A functional connection of *Dictyostelium* paracaspase with the contractile vacuole and a possible partner of the vacuolar proton ATPase

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Dictyostelium discoideum possesses only one caspase family member, paracaspase (*pcp*). Two separate mutant cell lines were first analysed: one cell line was an over-expressed GFP-tagged Pcp (GFP-Pcp), while the other cell line was a *pcp*-null (*pcp*-). Microscopic analysis of cells expressing GFP-Pcp revealed that Pcp was associated with the contractile vacuole membrane consisting of bladder-like vacuoles. This association was disrupted when cells were exposed to osmotic stress conditions. Compared with wild-type cells, the GFP-Pcp-over-expressing cells were susceptible to osmotic stress and were seen to be very rounded in hypo-osmotic conditions and contained more abnormally swollen contractile vacuole. Cells with *pcp*- were also rounded but had few, if any, contractile vacuoles. These observations suggest that Pcp is essential for *Dictyostelium* osmotic regulation via its functioning in the contractile vacuole system. Subjecting these cells to selected contractile vacuole inhibitor provided additional support for these findings. Furthermore, yeast two-hybrid system identified vacuolar proton ATPase (VatM) as the protein interacting with Pcp. Taken together, this work gives evidence for an eukaryotic paracaspase to be associated with both localization in and regulation of the contractile vacuolar system, an organelle critical for maintaining the normal morphology of the cell.

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1. Introduction

Programmed cell death is a genetically controlled mechanism that is regulated by a series of signals in the environment (Lu and Chen 2011). Caspases have been described as major regulators of apoptotic cell death (Vercammen *et al*. 2007). In 2000, two novel caspase-like proteins were discovered: paracaspases and metacaspases (Uren *et al*. 2000). Metacaspases were found in organisms missing caspases including fungi, plants and parasitic protozoa, while paracaspases were discovered in metazoans and *D. discoideum*. Caspases, metacaspases and paracaspases belong to the clan CD, family C14 cysteine proteases (Mottram *et al*. 2003; Carmona-Gutierrez *et al*. 2010). Both metacaspase and paracaspase genes encode proteins with caspase enzymatic active sites consisting of the conserved p20 subunit. This subunit is a structure conserved in all caspases, and it is necessary for substrate recognition and catalysis (Aravind and Koonin 2002; Lamkanfi *et al*. 2002). Despite the structural similarity between caspases, metacaspases and paracaspases, they differ in substrate specificity (Váchová and Palková 2007). Caspases cut their substrate at the specific aspartate residue at the C-terminal side, while metacaspases have been shown to cut with strict arginine/lysine substrate specificity or at the arginine substrate (Vercammen *et al*. 2004; Watanabe and lam 2005; Vercammen *et al*. 2006; Gonzalez *et al*. 2007). On the other hand, it has been shown that human paracaspase mucosa-associated lymphoid tissue 1 (MALT1) cleaves after an arginine residue in the P1 position (Coornaert *et al*. 2008). Typically, paracaspase protein in *Dictyostelium* does

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not have the caspase death domain or the immunoglobulin domains that are present in higher metazoan caspases (Uren *et al.* 2000). This difference in paracaspase structure may suggest a different function.

Numerous studies have presented incompatible data about metacaspase and paracaspase function in programmed cell death pathways. Functional analyses in different organisms have shown that metacaspases may play the same role that caspases do in apoptosis. It has been shown that the *Saccharomyces cerevisia* metacaspase Yca1 is involved in aged cell apoptosis. The overexpression of metacaspase Yca1 makes the yeast cells more sensitive to hydrogen peroxide stress (Madeo *et al*. 2002). In *Plasmodium falciparum,* metacaspase can induce apoptosis characterized by DNA fragmentation and the disruption of transmembrane mitochondrial potential (Meslin *et al*. 2007). Although caspases generally function in apoptosis, not all members of this family follow the trend. A study provided evidence that *Leishmania mexicana* metacaspase does not have a role in cell death; instead, this protein acts as a negative regulator of amastigote growth (Castanys-Muñoz *et al.* 2012). In comparison, it was demonstrated that MALT1 in humans also has an alternative caspase function. Diffuse large B cell lymphoma (DLBCL) is recognized by constitutive NF-κB activity. It was shown that treat DLBCL cell with the paracaspase MALT1 protease inhibitor blocks A20 and BCL10 cleavage, the negative regulator of NF-κB, which interne reduces the expression of NF-κB targets genes. Thus, the paracaspase MALT1 protease inhibition could be a novel drug target for lymphoma treatment (Ferch *et al*. 2009).

In *Dictyostelium,* a single-cell haploid amoeba, starvation causes the amoeba to aggregate and form multicellular structures better suited for survival. During this development process, cells differentiate, and some cells even undergo apoptosis. Interestingly, in *Dictyostelium,* paracaspase null cell lines (*pcp*-) demonstrate normal development when forming the multicellular stalk (Roisin-Bouffay *et al*. 2004). Additionally, double mutants, *pcp-atg1*-, do not differ from the wild type in autophagic and necrotic cell death processes (Lam *et al*. 2007). Thus, *Dictyostelium* caspase-like protein may have cellular functions other than cell death related caspase activity.

The contractile vacuole system in *Dictyostelium* is comprised of tubules and vacuoles (Gerisch *et al*. 2002). The tubules act as collecting structures to gather the excess water to insure the cells survival in a hypo-osmotic environment. The vacuoles fuse with the plasma membrane, open a pore to release water from the cell body, and then reconnect to the network (Gerisch *et al*. 2002; Heuser *et al*. 1993). During the discharge phase, the contractile vacuole collapses and flattens against the plasma membrane to maintain the distinct membrane components (Heuser 2006). Several proteins have been found to be essential for the contractile vacuole complex structure and function in *Dictyostelium* including Calmodulin (Ca^{2+}-binding protein), vacuolar proton pumps v-H^+-ATPase, Rab 11, as well as RabD (Zhu and Clarke 1992; Fok *et al*. 1993; Harris *et al*. 2001; Clarke *et al*. 2002; Bush *et al*. 1994).

D. discoideum is an appropriate model organism for paracaspase functional studies since it possesses only one caspase family member, paracaspase (Roisin-Bouffay *et al*. 2004). In this study, we tested the hypothesis that paracaspase may be activated in cellular functions other than apoptosis. To assess the localization and function of *pcp* in *Dictyostelium*, GFP-Pcp overexpression and *pcp*- cell lines were tested and their cellular functions were analysed. Our observation provides evidence that the paracaspase protein is associated with the contractile vacuole bladder. Supporting this idea, the yeast two-hybrid system was used to detect the Pcp protein partner within the contractile vacuole. We found that the paracaspase overlaps with vacuolar proton pump v-H^+-ATPase.

2. Materials and methods

2.1 *Cells and culture conditions*

For all experiments, *D. discoideum* cell lines (wild-type AX4, pDneo2a-GFP,GFP-Pcp, and *pcp*-) were grown axenically at 21°C and shaken at 150 rpm in HL5 medium (1% oxoid proteose peptone, 1% glucose, 0.5% yeast extract, 2.4 mM Na_2HPO_4, and 8.8 mM KH_2PO_4, pH 6.5). Cells were grown to a density of 1–4 × 10^6 cells/ mL. To minimize background fluorescence from the HL5 medium, cells were incubated in 'Loflo' medium for 24 hours before experimentation (*www.dictybase.org*). It should be noted that the *pcp*- clon was kindly provided by Dr Pierre Golstein.

2.2 *Cloning pcp and creation of a GFP-Pcp cell line*

The sequence of *D. discoideum* paracaspase was obtained from the online database, Dictybase.org (*http://dictybase.org/gene/DDB_G0293196*) and NCBI Gene Bank: AF316600). Based on this sequence, primers for PCR were designed with addition of *Sal*I and *Xho*I recognition sites DNA to the forward and reverse primers, respectively. The primer sequences (GTCGACA ATG GCA TAC CCC TAC GGA G) were the forward primer and (CTCGAGTTA CAT GAT GAA CTG GGC G) were the reverse primer. The PCR product was verified to be of the expected size (~1200 bp) by electrophoresis. It was then cloned using the TA vector (Invitrogen) and sequenced for both errors and confirmation for the presence of the N-terminal *Sal*I sites and C-terminal *Xho*I sites. The *pcp* PCR TA product was next digested with *Sal*I and *Xho*I, purified, and ligated into expression plasmid

pDneo2a-GFP previously cut by *Sal*I and *Xho*I (Dubin and Nellen 2010). This vector construct pDneo2a-GFP-*pcp* was sequenced for errors and reading frame conformation. The pDneo2a-GFP-*pcp* vector were then transformed into *Dictyostelium* cells as previously described (*www.dictybase.org*) and properly expressing cells selected via use of G418 antibiotic selection.

2.3 *Western blot*

Proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore Corp.) using a Hoefer transfer unit (Bush *et al*. 1994). Proteins were transferred for 3 h at 4°C and 100 V in transfer buffer. The resulting blots were then incubated with primary antibody (1: 2000 dilution of a mouse monoclonal anti-GFP antibody). The cells were then washed, incubated with goat anti-mouse secondary antibody conjugated to horse radish peroxidase (Phototope®-HRP Western Blot Detection Kit, New England Biolabs), and visualized by exposing the membrane to X-ray film for 60s. The developed film was checked for a protein band at 73 kDa (*pcp* 46KDa + GFP 27KDa), the predicted size of GFP-Pcp.

2.4 *Contractile vacuole visualization: GFP visualization and Calmodulin immunofluorescence*

Cells were harvested and allowed to settle on a glass cover slip. Fluorescence was viewed and photographed at 1000× magnification using the BrightLine® GFP filter set on a Nikon 2000SE microscope and IPLab 3.7 software. A primary antibody, anti-Calmodulin, was used to visualize Calmodulin in the cells. Cells were harvested then incubated at room temperature in a solution composed of 2% formaldehyde in PBS, 0.1% DMSO, and 1/3% protease peptone. Next, the cells were chilled in methanol containing 1% formaldehyde at −20°C for five minutes. Cells were washed three times in PBS for antibody staining (Bush *et al*. 1996; Fok *et al*. 1993; Heuser *et al*. 1993) and then blocked with a 1:20 dilution of normal goat serum (NGS) in a 0.1% BSA/ PBS solution at 37°C for 30 min. The primary antibody, mouse anti-Calmodulin, was diluted 1:400 in 0.1% BSA/ PBS (Hulen *et al*. 1991; Zhu *et al*. 1993). The cells were incubated with anti-Calmodulin at 37°C for 30 min and washed three times with PBS/ 0.5% Tween-20. The secondary antibody, RITC goat anti-mouse IgG (1 mg/ mL), was diluted 1:200 in PBS/0/1percentage BSA (Zhu *et al*. 1993) and added to the cells. Incubation with the secondary antibody was at 37°C for 30 min. The cells were then washed three times with PBS/ 0.5% Tween-20, once with water, and then air-dried (*http://dictybase.org/techniques/*). Calmodulin fluoresced red were visualized using the BrightLine® TXRED filters set. Comparisons were made to GFP localization, visible under the BrightLine® GFP filter set.

2.5 *Contractile vacuole visualization: FM® 1–43 staining*

To visualize the contractile vacuole for some experiments, cells were dyed with FM®1-43 (lipophilic styryl dye *N*-(3trietylammoniumpropyl)-4-(4-(dibutylamino) styryl) pyridiniumdibromide; Molecular probes). First, cells were harvested and allowed to settle on a glass cover slip. Next, FM®1-43 was added for a final concentration of 16 µM in KK2 phosphate buffer **(**1.6 mM KH_2PO_4 and 40 mM K_2HPO_4**)** and chilled for 1 min on ice. Cells were then washed and visualized using the BrightLine® GFP filter set and the BrightLine® TXRED filter set.

2.6 *Lysosomes visualization: LysoTracker® staining*

Ax4, pDneo2a-GFP, GFP-*pcp,* and *pcp*-cell lines were harvested and allowed to adhere on cover slips in HL5 medium. The medium then was replaced with fresh medium containing 100 nM LysoTracker®. Incubation time was 30 min at room temperature. Fluorescence in the cells was visualized using the BrightLine® DAPI filter set, where lysosomes fluoresced blue, and the BrightLine®GFP filter set for comparison with GFP localization.

2.7 *Endosome visualization: RITC-dextran loading*

RITC's red fluorescence was used to mark endosomes. First, cells were harvested and allowed to settle in a 35 × 10 mm Petri dish containing 2 mL of HL5 medium. Next, 40 µL of 100 mg/mL RITC-dextran were added to the cells with incubation for either 60 or 180 min. Cells were then collected, washed, and allowed to settle on a glass cover slip. All cell lines were photographed using the BrightLine® TXRED filter set to visualize endosomes and the BrightLine® GFP filter set to visualize GFP-Pcp.

2.8 *Golgi visualization: Wheat germ agglutinin staining*

Wheat Germ Agglutinin (Sigma Aldrich) is a Golgi-specific stain. Cells were harvested and allowed to settle on a glass cover slip. 50 µL of WGA (1.0 µg/mL) were added to the cells. The cells were incubated at 37°C for 10 min, then washed twice with Loflo medium. Fluorescence was visualized using the BrightLine® TXRED filter set, where the Golgi appeared red, and the BrightLine® GFP filter set for comparison with GFP localization.

2.9 *Osmotic stress assays*

The contractile vacuolar network was visualized under the microscope using the BrightLine® filter set. Comparisons were made for cells in an isotonic, hypotonic, and hypertonic environment. Cells were incubated for 60 min in HL5, water, or 100 mM sucrose in HL5 medium, respectively. The cells were harvested and allowed to settle on a glass cover slip, then viewed and photographed.

2.10 *Concanamycin A inhibitor assay*

Concanamycin A (CMA) is an ATPase inhibitor specific to the contractile vacuole (Temesvari *et al.* 1996). Inhibition effects on the different cell lines were studied under isotonic, hypotonic, and hypertonic environments. First, cells were harvested and allowed to adhere to a glass cover slip. Loflo medium, water, or 100 mM sucrose in Loflo medium were added, respectively. CMA (sigma) was added to all treatments at a final concentration of 5 μM. Additionally, AX4 and *pcp*- cell lines were treated for 1 min on ice with the dye FM®1-43 for visualizing the contractile vacuole. Fluorescence from either GFP or FM®1-43 was viewed and photographed using the BrightLine® GFP filter set.

2.11 *Yeast two-hybrid analysis*

The Gal4 yeast two-hybrid system was used to test four potential protein partners for paracaspase. The yeast strain PJ69-4A (kindly provided by Dr Fusheng Tang), a Leucine-Tryptophan strain, was used. The strain utilizes HIS3, ADE3, and lacZ as reporter genes regulated by Gal4 (James *et al.* 1996). *Dictyostelium pcp* was cloned into the *Sal*I and *Bgl*II recognition sites of the binding domain (BD) plasmid pGBD-C3. The *Dictyostelium* cDNA of suspected protein partners, *calA*, *rab11A*, and *rabD* was cloned into the *Sal*I and *Bgl*II recognition sites of the activation domain (AD) plasmid pGAD-C3. *VatM*, due to an internal *Bgl*II site, was cloned into the *Eco*RI and *pst*I recognition sites of pGAD-C3. Yeast-harboring pGBD-*pcp* was transformed with pGAD-*calA*, pGAD-*rabD*, pGAD-*rab11*, or pGAD-*vatM*, using the lithium acetate procedure (Gietz *et al.* 1995). The successfully transformed cells were selected in minimal medium lacking Tryptophan (Trp), Leucine (Leu), Histidine (His), and Adenine (Ade). Additionally, Trp-Leu plates supplemented with X-gal (Sigma) were used to confirm the expression of lacZ reporter gene.

Once interaction between Pcp and VatM was suggested by the yeast two-hybrid analysis, the experiment was repeated with truncated *pcp* in order to determine which regions are key to the interaction. First, *Spe*I and *Bgl*II sites were used to remove 478 bases on the 3′ end of *pcp*. Another truncated version of *pcp* was made using *Stu*I and *Bgl*II sites to remove 989 bases on the 3′ end. Restriction digestion was followed by gel purification using a Qiagen kit. Next, PFU DNA polymerase (Promega) was added to fill the overhangs. Incubation was at 72°C for 20 min. The DNA was then purified using a Qiagen PCR cleanup kit. The two truncated *pcp* versions were cloned into the pGBD plasmid separately, followed by transformation of *E. coli* cells. Plasmid-carrying colonies were confirmed by restriction digestion and gel electrophoresis of the DNA. Each pGBD plasmid, containing either truncated *pcp* (*pcp*-I and *pcp*-II), was then co-transformed with pGAD-*vatM* into yeast cells. Transformed cells were selected in minimal medium lacking Trp, Leu, His, and Ade. Additionally, Trp-Leu plates supplemented with X-gal (Sigma) were also used to confirm expression of lacZ as a reporter gene for the strong interaction.

2.12 *β-Galatosidase assay*

To confirm the interaction between VatM and Pcp proteins, β-galactosidase activity was quantified using the Beta-Glo® Reagent (Promega) kit. Yeast cultures were grown in Leu-Trp selective media to low density (A_{600} of 0.1–1.0). Cultures were diluted in water to an A_{600} of 0.1 (Hook *et al.* 2006) before usage in the Beta-Glo® Reagent kit. Samples were read by the Microtiter® Plate Luminometer B36580 using Revelation MLX software.

3. Results

3.1 *Confirmation of GFP-Pcp expression in D. discoideum*

D. discoideum paracaspase gene (Dictybase gene information, http://dictybase.org/gene/DDB_G0293196) encodes a 46.536 KDa of Pcp protein. Pcp visualization was conducted by genetically inserting a green fluorescent protein (GFP) sequence in frame with the paracaspase gene (pDneo2a-GFP-*pcp*). *Dictyostelium* cells have been transformed with pDneo2a-GFP-*pcp* plasmid via electroporation. The presence of the fusion protein was verified by Western blotting analysis using anti-GFP antibodies. A single band of 73 KDa corresponds to the total molecular weight for Pcp and the GFP fusion protein (Pcp 46 KDa + GFP 27 KDa) (Figure 1A).

3.2 *Dictyostelium paracaspase associates with the contractile vacuole system*

A variety of experiments was carried out to determine the location of paracaspase within the cell. In each experiment, a specific organelle was marked with a fluorescent dye. Overlap of fluorescence from GFP-Pcp and an organelle may

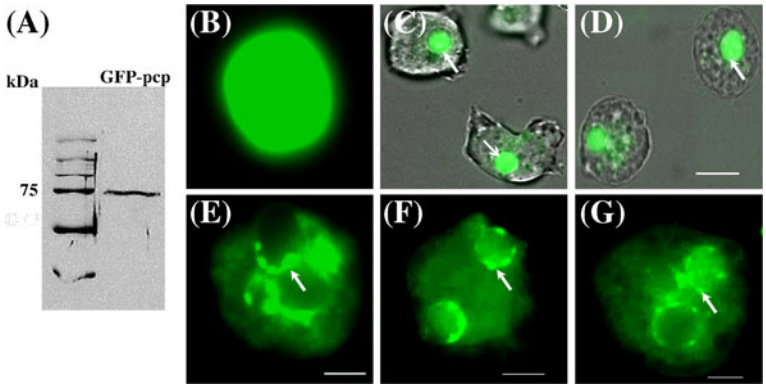

Figure 1. *Dictyostelium* Pcp is associated with the CV membranes. (**A**) Protein samples were subjected to SDS-PAGE followed by Western blot analysis to confirm the expression of GFP-Pcp (73 kDa) using an anti-GFP antibody. (**B**) Visualization of the GFP expressing in pDneo2a-GFP cells. (**C**, **D**) Overlaid GFP-Pcp visualization (green channel) with the bright field suggests a possible localization of GFP-Pcp with the CV system. (**E**, **F**, **G**) Visualize the GFP-Pcp location. The arrows indicate the contractile vacuole system where Pcp appears to be associated in the contractile vacuole membrane. Bar 5 μm.

suggest that the protein is located within that organelle. Initially, the location of GFP was discerned by overlaying bright field images and green fluorescence images. Figure 1 represents an example of images of cells expressing GFP-Pcp. As can be seen in figure 1C and D, GFP-Pcp is associated with a very dynamic CV compared with the images of cells that express GFP protein only (figure 1B). A very prominent co-localization of GFP-Pcp with CV can be seen in figure 1E– G.

Microscopic approaches were taken to confirm that Pcp associates with the CV. Calmodulin is a protein that is greatly enriched in the membranes of the contractile vacuole (Zhu and Clarke 1992). Immunofluorescence microscopy was used to confirm that GFP-Pcp co-localized with this established CV marker. Figure 2 shows the results of these co-localization studies, in which cells were incubated with a primary antibody, anti-Calmodulin. Cells were then washed and incubated with a secondary antibody coupled with rhodamine (RITC goat anti-mouse IgG). GFP visualization was shown in green, while calmodulin antibody immunofluorescence was shown in red images. A merger of the two images reveals the sites of co-localization (yellow-orange). Figure 2B shows that the GFP signal was co-localized with calmodulin signal. The white arrowhead indicates the area of overlap between Pcp and calmodulin in the CV elements. Thus, we conclude that Pcp and calmodulin overlap in the same membrane system, namely the CV complex.

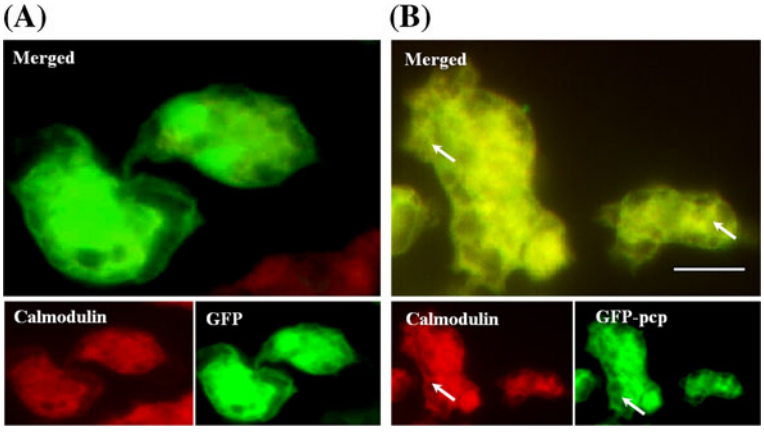

Figure 2. The expressed GFP-Pcp protein associates with the CV organelle marker protein, calmodulin. (**A**) Visualization of the GFP expressing in pDneo2a-GFP cells. Clearly, no overlapping between the GFP and calmodulin can be observed in these cells. (**B**) *Dictyostelium* Pcp is co-localized with the CV marker calmodulin. Overlaid GFP-Pcp visualization (green) and calmodulin antibody immunofluorescence (red) images show overlapping areas (yellow-orange) where the co-localization occurs (indicated by arrows). Bar 5 μm.

3.3 *GFP-Pcp was not found in lysosomes, endosomes or Golgi complex*

The presence of GFP-Pcp in other organelles has also been investigated. To determine whether GFP-Pcp is found in lysosomes, the marker LysoTracker (Molecular Probes) was used. LysoTracker® is a dye used in *D. discoideum* to mark acidic organelles, mainly lysosomes (Rodriguez-Paris *et al*. 1993). The fact that there was little to no overlap between GFP-Pcp (green) and Lysotracker (blue) suggested that GFP-Pcp is not associated with lysosomes (figure 3A).

To rule out the possibility that GFP-Pcp is present in endosomes, we examined the overlap of GFP-Pcp with endosomes that were marked with Rhodamineisothiocynate-dextran, a fluid that is internalized in endosomes but not degraded (Klein and Satre 1986). No overlap was discernible after RITC-dextran loading for 180 min. As controls, cell lines without GFP (AX4 and *pcp*-) were stained for 5 min with the dye FM®1-43 to confirm that there was no overlapping between the contractile vacuole and the endosomes (figure 3B). Next, to test if GFP-Pcp overlapped with the Golgi complex, cells were stained with Wheat Germ Agglutinin (WGA), a conventional Golgi marker (Iida and Page 1989). Cells displayed no overlap between GFP-Pcp and dyed Golgi components (figure 3C).

3.4 *Effects of different osmotic stress conditions on the CV function in Pcp over-expressing cell line*

The co-localization of GFP-Pcp and calmodulin (figure 2B) suggests that Pcp may have a functional role in the CV system in possible collaboration with CV localized proteins. To investigate the function of the Pcp protein in the CV system, cells were collected by centrifugation and suspended in hypo-osmotic medium (water) or hyper-osmotic (100 mM sucrose in HL5) for 1 h. Cells were spotted on cover slips for 10 min and then processed for immunofluorescence microscopy. Figure 4 presents both GFP-Pcp and *pcp*- cell lines compared to wild-type cells and pDneo2a-GFP (controls) under isotonic, hypotonic, and hypertonic environments.

In normal growth medium (isotonic condition), the wild-type AX4 and pDneo2a-GFP cell lines maintained an amoeboid shape. However, the GFP-Pcp cell line displayed an

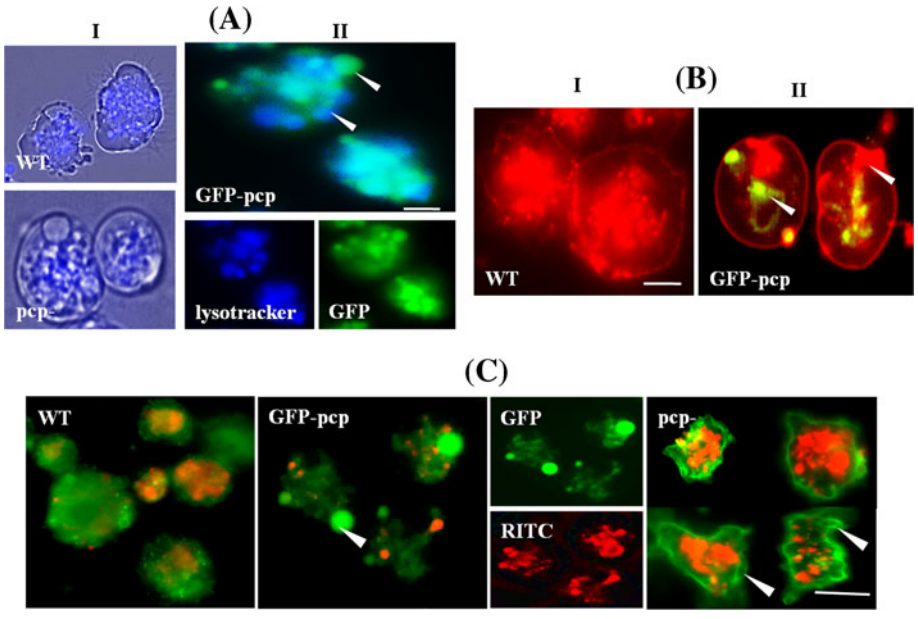

Figure 3. The expressed *Dictyostelium* GFP-Pcp protein does not associate with lysosomes, Golgi component markers or endosomes. (**A**) (**I**) Distribution of LysoTracker® dye in the AX4 and *pcp*- cell lines. (**II**) Overlaid images of LysoTracker® with GFP visualization in the GFP-Pcp cell line. GFP-Pcp (green) does not association with the lysosomal vesicles (blue). (**B**) Golgi complex stained with the Wheat Germ Agglutinin (WGA). GFP-Pcp cells showed that GFP signal (green) distinguish from WGA-staining Golgi vesicles (red) and they do not overlap (indicating by arrows). (**C**) The vesicles of the endosome shown in red by visualization of RITC-dextran after 180 minutes of treatment. The AX4 and *pcp*- cell line stained with FM®1-43 (green). Overlaid images of GFP-Pcp (green) and RITC-dextran (red) show no apparent association of GFP-Pcp with the endosome system. Cells expressing *pcp*-null mutant confirm stained CV, and the RITC-Dextran (endocytic marker) did not find inside the CV after a RITC-Dextran loading. Bar 5 μm.

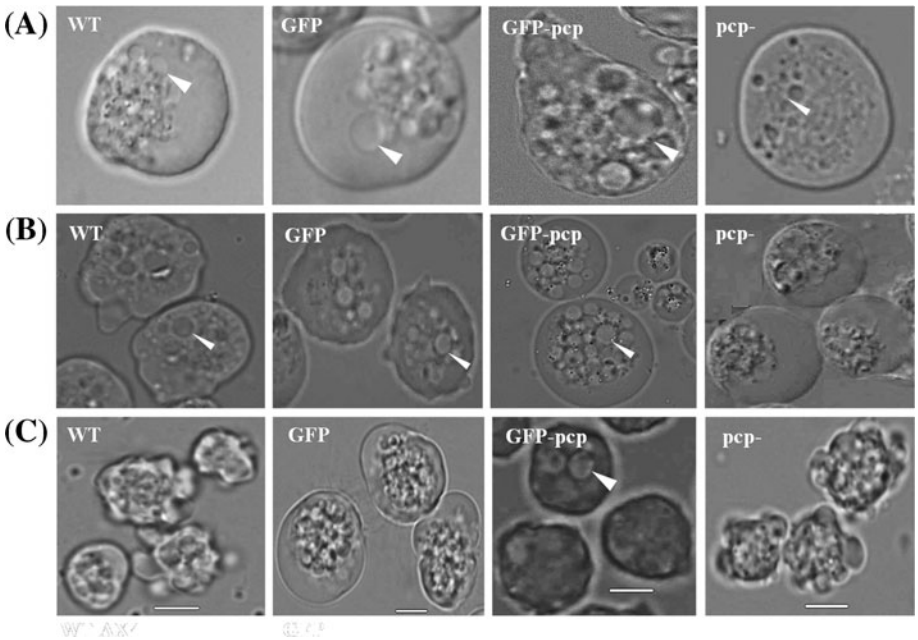

Figure 4. GFP-Pcp-expressing cells are defective in contractile vacuolar structure and function. (**A**) Cellular appearance in isotonic environment (HL5 medium). The GFP-Pcp cells appear different with enlarged contractile vacuoles compared to other cell lines. Cells with *pcp*- showed smaller contractile vacuoles. (**B**) Appearance in a hypotonic environment (60 minutes incubation in sterile water). The GFP-Pcp expressing cells were extremely rounded with more and larger contractile vacuoles compared to AX4 and pDneo2a-GFP cells. Cells with *pcp*- had circular shapes with fewer contractile vacuoles. (**C**) Appearance in a hypertonic environment (60 minutes incubation in HL5 medium containing 100 mM sucrose). Cells expressing GFP-Pcp had slightly enlarged vacuoles, while cells with *pcp*- appear similar to the control cells. Bar 5 μm.

increased number of enlarged contractile vacuoles, apparently due to the effects of the over-expression of the Pcp protein. It seems that these mutant cells have difficulty releasing water outside the cells. Interestingly, *pcp*-cells were smaller and aggregated into groups (figure 4A). In cells exposed to hypo-osmotic conditions (100% water), the shape of GFP-Pcp-expressing cells was abnormally large, rounded, and contained more enlarged CVs compared to both the control cultures of AX4- and GFP-expressing cells. Cells with *pcp*- were also rounded; however, they had few if any CVs (Figure 4B). For cells exposed to 100 mM sucrose in HL5 (hypertonic stress), GFP-Pcp appeared similar to the control cells with slightly enlarged vacuoles. The large CVs that were seen in the GFP-Pcp cells under hypotonic stress were no longer seen in conditions of hypertonic stress – most likely due to the loss of water from the cells. The *pcp*- cell line was smaller than the control cells and had a rounded appearance (figure 4C).

With respect to all of the data shown in figure 4, the Pcp over-expressing cells and *pcp*- cell lines were clearly unable to perform successful osmoregulation as demonstrated by the extreme appearances of the cells. It is reasonable to conclude that defects in the CV system function were due to Pcp over-expression or knockout mutant.

3.5 *GFP-Pcp expression under the CV inhibitor effect (Concanamycin A)*

Concanamycin A (CMA) is an inhibitor that affects vacuolar ATPases and has been shown to disrupt CV function (Temesvari *et al*. 1996). During the inhibitor disruption, phenotypic variations in the Pcp over-expressing and the *pcp*- cells may elucidate the role of Pcp in the contractile vacuole. The phenotypes were tested under different osmotic conditions. All cell lines were exposed to isotonic, hypotonic and hypertonic stress conditions. Additionally, the cell lines were treated with CMA to inhibit vacuolar function.

CMA effects on GFP distribution in cells over-expressing GFP-Pcp as compared to the FM®1-42 probe, a dye that stain the CV in the non-GFP expressing AX4 and *pcp*- cells, were investigated (figure 5). Under isotonic condition and CMA treatment, the disassociation of the spongiomal network of the CV system was evident in the wild-type cell line. In cells expressing pDneo2a-GFP plasmid (only GFP), the green signal was very bright. Cells over-expressing GFP-Pcp still had some CV (figure 5A). Surprisingly, in *pcp*- cells, the CVs appeared to fuse back with the cell membrane. Under hypotonic stress (figure 5B), the wild-type cells have been shown to disassociate in the CV network due to CMA

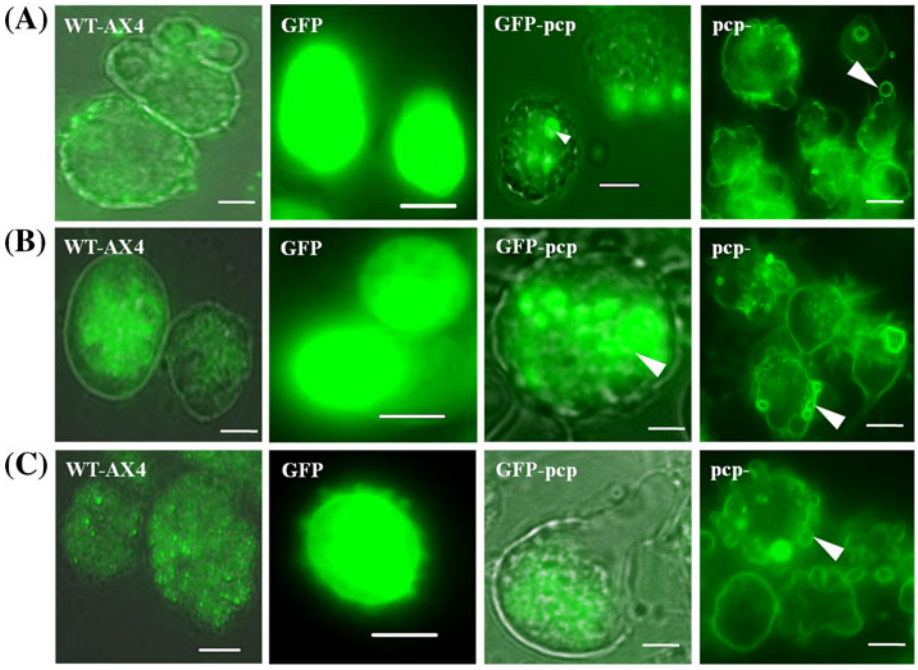

Figure 5. The contractile vacuole structure is altered in cells expressing GFP-Pcp after treating with Concanamycin A (CMA) inhibitor. Distribution of GFP in the GFP-Pcp cell line and FM®1-43 staining of the CV system in the AX4 and *pcp*- cells treated 60 minutes with 5 µM CMA. (**A**) Treat the cells with CMA in loflo media (isotonic condition). Disassociation of the spongiomal network of the CV system is evident in the WT-AX4. Cells with *pcp*- had some CV. (**B**) Treatment with water (hypotonic condition). Disassociation of the spongiomal network has seen in the CV system in WT-AX4 cells. Abnormally enlarged CVs have seen in the GFP-Pcp cell line (indicated by arrow) while the CV were smaller in *pcp*- cells and fused back to the cell membrane. (**C**) Treat the cells with CMA in loflo medium containing 100 mM sucrose (hypertonic condition). In the WT-AX4 and GFP-Pcp cell lines, little disassociated CVs network have seen due to the water lost by cells in the hypertonic environment. Again, cells with *pcp*- showed CVs that clearly fused back with the cell membrane. Bar 5 µm.

treatment. Abnormally large contractile vacuoles were clearly visible in the GFP-Pcp cell line, while the *pcp*- cell line contained small vacuoles fused to the cell membrane. Under hypertonic stress, CMA-treated cell lines all had similar phenotypes. The cellular morphology was normal, and the CV network was not prominent due to water expulsion. Cells that express *pcp*-mutant again show fused CVs within the cellular membrane (figure 5C).

3.6 *Identification of VatM as binding partner for Pcp protein using yeast two-hybrid system*

The data from this project have shown that Pcp protein associates with the contractile vacuolar system and causes differences in osmoregulation function. The alternation effect of *pcp* on these cellular functions causes us to believe that Pcp may have a partner protein(s) that also associates with the contractile vacuolar system. Previous studies have shown that CalA, RabD, Rab11 and VatM proteins are also associated with the contractile vacuolar system (Zhu and Clarke 1992; Fok *et al.* 1993: Bush *et al.* 1994; Harris *et al.* 2001). In order to determine whether Pcp has a cellular interaction with these contractile vacuolar proteins, the yeast two-hybrid system was used. The yeast strain PJ69-4A was co-transformed with a bait plasmid pGBD-*pcp* and the prey plasmid pGAD-*calA,* pGAD-*rabD,* pGAD-*rab11A* or pGAD-*vatM*. To confirm the positive interaction, we used pGBD-*pcp*+ pGAD, pGAD-a vacuolar protein+ pGBD and pGBD+ pGAD as a negative interaction protein for Pcp. All transformed yeast cell lines were cultured onto the Sc-Leu-Trp-His-Ade selected plate, an indicator for strong interaction. Positive protein interaction was only observed in yeast cells in which both the pGBD-*pcp* and pGAD-v*atM* were transformed (figure 6). In addition, the same clones were cultured in Sc-Leu-Trp plates that were supplemented with X-gal substrate to confirm expression of lacZ as a reporter gene for the protein interaction. A high expression for the β-galactosidase enzyme (green colour) was also observed in yeast cells in which both the pGBD-*pcp* and pGAD-*vatM* were transformed.

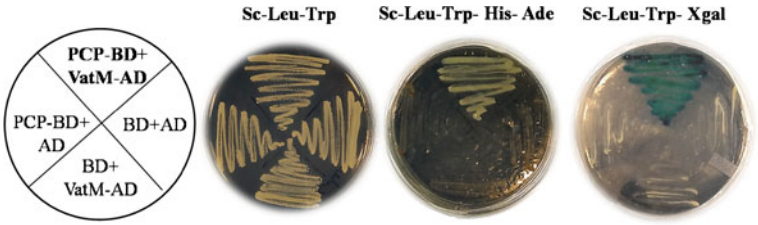

Figure 6. Identification the interaction of Pcp and VatM proteins using the yeast two-hybrid system. Pcp coding region was fused in-frame to the Gal4 DNA binding domain in pGBD-C3 'bait' plasmid. *VatM* that amplified from *Dictyostelium* cDNA library fused to the Gal4 activation domain in pGAD-C3 'pray' plasmid. Yeast strain PJ69-4A transformed with the indicated constructs (left). Transformed yeast cells containing both pray and bait plasmids streaked out on selective plates. A serial of graphical representation show the successful growth of pGBD-*pcp*+ pGAD-*vatM* transforming yeast cells on the selective media confirming the interaction between Pcp and VatM protein.

Next, we wanted to determine specifically which region of Pcp interacts with VatM protein. This information could aid in determining which domain is necessary for the Pcp protein to function. Our analysis of the *pcp* gene's sequence revealed two common cutting sites that were unique for both the *pcp* gene and the pGBD plasmid restriction enzymes that make it possible to cut and remove regions of the Pcp protein domain. Cutting out part of the *pcp* allowed us to see if it could still interact with the *vatM*. If the last region of the Pcp protein is needed to interact with the VatM protein, then the mutated Pcp protein should show negative results for interaction. On the other hand, if the middle region of Pcp protein is needed for interaction, then we would see positive results for the first mutation and negative results for the second mutation. This would give us a general idea of which part of the protein is needed for the interaction for future studies. Figure 6 shows that there is an interaction between the original Pcp and VatM protein. After the last 478 bp region or 989 bp region was cut out of the *pcp* (figure 7A), there was no growth in the Sc-leu-Trp-His-Ade selective plates. In addition, no colour expression was seen in the X-gal plates (figure 7B, C). Thus, we can conclude that VatM interacts with the last domain of Pcp.

Finally, in order to confirm the interaction between VatM and Pcp proteins, β-galactosidase activity was quantified in all yeast two-hybrid cell lines using the Beta-Glo® Assay System. In three independent experiments, Pcp and VatM showed meaningful interaction compared to other yeast cell lines ($P < 0.05$) (figure 8).

4. Discussion

Until recently, there were numerous conflicting data about the role of metacaspase and paracaspase in programmed cell death and their molecular function is unknown. *D. discoideum* has no caspase; instead, it has a single paracaspase, which does not have the caspase death domain that is present in higher metazoan caspases (Uren *et al*. 2000; Lam *et al*. 2007). For a unicellular organism capable of development from stalk to spores, such as *D. discoideum*, this development would seem to be an ideal alternative to apoptosis. Not much is known about *pcp* in *Dictyostelium*. However, it has been reported that the *pcp*- mutant in *Dictyostelium* cells does not change the cell death rate in either the developmental or vacuolar autophagy cell death processes (Roisin-Bouffay *et al.* 2004), and the double mutants, *pcp-atg1*-, do not affect necrotic cell death processes in *Dictyostelium* (Lam *et al*. 2007). Thus, we need to gain insight into the localization and cellular function of paracaspase in *Dictyostelium*.

Visualization of the GFP-Pcp showed an interesting pattern suggesting that Pcp co-localized within the CV network. This co-localization has been confirmed by using a CV marker, immunostained calmodulin (Zhu and Clarke 1992). This type of association of the CV network has already been observed for *Acanthamoeba castellanii* metacaspase in *Dictyostelium* (Saheb *et al*. 2013). The CV network controls intracellular water balance by accumulating and expelling excess water from the cell, thus allowing the cells to survive under hypotonic stress. Dysfunction of the CV complex can cause cell swelling and lyses (Gerisch *et al*. 2002). It was shown that the cell death caused by hyperosmotic stress in *S. cerevisiae* is not a lytic process; instead, it is a metabolic process characterized by metacaspase activation (Silva *et al*. 2005). Our results show that cells overexpressing Pcp protein were enlarged, rounded and have a highly active CV. However, these CV failed to fuse with each other or release excess water from the cell. On the other hand, cells with *pcp*- had also morphological alterations in their CV membrane structure and function. These cells had a defect in CV formation which caused a failure to release excess water from the cell. This differential response to the loss of the Pcp protein vs the over-expression of it under these stress conditions revealed abnormally functioning CVs in the mutant cell lines. Thus, this data suggest that Pcp is involved in CV membrane trafficking, fusion, and/or remodeling and is

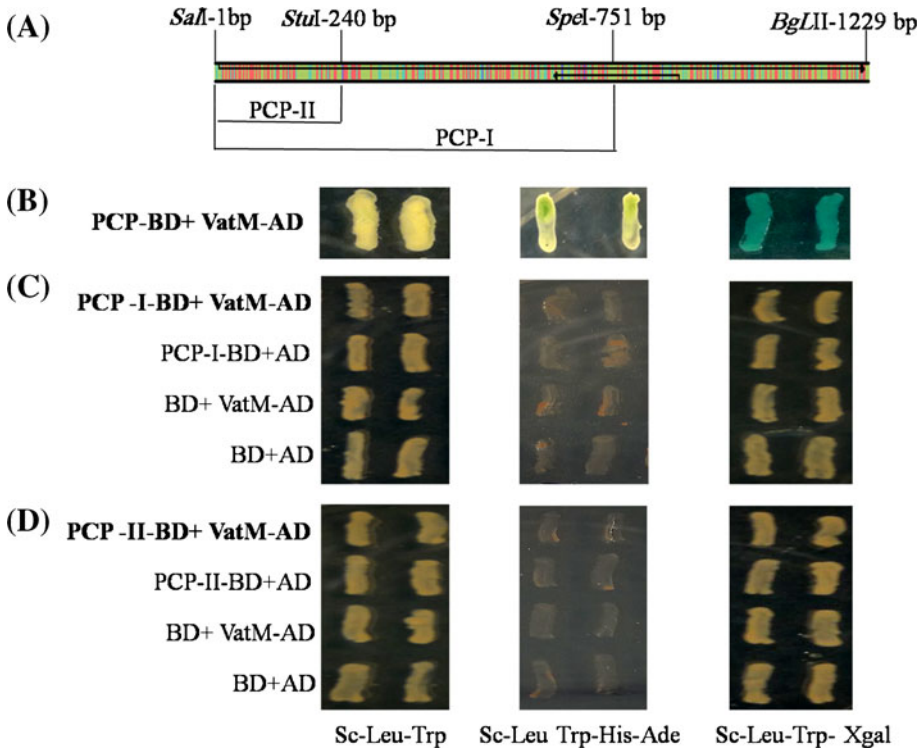

Figure 7. Two-hybrid expression studies have determined the essential protein domains required for Pcp protein to interact with vatM protein. (**A**) The construction of the truncated *pcp* gene mutants. Analysis of the restriction map with either *Spe*I+ *Bgl*II or *Stu*I+ *Bgl*II recognition sites of *pcp* DNA fragments using a freeware program pDRAW to be used to create the truncated version of *pcp* gene (*pcp*-I and *pcp*-II). (**B**) Successful growth of pGBD-*pcp*+ pGAD-*vatM* transforming yeast on the selective media. (**C**) Cut out the last region (478 bp) of *pcp* gene using *Spe*I and *Bgl*II restriction enzymes and then fused in-frame to the Gal4 DNA binding domain in pGBD-C3. Yeast strain PJ69-4A transformed with the indicated constructs (left). Transformed yeast cells containing both pray and bait plasmids streaked out on selective plates. No interaction was seen between mutated Pcp-I and VatM in the selective plates. (**D**) Cut out the last 989 bp of *pcp* gene using *Stu*I and *Bgl*II restriction enzymes to create the mutant *pcp*-II gene and then fused in-frame to the Gal4 DNA binding domain in pGBD-C3. No interaction had shown between Pcp-II and VatM on the selective media.

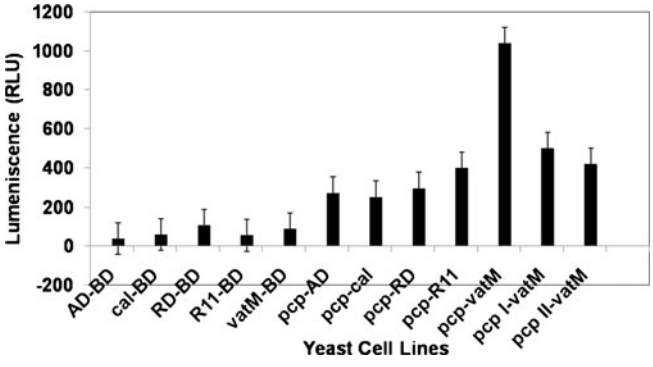

Figure 8. Two-hybrid-based β-galactosidase activity in transformed yeast cells expressing the Pcp protein along with proteins expressed from the denoted second gene constructs. Beta-Glo® Reagent added to yeast grown to an A_{600} of 0.1 and incubated for 60 min. β -galactosidase activity measured as luminescence and presented as relative light units (RLU).

important for proper formation and functioning of the CV in *Dictyostelium*. Additional studies were conducted in order to investigate the possibility that the Pcp protein associates with other organelles within the cell. However, Pcp did not appear to be associated with lysosomes, endosomes or Golgi components.

Owing to the close proximity of Pcp in the CV network, the effects of v-H^+-ATPase inhibition by CMA presented the possibility that Pcp has some function in the CV network in relation to v-H^+-ATPase. The v-H+−ATPase is a highly conserved enzyme that transports protons across the membrane. It is delivered to the plasma membrane by fusion with endosomes (Clarke *et al*. 2010). It has been shown that CMA in *Dictyostelium* reduces Ca^{+2} uptakes in the calcium sequestering vesicles containing v-H^+-ATPase in the CV complex (Xie *et al*. 1996). When cells were subjected to osmotic stress and under the effects of CMA, enlarged contractile vacuoles with Pcp association were seen in the GFP-Pcp cell

line (Figure 5B). This led to the conclusion that Pcp function may overlap with the v-H^+-ATPase within the contractile vacuole.

It has been reported that *Dictyostelium* Rab11 localizes with the CV complex and has a role in regulating membrane traffic to keep the CV function in osmotic regulation (Harris *et al.* 2001). Furthermore, a study showed that *T. brucei* metacaspases proteins co-localize with RAB11 (Helms *et al.* 2006). These facts led to the hypothesis that Pcp may have some function in collaboration with other proteins that also associate with the CV. As reported here, in yeast two-hybrid assays using full-length *pcp* as bait, the vacuolar proton ATPase (VatM) was identified as a protein partner of Pcp. The data also indicate that the last 478 bp are vital for Pcp to interact with VatM. When Pcp was fused with green fluorescent protein and expressed in *Dictyostelium*, the results showed that this protein localized with the contractile vacuole. The discovery that Pcp interacts with VatM, an enriched protein in the contractile vacuole membranes (Liu and Clarke 1996), would support this finding. It has been reported that the number of proton pumps v-H+−ATPase in the contractile vacuole membranes is ten times greater than in the endosomes and lysosomes (Rodriguez-Paris *et al.* 1993; Clarke and Heuser 1997) which suggests it is likely to play an important role in contractile vacuole function. This enzyme is responsible for the accumulation of water by the contractile vacuole (Liu and Clarke 1996; Mitchell and Hardham 1999). It energizes fluid accumulation of the contractile vacuole (Clarke *et al.* 2002). Cells over-expressing Pcp had highly active contractile vacuoles while mutant cells with the *pcp*- have been shown defective in the CV formation. These results suggest that Pcp interact with v-H+−ATPase to regulate the CV function. Thus, when Pcp is over-expressed in the cell, this may induce abnormally high v-H+−ATPase activity which ultimately causes highly active contractile vacuoles. On the other hand, *pcp*- may have decreased v-H+−ATPase activity leading to mutant cells that had little to no CV functional activity in these cells.

Not much is known about the molecular function of caspase-like proteins (metacaspases and paracaspase). However, some studies have demonstrated potential functional roles of the paracaspase protein. For example, Ferch *et al.* (2009) demonstrated that MALT1 protease inhibition may provide a treatment for lymphoma. Furthermore, MALT1 and caspase 8 have several similarities (Hachmann *et al.* 2012), and there is a functional relation between the caspase and paracaspase families to facilitate non-apoptotic phenomena within these cells (Kawadler *et al.* 2008). In comparison, it has been shown that *T. brucei* MCA4 is important for parasite virulence during mammalian infection because it is expressed mainly in the bloodstream form of the parasite (Proto *et al.* 2011). Thus, these studies suggest that paracaspase and metacaspase proteins have other functions in the cell – not merely an apoptotic PCD role. These involve instead cellular viability and/or stress signaling pathways. Data analysis from the current study has provided the first evidence that the eukaryotic caspase-like protein, *Dictyostelium* paracaspase, possibly associates almost exclusively with the CV system. Additionally, it appears that paracaspase is crucial in the formation of an organelle important in osmotic regulation via its role in interacting with the CV localized v-H+−ATPase. Confirmation of paracaspase playing a role in cellular stress response pathways in *Dictyostelium* provides an understanding of how these essential processes occur. In addition, findings concerning the location and function of *Dictyostelium* paracaspase within cells will provide new insight into its potential as a drug target.

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