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RESEARCH ARTICLE

Endosomal vesicle fusion machinery is involved with the contractile vacuole in *Dictyostelium discoideum*

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ABSTRACT

Contractile vacuoles (CVs), enigmatic osmoregulatory organelles, share common characteristics, such as a requirement for RAB11 and high levels of V-ATPase. These commonalities suggest a conserved evolutionary origin for the CVs with implications for understanding of the last common ancestor of eukaryotes and eukaryotic diversification more broadly. A taxonomically broader sampling of CV-associated machinery is required to address this question further. We used a transcriptomics-based approach to identify CV-associated gene products in *Dictyostelium discoideum*. This approach was first validated by assessing a set of known CV-associated gene products, which were significantly upregulated following hypo-osmotic exposure. Moreover, endosomal and vacuolar gene products were enriched in the upregulated gene set. An upregulated SNARE protein (NPSNB) was predominantly plasma membrane localised and enriched in the vicinity of CVs, supporting the association with this organelle found in the transcriptomic analysis. We therefore confirm that transcriptomic approaches can identify known and novel players in CV function, in our case emphasizing the role of endosomal vesicle fusion machinery in the *D. discoideum* CV and facilitating future work to address questions regarding the deep evolution of eukaryotic organelles.

KEY WORDS: Contractile vacuole, Rab, SNARE, Osmoregulation, Endomembrane, RNA-seq, Evolution

INTRODUCTION

Contractile vacuole (CV) complexes (CVCs) are morphologically diverse, but functionally comparable, osmo-regulatory organelles found in diverse freshwater protists (Patterson, 1980; Plattner, 2013). There are descriptions of CV structures in freshwater members of nearly every major eukaryotic lineage (Allen and Naitoh, 2002). Despite their morphological variation, unifying features of CV systems are apparent. In general, the CV system is formed from tubular-vesicular membrane structures, called the spongiome, carrying high levels of the vacuolar ATPase (V-ATPase). Under hypo-osmotic conditions, the V-ATPase creates an osmotic gradient across the CVC membrane driving the removal of water from the cytosol. Fusion of these tubular-vesicular compartments creates a large water containing vacuole. This subsequently undergoes fusion with the plasma membrane, thereby removing water from the cytosol and combating the effects of the hypo-osmotic environment (Allen and Naitoh, 2002; Plattner, 2013). This cycle is repeated, giving the appearance of pulsatile vacuolar swelling and contraction.

Beyond these gross morphological descriptions of various CVCs, our understanding of the molecular basis of CVC biogenesis and function remains patchy.

Aspects of CVC function have been examined in a range of model systems, most intensively in *Paramecium tetraurelia* and *Dictyostelium discoideum*, as well as *Trypanosoma cruzi*. However, with the exception of Rab11 paralogues, which have been shown to be important for CV function in multiple systems (Harris et al., 2001; Ulrich et al., 2011; Niyogi et al., 2014), these studies largely assess different protein families and pathways. This currently limits our ability to draw comparative conclusions. Their membrane-bounded nature and the common requirement for Rab11 does, however, suggest that CVCs have arisen through adaptations of the existing eukaryotic endo-membrane system, likely the endocytic and vacuolar branches specifically. Whether these organelles represent a conserved eukaryotic feature or multiple examples of convergent evolution remains an open question.

Extensive molecular characterisation and comparative genomics has revealed the common origin of the bulk of the membrane trafficking machinery found in extant eukaryotes (reviewed in Dacks and Field, 2018). This comparative evolutionary cell biological approach relies on broadly comparable inventories of organelle-associated gene products and knowledge of their functions across distantly related eukaryotic systems. As much of our understanding of endomembrane system organization is derived from studies in yeast and metazoan systems, organisms without CVCs, the data from which to base comparative studies of CV biogenesis and function is currently lacking. Furthermore, the diverse morphologies and kinetics of CV function, together with the paucity of genetic tools in many protistan lineages expressing these organelles make classical molecular cell biology approaches challenging. We therefore set out to use more broadly applicable transcriptomic approaches for the identification of a cohort of CV-associated genes. In this instance, we employed the best characterised and most experimentally tractable CV-expressing system, *D. discoideum*, in order to evaluate the relevance of the transcriptome changes observed. We show that a large-scale transcriptome response to hypo-osmotic stress includes the upregulation of nearly all known CV-associated genes in

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D. discoideum. Focusing on genes encoding proteins associated with the membrane-trafficking machinery, we observed that Rab GTPases and SNARE proteins were notably upregulated, whereas vesicle coat and multi-subunit tethering complex components were not. Moreover, of the differentially expressed genes, those paralogues associated with the late secretory or endocytic system were enriched versus those for the early secretory system. Furthermore, we demonstrate that this approach has predictive potential through the identification of a previously uncharacterized plasma membrane localised SNARE protein, which was found to be enriched at sites proximal to CVs. Finally, we provide phylogenetic evidence that CV-associated Rab11 paralogues from *D. discoideum* and *T. cruzi* represent lineage specific expansions of this protein family, and thus the involvement of specialized Rab11 paralogues in CV function represents a convergent evolutionary phenomenon.

RESULTS

CV gene transcripts are upregulated during hypo-osmotic stress

In order to examine the transcript-level response of CVC system induction, we subjected *D. discoideum* cultures to hypo-osmotic conditions and made a comparison to cultures in isotonic medium. We analysed transcriptomes of *D. discoideum* (DH-1) under normal growth conditions (HL5 medium) and following 1 h of hypo-osmotic shock (H_2O). Samples were generated in biological triplicates and transcript abundance assessed by RNA-sequencing (RNA-seq). In total, abundance profiles were obtained for over 13,000 transcripts. In line with previous findings (Na et al., 2007; Mathavarajah et al., 2018), differential expression analysis using a false discovery rate (FDR) cut-off of <0.01 revealed that hypo-osmotic shock led to a large-scale alteration of transcript abundance within this timeframe. We identified over 2200 and 1900 downregulated and upregulated transcripts, respectively (Fig. 1A; Table S1).

To assess whether genes associated with CV function were under transcript-level control, we generated a test set of genes previously characterized in *D. discoideum* as being implicated in CV function (Table S2). Examining our comparative transcriptome dataset, we saw that the majority of genes within this test set (16 of 18) were significantly upregulated during hypo-osmotic stress (FDR <0.01) (Fig. 1B). The high level of concordance in both the appearance of transcript regulation and its direction in this test set strongly supported our premise that comparative transcriptomics could reveal genes and larger networks of genes involved in CV biogenesis and function.

As we sought to obtain insight specifically into CV-associated genes, we focused our further attention on the upregulated gene set. As has been previously reported (Mathavarajah et al., 2018), analysis of significantly enriched GO: Biological Process terms identified within the upregulated gene-set revealed a number of terms associated with ‘cellular catabolic process’, predominantly protein ubiquitylation pathways (Fig. 1C; Table S3, yellow rows) and cyclic AMP-related signalling pathways (Fig. 1C, Table S3, blue rows). In addition, and perhaps due to our increased sampling depth, we identified a substantial proportion of GO terms relating to intracellular membrane trafficking (Fig. 1C). Finally, analysis of significantly enriched GO: Cellular Component terms revealed a striking number of terms relating to the vacuole, endosome, Golgi apparatus and SNARE complex, again supporting the validity of our approach for the identification of membrane trafficking machinery with potential relevance to CV function.

Subsets of membrane trafficking machinery are differentially regulated during hypo-osmotic stress

As we are interested in the biogenesis of the CV and the conservation of this process, we next examined in more detail the transcriptional control of membrane trafficking machinery during hypo-osmotic stress. We took two approaches, firstly by broadly categorizing the identified upregulated membrane trafficking-associated genes by their predicted site of action. Secondly, to obtain a more broadly comparable picture of the membrane trafficking machinery, we identified *D. discoideum* homologues of four evolutionarily well-conserved membrane trafficking machinery families and examined their expression levels between our experimental conditions.

Our identified upregulated gene set contained ∼130 genes annotated as having a function related to intracellular membrane trafficking (Fig. 2A; Table S4). Notably, these genes included several (e.g. Rab 1A, Rab8a, Rab11a, Rab14, Sec1, Copine, Drainin, Disgorgin, LvsA and LvsD) that had been previously implicated in CV function. We then categorized these genes by their predicted site of action (Fig. 2A). As would be expected, based on the broad characterisation of CVs as having an endo-lysosomal origin, we found that the most highly represented localisation was endosomal or vacuolar. This finding gives us confidence that we are indeed seeing transcriptional regulation of genes whose products are important for CV function. Of note, within this set of endosomal and vacuolar genes, there was a strong enrichment of ESCRT components (Chmp2a1, Chmp2a2, Chmp4, Vhmp5, VPS28 and the putative ESCRT0 analogue Tom1). Previous analysis (Mathavarajah et al., 2018), and our current data, suggest upregulation of ubiquitylation machinery in *D. discoideum*, and it is possible that this is reflected in the observed significant modulation of the ESCRT machinery. We saw relatively few genes associated with the biosynthetic pathway [Golgi and endoplasmic reticulum (ER)] supporting the notion that the majority of CV membrane is derived from the endolysosomal system.

We carried out a secondary analysis of members of four major classes of membrane trafficking machinery, namely, Rabs, SNAREs, coat proteins and tethers. Together, these proteins make up a well-characterised and functionally well-conserved core of the membrane trafficking machinery. *D. discoideum* genes belonging to these classes were identified, and their expression levels assessed across our experimental conditions (Table S5). Overall comparison between these classes revealed interesting differences. Expression of coat protein and multi-subunit tethering complex genes appeared to be largely unaffected by hypo-osmotic stress, with the possible exception of the Class-C HOPS tethering complex. Four subunits of the six that make up this complex were found to be mildly downregulated. Conversely, expression of multiple SNARE and Rab protein genes appeared to be upregulated during hypo-osmotic stress. The requirements for various Rab proteins during CV biogenesis and function in *D. discoideum* have been explored previously (Bush et al., 1996; Harris et al., 2001; Du et al., 2008; Essid et al., 2012). Among the upregulated Rab genes were those encoding the known CV-associated Rab11a, Rab8a and Rab14 (Fig. 2B). In addition to these known CV associated Rabs, the majority of upregulated Rabs were those associated with endosomal trafficking. These included Rab5b, Rabs7b and Rab7b, and Rab32a, which have been previously implicated in CV function in *T. cruzi* (Ulrich et al., 2011; Niyogi et al., 2015), and several *Dictyostelium*-specific Rab7 paralogues (*P*orfírio-Sousa et al., 2022) (Fig. 2C; Table S6). These Rab7 paralogues would be predicted to function in later endosomal compartments, but this prediction has not been tested. Overall, there appeared to be a balanced upregulation of Rabs acting at both early and late endosomal compartments.

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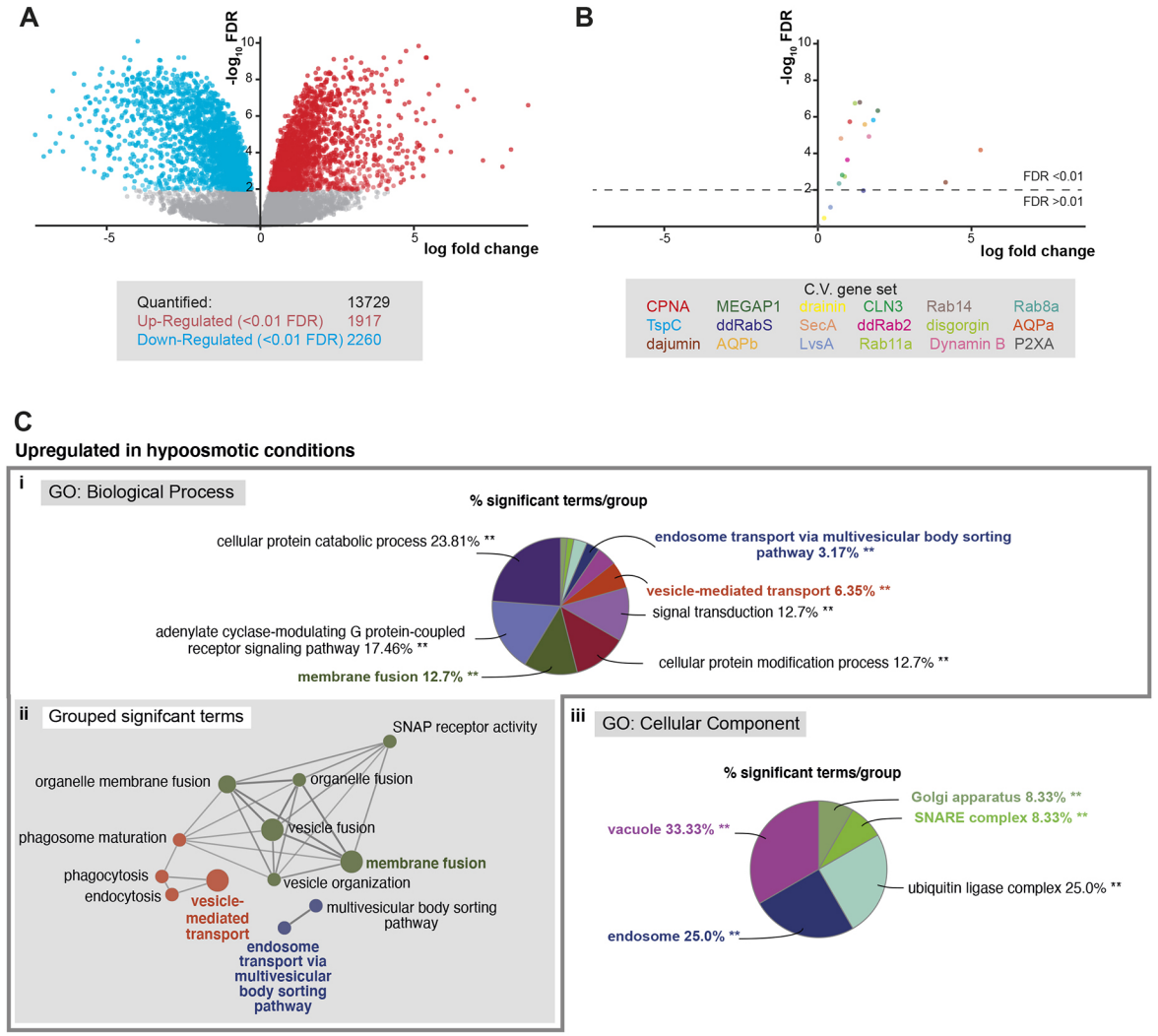

Fig. 1. Transcriptional response to hypo-osmotic stress. (A) Volcano plot of relative expression profiles (hypo-osmotic versus control, three biological replicates for each condition). Transcripts with significantly altered abundance (FDR <0.01) are coloured (red, upregulated; blue, downregulated). (B) Relative expression and FDR of CV-associated transcripts from dataset as shown in A. (C) (i) ClueGO (https://apps.cytoscape.org/apps/cluego; Bindea et al., 2009) grouping of Biological Process GO terms significantly overrepresented in the upregulated gene set. (ii) Expansion of GO term groups from (i) related to membrane trafficking (membrane fusion, green; vesicle-mediated transport, orange; endosome transport via multivesicular body sorting pathway, dark blue). (iii) ClueGO grouping of significantly enriched cellular component GO terms in the upregulated gene set. ***P*<0.01 (Benjamini–Hochberg corrected FDR).

SNAREs showed a similar pattern to the Rab proteins. Both Vti1B and, to a lesser extent, VAMP7B have been shown to localise to CVs of *D. discoideum*, although in the case of VAMP7B the abundance at the CV was lower than that found at endosomal membranes (Wen et al., 2009). To date, there is no data available on which SNAREs are required for CV function in *D. discoideum*. We saw upregulation of Vti1B but not VAMP7B (Fig. 2B). In addition to Vti1B, we identified a total of 17 SNARE genes with upregulated transcripts during hypo-osmotic stress. Among these it is interesting to note that each of the four SNARE subfamilies (Qa, Qb, Qc and R SNAREs; Fasshauer et al., 1998; Malsam et al. 2008) is approximately equivalently represented (Fig. 2C; Table S7). As one of each of these subfamilies of SNAREs is required to come together to produce a fusion competent SNARE complex it seems likely that hypo-osmotic stress is inducing upregulation of specific SNARE complexes. The majority of upregulated SNAREs were orthologues of those shown to be involved in post-Golgi endosomal trafficking pathways in other systems, including among others syntaxin 16, Vti1A and Vti1B, syntaxin 6 and syntaxin 8, with little to no upregulation of early biosynthetic pathway SNARE orthologues, such as Sec20, Sec22, Use1A and Bet1A (Table S6). In terms of cognate endosomal SNARE complexes, we saw upregulation of both early (Syx7, Vti1A and Vit1B, and Syx6) and late (Syx8 and Syp7) members with the exception of the R SNARE VAMP7. We did, however, note upregulation of two homologues of the R SNARE Ykt6. Although VAMP7 is predominantly associated with endosomal fusion pathways, Ykt6 is a promiscuous R SNARE that is able to compensate for VAMP7 when VAMP7 level are insufficient (Davis et al., 2021). Thus, we saw upregulation of potential fully functional SNARE complexes associated with early and late endosomal membrane fusion events. In addition to the endosomal SNAREs, we also saw upregulation of a cognate SNARE complex that would be predicted to act in secretory events at the plasma membrane (Syn1B, NPSNB, Syp7A and Syb1). This predicted SNARE complex is based on studies from plant systems and has not been characterised in *D. discoideum* (El Kasmi et al., 2013).

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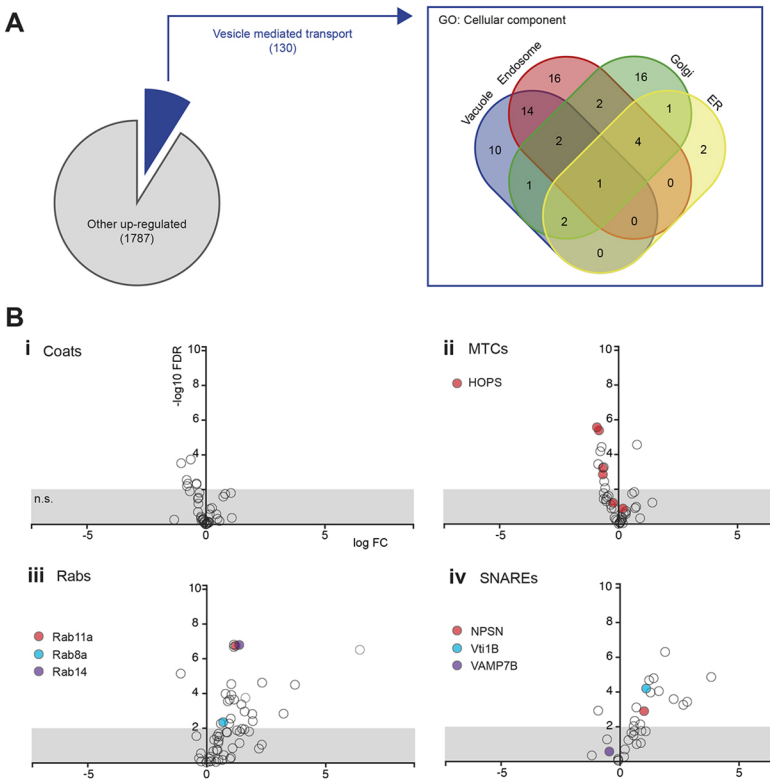

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ID	Gene name	FDR	Localisation
Rabs			
DDB_G0277867	Rab1B	1.55E-07	Early endosome, Golgi, ER
DDB_G0281337	Rab14	1.60E-07	Early endosome, Golgi, TGN,
DDB_G0269238	Rab11A	1.81E-07	Recycling endosome, TEN, TGN
DDB_G0269174	Rab1C	2.10E-07	Early endosome, Golgi, ER
DDB_G0276399	Rab8B	3.04E-07	Recycling endosome, TEN,TGN, Secretory vesicles
DDB_G0291233	RabA	2.38E-05	Rab1 paralogue
DDB_G0286553	Rab21	2.88E-05	Early endosome
DDB_G0290407	RabO	3.13E-05	Unknown
DDB_G0282847	Rab5B	1.04E-04	Early endosome
DDB_G0287553	Rab7B	1.23E-04	Autophagosome, Late endosome, Lysosome
DDB_G0287211	Rab11B	1.78E-04	Recycling endosome, TEN, TGN
DDB_G0269236	Rab7A	2.23E-04	Autophagosome, Late endosome, Lysosome
DDB_G0292268	Rab2A	2.26E-04	Golgi, Autophagosome
DDB_G0283757	Rab1A	2.80E-04	Early endosome, Golgi, ER
DDB_G0294571	RabX11	4.24E-04	Rab7 paralogue
DDB_G0282203	RabY	1.09E-03	Unknown
DDB_G0290779	RabL	1.45E-03	Rab7 paralogue
DDB_G0272138	Rab2B	1.51E-03	Golgi, Autophagosome
DDB_G0290829	RabM	2.79E-03	Rab7 paralogue
DDB_G0290789	RabN1	3.88E-03	Rab7 paralogue
DDB_G0280043	Rab8A	4.46E-03	Recycling endosome, TEN,TGN, Secretory vesicles
DDB_G0290783	RabG2	5.38E-03	Rab1 paralogue
DDB_G0283603	Rab32A	5.45E-03	Golgi, Lysosome related organelles, Secretory organelles
SNAREs			
Qa			
DDB_G0287733	Syn7A	1.60E-05	Early endosome, Late endosome
DDB_G0277565	Syn5	1.07E-04	Golgi, TGN
DDB_G0270556	Syn1B	2.55E-04	Plasma membrane (Secretory)
DDB_G0276469	Syn16B	5.47E-04	TGN, early endosome
DDB_G0279133	Syn7B	7.72E-04	Early endosome, Late endosome
Qb			
DDB_G0292974	Vti1A	6.27E-05	Early endosome, TGN,
DDB_G0285365	NPSNB	1.26E-03	Plasma membrane (Secretory) *predicted*
DDB_G0283363	Vti1B	9.21E-03	Early endosome, TGN, Late endosome, Plasma membrane
Qc			
DDB_G0289063	Syp7A	8.84E-05	Late endosome
DDB_G0284185	Syn6	5.95E-04	Early endosome, Late endosome, TGN
DDB_G0284385	Syn10	4.59E-03	Late endosome, TGN,
DDB_G0288439	Syn8B	7.27E-03	Early endosome, Late endosome, TGN
R			
DDB_G0280061	SybA	4.91E-07	Plasma mambrane (Secretory)
DDB_G0293712	YKT6 paralogue	1.37E-05	ER, Endosome, Golgi, Autophagosome, Plasma membrane
DDB_G0291656	Ykt6	2.09E-05	ER, Endosome, Golgi, Autophagosome, Plasma membrane
DDB_G0290363	SybB	3.64E-04	Plasma membrane (Secretory)

Fig. 2. Transcriptional regulation of membrane trafficking associated genes. (A) Organelle distribution (obtained via GO Cellular Component association) of significantly upregulated transcripts associated with vesicle mediated trafficking. (B) Expression profiles of curated gene sets corresponding to (i) heterotetrameric vesicle coat complexes, (ii) multisubunit tethering complexes (MTC), (iii) Rab family small GTPases, and (iv) SNARE proteins from dataset as in A. (C) Organelle associations of significantly upregulated Rab and SNARE gene products.

NPSNB is plasma membrane localised and enriched at sites of CV–plasma membrane apposition

NPSNB belongs to a poorly characterised family of SNARE proteins lost among animals and fungi, but that is widely conserved in other eukaryotes (Venkatesh et al., 2017). The NPSN SNARE family is currently only characterised in *Arabidopsis*, where they localise predominantly to the plasma membrane (Uemura et al., 2004). The upregulation of NPSNB in our dataset led us to ask whether its plasma membrane localisation is conserved in *Dictyostelium* and whether it might be associated with CV release.

To assess the localisation of NPSNB, we generated N-terminal fluorescent protein fusions and expressed them in *D. discoideum* AX2 cells via a non-integrating plasmid vector. Both GFP-tagged and mCherry-tagged NPSNB were predominantly plasma membrane localised (Fig. 3A; Fig. S1). The plasma membrane distribution was not homogenous, with enrichment of fluorescent signal in small regions of the plasma membrane adjacent to phase-light structures. This was particularly noticeable in cells with lower expression of the transgene (Fig. 3A). Given the increased expression of NPSNB upon CV induction, we reasoned that these structures might be plasma membrane-adjacent CVs. We therefore carried out live-cell imaging of NPSNB–GFP-expressing cells to monitor the behaviour of these NPSNB-associated phase-light structures. This showed that plasma membrane NPSNB foci were dynamic, and their appearance and disappearance corresponded to sites and times of close association between the plasma membrane and CVs (Fig. 3B; Movie 1). We then sought to quantify this association. For each GFP–NPSNB-expressing cell with a visible CV, we traced the cell periphery and identified the centroid of the CV (Fig. 3C). The fluorescence intensity around the cell periphery was measured (Fig. 3D) and plotted against the distance from the CV centroid (Fig. 3E). From these data, we extracted a mean fluorescence intensity value for each 0.5 µm of distance from CV centroid (Fig. 3F). By measuring 30 cells in this way, we were able to establish the normalised NPSNB fluorescence intensity distribution relative to the CV centroid across our cell population (Fig. 3G). We saw a clear inverse relationship between NPSNB intensity and distance from the CV centroid (Fig. 3G). We therefore suggest that NPSNB is enriched at the plasma membrane adjacent to CVs.

Phylogenetics of Rab11 are not consistent with organelle homology of CVs

Beyond the cell biology in *D. discoideum* specifically are evolutionary questions regarding CVs across eukaryotes. On the one hand, organelles classified as CVs share an osmoregulatory function and a similar physiological cycle. By contrast, the taxonomic distribution of CVs found in freshwater members of disparate lineage with no observable CVs in non-freshwater members, as well as differences in morphological features of the organelles, leaves open the question of whether the CVs of different eukaryotes are retained from an ancient homologous CV organelle or are convergent in nature, derived independently from endolysosomal organelles in different eukaryotic lineages. Homology between a wide range of endomembrane organelles has been demonstrated most conclusively through identification of conserved machinery involved in organelle biogenesis and function across the eukaryotic diversity.

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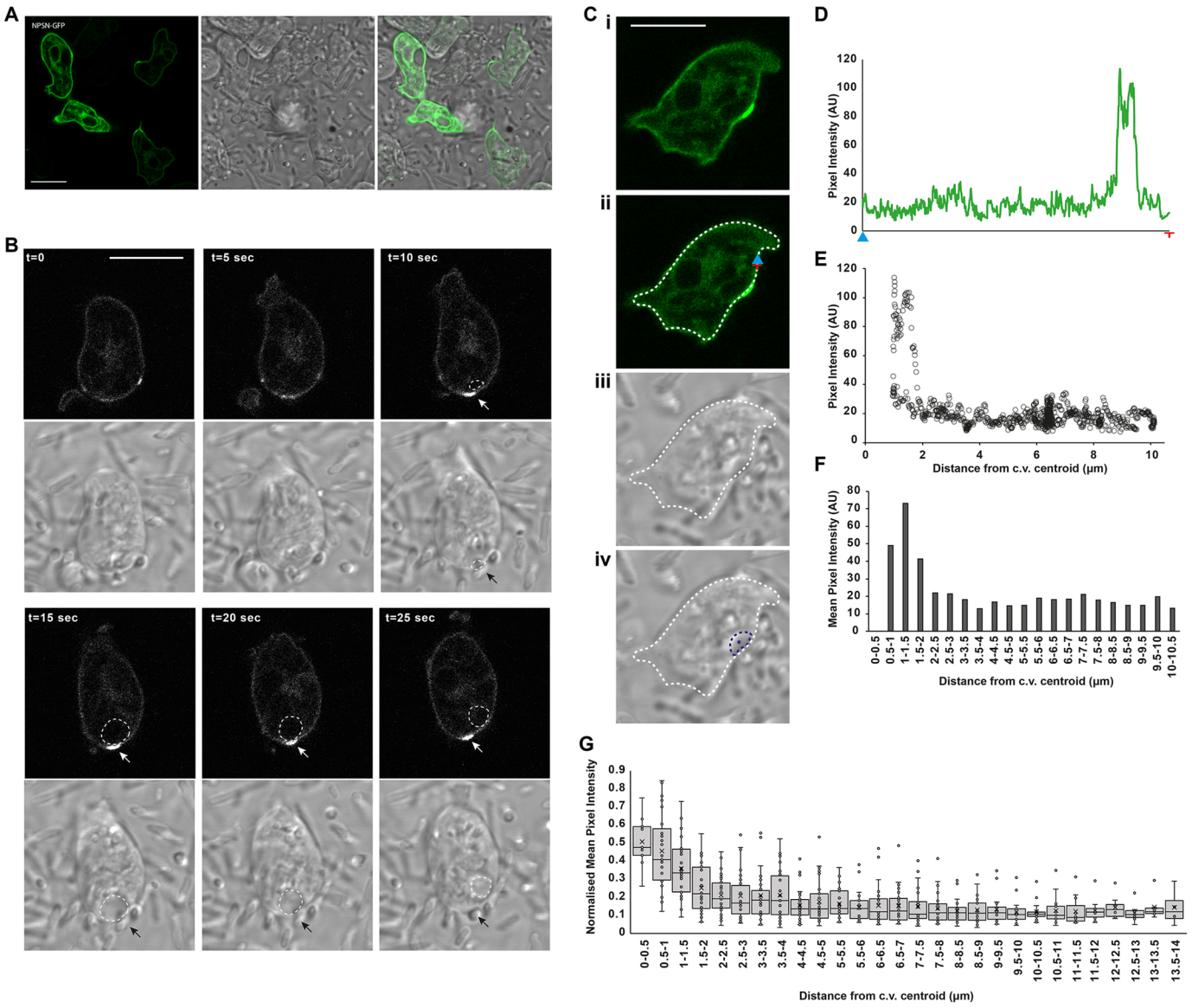

Fig. 3. The upregulated SNARE protein NPSNB is associated with the plasma membrane and sites of CV contact. (A) Localisation of N-terminally GFP-tagged NPSNB in AX2 cells. (B) Time series of NPSNB fluorescence intensity and CV appearance. Arrows indicate region of increased NPSNB fluorescence, white dashed line represents limiting membrane of CV visible in phase-contrast images. Timings are given relative to the first frame (*t*=0). (C) To relate NPSNB fluorescence to CV position, the intensity of NPSNB fluorescence was measured under a line-plot drawn around the cell limiting membrane (i,ii). CV limiting membrane and centroids were identified in phase-contrast images (iii,iv). (D–F) NPSNB fluorescence at the cell periphery (D) was plotted against (E) distance from CV centroid and (F) mean intensities were calculated from 0.5 µm bins. (G) Assembled and normalised data from 30 cells measured according to C–F. The box represents the 25–75th percentiles, and the median (line) and mean (cross) is indicated. Whiskers represent minimum and maximum values within 1.5× of the respective IQR. Points outside this range are shown and designated as potential outliers. Scale bars: 5 µm.

Rab11 proteins have been implicated in CV function in both *T. cruzi* and *Dictyostelium*. However, both *Dictyostelium* and *Trypanosoma* possess multiple paralogues of Rab11 (Elias et al., 2012). If the Rab11 paralogues that act at the organelle formed a clade containing both *Dictyostelium* and *Trypanosoma* orthologues, to the exclusion of the Rab11 paralogues that do not act at the CV, this would be strong evidence for a CV in the common ancestor of the taxa with organelle-specific paralogues, and thus homology of the CVs. If on the other hand, the Rab11 paralogues group by taxon, this would be most simply explained as the protein having been independently specialized to work at the organelle (i.e. convergence of CVs).

Phylogenetic analysis showed with robust support that the Rab11 paralogues group by lineage rather than by organelle (Fig. 4). Although there are several explanations for how these paralogues might have come to work at the organelle, this result means that although Rab11 paralogues can act at the CVs of *Dictyostelium* and *Trypanosoma*, they do not derive from a single primordial, pre-last eukaryotic common ancestor (LECA), CV-specific protein, and as such cannot be used as evidence for organelle homology between the *Dictyostelium* and *Trypanosoma* CVs.

DISCUSSION

One of the remaining open questions surrounding the evolutionary history of eukaryotic endomembrane organelles concerns the level of conservation versus convergence in CV origins. Answering this question has potential implications for our understanding of the

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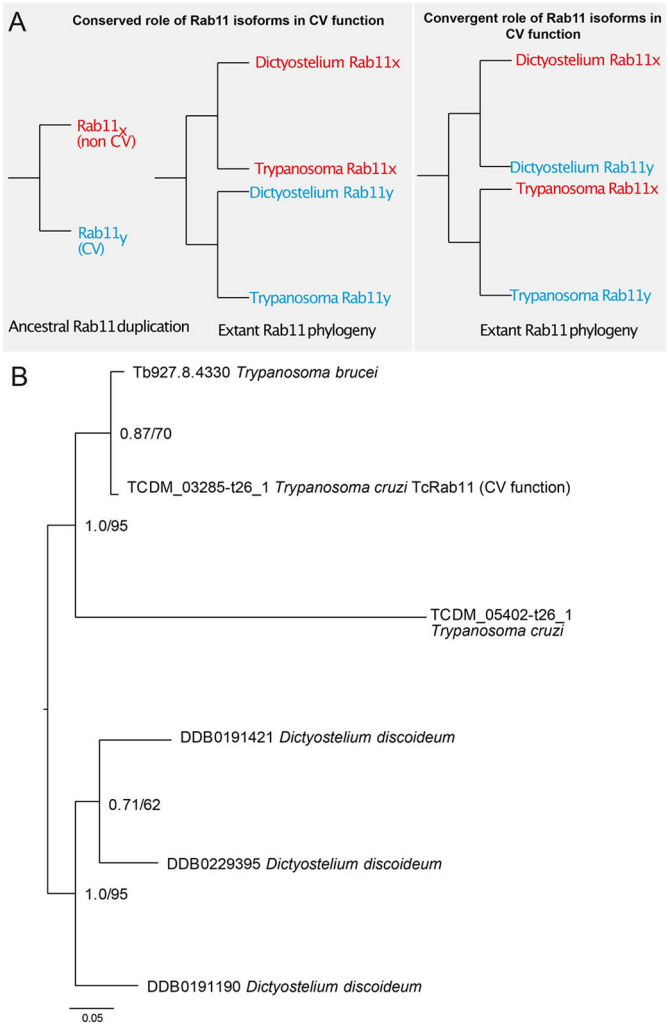

Fig. 4. Phylogeny of Rab11 paralogues from *T. cruzi*, *T. brucei* and *D. discoideum*. (A) Alternative expected phylogenetic patterns of Rab11 paralogues depending on whether the roles are conserved or convergent roles in CV function. (B) Phylogenetic reconstruction of the evolutionary relationship between all Rab11 paralogues from *T. cruzi* (possesses CV), *T. brucei* (lacks CV) and *D. discoideum* (possesses CV). Bayesian posterior probability and (ML) bootstrap likelihood support shown. Scale bar: 0.05 changes per site.

cellular physiology of a huge diversity of freshwater protists of various medical, agricultural and economic importance, as well as potentially revealing details about the lifestyle of the LECA of all extant eukaryotes. In comparison with other widely distributed organelles of the endomembrane system, our understanding of the molecular mechanistic basis of CV biogenesis and function is patchy at best.

Indeed, there is, by our reckoning, only a single paralogous gene family whose involvement in CV function has been assessed in multiple evolutionarily divergent model systems, namely Rab11. Obviously, a single data point such as this is insufficient to base broad statement of organelle paralogy or otherwise. However, our phylogenetic examination revealed that CV-associated Rab11 paralogues from *D. discoideum* and *T. cruzi* both represent lineage-specific expansions of the Rab11 family and therefore is not suggestive of a hypothesis of deep organelle homology of CVs. This echoes the recent demonstration that a Qa-SNARE implicated in CV function in *P. tetraurelia* (Plattner, 2013) is the product of a gene duplication of a plasma membrane clade (Syntaxin PM) specific to one clade of ciliates (Kaur et al., 2022) and thus cannot be homologous to CV machinery across eukaryotes, or even all ciliates for that matter. Both data points are, nonetheless, consistent with the hypotheses of deep organelle homology (but with convergent elaboration) or of convergent evolution. Far greater sampling depth of CV-associated proteins from multiple organisms is required to expand the comparative evolutionary studies of this organelle.

The most widely used yeast and metazoan model systems do not have the capacity to form CVCs. Instead, these organelles are found in an evolutionarily diverse range or freshwater protistan lineages. Historically, studies of CV function have been carried out in relatively tractable ciliates and, for the most part, *D. discoideum*. However, to answer deep evolutionary questions, it is vital to sample as broad a range of organisms as possible. Most suitable organisms are not amenable to classical cellular and molecular biology approaches, due to inherent technical limitation or a lack of developed molecular biology tools. Therefore, approaches which do not require extensive genetic modification will be important in building a picture of the diversity of CVs across the eukaryotes. As such we have assessed the suitability of a transcriptomic approach using the relatively well described *Dictyostelium* model system.

There are some additional considerations and confounding variables that deserve recognition from the outset. Hypo-osmotic incubation and nutrient withdrawal have long been known to induce starvation responses in *Dictyostelium*, which in turn stimulate differentiation (Raper, 1940). Transcriptomic analysis of differentiation and development of *Dictyostelium* in response to nutrient withdrawal in a moderately hypo-osmotic environment has demonstrated wide ranging modulation of gene expression and established the temporal ordering of these responses (Rosengarten et al., 2015). Based on this data, we elected to use a very early timepoint (1 h post hypo-osmotic incubation) for analysis; at this point, the transcriptional response to starvation is somewhat minimised relative to later time points. It is clear, however, that any transcriptomic alterations we observe likely represent not only adaptation to hypo-osmotic conditions but also the beginning of the cellular response to nutrient withdrawal and the initiation of a differentiation and development programme. In recognition of this problem, we have not attempted to draw conclusions about overall cellular responses to hypo-osmotic stress specifically, but limited our focus to membrane trafficking machinery, which as a system is known to be required for CV biogenesis. Indeed, it has not previously been shown the extent to which CV induction is reflected by a transcriptional response. We were able to utilize existing knowledge of CV-associated genes to generate a reference set against which we could compare our observed transcriptional changes. We found that almost all of our reference set were identified as significantly upregulated during hypo-osmotic stress. Moreover, we identified a SNARE protein NPSNB as being upregulated during hypo-osmotic stress and showed that it is associated with sites of contact between CVs and the plasma membrane. This is an important and encouraging result that suggests that transcriptomic approaches might be more broadly applicable in the characterisation of CV-associated genes from organisms which remain, to date, entirely unexplored.

Employing this membrane trafficking-focused approach, we also saw interesting patterns in the regulation of membrane trafficking machinery as a whole. Vesicle coats and tethers remained largely

unaffected, whereas SNARE proteins and Rabs appeared to undergo a large amount of transcriptional regulation. It is tempting to hypothesize that this might represent an increase in membrane flux, driven by increased rates of Rab-mediated membrane identity conversion and SNARE-mediated fusion, while maintaining the veracity of cargo sorting and organelle fusion through coat and tether driven recognition.

Overall, we saw a marked upregulation of Rab and SNARE proteins predicted to function at early and late endosomal membranes, and those associated with secretion at the plasma membrane. We therefore speculate that the CV membrane of *D. discoideum* is drawn from these sorting pathways with likely little involvement of membrane derived from the early biosynthetic pathway. We recently reported an apparent CV-related expansion of plasma membrane secretory SNAREs in ciliates, suggesting the involvement of these gene products in CV function (Kaur et al., 2022).

In summary, we have demonstrated regulation of CV-associated transcripts in *D. discoideum*, implicating specific sets of membrane-trafficking machinery in CV function and with implications for the organelle homology of the *Dictyostelium* CV. These general observations form a framework against which other organisms can be assessed, via this broadly applicable transcriptomic approach, coupled with focused assessment of membrane trafficking machinery, which has the potential to capture this regulation and provide predictive detail of novel CV-associated gene products.

MATERIALS AND METHODS

Cells and culture conditions

For transcriptomic studies, *D. discoideum* DH1 (Dicty Stock Center, Northwestern University, Chicago, IL, USA) were grown axenically at 21°C in shaking culture at 150 rpm in HL5 medium, which has an osmolarity of ∼0.1 M (Gamper et al., 1999). For induction of hypo-osmotic stress, medium was replaced with Milli-Q reverse osmosis H_2O and cells cultured for 1 h at room temperature. CV induction and cell viability post hypo-osmotic exposure was confirmed by live-cell imaging (Movie 2).

NPSNB cloning

The NPSNB gene (DDB G0285365) was amplified from *Dictyostelium discoideum* AX2 (Kay laboratory) genomic DNA using PCR. The forward (5′) primer (5′-GCAGTCAGATCTATGGCCGATTTACAAGAAAATG-3′) contained an additional BglII restriction site, and the reverse (3′) primer (5′-CT GACGACTAGTTTATTGGGTTGAACCAGTAACAT-3′) contained a SpeI restriction site. The product was ligated into non-integrating (extra-chromosomal) expression vectors (Veltman et al., 2009), amplified using *Escherichia coli* DH5α, and verified by Sanger sequencing. Specifically, we used the pDM1207 (for N-terminal GFP chimeras) plasmid, which drives overexpression of chimeras with the actin15 promoter and drive expression of a G418 resistance cassette with the cofillin A promoter.

Ectopic NPSNB expression

D. discoideum AX2 (Kay laboratory) cells were cultured in Erlenmeyer flasks at 22°C suspended in liquid HL5 medium (Formedium, Hunstanton, UK; Ashworth and Watts, 1970) in an orbital shaker at 180 rpm. Amoebae were transformed via electroporation as described previously (Gaudet et al., 2007). For this, 3×10^7 cells were harvested from liquid culture at a density of $\sim1\times10^6$–5×10^6 cells/ml (log phase) by centrifuging at 300 ***g*** for 3 min, resuspended in H-50 electroporation buffer and put on ice. For each transformation, 5 µg of plasmid DNA was pipetted into a sterile 1 mm electroporation cuvette (at 4°C), to which 100 µl of cell suspension (also at 4°C) was added. This mixture was electroporated using a Bio-Rad gene pulser electroporator (exponential voltage curve, 750 V, 25 µF, ∞ Ω resistance). Two pulses were applied to the cells with 5 s between pulses. The cuvette was then set on ice for 5 min, and then the electroporated cells were incubated in HL5 medium in Petri dishes at 22°C overnight. Transformants were then selected using G418 at 10 µg/ml.

Imaging

Prior to imaging, transformed *D. discoideum* AX2 cells were grown to confluence in plastic tissue-culture plates, with KK_2 buffer (16.5 mM KH_2PO_4, 3.9 mM K_2HPO_4 and 2 mM $MgSO_4$) containing a suspension of heat-killed *Klebsiella pnumoniae* at an optical density at 600 nm (OD_{600}) of 5. Also, 50 µg/ml tetracycline and 100 µg/ml dihydrostreptomycin were added to prevent contamination, and again 10 µg/ml G418 was added for selection. Imaging was performed using a Zeiss LSM 710 confocal microscope with a 63× (oil) objective lens. Time series (videos) were captured by sampling at 5 s intervals.

Image analysis

For measurement of the relationship between NPSNB fluorescence and CV positioning, a macro (available upon request) was written for FIJI to extract coordinates and associated fluorescence intensity from line plots drawn manually around the limiting membrane of the cell. The CV was identified and traced in corresponding phase images and the centroid was calculated (Fiji). These measurements were used to derive the spatial relationship between NPSNB distribution and CV position. In order to compare multiple cells with differing expression levels, NPSNB fluorescence was normalized to peak fluorescence along the line-plot and background subtracted. The mean of normalised fluorescence intensity for each 0.5 µm bin of distances was then calculated.

Transcriptomics

Total RNA from *D. discoideum* was extracted from six samples, with three biological replicates per condition, using the Qiagen RNeasy mini kit. RNA concentration was measured using a NanoPhotometer spectrophotometer. Samples were submitted to The Applied Genomics Core (TAGC), University of Alberta. Libraries were constructed using the Illumina TruSeq RNA Library Preparation kit and run on an Illumina NextSeq 500 system. Reads were aligned and quantified using the Galaxy web platform (https://usegalaxy.eu/). Reads were mapped to the *D. discoideum* genome (release 2.7) using HISAT2. Mapping quality was assessed using MultiQC. Aligned reads were assigned to genes (release 2.7.50) using FeatureCounts. Statistical analysis was carried out with Voom/Limma. A false discovery rate cut off of <0.01 was applied to assign differentially expressed transcripts. The raw reads for this project have been deposited in the NCBI Sequence Read Archive as BIOPROJECT PRJNA909995 (http://www.ncbi.nlm.nih.gov/bioproject/909995).

Phylogenetic analysis

Amino acid sequences were aligned using MUSCLE v3.8.31 with default parameters (Edgar, 2004). Positions that did not exhibit clear homology were removed from the alignments prior to phylogenetic analysis. Models of sequence evolution were selected using ModelFinder (IQtree) (Kalyaanamoorthy et al., 2017). Maximum likelihood analysis was performed using IQtree (Nguyen et al., 2015). Bayesian analysis was performed using MrBayes (Ronquist and Huelsenbeck, 2003). For this, 10^7 MCMC generations were run with a burnin of 25%, and a splits frequency of less than 0.01 was reached indicating convergence. IQtree and MrBayes analyses were run on the CIPRES server (Miller et al., 2010). The resulting trees were visualized using FigTree v1.4.0.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.B.D.; Formal analysis: P.T.M., I.R.-M., E.K.H.; Investigation: P.T.M., L.D.B.; Writing - original draft: P.T.M., J.B.D.; Writing - review & editing: P.T.M., L.D.B., I.R.-M., E.K.H., J.B.D.; Visualization: L.D.B.; Supervision: J.B.D.; Project administration: J.B.D.; Funding acquisition: J.B.D.

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Data availability

The raw reads for this project have been deposited in the NCBI Sequence Read Archive as BIOPROJECT PRJNA909995 (http://www.ncbi.nlm.nih.gov/bioproject/909995).

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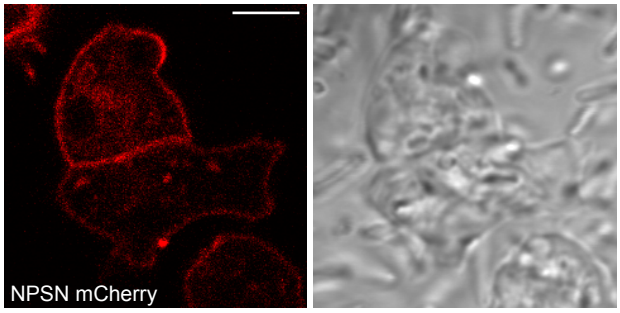

Fig. S1. NPSN mCherry distribution.

Localisation of ectopically expressed NPSN mCherry in AX2 cells. Scale bar = 5 µm.

Table S1. All significantly upregulated (A) and downregulated (B) genes following exposure to hypoosmotic conditions.

Click here to download Table S1

Table S2. Candidate list of previously identified contractile vacuole associated genes in *D. dictyostelium*

Click here to download Table S2

Table S3. All significantly upregulated genes associated with membrane trafficking function on the Gene Ontology

Click here to download Table S3

Table S4. Manually curated list of *D. dictyostelium* genes corresponding to well characterised and conserved membrane trafficking machinery

Click here to download Table S4

Table S5. All significantly upregulated Rab protein genes

Click here to download Table S5

Table S6. All significantly upregulated SNARE protein genes

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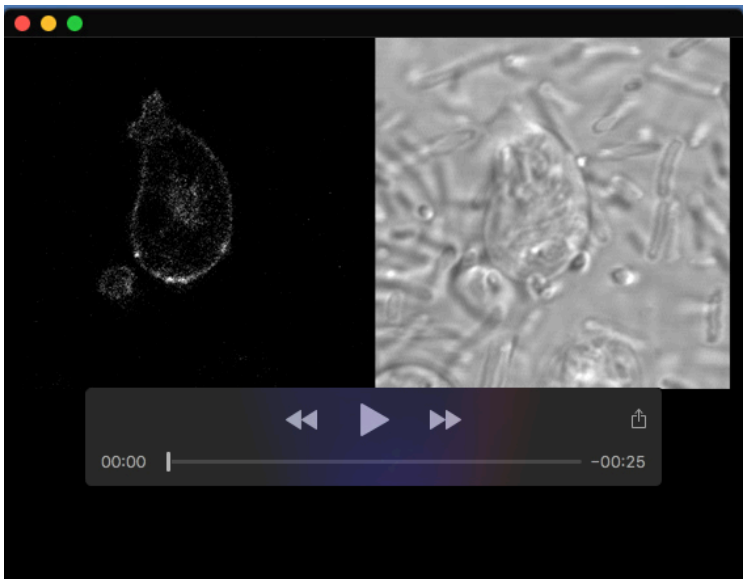
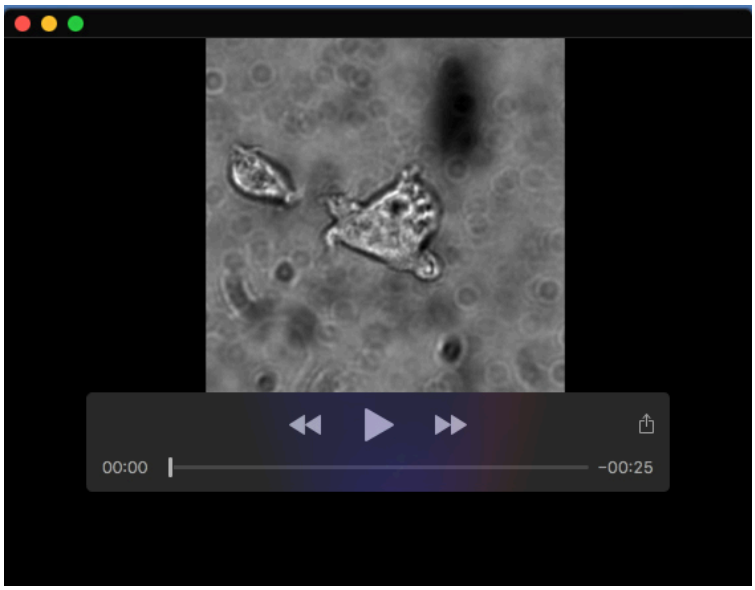
Table S7.

Click here to download Table S7



Movie 1. NPSN GFP dynamics.
Time lapse video microscopy of NPSN GFP ectopically expressed in AX2 cell. Contractile vacuole is visible in phase contrast imaging. Related to data in Figure 3B.



Movie 2. Amoeba viability under hypo-osmotic conditions. Time lapse video microscopy of *Dictyostelium discoideum* DH-1 after exposure to hypo-osmotic conditions as described, demonstrating that the amoebae were intact and viable as shown by movement, cellular integrity, and contractile vacuole action. Related to the Material and Methods section "Cells and Culture conditions".