February 2008; accepted: 16 June 2008; published online: 17 July 2008

Rab14 localizes to the endo-lysosomal and the CV systems. Cells expressing dominant-negative Rab14 are defective in endocytosis, endosomal membrane flow, homotypic lysosome fusion, and hypo-osmotic regulation (Weeks *et al*, 2005).

Similar to other small GTPases, Rabs cycle between GDP-bound (inactive) and GTP-bound (active) states. The hydrolysis of GTP is stimulated by GTPase-activating proteins (RabGAPs), most of which contain a conserved catalytic TBC (Tre/Bub2/Cdc16) domain (Bernards, 2003). While examining the regulation of *Dictyostelium* CV function, we identified a novel Rab8A-GAP, Disgorgin, that along with Drainin and its regulator Rab11A control CV discharge. Drainin and Disgorgin/Rab8A sequentially localize to the CV membrane at the late charging stage and control different stages in the process. We show that two BEACH family proteins, LvsA and LvsD, exert an effect as a suppressor and an enhancer of *disgorgin*[−] phenotypes, respectively, to regulate CV formation. Through the examination of the cellular phenotypes of different mutant strains and the genetic and biochemical interactions of the different CV components, we provide new insights into the pathways regulating CV function and formation.

Results

Loss of Disgorgin causes the formation of large vacuoles

In a genetic screen for cell morphology mutants, we identified a new CV regulator, which we named Disgorgin (DDB0218275, *Dictybase*). Disgorgin contains an F-box domain near its N-terminus and a TBC (RabGAP) domain with the conserved catalytic Arg and Gln residues required for GAP activity (Figure 1A and B; Pan *et al*, 2006). *disgorgin* knock-out strains, generated by homologous recombination and confirmed by Southern and Northern blot analyses (Supplementary Figure S1A and B), exhibit a large vacuole morphology (up to 7 µm) that is easily observed under phase contrast or DIC microscopy, whereas no vacuole is observed in most wild-type cells (strain Ax2; Figure 1C and Supplementary Figure S2A).

Expressing full-length Disgorgin or Disgorgin lacking the F-box (Disgorgin^{ΔF-box}; Figure 1A) in *disgorgin*[−] cells complements the large vacuole phenotype and causes no over-expression phenotype when expressed in wild-type cells (Figure 1D, data not shown). However, Disgorgin carrying amino-acid substitutions in either of the conserved residues required for GAP activity (Disgorgin^{R515A}; Disgorgin^{Q551A}) does not complement the null phenotypes and produces even larger vacuoles when expressed in *disgorgin*[−] or wild-type cells (up to 11 µm; Figure 1D and Supplementary Figure S2A), suggesting that the mutant proteins exert an effect as dominant-negative mutants, possibly by further blocking the Rab intrinsic GTPase activity and/or competing for common substrates or essential components of the pathway. These findings indicate that Disgorgin RabGAP activity is required in the pathway regulating vacuoles and that the F-box domain is not essential for this process.

Structure and activity of the CV is abnormal in *disgorgin*[−] cells

To identify the properties of the large vacuoles, we labelled *disgorgin*[−] cells with markers for different types of organelles:

TRITC-dextran (endosomes), LysoTracker (lysosomes), or RFP-Dajumin (CV system) (Gabriel *et al*, 1999; Insall *et al*, 2001). RFP-Dajumin, but not the other markers, clearly labelled the large vacuole structures corresponding to the ones observed under phase-contrast microscopy, indicating that the large vacuoles in *disgorgin*[−] cells are enlarged CVs (Figure 2A and Supplementary Movie S1). Interestingly, when we placed *disgorgin*[−] cells in low-salt buffer, the large vacuoles were no longer present; instead, we observed many smaller bladder structures (Figure 2A and Supplementary Movie S1), suggesting that the CV activity dramatically changed in *disgorgin*[−] cells under hypotonic stress. On the other hand, tonicity of the medium did not affect the CV structures in wild-type cells (Figure 2A and Supplementary Movie S1).

To understand the role of Disgorgin, we compared the CV cycle in wild-type and *disgorgin*[−] cells by labelling cells with RFP-Dajumin and placing the cells in water to activate the CV system. As previously described (Gabriel *et al*, 1999; Heuser, 2006), when cells are placed in hypotonic conditions, the activity of the CV network dramatically increases and the dynamic charging and discharging of the vesicles can be readily monitored (Figure 2B). After vacuole discharging, RFP-Dajumin is visible as a patch on the plasma membrane, suggesting that the CV membrane is flattened against the plasma membranes. Furthermore, new CV bladders preferentially form at these sites, suggesting that the collapsed CV bladder remains distinct from the majority of the plasma membrane whereupon it regenerates into a new CV (Figure 2B) (Heuser, 2006). To examine this in more detail, we used the styryl dye FM4-64, which labels both the plasma membrane and CVs (Heuser *et al*, 1993). In wild-type cells, we observed the complete collapse of the CV against the plasma membrane. Upon collapse, this region of the plasma membrane appears as a brighter domain compared with the surrounding region, suggesting a double layer of membrane (Figure 2C).

When placed in water, the large CVs in *disgorgin*[−] cells disappeared within 5 min by discharging their contents (Supplementary Movie S2). After the initial discharging, the large vacuoles did not reform. Instead, we observed numerous smaller vacuoles (Supplementary Movie S3), consistent with the RFP-Dajumin labelling (Figure 2A). These smaller vacuoles discharged through an abnormal process in which the CVs round up and push out of the membrane forming a bleb (Supplementary Movie S3). Then these vacuoles discharged, but the process was extended and incomplete with no observed flattening of the CV against the plasma membrane (Figure 2B and Supplementary Movie S3). At the end of this process, residual, small CV bladders remained, which then withdrew inside the cell (Supplementary Movie S3), a process not observed in wild-type cells. Using FM4-64 to visualize the discharging of the CVs, we clearly observed the membrane protrusion caused by charged CVs in *disgorgin*[−] cells. In contrast to wild-type cells, we did not observe the collapse of the CVs onto the plasma membrane. Instead, the partially discharged CVs withdrew inside the cells (Figure 2C).

Given the strong discharging phenotype we observed in *disgorgin*[−] cells, we suggest that Disgorgin mediates CV discharging by regulating efficient fusion between the CV and plasma membranes. We hypothesize that the mechanism underlying the formation of large vacuoles in *disgorgin*[−] cells in isotonic medium is that the vacuoles continue to grow (and possibly fuse), as they are unable to fuse with the plasma membrane.

Figure 1 Sequence analysis of *Dictyostelium* Disgorgin and cell morphology of *disgorgin*⁻ cells. (A) Schematic diagram of the domain structure of Disgorgin and the relative position of the truncation constructs used in the study. (B) Amino-acid sequence alignment of the TBC domain of Disgorgin (DDB0218275, *D. discoideum*); Drainin (AAD00520, *D. discoideum*); TRE2 (P35125, *Homo sapiens*); RN-tre (Q92738, *H. sapiens*); Bub2 (P26448, *Saccharomyces cerevisiae*); Cdc16 (CAA50606, *Schizosaccharomyces pombe*). The two conserved catalytic arginines and glutamines for GAP activity are marked with asterisks. The red box shows the hydrophobic stretch. (C) The cell morphology of wild-type Ax2 and *disgorgin*⁻ cells. Large vacuoles are indicated by open arrowheads. (D) The cell morphology of different strains. Cells expressing exogenous proteins are indicated by arrows. Scale bars, 10 μm. A full-colour version of this figure is available at *The EMBO Journal* Online.

Disgorgin localizes to the CV membrane at the late charging stage

To determine the subcellular localization of endogenous Disgorgin, we created a Disgorgin knock-in (*disgorgin*^{KI})

strain in which a v5 tag is inserted upstream of the stop codon of the endogenous *disgorgin* gene by homologous recombination. By indirect immunofluorescence staining with anti-v5 and antibodies against the CV markers

Figure 2 Structure and activity of CVs are abnormal in *disgorgin*⁻ cells. **(A)** CV structure in Ax2 cells and *disgorgin*⁻ cells in isotonic buffer or in hypotonic buffer. Localization of RFP-Dajumin represents a reconstruction of the 3D structure. **(B)** Time course of localization of RFP-Dajumin in Ax2 and *disgorgin*⁻ cells in water. Solid arrows indicate discharge events. Open arrowheads indicate a full charge and discharge cycle. **(C)** Time course of CV discharging in cells labelled with FM4-64. Arrows indicate the complete collapse of CV in Ax2 cells. The open arrowheads indicate CVs undergoing discharge. Scale bars, 5 μ m.

V-ATPase or calmodulin (Gerald *et al*, 2002), we found Disgorgin localized to the CVs as well as being found in the cytosol (Figure 3A and data not shown). However, Disgorgin localized only to the vacuoles and not to the tubule components of the CV, and, interestingly, Disgorgin marked a subset of the CV vacuoles (Figure 3A). Cell fractionation assays suggested that only 5% of the endogenous Disgorgin was

associated with membranes (Supplementary Figure S1C). GFP-Disgorgin, which complements the *disgorgin*⁻ phenotypes, exhibited a similar subcellular localization as endogenous Disgorgin and did not colocalize with either lysosomes or endosomes (Figures 3B and Supplementary Figure S1D). Furthermore, Disgorgin overexpression did not change the localization of the V-ATPase as determined using

the subcellular fractionation assay, suggesting that Disgorgin overexpression did not alter the CV system (Supplementary Figure S1C). We therefore used GFP–Disgorgin for our live-imaging studies. Under hypertonic conditions, GFP–Disgorgin localized to shrunken CVs (data not shown).

To understand the differences between the CVs with and without Disgorgin, we placed the cells co-expressing GFP–Disgorgin and RFP–Dajumin in water. RFP–Dajumin marked all of the vacuoles and tubules evenly, and we observed the charging and discharging of these CVs (Figure 3C). However, we found that Disgorgin localized to the membrane of CVs only at the late charging stage. Coincident with the recruitment of additional Disgorgin, the CV stopped growing, became spherical, and initiated its discharge (Figure 3C). We found that, in contrast to Dajumin, which remained on the CV patch, Disgorgin dissociated from the membrane immediately after vacuole fusion (Figure 3C; 60, 72, 120, and 132 s time points). The kinetics of Disgorgin association with CVs are consistent with Disgorgin's potential role in regulating CV–plasma membrane fusion.

To determine the domains required for Disgorgin's vacuolar localization, we made a series of deletion mutants (Figure 1A). GFP–Disgorgin^{ΔF-box}, GFP–Disgorgin^{Q551A}, and GFP–Disgorgin^{R515A} showed a similar pattern of localization as wild-type Disgorgin (data not shown), suggesting that the F-box domain and the catalytic residues are not required for CV membrane localization. We found that the TBC domain with its 70 upstream residues (construct C^{382–717}; Figure 1A) but not the TBC domain alone complements the *disgorgin*[–] cell large vacuole phenotype and localizes not only to cytosol but also weakly to the CVs and the plasma membrane (Figure 3D and E), suggesting that this 70 (residues 382–452)-residue region upstream of TBC domain, which is Arg and Lys rich, is required for membrane localization. Sequence analysis suggests that Drainin, a previously identified CV association protein in *Dictyostelium*, and several human RabGAPs have a similar Arg/Lys-enriched region upstream of their TBC domains, indicating this may be a conserved localization motif (Supplementary Figure S3).

We also identified a nine-residue hydrophobic stretch (LAAVFLIL) within the TBC domain (Figure 1A and B) that is required for Disgorgin CV localization. Disgorgin lacking these nine residues (Disgorgin^{Δ9}) is cytosolic, does not rescue the *disgorgin*[–] cell vacuole phenotype, and produces large vacuoles when expressed in wild-type cells (Figure 3D and E). A similar hydrophobic region was found previously in Drainin, and shown to be required for Drainin vacuole localization (Becker *et al*, 1999). Further sequence comparison indicates that the TBC domains of RabGAPs from yeast and mammals also have at least five hydrophobic residues in this region, I/L/VXXXF/I/LLL/M/YXL/M/C (Figure 1B). We suggest that this hydrophobic domain and the Arg/Lys-enriched domain are conserved membrane localization motifs that mediate vacuole targeting.

Drainin functions upstream of Disgorgin

Disgorgin shares some similarities with Drainin, another regulator of CV discharging. They both contain TBC domains and ablating either protein causes enlarged CV formation. However, the cell morphologies and the discharge of the CVs are different in the two null strains. The CVs are less enlarged and the vacuole sizes are more uniform in *disgorgin*[–] cells

than in *drainin*[–] cells (Figure 4A and Supplementary Figure S2A). *drainin*[–] cells are partially hypo-osmotic sensitive and exhibit two types of abnormal discharge, one of which is similar to that seen in *disgorgin*[–] cells (vacuole forms a bleb) (Becker *et al*, 1999). In *disgorgin*[–] cells, although active CV–plasma membrane fusion is absent as described above, cells can discharge and are insensitive to hypotonic stress (Figure 2C; data not shown).

To investigate the relationship between these two proteins, we performed an epistasis experiment. Overexpressing Disgorgin suppressed the large CV phenotype in *drainin*[–] cells (Figure 4A and Supplementary Figure S2A). Although the discharging was still abnormal, the *disgorgin*[–]-type of bladder discharging was significantly reduced (data not shown), suggesting Disgorgin is involved in one of the Drainin pathways. Overexpressing Drainin in *disgorgin*[–] cells, however, did not suppress the *disgorgin*[–] large CV phenotype and the discharging was unaltered (Figure 4A and Supplementary Figure S2A; data not shown), suggesting that Disgorgin might lie downstream of Drainin. We also found that Disgorgin still localized to CV bladders in *drainin*[–] cells and vice versa, indicating that the two proteins localize to the CVs independently (data not shown). Time-lapse video microscopy of CV formation and discharge in wild-type cells co-expressing GFP–Disgorgin and RFP–Drainin showed that Drainin localized to the CV bladder prior to Disgorgin under hypotonic conditions (Figure 4B). This result and the complementation data strongly suggest that Drainin lies upstream from Disgorgin in the CV discharge pathway. Consistent with this model, *disgorgin*[–]/*drainin*[–] cells exhibited cell morphologies and discharging defects similar to those observed in *drainin*[–] cells (data not shown).

Rabs involved in the Disgorgin and Drainin pathway

As RabGAP activity is required for normal vacuolar size in the Disgorgin pathway, we expect that some *disgorgin*[–] cell phenotypes result from specific Rab GTPases remaining in the GTP-bound form for an extended time. Both Rab11A and Rab14 were shown previously to localize to CV structures in *Dictyostelium*. Rab11A localizes to both tubules and bladders, whereas Rab14 only localizes to the tubular structures (Harris *et al*, 2001; Weeks *et al*, 2005). As expected, we discovered that Rab11A colocalizes with Disgorgin and, similar to Dajumin, is found constitutively on both CV tubules and bladders (Figure 5A). We also examined nine other Rabs (Rab5A, Rab5B, Rab6A, Rab7A, Rab7B, Rab8A, Rab11C, Rab21, and Rab32), all of which had been linked to vacuolar structures in *Dictyostelium* and/or other systems (Weeks *et al*, 2005; Heo *et al*, 2006). Of these nine Rabs, two, Rab8A and Rab11C, localize to CV structures. Rab8A exhibits the same localization as Disgorgin and, to the limit of the resolution of our studies, localizes coincidentally with Disgorgin on the CVs during CV-charging and -discharging cycles, whereas Rab11C exhibits localization similar to that of Rab11A, being found on all CV structures (Figure 5A and Supplementary Movie S4; data not shown).

To determine whether Rab8A, Rab11A, Rab11C, or Rab14 are the potential regulators or effectors of Disgorgin or Drainin, we performed a pull-down assay using GST-tagged Rabs and GST alone as a negative control. Figure 5B illustrates that Rab8A and Rab11A, but not Rab11C or Rab14 or GST alone, pulled down v5-tagged Disgorgin. However,

Figure 3 Disgorgin localizes to the CV membrane. (A) Localization of endogenous Disgorgin and V-ATPase (VatM). Colocalization of Disgorgin and V-ATPase is indicated by arrows. (B) Localization of GFP-Disgorgin with RFP-Dajumin in Ax2 cells. Colocalization of Disgorgin and Dajumin is indicated by arrows. (C) Localization of RFP-Dajumin (left panel) or GFP-Disgorgin (right panel) in Ax2 cells under hypotonic stress. Pictures were taken simultaneously for both colours. Solid arrows indicate a discharge event and open arrows indicate a full charge and discharge cycle. (D) Cell morphology of *disgorgin*⁻ cells or Ax2 cells expressing truncated Disgorgin. Arrows indicate enlarged CVs. (E) Localization of truncated Disgorgin in *disgorgin*⁻ cells or Ax2 cells and FM4-64 in GFP-C³⁸²⁻⁷¹⁷/*disgorgin*⁻ cells. To illustrate the localization better, additional image of GFP-C³⁸²⁻⁷¹⁷/*disgorgin*⁻ is shown. Arrows indicate the vacuoles to which GFP-C³⁸²⁻⁷¹⁷ and FM4-64 localize. Scale bars, 5 μm.

Figure 4 Drainin lies upstream of Disgorgin. (A) Cell morphology of different strains. In GFP-Drainin/*disgorgin*⁻ cells, the cells expressing GFP-Drainin are indicated by arrows. (B) Localization of GFP-Disgorgin and RFP-Drainin in Ax2 cells under hypotonic stress. Arrowheads indicate the time frames of Disgorgin and Drainin association with CV bladders during two CV-charging and discharging cycles. Pictures were taken simultaneously for both colours. Scale bars, 5 μ m.

Rab11A and Rab14 bound strongly with Drainin (Figure 5B). Using GST-fused constitutively active (Rab^{CA}) or dominant-negative (Rab^{DN}) Rabs in pull-down assays, we examined the GTP or GDP specificity of the interactions. We found that Drainin bound the strongest to Rab11A^{CA} and had little interaction with Rab11A^{DN}. However, Drainin bound equally to all three forms of Rab14. These data suggested that Rab11A but not Rab14 interacted with Drainin in a GTP-dependent manner (Figure 5C). Disgorgin bound both the Rab8A^{CA} and Rab8A^{DN} more strongly than the wild-type form of Rab8A (Figure 5C). Surprisingly, Disgorgin bound the strongest to Rab11A^{DN} (Figure 5C).

To determine whether Disgorgin is the *bona fide* GAP for any of the Rabs, we performed a Rab GTPase assay. As shown in Figure 5D, Disgorgin exhibited strong GAP activity to the Rab8A, and much less GAP activity to Rab11A, Rab11C, and Rab14, suggesting that Disgorgin is a specific RabGAP for Rab8A. Our sequence analysis suggests that Drainin lacks the putative catalytic residues (Figure 1B). Consistent with this observation, Drainin did not exhibit GAP activity on any of the Rabs tested (Figure 5D), suggesting Drainin is not a *bona fide* RabGAP.

Although Disgorgin is a Rab8A-GAP, overexpression of Rab8A in *disgorgin*⁻ cells suppresses the steady-state accumulation of large vacuoles (i.e. large vacuoles do not accumulate; data not shown), possibly by supplying a sufficient level of Rab8A-GDP to the CV system that might compete with Rab8A-GTP for sites on CV membranes. However, Rab8A does not suppress the abnormal discharge of CV in *disgorgin*⁻ cells (data not shown), indicating that the transition from the GTP-bound to the GDP-bound form of Rab8A is

important to fulfill its function in CV-plasma membrane fusion.

As Drainin interacts with Rab11A in a GTP-dependent manner and Drainin is not a RabGAP for Rab11A, we suggest that Drainin is a Rab11A effector. When we overexpressed GFP-Drainin alone, we observed Drainin vacuolar but not tubular localization (Figure 5E). However, when co-overexpressed with Rab11A, Drainin localized to the CV tubules as well (Figure 5E). Interestingly, co-expressing Drainin with Rab11A^{CA} led to the plasma membrane localization of Drainin, possibly due to active fusion between the CV and the plasma membrane. Co-expression of RFP-Rab11A^{DN}, which was found only in the cytosol, blocked Drainin localization to the CV or other membranes (Figure 5E). Together with the pull-down assay and GAP assay data, these data suggest that Drainin is the effector of Rab11A and that Rab11A recruits Drainin to the CV membrane. We suggest that Rab11A^{DN} blocks Rab11A activation and, in return, prevents Drainin CV localization. Consistent with this conclusion, expressing Rab11A did not rescue the large CV phenotype in *drainin*⁻ cells (data not shown), whereas Rab11A^{DN} expression mimicked a *drainin*⁻ cell phenotype (Harris *et al*, 2001). Rab8A, however, suppressed the large CV phenotype in *drainin*⁻ cells (data not shown), further supporting the hypothesis that Rab8A and Disgorgin lie downstream of Drainin pathway.

REMI suppressor/enhancer screening of *disgorgin*⁻ cells

To identify other potential components in the Disgorgin pathway, we undertook an insertional mutagenesis (REMI) screen for second site suppressors and enhancers of the

Figure 5 The Rabs regulated by Disgorgin and Drainin. (A) Colocalization of GFP-Disgorgin with RFP-Rab11A, RFP-Rab11C, and RFP-Rab8A. Arrows indicate the colocalization of Disgorgin and Rab11A or Rab11C. Scale bar, 5 μ m. (B, C) GST pull-down assay of Disgorgin and Drainin. Purified glutathione beads conjugated with GST, GST-Rab8A, GST-Rab11A, GST-Rab11C, and GST-Rab14 (B), GST-Rab^{CA}, or GST-Rab^{DN} (C) were incubated with a cell lysate expressing v5-Disgorgin or v5-Drainin. The products were probed with an anti-v5 antibody. Inputs are indicated in the two bottom panels. (D) Kinetics of GTP hydrolysis for Rab8A, Rab11A, Rab11C, and Rab14 in the presence and absence of 250 nM GST-C³⁸²⁻⁷¹⁷ of Disgorgin or GST-Drainin (see Materials and methods). Results are the means for three independent experiments. (E) Localization of GFP-Drainin with or without Rab11A, or Rab11A^{CA}, or Rab11A^{DN} in Ax2. Scale bar, 5 μ m. Vacuolar structures are indicated by arrows.

disgorgin[−] cell vacuolar phenotype by identifying strains exhibiting a change in vacuolar size. Visual screening of ~7000 clones yielded five candidates with a changed vacuolar morphology. After cloning the insertion site of the suppressor and enhancer clones, we found that *lvsA* (DDB0191124) had been disrupted in the strain that exhibited no vacuoles and *lvsD* (DDB0185108) was disrupted in the two strains exhibiting larger vacuoles. We were unable to clone the insertion site of the other two strains. To confirm the REMI clone phenotypes, we disrupted *lvsA* and *lvsD* in *disgorgin*[−] cells. As shown in Figure 6A, disruption of *lvsA* in *disgorgin*[−] cells suppressed the large vacuole phenotype of *disgorgin*[−] cells, whereas the phenotype of *disgorgin*[−] cells was enhanced when *lvsD* was disrupted (Figure 6A and Supplementary Figure S2A).

LvsA and LvsD are BEACH family proteins, which contain an evolutionarily conserved domain and are involved in a range of cellular processes (De Lozanne, 2003). We confirmed the results of De Lozanne, 2003 that disruption of *lvsA* causes a loss of visible CVs and tubules, whereas loss of *lvsD* (in a wild-type background) does not produce a visible phenotype (Figure 6A and B). An *LvsA* gene replacement strain in which the endogenous *LvsA* open reading frame fused to GFP is overexpressed from a more active actin promoter (Gerald *et al*, 2002) does not exhibit a vacuolar phenotype. Interestingly, disruption of Disgorgin in this background (*LvsA*^{OE}/*disgorgin*[−]) causes an enhanced, enlarged vacuole phenotype, similar to observations in cells lacking Disgorgin and LvsD (*lvsD*[−]/*disgorgin*[−]; Figure 6A and Supplementary Figure S2A). Overexpressing GFP-LvsD in *disgorgin*[−] cells, similar to disrupting *lvsA*, suppresses the large vacuole phenotype of *disgorgin*[−] cells (Figure 6A). These results indicate that LvsA and LvsD regulate the CV system but have distinct and possibly opposite functions.

LvsA maintains the integrity of the CV

Using RFP-Dajumin to visualize the CV, we compared the CV structures in different mutant strains. We confirmed that no bladder or tubular structures were observed in *lvsA*[−] cells; only small punctate structures were seen, suggesting that a functional CV system was absent (Gerald *et al*, 2002). *lvsA*[−]/*disgorgin*[−] cells exhibit a similar phenotype which explains how disrupting *lvsA* suppresses the large vacuole phenotype in *disgorgin*[−] cells (Figure 6B). V-ATPase, which predominantly localizes to CVs, still localizes to these punctate structures, suggesting that in the absence of LvsA, immature CV structures form but cannot mature or enlarge (Gerald *et al*, 2002). Thus, *lvsA*[−]/*lvsD*[−] and *lvsA*[−]/*lvsD*[−]/*disgorgin*[−] cells lack enlarged bladders and exhibit the punctate CV structures (data not shown). Furthermore, these two strains exhibit all of the phenotypes of *lvsA*[−] cells, including sensitivity to hypotonic stress as well as phagocytosis and cytokinesis defects (data not shown) (Kwak *et al*, 1999; Gerald *et al*, 2002).

As visualized using RFP-Dajumin, there is an increase in the number of CV bladder structures in *LvsA*^{OE} cells compared with wild-type cells (Figure 6B and Supplementary Figure S2B). Thus, the combination of overexpressing LvsA and disrupting *disgorgin* (*LvsA*^{OE}/*disgorgin*[−] cells) leads to extended enlargement of CVs (Figures 6B). In *lvsD*[−] cells, the CV bladder size is comparable to wild-type cells (Figure 6B). However, in *lvsD*[−]/*disgorgin*[−] cells, the CV bladder is greatly

enlarged (Figure 6B). Overexpression of LvsD in wild-type cells does not result in any observed change in the CV structure (Figure 6B).

In *lvsA*[−] cells, Disgorgin localized to punctate structures adjacent to the plasma membrane, which colocalized with RFP-Dajumin, indicating that these are CV structures (Figure 6C; data not shown). As Disgorgin only localized to the CV at the late charging stage, it suggests that the aberrant CV structures in *lvsA*[−] cells are remnants of CVs that stopped growing after the discharging. We suggest that LvsA functions to maintain the integrity of the CV during the discharging stage. As previous reports showed that LvsA translocates to the CV membrane after the vacuole reaches its maximum diameter (De Lozanne, 2003), we examined the detailed kinetics of the LvsA association with the CV membrane to better understand LvsA's function. Time-lapse video microscopy of cells co-expressed GFP-LvsA and RFP-Dajumin revealed that GFP-LvsA translocated to the CV membrane only at the very last stage of discharge immediately prior to the flattening of the CV bladders against the plasma membrane (Supplementary Movie S5). The period during which LvsA was associated with CVs was brief (18 ± 5 s ($n = 30$ cells)) in contrast to the 57 ± 18 s ($n = 30$ cells) between the time of Disgorgin localization to CVs and the complete discharging of the vacuole. The full cycle from the start of CV growth to discharging is $\sim 100 \pm 22$ s ($n = 30$ cells). The kinetics of LvsA association with CV membrane are consistent with a role for LvsA in maintaining the membrane integrity during the fusion process. This CV membrane localization of LvsA is Disgorgin independent (Figure 6C). Surprisingly, LvsD did not localize to CV membranes and was always cytosolic in all cell lines tested, whether the cells were in isotonic or hypotonic medium (data not shown).

To identify possible regulators and effectors of Disgorgin, we purified a Disgorgin-containing complex and analysed it by mass spectrometry (Supplementary Figure S4A). None of the proteins discussed above was identified in the Disgorgin complex. We found SKP1 orthologues FpaA and FpaB, the components of the SCF ubiquitination complex, ubiquitin, and the vacuolar H⁺-ATPase A subunit VatA. Co-immunoprecipitation experiments confirmed the *in vivo* interaction of Disgorgin, FpaA, and FpaB in an F-box domain-dependent manner (Supplementary Figure S4B). These findings are discussed in the Discussion and Supplementary data.

Discussion

A model for CV regulation

In this study, we report four new regulators of the CV in *Dictyostelium*, Disgorgin, Rab8A, Rab11C, and LvsD. By the analysis of these and three previously described CV proteins, Drainin, Rab11A, and LvsA, our results revealed a new pathway for the control of CV function in *Dictyostelium* (Figure 7A and B). On the basis of our findings, we propose that upon hypotonic stress, Rab11A is activated on the CV bladders and, in a GTP-bound form, recruits Drainin to the CV bladders. We discovered that Disgorgin colocalizes with Rab8A and exerts an effect as a Rab8A GAP in a pathway that lies downstream of Drainin. Our finding that Disgorgin GAP activity is required for Disgorgin function argues that the hydrolysis of Rab8A-GTP is required to mediate CV-plasma membrane fusion, possibly by releasing a Rab8A effector, maybe Disgorgin

Figure 6 *LvsA* maintains the integrity of CVs. (A) Cell morphology of different strains. (B) Localization of RFP-Dajumin in different cell lines. Each picture represents a reconstruction of the 3D structure. (C) Localization of GFP-Disgorgin in *Ax2* and *lvsA*⁻ cells and GFP-*LvsA* in *disgorgin*⁻ cells. Scale bars, 10 μm.

itself. As the interaction between Disgorgin and Rab11A^{DN} is much stronger than the one between Disgorgin and other forms of Rab11A or Rab8A, we postulate that there may be a feedback pathway between Disgorgin and Rab11A (Figure 7A).

By examining the kinetics of different proteins' association with the CV system, we assigned different CV proteins along the CV-charging and -discharging cycles, which help define CV regulation (Figure 7B). Under isotonic conditions, the CV is not very active and Disgorgin, *LvsA*, *LvsD*, Rab8A, and Drainin are predominantly cytosolic, whereas Dajumin, Rab11A, and Rab11C are associated with the CV tubular and vacuolar structures. In response to hypotonic stress, the CVs charge and enlarge by water uptake. Late in this process Drainin is recruited to the CV membrane. We propose this is mediated by Rab11A-GTP, as this does not occur in cells expressing Rab11A^{DN}. Drainin recruitment is followed, contemporaneously, by Disgorgin and Rab8A recruitment. Once Disgorgin and Rab8A are associated with the CV membrane, the vesicles stop charging and become spherical. Shortly before CV-plasma membrane fusion, *LvsA* is recruited to CV whereupon the CV bladder and plasma

membranes fuse forming a hole, which allows the CV contents to be released into the extracellular medium. Rab8A, Disgorgin, Drainin, and *LvsA* dissociate from the plasma membrane, whereas Dajumin, Rab11A, and Rab11C remain on the CV patches, which become re-filled with water, and the next charging begins.

The regulation of CV activity by Disgorgin and Drainin pathways

In wild-type cells, we suggest, as has Heuser (2006), that CV vacuoles are discharged through the fusion of the CV and plasma membranes. In *disgorgin*⁻ cells, CV discharge is defective, and we suggest that the bladder and plasma membrane fusion is mis-regulated. In the absence of Disgorgin, the fully charged bladders push against the plasma membrane, forming blebs under hypotonic conditions. As soon as some of the content is released from the bladder, the bladder retracts from the plasma membrane, even though the discharge is not completed. The formation and the kinetics of these blebs suggest that membrane tension may be involved in CV discharging in *disgorgin*⁻ cells. We suggest that in *disgorgin*⁻ cells, possible tension of the bladders against the

Figure 7 Model of the Disgorgin pathway. (A) The signalling pathway of Drainin and Disgorgin to regulate the CV. (B) CV development in *Dictyostelium* cells. For detailed description, see text.

plasma membrane may lead to localized, small ruptures in the plasma membrane allowing the release of the CV contents. Once some of the content is released, membrane tension may be reduced, the bladders then retract, and the cell reseals the gaps in the CV and plasma membranes. Such a resealing process was suggested previously (Becker *et al*, 1999). To date, there is no direct evidence whether membrane tension is required for the normal discharging process in wild-type *Dictyostelium* cells as suggested for *Paramecium* cells (Tani *et al*, 2001). Membrane tension has been suggested to be the primary force driving CV discharge (Heuser, 2006). Our data, however, suggest that membrane tension alone is insufficient for CV discharging as large CVs accumulate in *disgorgin*[−] cells under isotonic conditions when CV activity is reduced and discharging is inefficient in the absence of Disgorgin under hypotonic conditions. Our data argue that Disgorgin-mediated Rab8A-GTP hydrolysis mediates the fusion event.

Our findings indicate that Disgorgin is a GAP for Rab8A but not for other Rabs known to localize to the CV and that Disgorgin's GAP activity is required for its function. Further, Rab8A and Disgorgin contemporaneously localize to CV bladders. We suggest that CV–plasma membrane fusion, which permits the discharge of the CV bladder's content into the extracellular medium, is mediated by Disgorgin and Rab8A and this event requires the cycling of Rab8A from the GTP- to the GDP-bound state. In yeast, Sec4 (a homologue of Rab8) binds to Sro7p, which interacts with the t-SNARE protein Sec9p and is involved in membrane fusion (Grosshans *et al*, 2006a). Another Sec4 effector, Sec15p, is a component of the exocyst, which mediates the docking and fusion of exocytic vesicles to the plasma membrane and interacts with the Sro7p pathway (Guo *et al*, 1999; Grosshans *et al*, 2006a). Rab8A may have a similar function in CV–plasma membrane fusion in *Dictyostelium*. Our proposed role for Rab8A is inferred by its colocalization with

Disgorgin and the fact that Rab8A-GTP is a Disgorgin substrate. We were unable to obtain definitive genetic evidence that Rab8A is required for this process as we could not create a Rab8A null strain, most likely because Rab8A is essential for growth. Similarly, we were unable to test a dependency for Disgorgin's localization on Rab8A.

Both Drainin and Disgorgin contain a TBC domain. However, Drainin lacks the conserved catalytic Arg and Gln required for Rab GAP activity (Bos *et al*, 2007). Consistent with this, we find that Drainin lacks GAP activity (on the Rabs tested) but does bind to Rab11A-GTP *in vitro* and is recruited to CVs by Rab11A. We suggest that Drainin acts as an effector of Rab11A, and functions upstream from Disgorgin and Rab8A. Evi5 is a mammalian TBC-domain-containing protein that binds Rab11 in a GTP-dependent manner. Instead of being a GAP for Rab11, Evi5 competes with the Rab11 effector FIP3 for Rab11 binding (Westlake *et al*, 2007). Drainin might function similarly to compete with other Rab11A effectors. We suggest that Drainin and Evi5 represent a group of TBC-domain-containing proteins that lack GAP activity but bind Rabs and control Rab-mediated pathways.

Harris *et al* (2001) found that Rab11A does not localize on the CV under hypotonic conditions. However, our studies demonstrate that Rab11A remains associated with the CV membranes under hypotonic conditions during both the charging and discharging stages. The different observations might be due to different expression levels of the proteins or sensitivities of the detection systems. Although constitutively activated Rab11A exhibits localization similar to that of wild-type Rab11A, Rab11A^{CA} causes Drainin to localize to the plasma membrane. This suggests that whereas Drainin binds Rab11A in a GTP-dependent manner, an inability of Rab11A to cycle may result in active fusion between the CV and the plasma membrane. Cells expressing dominant-negative Rab11A are sensitive to hypotonic stress as described previously (Harris *et al*, 2001). We suggest this results from

Rab11A^{DN} blocking Drainin recruitment to the CV. The exocyst component Sec15p interacts not only with Sec4 (Rab8) but also with Rab11 in a GTP-dependent manner and mediates some of the phenotypes regulated by Rab11 in *Drosophila* (Guo *et al*, 1999). We suggest that exocysts may have a function in *Dictyostelium* CV–plasma membrane fusion.

When expressed as a tagged protein, Rab11C also associates with the CV. However, Rab11C does not interact with Disgorgin or Drainin and overexpressing Rab11C only partially suppresses the large vacuole phenotype in *disgorgin*[−] cells (unpublished data). Whereas Rab11A is expressed at a high level during the vegetative stage, Rab11C is expressed mostly in 18-h developmental cells (*Dictybase*) and may therefore not be relevant to CV function in vegetative cells.

We know that GAP activity and membrane localization are required for Disgorgin function, but we do not know the function of the Disgorgin F-box domain. Loss of the F-box does not affect the ability of overexpressed Disgorgin to complement the *disgorgin*[−] phenotypes. We found two *Dictyostelium* SKP1 orthologues in Disgorgin-containing complexes and demonstrated that they interact with Disgorgin in an F-box-dependent manner. Both F-box-containing proteins and the SKP proteins are components of the SCF (Skp–cullin–F-box) ubiquitin E3-ligase complex. Disgorgin is most likely either a target of ubiquitination or it functions as an adaptor to bring another protein to the SCF complex, which is then ubiquitinated. Alternatively, the Disgorgin F-box domain may mediate the formation of a non-SCF-containing complex (Kipreos and Pagano, 2000). However, we do not have direct evidence for either model and were unable to detect Disgorgin ubiquitination.

Regulation of the CV by BEACH proteins

We identified the BEACH proteins LvsA and LvsD in a second site suppressor/enhancer screen for genes that genetically interact with Disgorgin to control CV morphology and function. We and Wu *et al* (2004) found that LvsA is required for the formation of a functional CV system. In *lvsA*[−] cells, what remains are small punctate structures that have Disgorgin, V-ATPase, and Dajumin. As Disgorgin only associates with the CV membrane at the discharge stage, these observations suggest that the punctate structures in *lvsA*[−] cells are post-discharge CVs that arrest at this stage. Our results suggest that LvsA is required to maintain the integrity of the CV presumably during the fusion and resealing process.

We demonstrate that disrupting *lvsA* or overexpressing LvsD suppresses the large CV in *disgorgin*[−] cells, whereas overexpressing LvsA or disrupting *lvsD* leads to the extended enlargement of CVs in *disgorgin*[−] cells. We demonstrate for the first time that two BEACH proteins regulate same physiological process, which might be a general mechanism by which BEACH proteins function. LvsD's null and overexpression phenotype effects in a *disgorgin*[−] background are consistent with a model in which LvsD antagonizes the function of LvsA. As LvsD does not associate with the CV, we speculated that LvsD might interact with and inhibit LvsA to fulfill the regulation of the CV or these two proteins may compete for the same binding proteins in the cytosol. The connection between the two BEACH proteins and the Disgorgin protein strongly suggests that BEACH proteins and Rab proteins might function together. Another BEACH protein, Bchs, antagonizes Rab11 in synapse morphogenesis in *Drosophila* (Khodosh *et al*, 2006). Bchs has also been

shown to genetically interact with another Rab, Rab-RP1 (Simonsen *et al*, 2007). Future studies will be required to understand the role of the BEACH proteins at a biochemical level.

Materials and methods

Cell culture and constructs

The cell culture and all the constructs were made using standard approaches. See Supplementary data for details.

Indirect immunofluorescence staining

Indirect immunofluorescence staining was performed as described (Sesaki *et al*, 1997). See Supplementary data for details.

Microscopy

Cells were plated in HL5 medium on glass-bottomed microwell plates for 15 min. The medium was then replaced by either hypotonic conditions such as water or Na/K phosphate buffer (9.5 mM KH₂PO₄, 2.5 mM Na₂HPO₄, pH 6.1) or isotonic buffer (65 mM glucose, 15 mM KH₂PO₄, 20 mM Na₂HPO₄, pH 6.1). The styryl dye FM4-64 (Invitrogen) was used at 1 µg/ml and the images were recorded between 2 and 15 min after staining to visualize both the CV and the plasma membrane. Z-Stack images or time-lapse videos were collected on a microscope (model DMIRE2; LEICA) with DIC and/or fluorescence imaging. The bright-field pictures were taken by a Hamamatsu C4742–95 camera with SimplePCI software under a 100×1.40 objective. We took the fluorescence pictures under a 60×1.40 objective with either a Hamamatsu ORCA-ER C4742–80 camera for monochrome images or a Hamamatsu c9100 camera for two-colour images.

GST pull-down and GAP assays

GST or GST-fused proteins were produced in BL21(DE3) bacteria and purified on glutathione-sepharose (Amersham Biosciences) according to the manufacturer's instructions. The GST pulldown assay was a modified form of the Ras-binding assay described previously (Sasaki *et al*, 2004). Briefly, the cell extract from Ax2 cells expressing v5–Disgorgin or v5–Drainin was incubated with 10 µg of GST or GST–Rabs on glutathione-agarose beads at 4°C for 1 h. The beads were washed three times. Proteins were separated on an SDS–PAGE gel and immunoblotted with the anti-v5 antibody (Sigma). We performed the GAP assay with or without 250 nM GAPs using the EnzChek[®] Phosphate Assay Kit (Invitrogen) according to the protocol described previously (Pan *et al*, 2006). The absorbance at 360 nm was monitored with microplate detection system SpectraMax Plus384 using SoftMax Pro 4.3LS (Molecular Devices). Three independent experiments were performed. The kinetic data were analysed by simultaneously fitting to the pseudo-first-order Michaelis–Menten model function using MATLAB (MathWorks Inc.).

REMI screening

REMI suppressor/enhancer screening is a modification of the protocol used previously (Dynes *et al*, 1994). See Supplementary data for details.

Detection of the Disgorgin complex and mass spectrometry

We performed the purification of the Disgorgin complex and mass spectrometry as described previously (Lee *et al*, 2005). See Supplementary data for details.

Supplementary information

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank members of the Firtel laboratory for helpful suggestions over the course of this study, especially Susan Lee for excellent technical assistance, and Jennifer Roth for help in preparing this paper. We thank Colin Jamora for helpful suggestions for the paper. We thank Margaret Clarke for providing us with anti-calmodulin and anti-V-H⁺-ATPase antibodies. These studies were supported by United States Public Health Service grants to RAF.

References

- Becker M, Matzner M, Gerisch G (1999) Drainin required for membrane fusion of the contractile vacuole in *Dictyostelium* is the prototype of a protein family also represented in man. *EMBO J* **18**: 3305–3316
- Bernards A (2003) GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and *Drosophila*. *Biochim Biophys Acta* **1603**: 47–82
- Beyenbach KW, Wiczorek H (2006) The V-type H⁺ ATPase: molecular structure and function, physiological roles and regulation. *J Exp Biol* **209**: 577–589
- Bos JL, Rehmann H, Wittinghofer A (2007) GEFs and GAPs: critical elements in the control of small G proteins. *Cell* **129**: 865–877
- Bush J, Temesvari L, Rodriguez-Paris J, Buczynski G, Cardelli J (1996) A role for a Rab4-like GTPase in endocytosis and in regulation of contractile vacuole structure and function in *Dictyostelium discoideum*. *Mol Biol Cell* **7**: 1623–1638
- De Lozanne A (2003) The role of BEACH proteins in *Dictyostelium*. *Traffic* **4**: 6–12
- Dynes JL, Clark AM, Shaulsky G, Kuspa A, Loomis WF, Firtel RA (1994) LagC is required for cell–cell interactions that are essential for cell-type differentiation in *Dictyostelium*. *Genes Dev* **8**: 948–958
- Gabriel D, Hacker U, Kohler J, Muller-Taubenberger A, Schwartz JM, Westphal M, Gerisch G (1999) The contractile vacuole network of *Dictyostelium* as a distinct organelle: its dynamics visualized by a GFP marker protein. *J Cell Sci* **112**: 3995–4005
- Gerald NJ, Siano M, De Lozanne A (2002) The *Dictyostelium* LvsA protein is localized on the contractile vacuole and is required for osmoregulation. *Traffic* **3**: 50–60
- Gerisch G, Heuser J, Clarke M (2002) Tubular-vesicular transformation in the contractile vacuole system of *Dictyostelium*. *Cell Biol Int* **26**: 845–852
- Grosshans BL, Andreeva A, Gangar A, Niessen S, Yates III JR, Brennwald P, Novick P (2006a) The yeast Igl family member Sro7p is an effector of the secretory Rab GTPase Sec4p. *J Cell Biol* **172**: 55–66
- Grosshans BL, Ortiz D, Novick P (2006b) Rabs and their effectors: achieving specificity in membrane traffic. *Proc Natl Acad Sci USA* **103**: 11821–11827
- Guo W, Roth D, Walch-Solimena C, Novick P (1999) The exocyst is an effector for Sec4p, targeting secretory vesicles to sites of exocytosis. *EMBO J* **18**: 1071–1080
- Harris E, Yoshida K, Cardelli J, Bush J (2001) Rab11-like GTPase associates with and regulates the structure and function of the contractile vacuole system in *Dictyostelium*. *J Cell Sci* **114**: 3035–3045
- Heo WD, Inoue T, Park WS, Kim ML, Park BO, Wandless TJ, Meyer T (2006) PI(3,4,5)P₃ and PI(4,5)P₂ lipids target proteins with polybasic clusters to the plasma membrane. *Science* **314**: 1458–1461
- Heuser J, Zhu Q, Clarke M (1993) Proton pumps populate the contractile vacuoles of *Dictyostelium amoebae*. *J Cell Biol* **121**: 1311–1327
- Heuser J (2006) Evidence for recycling of contractile vacuole membrane during osmoregulation in *Dictyostelium amoebae*—a tribute to Gunther Gerisch. *Eur J Cell Biol* **85**: 859–871
- Insall R, Muller-Taubenberger A, Machesky L, Kohler J, Simmeth E, Atkinson SJ, Weber I, Gerisch G (2001) Dynamics of the *Dictyostelium* Arp2/3 complex in endocytosis, cytokinesis, and chemotaxis. *Cell Motil Cytoskeleton* **50**: 115–128
- Jordens I, Marsman M, Kuijl C, Neefjes J (2005) Rab proteins, connecting transport and vesicle fusion. *Traffic* **6**: 1070–1077
- Khodosh R, Augsburger A, Schwarz TL, Garrity PA (2006) Bchs, a BEACH domain protein, antagonizes Rab11 in synapse morphogenesis and other developmental events. *Development* **133**: 4655–4665
- Kipreos ET, Pagano M (2000) The F-box protein family. *Genome Biol* **1**, REVIEWS3002
- Kwak E, Gerald N, Larochelle DA, Vithalani KK, Niswonger ML, Maready M, De Lozanne A (1999) LvsA, a protein related to the mouse beige protein, is required for cytokinesis in *Dictyostelium*. *Mol Biol Cell* **10**: 4429–4439
- Lee S, Comer FI, Sasaki A, McLeod IX, Duong Y, Okumura K, Yates III JR, Parent CA, Firtel RA (2005) TOR complex 2 integrates cell movement during chemotaxis and signal relay in *Dictyostelium*. *Mol Biol Cell* **16**: 4572–4583
- Pan X, Eathiraj S, Munson M, Lambright DG (2006) TBC-domain GAPs for Rab GTPases accelerate GTP hydrolysis by a dual-finger mechanism. *Nature* **442**: 303–306
- Sasaki AT, Chun C, Takeda K, Firtel RA (2004) Localized Ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement. *J Cell Biol* **167**: 505–518
- Sesaki H, Wong EF, Siu CH (1997) The cell adhesion molecule DdCAD-1 in *Dictyostelium* is targeted to the cell surface by a nonclassical transport pathway involving contractile vacuoles. *J Cell Biol* **138**: 939–951
- Simonsen A, Cumming RC, Lindmo K, Galaviz V, Cheng S, Rusten TE, Finley KD (2007) Genetic modifiers of the *Drosophila* blue cheese gene link defects in lysosomal transport with decreased life span and altered ubiquitinated-protein profiles. *Genetics* **176**: 1283–1297
- Tani T, Allen RD, Naitoh Y (2001) Cellular membranes that undergo cyclic changes in tension: direct measurement of force generation by an *in vitro* contractile vacuole of *Paramecium multimicronucleatum*. *J Cell Sci* **114**: 785–795
- Weeks G, Gaudet P, Insall RH (2005) The small GTPase superfamily. In *Dictyostelium Genomics*, Loomis W, Kuspa A (ed), pp 173–210. Norfolk, UK: Horizon Bioscience
- Westlake CJ, Junutula JR, Simon GC, Pilli M, Prekeris R, Scheller RH, Jackson PK, Eldridge AG (2007) Identification of Rab11 as a small GTPase binding protein for the Evi5 oncogene. *Proc Natl Acad Sci USA* **104**: 1236–1241
- Wu WI, Yajnik J, Siano M, De Lozanne A (2004) Structure-function analysis of the BEACH protein LvsA. *Traffic* **5**: 346–355