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Superoxide dismutase C and Ras affects Dictyostelium Contractile vacuole morphogenesis and function

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

SUPEROXIDE DISMUTASE C AND RAS AFFECTS *DICTYOSTELIUM*
CONTRACTILE VACUOLE MORPHOGENESIS AND FUNCTION

A dissertation submitted in partial fulfillment of
the requirements for the degree of
DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Adwait Kabra

2017

To: Dean Michael R. Heithaus
College of Arts, Sciences and Education

This dissertation, written by Adwait Kabra, and entitled Superoxide Dismutase C and Ras Affects *Dictyostelium* Contractile Vacuole Morphogenesis and Function, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2017

DEDICATION

To my Family and friends for their support

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ABSTRACT OF THE DISSERTATION
SUPEROXIDE DISMUTASE C AND RAS AFFECTS *Dictyostelium*
CONTRACTILE VACUOLE MORPHOGENESIS AND FUNCTION.

by

Adwait Kabra

Florida International University, 2017

Miami, Florida

Professor Lou W. Kim, Major Professor

Dictyostelium cells lacking SodC displayed severely compromised CV morphogenesis and function. The *sodC*⁻ cells were vulnerable to hypotonic stress and showed poorly developed CV tubules, significant increase in the number of CV bladders, and a prolonged charging phase. Alleviation of the intracellular superoxide level in *sodC*⁻ cells with the cytoplasmic Superoxide dismutase A (SodA) or superoxide scavenger XTT significantly reduced the number of CV bladders to the level of the wild type, but the discharging step was still hampered. Introduction of redox insensitive RasG(C118A) mutant in *sodC*⁻ cells or inhibition of RasG target PI3K with LY204002 also significantly reduced the number of the CV bladders. In contrast to the superoxide scavenging, obstruction of the RasG downstream PI3K signaling aggravated the discharging step. It is plausible that other superoxide sensitive small GTPase's such as Rab8 is likely persistently activated in LY204002 treated or RasG(C118A) expressing *sodC*⁻ cells. In addition, we report that *sodC*⁻ cells not only suffer from high level of RasG activity, but also from low level of

RacC. Furthermore, *sodC*⁻ cells treated with amiloride EIPA, which inhibits sodium proton exchange, rescued aberrant regulation of Ras, but not RacC. The EIPA treated *sodC*⁻ cells displayed a significant increase in directional cell migration, reduction of the bladder number to the wild type, and significantly improved bladder charging and discharging. We also found that heavy chain of Myosin II is essential for normal architecture of CV system as *mhcA*⁻ cells displayed defects in both, morphogenesis and function of contractile vacuole.

Thus, rectifying the dysregulation of RasG and PI3K signaling, global suppression of superoxide level with XTT or SodA expression in *sodC*⁻ cells rescued CV morphogenesis. In contrast, inhibition of NHE proteins with EIPA in *sodC*⁻ cells induced near complete rescue of both CV formation and function. EIPA may not only affect the RasG/PI3K signaling, but may also modulate other unidentified signaling components of the functional CV cycle.

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ABBREVIATIONS AND ACRONYMS

ABBREVIATION	FULL NAME
BSA	Bovine serum albumin
cAMP	3'-5'-cyclic adenosine monophosphate
CV	Contractile vacuole
DNA	Deoxyribose nucleic acid
EIPA	(5-(N-Ethyl-N-isopropyl) amiloride
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathion S-tansferase
GTPase	Enzyme that hydrolyzes guanosine triphosphate
Hr	Hour
JH10	<i>Dictyostelium</i> auxotrophic wild type strain
I-BARa	Inverse-Beach Bin/Amphiphysin/Rvs
kD	Kilo Dalton
L	Liter

LB	Luria broth
LvsA	Large Volume Sphere protein
LY294002	Inhibitor of phosphoinositide 3-kinases
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
PAKa	p21 Activated Protein A
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
<i>sodC</i>	Ablation of Superoxide Discmutase C
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NHE	Na ⁺ /H ⁺ exchanger
NKCD	Asparagine, Lysine, Cysteine, Aspartic acid
NOX	NADPH oxidase
RBD	Ras binding domain
Ras	Rat sarcoma

μg	Microgram
μl	Microliter
μm	Micrometer
μM	Micromolar
μ	Micro-
<i>Wt</i>	Wild type
XTT	$\text{C}_{22}\text{H}_{16}\text{N}_7\text{NaO}_{13}\text{S}_2$

CHAPTER 1 INTRODUCTION

1.1 Role of Contractile vacuole in Protists.

A unicellular eukaryotic cell may encounter several distinct environmental conditions which can either be isotonic, hypotonic or hypertonic compared to their cytoplasm. The fluctuations of osmolality in the environment can be stressful for cells and could even be lethal if persistent, which is why cells are equipped with specialized organelles that mediate the regulation of these fluctuations and protect cells from osmotic damage. In response to hyposmotic and hyperosmotic stresses, animal cells do swell and shrink mainly by utilizing Na^+/K^+ transporters (Tosteson and Hoffman, 1960). However, equipped with the protective barrier such as the skin, these cells are better protected from varying osmotic stresses than unicellular amoeboid cells.

Unicellular eukaryotic organisms often encounter fluctuating osmolality in their habitats. The isotonic condition is the last thing they would expect, and instead rainfall or drought would be more normal circumstances. To survive such hostile environments, they either developed a cell wall or contractile vacuole, a specialized organelle dedicated to control osmolality. Contractile vacuoles are found in protists such as *Dictyostelium*, *Paramecium*, *Trypanosomes*, *Leishmania* and, to a lesser extent in unicellular green algae such as *Chlamydomonas* (Docampo, et al., 2013). Although the structure of the contractile vacuole (CV) differs in the organisms it is found, the function of the CV is conserved among all. *Paramecium* was one of the first organisms studied in great length to elucidate the CV biogenesis and function. Previous studies demonstrated that there are two contractile vacuoles found in *Paramecium*, one at each end, which function by filling and

discharging alternatively (McKanna, 1976). The two vacuoles were found to be connected by radiating canals that would fill by collecting the water from the cytoplasm and discharge through a tiny orifice which is 1.2 μ m long and 1 μ m in diameter (McKanna, 1974). Furthermore, studies conducted on *Chlamydomonas reinhardtii* demonstrated that there are two contractile vacuoles situated at the anterior of the cell, closer to the basal bodies (Luykx, et al., 1997). The size of the contractile vacuole increases during cell growth, and contraction of the CV depends on the osmotic strength of the environment. Since *Chlamydomonas* utilizes contractile vacuoles to adapt to the media of different osmotic strength but does not vary the cytosolic osmolarity or membrane permeability, under an isotonic environment there are no contractile vacuoles seen in *Chlamydomonas* (Komsic-Buchmann, et al., 2014). Interestingly, *Trypanozome cruzi*, a parasite which uses mammalian and insects as host, must resist severe osmotic challenge faced during its developmental cycle. Everytime the parasite passes through the kidney of a mammalian host it must overcome an osmotic pressure which is five times more than its isotonic conditions (Lang, 2007). The parasite happens to live in the gut of mammals, the intestinal fluid and shuttles between the phagolysosomes and the cytosol (Kollien, et al., 2001). The parasite can cope with such diverse changes in the osmolarity of the environment with the help of their strong osmoregulatory system.

To induce water influx into the CV under hypotonic conditions, these protists manage high concentration of osmolytes in their CV system either using ion channels and transporters on the CV or indirectly through fusion of the CV with smaller vesicles called acidocalcisomes enriched with calcium and polyphosphates (Docampo, et al., 2005). Acidocalcisome were first discovered in Trypanosomatids (Vercesi, et al., 1994, Docampo,

et al., 1995) and later in *Toxoplasma gondii* (Moreno and Zhong, 1996), *Plasmodium falciparum* (Garcia, et al., 1998, Luo et al., 1999, Marchesini et al., 2000), *Chlamydomonas reinhardtii* (Ruiz, et al., 2001) as well as in *Dictyostelium discoideum* (Marchesini, et al., 2002). Recently acidocalcisomes have also been identified in bacteria (Seufferheld, et al., 2003) and human platelets (Ruiz et al., 2004), which suggest that these organelles have been conserved from bacteria to humans. Electron microscopy revealed high concentration of phosphorous, pyrophosphates and polyphosphates complexed with calcium and other elements (Docampo and Moreno., 1999) in the lumen of acidocalsiome while the membrane of acidocacisome contained pumps (proton pumps and Calcium pumps) (Lu, et al., 1998), exchanger (Na^+/H^+ and $\text{Ca}^{2+}/\text{H}^+$) (Vercesi and Docampo., 1996) and aquaporin (Rohloff and Docampo., 2008) which suggest their role in metabolic functions.

A previous study conducted on *Toxoplasma gondii* demonstrated that knocking down of Ca^{2+} -ATPase from the acidocalcisomes resulted in defects of virulence and invasion of the parasite (Luo, et al., 2001). Apart from serving as storage compartment for elements such as Calcium, magnesium, sodium, potassium, zinc, iron, phosphorous and basic amino acids, acidocalcisome play key role in regulating intracellular pH and osmoregulation (Docampo, et al., 2005). Previous study on *T.brucei* showed that knocking down of the Vacuolar proton pump of acidocalcisomes resulted in failure of the parasite to achieve pH homeostasis when kept in basic pH (Lemercier, et al., 2002). *Dictyostelium* cells also show the presence of acidocalcisome and can be identified as small granular particles localized near the contractile vacuole membrane (Marchesini, et al., 2002a). By subcellular fractionation technique, it was found that the buoyant fraction contained the markers specific to contractile vacuoles while the other fraction contained mass-dense

granules specific to acidocalcisome (Marchesini, et al., 2002a). Acidocalcisome and contractile vacuole together assist the *Trypanosomes* survive under extreme hyposmotic and hyperosmotic conditions. A previous study on *T.cruzi* found that there is a surge in the levels of cyclic AMP when the parasite is subjected to hypoosmotic stress. The increased cAMP resulted in modification of microtubule function leading to the trafficking of TcAQP1 from acidocalcisome to the contractile vacuole (Rohloff and Docampo, 2008). Transfer of TcAQP1 to the CV assisted in sequestering of water from cytoplasm in to the contractile vacuole which eventually helped in expelling the water in the flagellar pocket.

1.2 Dictyostelium as a model organism to study contractile vacuole system.

Dictyostelium discoideum has been widely used to study the signaling pathways in the cell since it shows significant level of conservation with its mammalian counterparts (Eichinger, et al. 2005, Muller-Tauberger, et al. 2013). Signaling pathways regulated by G proteins, small GTPases Ras, PI3K/Akt signaling, F-Actin remodeling by modulation of Arp2/3 as well as Formin dependent Myosin II activity are some of the examples which are well studied using *Dictyostelium* as a model organisms.

It is an ideal model organism for studying proteins and their effector molecules of the contractile vacuole system because it is a social amoeba which often inhabits hypotonic environments and thus needs to have a strong contractile vacuole system. It can be easily cultured in the laboratory in vegetative stages and suspension cultures for biochemical analysis which makes it an outstanding candidate to study the regulatory circuits of contractile vacuole complex (CVC). Furthermore, Because of its fully sequenced haploid genome it is possible to create knockouts and knockdowns to study the effect of desired

genes or proteins in the cell. Proteins tagged with GFP can be expressed in the cell as a fusion protein which help us to study the localization of the desired protein in the cell by microscopic analysis.

1.3 Generation of Reactive Oxygen Species and their effect on small GTPases

Reactive oxygen species such as superoxide, hydrogen peroxide and hydroxyl radical are constantly generated inside the body by the metabolic reactions (Palmer and Paulson, 1997). Some enzymes and enzyme complexes also produce oxygen radicals as their major activity or as a byproduct of it. Some examples are the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), the xanthine oxidase and the electron transport chain (Guzik, et al., 2002, Warnholtz, et al., 1999). Environmental changes can also affect the generation of reactive oxygen species, as a recent study demonstrated that rat cardiomyocytes when kept under hypoosmotic environment resulted in increased production of reactive oxygen species in the cell (Díaz-Elizondo, et al., 2006). There is substantial evidence which suggest involvement of reactive oxygen species in many human diseases such as cancer (Dreher and Junod, 1996), cardiovascular disease (Westhuyzen, 1997), neurodegenerative diseases such as Alzheimer's and Parkinson's (Knight, 1997). Additionally, some viral infections are also linked with surge in the production of ROS (Peterhans, 1997). Cells are equipped with anti-oxidant systems to attenuate the reactive oxygen species and act as a defense system for the cell by maintaining redox homeostasis in an event of oxidative stress (Martilaa, et al., 1988) but any dysregulation in their activity often leads to aberrancies in the cell behavior (Bloomfield and Pears, 2003).

Superoxide acts as a secondary messenger in the cell by targeting various transcription factors which would bring the changes in gene expression (Kamata & Hirata, 1999). One of the targets of superoxide is the Ras superfamily of GTPases which are involved in cell motility, proliferation, differentiation and apoptosis (Campbell et al., 1998, Cox and Der, 2003). Activity of these GTPases is determined by the binding of Guanosine-5'-triphosphate (GTP) or Guanosine diphosphate (GDP). The process is facilitated by Guanine nucleotide exchange factors (GEFs), which promote the exchange of GDP with GTP, thus activating Ras GTPases or GTPase-Activating Proteins (GAPs) which inactivate Ras proteins by exchanging the GTP with GDP (Boguski and McCormick, 1993). *In vitro* studies reviewed by (Heo, 2011) suggested the presence of redox sensitive motifs, either NKxD or P-loop or both, which interact with the bound guanine nucleotide. These motifs have cysteine at phosphate binding P-loop or the guanine base binding NKxD motif, both of which could be direct targets of superoxide and result in misregulation of these GTPases either by increasing or decreasing their activity. It has been reported that when Ras with a Cys118 in the NKCD motif comes in contact with the superoxide, it forms a thiol radical intermediate, Ras-S[•] 118. The radical acquires electron from the GDP which leads to the formation of G⁺DP. The superoxide then forms a 5-oxo-GDP which leaves Ras with not enough affinity to bind to GDP. The affinity of Ras is quenched by glutathione (GSH), but would result in Ras activation since GTP is more abundant in the cell as compared to GDP (Heo, 2011).

One of the member of Ras superfamily is Rab GTPase which plays prominent role in vesicular trafficking, where distinct members of Rab GTPase are involved in the steps of delivery, docking and fusion of intracellular vesicles (Somsel Rodman and Wandinger-

Ness, 2000). Out of the 54 different *Dictyostelium* Rab proteins, Rab8, Rab11a and Rab14 were shown to affect CV function. On the basis of *in vitro* studies, it was reported that Rab GTPases have redox sensitive motif (Heo, 2011) which can be misregulated under hypoosmotic stress. In a recent finding it was reported that in *Dictyostelium* hypoosmotic environment resulted in increased ROS production which had further effects on important regulatory molecules in the cell (Castillo, et al., 2016).

1.4 Function of Contractile vacuole in *Dictyostelium*

Dictyostelium discoideum is a social amoeba which lives in osmotically variable environments and thus needs to have a strong osmoregulatory organelle which can expel the excess water out of the cell quickly. In *Dictyostelium* the CV system consists of a central vacuole surrounded by the network of loose tubules and bladders also known as spongione. These tubules assist in collecting the water and fill the central bladder which prevents the vacuoles from bursting (Allen and Naitoh, 2002). The early phase of CV function, filling and enlargement of the contractile vacuoles, is assisted by the V-ATPase, a vacuolar proton pump localized at the membrane of the CV, which transfers protons into the CV lumen using ATP (Figure 1, step 2). The localization of V-ATPase to the membranes of CV tubules has also been reported in *Paramecium* (Fok, et al., 1995) where the vacuolar proton pumps ensure proper fluid segregation. The transferred protons combine with the ammonia, which is released as the byproduct of the protein degradation in the cytosol (Heuser, et al., 1993). Ammonia present in the cytoplasm can move across the membrane which combines with the protons coming inside the CV transported by CV membrane bound V-H⁺-ATPase to form Ammonium ion (NH₄⁺), which is one of the

osmolyte and attracts the water inside the CV (Docampo, et al., 2005). Presence of ammonium transporter (AmtB) on the CV membrane have been previously reported by (Kirsten, et al., 2008) which suggests the role of ammonium balance in the cell. There is evidence suggesting localization of chloride ion channels and an anion exchanger on the CV membrane (Cabantchik and Greger, 1992). The anion exchanger mediates influx of bicarbonate ions (HCO_3^-) into the lumen and efflux of chloride ions toward the cytoplasm. The bicarbonate ion is also an osmolyte which can induce water influx to the CV lumen.

The last phase is expulsion or discharge, where the contractile vacuoles move towards the plasma membrane and expel out the contents. Some proteins are associated with the CV membrane more than one phase while others are transiently involved at specific stages. During this discharging phase, protons associate with the carbonate ion, releasing carbon dioxide (CO_2) and water. The CO_2 transporter RH50 protein, transports CO_2 outside the CV (Benghezal, et al., 2001) and the ammonium ions are transported by the ammonium ion transporter (AmtB) (Kirsten, et al., 2008). The remaining water is then expelled outside the cell through transiently formed pore, which allows the discharge of CV contents but prevents the diffusion of the membrane components with the contractile vacuole membrane. The process was reported to be dependent on the function of Rab8 GTPase (Essid, et al., 2012).

1.5 Markers to study the Contractile vacuole

Apart from endoplasmic reticulum and Golgi apparatus, *Dictyostelium* has two major vesicle systems; Endosomal system and Contractile vacuole network. The endosomal pathway begins the particle uptake by phagocytosis or liquid uptake through

micropinocytosis (Hecker, et al., 1997) while the contractile vacuole network is a network of interconnected bladders and tubules (Heuser, et al., 1993). Various studies found that membranes of contractile vacuole and endosomes share two proteins; Vacuolar H⁺-ATPase (Temesvari, et al., 1996) and rabD, which is a rab14-like small GTPase (Bush, et al., 1994). The presence of common markers in their membranes suggest a relation between the two-vesicle system which could result from the fusion of their membranes (Bush, et al., 1996). Thus, to distinguish the CV system from the endosomal system, specific markers are required. The most commonly utilized marker to study the complete CV system is Dajumin-GFP, having GFP tag at the C terminal which labels both bladders and the tubules of the CV system (Gabriel, et al., 1999). It is a chimeric protein which is trafficked via cell membrane and is identified as a cargo, which is internalized by clathrin dependent mechanism (Macro, et al., 2012).

To study the contractile vacuoles before the discovery of Dajumin-GFP, lipophilic styryl dye FM4-64 and GFP-Drainin were utilized. The fluorescent dye, FM 4-64 labels only the bladders of the contractile vacuole but not the tubules (Heuser, et al., 1993). Also, these dyes are found to be associated with the CV up to 18-20 mins post exposure, after which they move to label non-specific organelle such as endosome. The GFP-Drainin on the other hand only labels the bladders of CV system since it is a peripheral membrane protein which is associated with the bladders during the discharge (Becker, et al., 1999). In order to visualize the CV bladders, different strategies have been applied such as raising antibodies specifically against the calcium binding protein, calmodulin (Zhu, et al., 1993) or GFP fusion proteins have been created with the gene encoding for vatM, a transmembrane subunit of the vacuolar proton pump, V-ATPase (Clarke, et al., 2002).

1.6 Contractile vacuole morphogenesis

The Contractile vacuole system is considered to be a post Golgi organelle which is evident from the CV localization of the Golgi-specific O-glycosylated proteins (Gabriel, et al., 1999). Most of the membrane proteins are commonly found in the CV membrane as well as other post-Golgi compartments such as endolysosomal system. It was found that there are 10 times more vacuolar proton pumps found in the membrane of CV as compared to the endo lysosomal system (Temesvari, et al., 1996, Neuhaus, et al., 1998) while another protein Golvesin, localizes only to the endosomal compartments not to the CV system (Schneider, et al., 2000). Previous studies have reported that the transfer of proteins from the Golgi network to the CV membrane occurs by the clathrin coated vesicles budding from the trans Golgi network (Boehm and Bonifacino, 2001). Clathrin is one of the coat proteins that facilitate endocytosis of vesicles from the plasma membrane or trans-Golgi to endosomes. In *Dictyostelium*, clathrin is also involved in the trans-Golgi trafficking together with the adaptor protein AP1. *Dictyostelium apm1⁻* cells, displayed mis-localization of the CV components and dysregulation of the CV system (Lefkir, et al., 2003). Furthermore, *chcA⁻* cells, which lacks Clathrin heavy chain, displayed no CV system, suggesting that clathrin is essential for the biogenesis of contractile vacuole (Macro, et al., 2012). The authors identified CV specific marker, Dajumin, only on the plasma membrane. In *Dictyostelium*, RabD is related to the mammalian Rab 14 which is responsible for the transport of cargo from Golgi to the endosomal compartments (Junutula, et al., 2004). The transport is facilitated by the vesicle budding adaptor protein AP-1 in mammals while it is utilized by *Dictyostelium* for the CV morphogenesis (Essid, et al., 2012, Lefkir, et al., 2003). In *Paramecium*, the spongiome of contractile vacuole is seen

decorated with the cisternae of rough endoplasmic reticulum (Patterson, 1980) suggesting a possible involvement of ER (directly or indirectly through Golgi) in the CV biogenesis but the role of Golgi in the CV biogenesis has been poorly understood.

As discussed previously, The contractile vacuole system consists of bladders and tubules which are interconvertible. The number of vacuoles depends on the osmotic challenge faced by the cells as well as cell stage (Gerisch, et al., 2002) while in the *Paramecium* contractile vacuole system is more stable with fixed differentiation of cisternae, ion channels and fusion pores (Plattner, 2013). The *Dictyostelium* CV system comprises of asynchronously contracting vacuoles which are connected by network of tubules which lies on the inner surface of the plasma membrane (Figure 1). The tubules can elongate, branch and fuse with one another or other bladders to collect excess water. The tubules are anchored to the actin network along which they extend or branch. The discharge phase of the CV is accompanied by several shape changes at the plasma membrane. At the time of filling, the cisternae take on a lenticular shape and remains attached to the cell cortex while during the discharge phase they fold themselves, eventually converting into tubules (Gerisch, et al., 2002).

Cells maintain unidirectional transport of water in the cisternae to avoid the back flow of water from cisternae to the tubules. It was found that during the discharge phase, the cisternae disconnects themselves from the lumen of the channel allowing them to empty the contents without any hindrance (Gerisch, et al., 2002). Once the discharge is completed, they convert themselves into the tubules which are connected to the entire system. The above phenomenon has also been well studied in the *Paramecium* (Allen and Tresini, 2000).

1.7 Proteins involved during contractile vacuole cycle

Previous studies using *Dictyostelium* uncovered multiple regulatory proteins that can be classified by their roles during the three main stages of the CV cycle as mentioned earlier: charging phase, enlargement or filling stage, and expulsion or discharge. Some proteins are associated with the CV membrane in more than one phase while others are transiently involved at specific stages.

Most of the proteins associated with the CV compartment have been found related to human proteins which suggests the preservation and function of these proteins has been conserved during evolution from lower to higher eukaryotes. One such protein, Drainin, which functions as an effector of Rab 11a (Figure 1, step 3), is associated with the CV during its discharge phase in *Dictyostelium*. The gene responsible for Drainin has also been mapped in the genome of *C. elegans* and humans (Becker, et al., 1999). Interestingly, Drainin lacks GAP activity because of the absence of arginine and glutamine in the catalytic subunit, but binds to the Rab11a *in vitro*. Drainin plays two important roles in CV cycle, upon activation it recruits inactivated Rab8 from the cytoplasm on to the CV membranes (Figure 1, step 3) which concludes the process of charging (Essid, et al., 2012) and secondly it stimulates the activity of Rab specific GAP, disgorgin (Du, et al., 2008). It was reported that GAP activity of disgorgin leading to hydrolysis of GTP-Rab8, is a necessary step required for tethering of CV bladder to the plasma membrane (Essid, et al., 2012) (Figure 1, step 4). Another important class of proteins, which are localized parallel to the localization of disgorgin are Lvs (Large Volume Sphere) proteins. There are 6 different Lvs proteins found in *Dictyostelium* (Gerald, et al., 2002) out of which LvsA is recruited from the cytoplasm to the CV membrane once it reaches its maximum diameter

(Figure 1, Step5), thus regulating the CV discharge and its morphogenesis (De Lozanne, 2003). Cells lacking LvsA were found incompetent in CV discharge and had abnormally large bladders (Du, et al., 2008).

The contractile vacuole system is not only required to maintain the water balance but is also essential for the calcium homeostasis. Cells need to maintain low intracellular Ca^{2+} ion concentration since high Ca^{2+} ion concentration can be lethal as well as basic environment is required for Ca^{2+} to act as a secondary messenger. Protists such as *Paramecium* and *Trypanosome* do not have calcium ion channels to sequester and extrude the calcium ions but in *Dictyostelium*, the Calmodulin protein binds to the Ca^{2+} ions and releases it outside the cell thus maintaining the homeostasis (Sriskanthadevan, et al., 2013). Previous studies also suggests the presence of gated calcium ion receptor, P2XA on the membrane of CV which play important role during the CV discharge (Parkinson, et al., 2014).

The force providing the capability of contraction to the CV has been a topic of discussion from long time. A study conducted by Heuser and others (2006), suggested that there is no involvement of the actin or myosin during the contraction of CV bladders which was drawn from electron microscopy data. Instead, their proposed model confides on the fact that the membranes of the CV have natural tendency to gain tubularity which causes the CV bladders to contract once they become spherical. They also argue that this would be a better explanation of the contractility as the arrangement of the phospholipids would be assymetrical. On the contrary, a recent study conducted by Betapudi and Eggelhoff (2009), suggests the involvement of conventional myosin II in this process. They argue that instead of driving the CV bladder toward the plasma membrane, these molecular

motors might directly assist in contraction during the discharge phase. They demonstrated that the myosin II null cells have defective CV bladder biogenesis as well as the bladders were defective in discharge which suggest a possible role of myosin II in CV discharge. Their proposed CV discharge model suggests that myosin II does not provides direct contractility to the CV instead it creates the cytosolic pressure which drives the CV bladders to collapse at the membrane, a process which is accompanied by the localization of LvsA at the CV membrane just before the discharge (Gerald, et al., 2002). The LvsA induces curvature in the membrane which acts as a source of tension for the CV bladders to discharge. During the contraction of the CV there are many changes occurring at the plasma membrane regarding the membrane curvature. This process is accompanied by the members of the superfamily of Bin/Amphipysin/Rvs (BAR)-domain proteins such as IBARa and F-BAR proteins (Linkner, et al., 2014). Another class of proteins, Mental Retardation GTPAse activating proteins, MEGAP are also involved with the CV during discharge phase. The MEGAP1 and MEGAP2 molecules play key role in the cytoskeletal arrangements and osmoregulation (Heath and Insall, 2008). The IBARa protein is localized at the CV tubules membrane (Figure 1, step 6) and is required to produce the necessary invaginations in the membrane as cells lacking IBRa show multiple CV phenotype (Linkner, et al., 2014a) while the MEGAP 1 and MEGAP 2 are associated with the tubules of the CV system and are responsible for the tubulation of the collapsed CV bladders (Heath and Insall, 2008), (Figure 1, step 6).

As the electron microscopic data by Heuser and others (2006) not reports the presence of cytoskeleton, Betapudi & others (2009) proposed that VwkA kinase removes the cytoskeletal barrier during the final stages of CV discharge which facilitates the

transient fusion of the CV membrane with the plasma membrane for expelling the water out of the cell through kiss-and-run mediated discharge as described by Essid and others (2008).

Contractile vacuole membranes are found to be closely associated with the actin-rich cortex and the CV tubules move along this cortex in *Dictyostelium* (Zhu and Clarke, 1991). In a study, it was demonstrated that *Dictyostelium* recruits another myosin, Type V myosin, MyoJ to the CV membrane. The MyoJ facilitates the movement of CV tubules, which are generated by the collapsed CV bladders at the plasma membranes after the discharge (Lung, et al., 2009). It was reported that MyoJ does not take part in providing contractility to the CV instead it navigates the branched actin filaments, which are formed on the plasma membrane at the base of CV bladders just before discharge (Heuser, et al., 2006), (Figure 1, step 5). The array of radiating actin filaments has pointed ends near the pore while the barbed ends are away from the pore. Since MyoJ is a barbed end directed motor it drives the tubules away from the site of bladder discharge.

The contractile vacuole system is a complex network of bladders and tubules. Specific proteins localize to CV bladders or tubules or both which regulate their morphogenesis and function. A summary of the proteins and their effector molecules involved is compiled in the table below.

Protein	Localization	Associated Function	Reference
	Bladder/Tubules		
V-H ⁺ - ATPase	Both	Responsible for the transfer of H ⁺ ions in CV lumen	(Heuser et al., 1993)
Calmodulin	Bladder	A calcium binding protein localized to the CV membrane facilitating the release of calcium.	(Zhu & Clarke, 1993)
Copine A	Both	Calcium Ion Binding protein	(Damer et al., 2007)
P2X receptor	Both	Calcium Ion channel gated by ATP. P2X null cells exhibit hypoosmotic shock defects and defects in discharge.	(Fountain et al., 2007) (Ludlow et al., 2009)
Ammonium Transporter (AmtB)	Both	Regulates/Balances Ammonium in CV	(Kirsten et al., 2008)
CO ₂ Transporter (RH50)	Both	Releases CO ₂ in the cytoplasm after the water is discharged	(Benghezal et al., 2001)
Rab8A	Both	Association of Rab8 to the CV membrane initiates the discharge phase of CV	(Essid et al., 2012)

Rab11a	Both	Controls the CV discharge. Dominant Negative Rab11a cells show enlarged CV.	(Harris and Cardelli, 2002)
Rab14 (RabD)	Tubules	Regulate Vesicular Trafficking between the CV and endocytic Pathways.	(Harris and Cardelli, 2002)
Disgorgin	Both	Rab8-GAP	(Du et al., 2008)
Drainin	Both	Recruited by Rab11a-GTP which localizes to the CV membrane during discharge phase.	(Becker et al., 1999)
LvsA	Tubules	Regulates CV biogenesis and discharge. Becomes localized to the CV once it reaches its maximum diameter.	(Gerald et al., 2002)
Clathrin	Both	Formation of CV membrane. Heavy and light chain null cells show defects in CV morphogenesis	(Heuser, 2006) (Stavrou and O'Halloran, 2006)
AP180	Bladder	Clathrin assembly protein regulating	(Wen et al., 2009)

the contractile
vacuole size.

I-BAR	Bladder	Associated with CV during its discharge phase. Proposed to provide invaginations to the CV membrane.	(Linkner et al., 2014b)
MEGAPs	Tubules	Involved in regulation of discharge event of contractile vacuole.	(Heath and Insall, 2008)
Myosin II	Both	Provides contractility to the CV. Myosin II null cells display large CV under hypoosmotic conditions.	(Betapudi and Egelhoff, 2009)
Golgesin	Both	Marker to study the post Golgi events	(Gerisch,et al., 2004, Schneider et al., 2000)
Dajumin	Both	Marker labeling the bladders and tubules. (Complete CV network).	(Gabriel et al., 1999)
Procaspase	Bladder	Associated with bladder. Overexpressing GFP-Pcp show swollen contractile vacuole and	(Saheb, et al., 2013)

rounded cell
morphology.

Table 1. The proteins listed above are either associated with bladders or tubules or both. The above experiments results are either from overexpression studies, null mutants or use of specific drugs which affects the CV system.

Figure. 1 Stepwise representation of contractile vacuole morphogenesis and proteins involved in regulating CV function.

1.8 References

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CHAPTER 2. ROLE OF SODC AND RAS ON CONTRACTILE VACUOLE MORPHOGENESIS AND FUNCTION.

2.1 Introduction

Dictyostelium discoideum is a soil dwelling amoeba which is found living in hypoosmotic environments. To survive under such drastic environments, *Dictyostelium* cells utilizes an osmoregulatory organelle, The contractile vacuole which ensures the proper discharge of the excess water coming inside the cell. Since the contractile vacuole protects the cell from bursting and is also utilized by many protists (Docampo, et al., 2013), the mechanisms of CV biogenesis and function drew significant attention. The contractile vacuole system consists of bladders and tubules which are responsible for its appropriate function (Heuser, et al., 1993). Previous studies uncovered the fundamental principles of CV morphogenesis and function (Gabriel, et al., 1999, Gerisch, et al., 2002, Maniak, 2003). Studies to uncover the mechanisms of CV function have mostly often been performed under hypotonic solutions such as water. However, it was recently shown that such condition induces the generation of reactive oxygen species (ROS) such as superoxide (Castillo, et al., 2016, Díaz-Elizondo, et al., 2006). Thus, cells actively discharging excess water through the CV system are under the influence of enhanced level of superoxide radicals.

In general, reactive oxygen species are considered as signaling messengers in various organisms including humans (Finkel, 1998). Previous studies suggests that cells require defined levels of superoxide for the optimal function of the cellular processes (Bloomfield and Pears, 2003). Superoxide is produced intracellularly as well as extracellularly by NADPH oxidase and is also released as by product from various

biochemical reaction taking place inside the cell (Thannickal and Fanburg, 2000). To counterbalance the oxidative stress, cells are equipped with anti-oxidant system which comprises of the non-enzyme (glutathione and vitamins) system and enzymes system such as SOD (superoxide dismutase), catalase, glutathione peroxidase, thioredoxin, and the peroxyredoxin family of proteins (Finkel, 2003, Pinkus, et al., 1996, Valko, et al., 2006). Superoxide dismutase is a membrane bound enzyme which causes dismutation of superoxide into hydrogen peroxide and molecular oxygen in two set of reactions with the use of metal ions as the co-factor. In the first reaction, the SOD bound metal ion is reduced which releases molecular oxygen while in the second reaction, reduced metal ion is oxidized in the presence of a proton which releases hydrogen peroxide (H_2O_2) and is recycled back to its original form (Fridovich, 1986, McCord and Fridovich, 1988, Mruk, et al., 2002). There are three different families of Superoxide dismutase which can be classified on the basis of their protein folding and metal co-factor binding. First is Cu/Zn Superoxide which binds to both copper and zinc and is most commonly utilized by eukaryotes (Richardson, et al., 1975), Second is iron/manganese SOD which is found either bound to iron (Fe-SOD) or manganese (Mn-SOD) and is most commonly found in mitochondria, chloroplast and in many bacteria (Borgstahl, et al., 1992; Crosa, 1989), Last is the nickel binding SOD which is only found in the prokaryotes (Barondeau, et al., 2004).

Extracellular superoxide dismutases are present from bacteria to humans and they protect the organism from various oxidative stresses. Many bacteria have periplasmic superoxide dismutase which are utilized by them during infection. Presence of SOD confers resistance from the oxidative stress created by enormous amounts of superoxide produced by the intracellular NADPH-oxidase (De Groote, et al., 1997). Mammalian cells

also utilize extracellular SOD to combat various oxidative stress. Endothelial cells are the main source of SOD in mammalian system but recently it was found that neutrophils also possess extracellular SOD through endocytosis (Iversen, et al., 2016). Immune cells utilizes the extracellular SOD to protect the tissue under inflammation. Previous studies have reported that the role of extracellular SOD is not limited to protect the cell from extracellular oxidative environment but also acts as an inducer of intracellular responses pertaining to changes in gene expression and suppression of cell cycle (Laurila, et al., 2009, Break, et al., 2012), as it was reported that loss of extracellular SOD results in progression of certain type of cancer (Teoh-Fitzgerald, et al., 2014, O'Leary, et al., 2015).

Dictyostelium has 5 isoforms of the SOD enzyme, namely SodA, SodB, SodC, SodE and SodF of which SodC is GPI anchored to the outer leaflet of plasma membrane (Veeranki, et al., 2008). Any disturbance in the balance between the anti-oxidant system and reactive oxygen species generation may have prominent effect on the intracellular signaling processes resulting in changes in gene expression (Palmer and Paulson, 1997). In one of the recent studies, it was found that *sodC*⁻ cells display only modest increase in the intracellular superoxide levels but it was sufficient enough to dysregulate the signaling events pertaining to Ras/PI3K (Veeranki, et al., 2008) while a previous study reported that overexpressing a cytosolic superoxide dismutase in the cell resulted in impaired cell migration (Bloomfield and Pears, 2003).

Previous studies in the mammalian system found that Ras proteins can localize from membrane to the endocytic vesicles, ER, Golgi or cytoplasm (Bivona, et al., 2003, Caloca, et al., 2003, Rocks, et al., 2005, Eisenberg, et al., 2011). Refer to Figure 2 for the following text. Active Ras on the membrane can initiate downstream signaling pertaining

to cell development and chemotaxis. A recent study suggested a mode of superoxide generation in the endosomes. Anionic channels such as ClC-3, are localized at the plasma membrane, which electrogenically mediates the extracellular efflux of a proton and influx of two chloride ions into the cytoplasm of the cell. NADPH oxidase which produces superoxide extracellularly also localizes at the plasma membrane. In the extracellular environment, the superoxide has a higher chance of converting into hydroperoxyl radical ($\text{HO}_2\cdot$) (Lamb, et al., 2009, Liu, et al., 2013), which is neutral and can move inside the cell. In the cytoplasm of the cell, the hydroxyl radical ($\text{HO}_2\cdot$), gets dissociated into superoxide and hydrogen due to alkaline pH created by the activity of NHE. Another proposed model suggests that superoxide can directly utilize the chloride ion channel to transpass inside the cell (Hawkins, et al., 2007). A recent study by Castillo and others (2016) found that the presence of superoxide dismutase C (SodC) is essential for the localization of superoxide activated RasG protein to cytoplasmic vesicular compartment. It was reported that these redox activated Ras proteins, localized at the vesicles, are spatially separated from the PI3-K as they were unable to activate downstream targets in wild type cells under oxidative stress and PIP3 localized only on the local plasma membrane and macropinosome like structures. In contrast to wild type cells, *sodC*⁻ cells displayed active RasG persistently at the plasma membrane and activation of its downstream targets. Together, these observations suggest that SodC not only functions as a superoxide scavenger but is also required for the localization of active Ras to the cytoplasmic vesicles under oxidative stress conditions. It was proposed that by localizing the active Ras to the cytoplasmic compartment, cells prevent the activation of Ras downstream targets which would lead to chemotaxis and cell development as under oxidative stress environment apart from aberrant

signaling, cells are more prone to accumulate mutation (Muid, et al., 2014) which might result in genetically mutated gene pool of *Dictyostelium*.

Furthermore, several *in vitro* and *in vivo* studies showed that certain small GTPases are targets of superoxide anions. These superoxide sensitive small GTPases include, but not limited to, Ras, Rac, and Rab GTPases (Castillo, et al., 2016, Heo, 2011, Veeranki, et al., 2008). Many members of the Ras superfamily have redox sensitive motifs, NKCD or P-loop, which makes them easy target of the superoxide causing their aberrant activity (Heo, 2011).

Previous studies conducted on Rab GTPases suggest their involvement in the vesicular trafficking and endocytic events (Somsel Rodman and Wandinger-Ness, 2000). There are 54 different Rab GTPases found in *Dictyostelium* and most of them are known to have a human orthologue, out of which eleven Rabs (Rab5A, Rab5B, Rab6A, Rab7A, Rab7B, Rab8A, Rab11A, Rab11C, Rab14, Rab21 and Rab32) have been identified to be associated with vacuolar structure in *Dictyostelium* (Heo, et al., 2006, Weeks, et al., 2005). Amongst these, four Rabs, Rab8, Rab11a, Rab11c and Rab14 have been found to be associated with the CV (Du, et al., 2008). Previous studies have reported that Rab GTPases and Rab GAP domain proteins localize to the CV system (Parkinson, et al., 2014) and Rab GAP activity is necessary normal vacuolar size. The CV localization suggests that Rab proteins and their regulators may constitute a regulatory circuit for the CV cycle. Rab11a is associated with the CV bladder as well as tubules (Du, et al., 2008) while Rab11c is found to be associated with the CV membranes all the time. On the other hand, Rab 14 localizes only to the tubular structures and cells expressing dominant negative Rab14 were found to be defective in endocytosis and hypo-osmotic regulation (Harris and Cardelli,

2002). Under hypo-osmotic condition, Rab11a became activated and recruited another regulatory protein, drainin, to the CV membrane during the early steps of the discharge phase. As the CV reaches its maximum diameter, drainin recruits inactivated Rab8 to the CV membrane. Once Rab8a-GDP localizes to the CV membranes, it recruits the exocyst complex which acts as GEF for Rab8a and ensures GTP-bound Rab8a to the CV membrane (Essid, et al., 2012). The association of Rab8a-GTP is necessary for the tethering of CV to the plasma membrane. Once the CV is tethered to the plasma membrane, drainin stimulates the activity of disgorgin, a specific GAP for Rab8a, which leads to hydrolysis of the Rab8a-GTP again to GDP bound inactivated Rab8 (Du, et al., 2008). It was proposed that this step of hydrolysis by disgorgin is necessary for initiation of the discharge phase. Upon inactivation of Rab8, the CV bladder initiate the discharge phase (Jung, et al., 2009) where the CV contents are expelled through a kiss and run mediated discharging mechanism (Essid, et al., 2012).

Contractile vacuoles are considered as an acidic calcium storage compartment (Patel and Docampo, 2010). Previous studies have reported that calcium channels and calmodulin are localized to the CV in protists such as *Paramecium*, *Tetrahymena* (Bergquist, 1989). Recent study uncovered that that proteins involved in the regulation of Ca^{2+} ions such as Ca^{2+} -ATPase, Calmodulin, and P2XA receptors are crucial for proper CV fusion with the plasma membrane (Fountain, et al., 2007). In a study it was demonstrated that, *Dictyostelium* cells when grown in calcium rich media lead to upregulation of Ca^{2+} -ATPase of contractile vacuole as well as conditions which impair the contractile vacuole function found reduced rates of Ca^{2+} secretion, suggesting an important role of calcium in contractile vacuole function (Moniakakis, et al., 1999). In an aim to study the release of Ca^{2+} from the

contractile vacuole, it was proposed that P2X receptors of *Dictyostelium* are the Ca^{2+} release channels which are stimulated by the changes in luminal ATP (Ludlow, et al., 2009). The change causes the translocation of P2X receptors into the contractile vacuole lumen with the help of ATP specific transporter which would ultimately facilitate the release of calcium (Sivaramakrishnan and Fountain, 2013). Contractile vacuoles have high calcium concentration and during fusion event the calcium concentration increases 2 folds (Parkinson, et al., 2014). An increase in calcium concentration is responsible for activating other important regulators of the CV system, one of which is the RabGAP. It was reported that activated P2XA receptors increased the calcium efflux from the contractile vacuole, which in turn activate Rab11a specific GAP proteins and thus inactivate Rab11a (Parkinson, et al., 2014). Inactivation of Rab11a affects the CV phenotype as *Dictyostelium* cells expressing dominant negative Rab11a were found to display more number of contractile vacuoles which were bigger in size when kept under hypoosmotic environment (Harris and Cardelli, 2002), which suggest important role of Rab11a during the discharge phase of contractile vacuole. Cells lacking P2XA displayed higher level of Rab11a (Fountain, et al., 2007), which is consistent with its role of activating Rab11a GAP.

In this study, we report that SodC and Ras proteins are essential for the proper CV formation and function in *Dictyostelium* cells. We discovered that cells lacking SodC have abnormal contractile vacuole morphogenesis and discrepancies in the CV function which were partially rescued by reducing the extracellular superoxide load by treating the cells with superoxide scavenger XTT. Since active RasG levels are found to be more in *sodC*⁻ cells (Veeranki, et al., 2006), a redox insensitive mutant of RasG (C118A) displayed promising results on CV morphogenesis. Furthermore, we discovered that *sodC*⁻ cells

when treated with PI3K inhibitor, LY294002 had effect on CV morphogenesis but there was no effect on function of contractile vacuole.

Figure 2. Representation of extracellular superoxide mediated Ras activation.

2.2 Materials and Methods

2.2.1 Cell Culture

The different strains would be grown in D3-T media (15.3 g Peptone #3, 7.15 g Yeast Extract, 15.4 g Glucose, 0.525 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.48 g KH_2PO_4 , 0.53 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ to 1L of water, pH 6.5–6.9). All the strains were complemented with 0.5 mg/ml of thymidine for cells derived from *JH10* wild type strain. In addition, 5 ng/ μL of Blasticidin was added to the cells from *sodC* background and for all the other overexpressors 20 $\mu\text{g/ml}$ of G418 was supplemented to select the transformants.

Cells were kept in Nunc stationary culture flasks (Thermo scientific cat# 136196) at 20 degrees Celsius. For most experimental purposes cells were grown in shaken suspension at 150 rpm (Wats and Ashworth, 1970) to a density of $\sim 2.5 \times 10^6$ cells/mL. The cells number will be obtained using a hemocytometer. After this, cells were harvested and used for the different experiments.

2.2.2 Western Blot

To prepare the cell lysates, *Dictyostelium* cells were harvested by centrifugation and subjected to complete lysis buffer TTG (1% Triton, 5% glycerol, 0.15 M NaCl, 20 mM Tris.HCl pH 7.7, 1mM EDTA, 1 mM sodium orthovanadate, 40 mM ammonium molybdate, 1% BME, 1 tablet of protease inhibitor/10 mL TTG). 1ml of complete TTG was used to lyse 25 million cells. Protein lysates were mixed with 4x sample loading buffer and boiled at 100°C for 3 min. SDS–polyacrylamide gel electrophoresis (Sambrook, et al., 1989) was utilized to fractionate and normalize the proteins lysates. Proteins were transferred onto nitrocellulose membranes (Millipore) once the electrophoresis was done,

blocked with a 2% Bovine serum albumin (BSA) for 25 min. A suitable primary antibody was probed overnight at 4 degrees C or 2 hours at room temperature following a treatment with the enzyme linked secondary antibody specific to primary antibody for 3 hours. The bound antibody was detected by means of enhanced chemiluminescence (ECL, Cell Signaling Technology).

2.2.3 Antibodies

The primary antibodies that were used are the monoclonal anti-PanRas produced in mice (Sigma), and monoclonal anti-CFP (Covance). The dilution used for both antibodies was 1 : 1000. For secondary we used the Goat anti-mouse IgG-(H+L)-HRP from Southern Biotech.

2.2.4 Bacterial cell culture

The *E. coli* bacterial cells were grown overnight in 100ml LB medium with 50ug/mL ampicillin for about 16 hours at 37 degrees C and 200 rpm. Cell stock for the different plasmids was made in final 30% glycerol solution and stored at -80 degrees C for future use. After that, cells were pelleted using a IEC CL30R centrifuge at 4000 rpm for 25 minutes. The DNA was extracted from the pellet using the Midi-Prep kit from Quiagen. After measuring optical density at 260 nm using the GENESYS 10uv spectrophotometer, the pellets were stored at -20 degrees C for future use.

2.2.5 Transfection

Five million of *Dictyostelium* exponentially growing cells were centrifuged for 5 min at 2000 rpm (4° C), washed once with H-50 and resuspended in 100 µl of H-50. The cells were mixed with 10 µg of desired DNA, and placed in a cold 0.1 cm Fisher electroporation cuvette (cat# FB101). Electroporation was done at 1 kV two times with a pause of 5 secs between pulses. Cells were placed on ice for 5 minutes and then transferred into a 10 cm Petri dish with 10 ml of D-3T media, supplemented with 0.5 mg/mL thymidine. After 24 h, G418 was added as a means of selection. The method was modified from Schauer and others (1993).

2.2.6 RBD and GBD binding assay

The GST-Raf1-RBD recombinant protein was expressed in *E. coli* and purified as described (Kae, et al., 2004). The RBD binding assay has been used previously to identify active Ras in *Dictyostelium discoideum* (Sasaki, et al., 2004; Veeranki et al., 2008). Basically, each sample's protein lysates were incubated with 5 µg of purified recombinant GST-RBD bound to GTT-sepharose beads (GE healthcare) and the mixture was shaken at 4° C for 1:30 hr. Beads with the proteins attached were centrifuged and washed three times in 1x TTG. The pelleted beads were mixed with LDS (lithium dodecyl sulfate) 4x sample loading buffer and the mixture was boiled for 3 min. Then the procedure continues as described in the section "Western Blot".

2.2.7 Microscopy

Log phase growing cells in D3-T media were plated at a concentration of 5×10^6 cells/ml in a plastic chamber for 10 min to allow them to settle at the bottom. The media was then replaced with water (hypotonic condition). To visualize contractile vacuole, styryl dye FM 4-64 (ThermoScientific) was used with a final concentration of $1\mu\text{g/ml}$ and the images were taken between 2 and 18min post staining by CoolSNAP-Pro^{cf} digital camera on Leica DM IRB epifluorescence microscope. To visualize the complete CV system, Dajumin-GFP expressing cells were harvested at log phase and plated as described above. Images were recorded at 100X magnification from DV ELITE (Delta Vision Elite Imaging System) at FIU imaging core facility. Data processing of the images was accomplished by using Softworks.

2.2.8 XTT and LY294002 treatment

Log phase growing *Dictyostelium* cells were harvested and plated at a concentration of 5×10^5 cells/ml as described above. The media was replaced with water and supplemented with XTT to a final concentration of 4mM. The same procedure was followed for treating the cells with LY294002 to a final concentration of $25\mu\text{M}$. The images were then recorded using the DV ELITE microscope.

2.2.9 3D Image reconstruction

Log phase growing *Dictyostelium* cells were harvested and plated at a concentration of 5×10^5 cells/ml as described above. The media was replaced with water and cells displaying adequate GFP-expression were subjected to Z-stack imaging by marking the top and

bottom of the cell using Softworks in DV ELITE microscope. Continuous images of the cells were taken with each image having a difference of 0.35 μm in depth with the previous one until the desired marking was completed. The saved file for these images were then combined in a tool, Volume viewer, where these thin layers were combined to form a 3-D image either 360° around X- axis or Y-axis.

2.2.10 Survival assay:

Log phase growing *Dictyostelium* Wild type and *sodC* cells were harvested and suspended in water at a concentration of 1million cells/ml. The cells were kept on a shaking culture at 150 rpm, room temperature and harvested at 0min, 30min and 120min from the suspension. These harvested cells were diluted with the overnight grown live *Klebsiella* and 100 cells were plated on SM-agar plates. These plates were kept at 20°C for 1 week and the plaques formed were analyzed for their size and number.

2.2.11 Data Processing

The films with the images from western blot were scanned and saved as JPEG images. Band density of the protein bands was determined using the “UN-SCAN-IT gel” software version 5.3 (Silk Scientific Corporation). The numerical values obtained this way were used for statistical analysis of the results of the western blots. For the statistical analysis, we used $\alpha = 0.05$. Each experiment was performed at least 3 times with different cell cultures and lysates every time.

2.3 Results

2.3.1 *sodC* cells displayed aberrant responses to hypotonic stress.

When growing under nutrient rich axenic media, *Dictyostelium* cells gain nutrients from extracellular mostly through macropinocytosis. Wild type cells generally display a few vesicular structures under rich medium. However, upon incubation with hypotonic medium, wild type cells showed swelling which was accompanied with emergence of enlarged vesicles visible under phase contrast microscope (Fig. 3A). However, unlike wild type cells, *sodC* cells displayed enlarged vesicles even under rich media. Upon transition to hypotonic medium, *sodC* cells displayed a significantly larger enlarged vacuole, which is a possible consequence of aberrant regulation of contractile vacuole system. *Wild type* and *sodC* cells were incubated under hypotonic medium and were mixed with excessive *Klebsiella aerogenes* (Ka). Healthy cells capable of phagocytizing Ka grew and formed plaques on SM agar plate. Only 5 % of *sodC*- cells were able to grow on Ka plate after 2 hours of incubation under hypotonic medium, whereas 95% of wild type cells did. However, *sodC* cells exposed to no hypotonic medium exhibited 37 % reduction in the number of plaques on Ka compared to the wild types (Fig. 3B), suggesting that *sodC* cells are not as efficient as wild type cells in growing on Ka plate. In addition, *sodC* cells displayed 75% reduction in the average size of the plaques compared to that of the wild type, suggesting compromised growth on Ka lawn plate, which is consistent with the severe defects of directional migration of *sodC*- cells as previously reported (Veeranki, et al., 2008) and their defects in phagocytosis (In preparation, Cong, et al., 2017). These together

suggest that *sodC*⁻ cells were likely compromised in handling hypotonic medium, which is largely mediated by CV system in *Dictyostelium* cells.

2.3.2 *sodC*⁻ cells displayed aberrant CV morphogenesis and function.

To corroborate if the CV system is indeed compromised in *sodC*⁻ cells, GFP-Dajumin, a complete CV marker which labels the bladders and the tubules (Gabriel, et al., 1999), was introduced in wild type and *sodC*⁻ cells. *sodC*⁻ cells expressing GFP-Dajumin displayed over 300% increase in the number of contractile vacuole bladders (Fig. 4A). Wild type cells generally contain one or two bladders, but *sodC*⁻ cells often showed five or more bladders and their average size was 1.5 times smaller than that of the wild type (Fig. 4A). To unravel the details of the overall organization of the CV system, the GFP-Dajumin images were reconstructed in three dimensions (Fig. 4B). In this reconstructed images, not only the bladders, but also the tubular structures emerged. *Wild type* cells displayed a bladder with a dominant tubule with short radiating projections (marked with arrow heads, Fig. 4B). *sodC*⁻ cells, however, displayed unconnected bladders, which were significantly smoother than that of the *wild type*. Furthermore, tubular structures emerged as a series of dots unlike the continuous tubular structure of the wild type (Fig. 4C). These observations showed that the single enlarged vacuole of *sodC*⁻ cells under hypotonic medium (Fig. 3A) is unlikely the bladder of the contractile vacuole system. It is plausible that *sodC*⁻ cells might have rerouted excess water into certain types of endosomal system when the CV system operates inefficiently. In any case, the enlarged vacuole in *sodC*⁻ cells was not the part of the CV system.

The contractile vacuole system in *Dictyostelium* cells functions by charging and discharging the bladder to expel the excess water out of the cell. To investigate the effect of SodC on the functional cycle of the CV system, GFP-Dajumin expressing wild type and *sodC*⁻ cells were kept in hypotonic medium and the GFP images were captured with 15 seconds of intervals as indicated in the figure. Wild type cells completed one CV cycle in about a minute. During the charging phase, the wild type bladder adopted a shape of half moon, which rapidly evolved to that of a sphere (at 15, 75, 120 seconds' images, Fig. 4D) as charging process neared to a completion. The *sodC*⁻ cells, in contrast, displayed significantly delayed charging process, which often exceeded more than five to six minutes (marked with arrows, Fig. 4E). Furthermore, some of the bladders failed to be fully charged and prematurely discharged (marked with arrow heads, Fig. 4E).

2.3.3 Scavenging superoxide alleviated the CV phenotypes of *sodC*⁻ cells.

Our previous report (Veeranki et al., 2008) demonstrated that superoxide radical scavenger XTT (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2*H*-Tetrazolium-5-Carboxanilide) reduced the level of intracellular superoxide and active RasG in *sodC*⁻ cells. To determine if superoxide dependent RasG misregulation is at least partially responsible for the CV phenotype of *sodC*⁻ cells, GFP-Dajumin expressing *sodC*⁻ cells were treated with 4mM of XTT for 10 min and the fluorescent images were captured. The XTT treated *sodC*⁻ cells displayed ~50 % reduction in the number of bladders of which size increased to the level of wild type (Fig. 5A & 5B). On the other hand, the wild type cells showed no significant change in average number of bladders and their average size. Given that the XTT treatment induced significant rescue of the defective CV morphogenesis of *sodC*⁻ cells, we

investigated the effect of XTT on CV function as well. The GFP-Dajumin expressing wild type cells were exposed to 4mM of XTT under hypotonic medium for 10 min and the fluorescent images were captured with 15 seconds' intervals (Fig. 5C). The XTT treated wild type cells exhibited comparable CV cycle to that of the wild type with no XTT treatment. However, when treated with XTT, *sodC*⁻ cells displayed bladders that were better connected with each other and comparable in size to that of the wild type cells. The bladders of XTT treated *sodC*⁻ cells clearly demonstrated discharging steps although modestly delayed to that of the wild type (Fig. 5D).

The above results strongly suggested that the increased level of intracellular superoxide affects the CV morphogenesis and function. To further gain the evidence that intracellular superoxide anions affect the CV morphogenesis and function, the cytosolic superoxide dismutase, SodA, was introduced into *sodC*⁻ cells. The CV bladder of the CV system in SodA expressing *sodC*⁻ cells was visualized with a lipophilic styryl dye, FM 4-64. Previous report showed that this lipophilic dye is absorbed by the plasma membrane first and is associated with the contractile vacuole for 20 min post exposure (Du, et al., 2008) after which it labels other non-specific organelles inside the cell. In comparison to the *sodC*⁻ cells, SodA expressing *sodC*⁻ cells displayed a three-fold decrease in the average number of contractile vacuole when compared to *sodC*⁻ cells and 1.5-fold increase in their average size (Fig. 6A & 6C). Next, we compared the level of active Ras proteins in *sodC*⁻ and SodA expressing *sodC*⁻ cells and uncovered that Ras activity dramatically decreased upon SodA expression, suggesting that the SodA mediated modulation of CV may involve Ras proteins (Fig. 6B).

2.3.4 Aberrant Ras regulation affected CV morphogenesis in *sodC* cells.

Our previous study uncovered that the Cysteine 118 of RasG is a critical target of superoxide in *sodC* cells and RasG(C118A) substitution mutant was shown to be superoxide resistant in *sodC* cells (Castillo, et al., 2016). The *sodC* cells expressing RasG(C118A) were incubated under hypotonic medium and the CV system was visualized with the styryl dye FM 4-64. As shown in figure 7, the average number of bladder decreased from five to two and the average size of the bladder increased to the level of the wild type, strongly suggested that superoxide mediated misregulation of RasG activity is largely responsible for the aberrantly increased numbers of the CV bladders in *sodC* cells. The results described so far strongly argue that RasG regulates the CV morphogenesis. Although *sodC* cells expressing SodA or RasG(C118A) displayed CV bladders similar to those of the wild type, we were unable to determine the functional cycle of the filling and discharging due the unusual vertical movement of the CV system, which significantly hampered microscopic analysis.

To further gain the mechanism of Ras mediated CV regulation, one of the Ras downstream effectors, PI3K was inhibited with the pharmacological inhibitor LY294002. Previous studies showed that *sodC* cells showed significantly improved chemotaxis upon LY294002 treatment (Veeranki et al., 2008). The PIP3 generation in the cell depends on the action of PI3-Kinase, which converts PIP2 into PIP3, which is required for directional sensing and cell polarization (Takeda, et al., 2007). The GFP-Dajumin expressing wild type and *sodC* cells were treated with 25 μ M LY294002 and the numbers and sizes of the CV bladders were counted. Wild type cells demonstrated no significant difference in the number and size of the CV bladder but *sodC* cells showed 50% decrease in the number of

the bladders and increased size of the bladders comparable to those of the wild type (Fig. 8A). Although the significantly improved morphogenetic attributes of the CV system in LY294002 treated *sodC*⁻ cells, the bladders of *sodC*⁻ cells were still inefficient in the final stage of discharging. The bladders were completely filled with the peculiar spherical shape, yet they did not proceed to the final fusion event of discharging (Fig. 8C). In contrast, wild type cells treated with LY294002 showed no such defect (Fig. 8B), indicating that the inhibition of PI3K rescued the morphogenesis of the CV system, but aggravated the defective discharging phase of *sodC*⁻ cells as shown earlier in figure 4E.

2.4 Figures

Figure 3. *sodC*⁻ cells displayed aberrant responses to hypotonic stress.

(A) Wild type and *sodC*⁻ cells were incubated under nutrient rich axenic and hypotonic medium. Upon incubation under hypotonic medium, wild type cells displayed swelling which was accompanied with emergence of multiple enlarged vesicles. *sodC*⁻ cells, in contrast, displayed enlarged vesicles even under rich media, which upon transition to hypotonic medium, transformed into a single large vacuole. (B) Wild type and *sodC*⁻ cells were incubated under hypotonic medium up to 2 hours as marked, and were mixed with excessive *Klebsiella aerogenes* (Ka) on SM agar plate. The numbers and sizes of plaques formed on the Ka plates were counted from three independent experiments. (C) 90 % of wild type cells survived 2 hours of hypotonic stress, while only 6% of *sodC*⁻ cells survived. Furthermore, the average size of plaque of *sodC*⁻ cells was only 24% of the size of the plaques formed by wild type cells.

Fig. 3

A

Wt

***sodC*⁻**

B

Time 0 min

Wild type

sodC-

Time 30 min

Wild type

sodC-

Time 120 min

Wild type

sodC-

Figure 4. Aberrant contractile vacuole system in *sodC⁻* cells. (A) GFP-Dajumin, a complete CV marker, was introduced into *Wt* and *sodC⁻* cells. (B) Average number of the CV bladders in *sodC⁻* cells was 3 times higher than *Wt* cells while their average size was 1.5 times smaller than *Wt* cells. These values were from 3 independent experiments and the p values (Student's t-test) was smaller than 0.001. (C) 3-dimensional reconstruction of the Z-stack images by Volume viewer software (DV ELITE microscope) revealed poorly interconnected bladders and defective (punctate, instead of contiguous) tubule structures in *sodC⁻* cells. (D) *Wt* cells displayed a complete charging and discharging events in a minute. (E) *sodC⁻* cells exhibited either incomplete filling followed by premature discharge or full charging with significant delay as shown. (Scale=10 μ m).

Fig. 4

B

Wt

sodC⁻

GFP-Dajumin

C

Wt

sodC-

GFP-Dajumin

D

Wt

E

sodC⁻

Figure 5. Alleviation of extracellular superoxide rescues CV bladder phenotype.

(A) *sodC*⁻ cells expressing GFP-Dajumin treated with 4 mM XTT, a superoxide scavenger, displayed 3 times reduced number of the CV bladders and 1.5 times increase in size. (B) The average values of the number and size of the bladder from *sodC*⁻ cells with and without treatment were obtained from three independent experiments, and the p value was smaller than 0.01. No such effects were observed from XTT treated wild type cells. (C) *Wild type* cells treated with XTT completed one CV cycle in 60 seconds, which is comparable to that of untreated wild type cells. (D) XTT treated *sodC*⁻ cells still displayed delayed CV cycle compared to that of wild type cells as they still took more than 2 minutes to complete the CV cycle which was quite similar to that of the untreated cells). (Scale= 10µm)

Fig. 5

B

C

***Wt::GFP-Dajumin* + XTT**

D

***sodC- :: GFP-Dajumin* + XTT**

Figure 6. Overexpression of SodA in *sodC*⁻ cells rescues CV morphogenesis. (A) The representative images the CV system of *sodC*⁻ and *sodC*⁻ cells expressing the cytoplasmic superoxide dismutase SodA are shown. (Scale= 10μm) (B) The levels of active Ras proteins in *sodC*⁻ and SodA expressing *sodC*⁻ cells were determined with GST-RBD pull-down assay. (C) The average values of the number and size of the bladder from *sodC*⁻ cells with and without overexpressing SodA were obtained from three independent experiments, and the p value was smaller than 0.01. The number of bladder decreased 66% and the average size of the bladder increased 23%.

Fig. 6

B **C**

Figure 7. Redox insensitive mutant, RasG(C118A) rescues CV morphogenesis in *sodC*⁻ cells. (A) The redox insensitive RasG(C118A) substitution mutant was introduced into *sodC*⁻ cells and the representative images of the CV systems of wild type, *sodC*⁻, and *sodC*⁻ expressing RasG(C118A) were visualized with the styryl dye FM 4-64 (1μg/ml of). The redox insensitive motif shows comparable phenotype to *Wt* cells. (B) Average number of the CV bladders in RasG(C118A) expressing *sodC*⁻ cells was reduced 66% while their average size increased 29%. These values were obtained from 3 independent experiments and the p values (Student's t-test) was smaller than 0.001. (Scale= 10μm)

Fig. 7

Figure 8. PI3K Inhibition rescued CV bladder phenotype in *sodC*⁻ cells. (A) Wild type and *sodC*⁻ cells were treated with the PI3K inhibitor LY294002. Representative images of the CV system from wild type and *sodC*⁻ cells with and without LY294002 treatment were shown. (B) Average number of the CV bladders in LY294002 treated *sodC*⁻ cells was reduced 52% while their average size increased 35%. These values were obtained from three independent experiments and the p values were smaller than 0.01. Wild type cells displayed no significant differences with and without LY294002 treatment. (C) Wild type cells treated with LY294002 displayed the CV cycle in 75 seconds. (D) *sodC*⁻ cells treated with LY294002 displayed a single fully charged bladder that persistently displayed extended delay of fusion. The images show persisting bladder at 210 seconds. (Scale=10μm).

Fig. 8

***sodC* -**

B

Wt + LY294002

C *sodC*⁻ + LY294002

2.5 Discussion

The current understanding of contractile vacuole morphogenesis finds it to be a post Golgi organelle as most of the proteins associated with the membrane of CV are also found localized at the plasma membrane. Our first observation of *Wild type* and *sodC*⁻ cells under Brightfield microscope revealed that under media *Wild type* cells displayed multiple small vesicles/vacuoles which were consistent for prolonged time periods while *sodC*⁻ cells displayed multiple vacuoles which were bigger in size. When the media was replaced with water, there was no change in the vesicles/vacuole in wild type cells initially but gradually the cells started to swell up at the edges and displayed bigger vesicle/vacuoles. On the contrary, for *sodC*⁻ cells, the multiple vesicle/vacuoles phenotype started to disappear instead we found fusion of these vesicle/vacuoles to form a single big vacuole in the cell. Previous studies on *Dictyostelium* have also reported appearance of bigger vesicle/vacuoles which were distinguished from contractile vacuoles (Uchikawa, et al., 2011). They argue that these big vacuoles are a result of transformation of cell from vegetative to pre-stalk stage where the autolysosome prepare the cell to form a multicellular aggregate during stress or starvation conditions. Another study suggests that during autophagic cell death, the cells become round due to loss of F-actin polymerization and transforms into paddle cell which is followed by vacuolization ultimately leading to cell death (Giusti, et al., 2009, Levraud, et al., 2003).

But to our surprise, when we transformed the cells with complete CV marker, Dajumin-GFP, we found that *sodC*⁻ cells are osmosensitive, defective in the CV morphogenesis, and the dynamics of the CV functional cycle. Compared to the wild type, *sodC*⁻ cells contained three times more bladders that were poorly interconnected. *sodC*⁻

cells also displayed premature dischargement with incomplete filling, which could arise because of the inefficient water flow through the poorly connected tubules. The *sodC*⁻ cells often also displayed significantly delayed dischargement, which could be a consequence of a hampered fusion between the bladder and the plasma membrane.

In *Dictyostelium*, interconnected bladders and tubules of contractile vacuole system allow proper channeling of the water into the CV and helps the cell survive extreme osmotic environments. On comparing survival efficiency of wild type and *sodC*⁻ cells of under hypoosmotic stress, we found that number of plaques formed by *sodC*⁻ cells were 40% less than wild type cells at time 0. Interestingly, the number of plaques formed by *sodC*⁻ cells reduced to 6% after 2 hours of exposure to water while 90% of the wild type cells survived and formed plaques. On comparing the relative size of the plaques, which is also an indication of chemotaxis efficiency, our results were coherent with the previous studies that *sodC*⁻ cells have defective chemotaxis (Veeranki et al., 2008), as the size of the plaques formed by *sodC*⁻ cells were 80% smaller as compared to the wild type cells with no significant difference among the plaques formed at 0min, 30min and 120 min. Together these observation indicate that although *sodC*⁻ cells have more number of contractile vacuole as compared to wild type cells, CV morphogenesis and function in these cells is mis-regulated causing failure of cells to survive under hypoosmotic stress.

sodC⁻ cells treated with a superoxide scavenger XTT displayed decreased number of bladders, which were better connected through the CV tubules. However, the final fusion step was still not as efficient as that of wild type cells. *sodC*⁻ cells expressing the cytoplasmic SodA protein also displayed decreased number of bladders, suggesting that the global alleviation of superoxide radicals contributed appropriate CV morphogenesis. Since

previous results from our lab suggest that *sodC*⁻ cells when treated with XTT showed decrease in the active Ras levels (Veeranki, et al., 2008), we quantified the active Ras levels in *sodC*⁻ cells overexpressing *sodA*. We found that compared to *sodC*⁻ cells, there was significant decrease in the Ras levels in *sodA* overexpressor which is in consistent with our theory that RasG might be affecting the CV morphogenesis as *sodA* overexpressor cells had normal CV formation which were comparable to the wild type cells.

Previous studies show that RasG and RasS are upregulated in *sodC*⁻ cells, out of which RasG showed bigger increase as compared to the RasS, which showed only a modest increase in *sodC*⁻ cells (Castillo, et al., 2016). To confirm our hypothesis, we introduced superoxide insensitive RasG(C118A) in *sodC*⁻ cells. The redox insensitive mutant showed decreased number of bladders, suggesting that superoxide mediated activation of RasG hampers CV morphogenesis.

Previous investigation on the CV bladder biogenesis uncovered that MEGAP, IBAR, and VatM proteins are involved in the regulation of the number of bladder in *Dictyostelium* (Heath and Insall, 2008, Linkner, et al., 2014, Temesvari, et al., 1996). MEGAP and IBAR proteins were suggested to modulate the transition between tubular-bladder transition, but potential mechanisms of VatM mediated modulation of bladder biogenesis is unclear. In contrast to the CV system of ciliates, the *Dictyostelium* bladder does not maintain a fixed location near the dedicated pore on the plasma membrane. Furthermore, two bladders with branched radial tubules seem to alternate the filling and collapsing cycle, which contrast that of the ciliates such as *Paramecium*, *Tetrahymena*, and *Chlamydomonas* (Plattner, 2015). The regulatory mechanism of CV biogenesis in protists thus seems to contain multiple versions. In any case, the number of the CV bladder in *Dictyostelium* is a subject

of regulation and SodC and Ras proteins in addition to the previously reported MEGAP, IBAR and VatM are the members of the regulatory network.

Consistently, *sodC*⁻ cells treated with a pharmacological inhibitor LY294002 for the RasG downstream target PI3K also displayed decreased number of bladders. Interestingly, however, the final fusion step was even more severely hampered than the *sodC*⁻ cells without LY294002 treatment. Previous studies showed that PI3K was involved in the vesicle trafficking and water discharging of the CV system of *Trypanosoma cruzi* (Schoijet, et al., 2008). LY294002 treated *Trypanosoma cruzi* displayed inhibited water expel under hypotonic conditions. As previously discussed, Rab GAP proteins and Rab GTPase have been involved in regulating the CV discharge events. It was found that cells lacking Disgorgin have enlarged CV bladders and show defects in discharging of the CV suggesting that the hydrolysis of Rab8a GTP is crucial for the fusion on CV membrane with the plasma membrane which is responsible for proper discharging of CV (Du, et al., 2008). As discussed in the previous chapter, Rab11a is also associated with the CV membrane just before discharging and is responsible for the recruitment Drainin which is also Rab-GAP like protein and associated with CV discharge (Bos, et al., 2007, Clarke, et al., 2002). It was found that cells lacking Drainin have abnormally inflated bladders and those cells had premature discharge (Becker, et al., 1999), a similar phenotype also observed in *sodC*⁻ cells. As previously reviewed by Heo, et al., 2005, many Rab GTPases have redox sensitive motifs which can be the target of reactive oxygen species generated under hypoosmotic environment. Thus, it would be interesting to study the redox sensitive motifs of Rab GTPases and their activity under hyposmotic environment.

Thus, we propose that hyposmotic environment generates reactive oxygen species which affects the contractile vacuole morphogenesis, directly or indirectly through Ras GTPases which have redox sensitive motifs. The nature of superoxide mediated CV morphogenesis and function needs to be further studied as there are other members of Ras GTPases which might be misregulated under oxidative stress. Those studies would be beneficial in understanding contractile vacuole functions which would in turn help in understanding the survival strategies of *Trypanozomes* and other organism which utilize contractile vacuoles to survive under extreme hyposmotic stress conditions.

2.6 References

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CHAPTER 3. ROLE OF NHE AND RAS ON CONTRACTILE VACUOLE MORPHOGENESIS AND FUNCTION.

3.1 Introduction

Dictyostelium discoideum is a soil dwelling amoeba which has been extensively studied to understand eukaryotic mechanisms such as chemotaxis and phagocytosis, whose principles are common to both *Dictyostelium* and mammalian cells (Parent, 2004). The *Dictyostelium* Na^+/H^+ exchanger, NHE, is an integral membrane protein transporter which is responsible for the counter transport of the protons and the sodium ions across the membrane (Denker, et al., 2000). Many mammalian studies have identified the potential role of NHE and its isoforms. Myocardial cells during ischemia release large number of protons intracellularly which are removed by the activity of NHE by transporting the protons outside the cell with the exchange of a sodium ion thus helping the cells in overcoming the damage caused by the ischemia (Karmazyn, et al., 2008). *Dictyostelium discoideum* has two NHE, NHE1 and NHE2, of which NHE1 is widely studied and found to be involved in regulation of cell polarity mediated by facilitation of K^+/Na^+ ions during the process of chemotaxis (Lusche, et al., 2011). One of the functions of NHE is to assess the difference between steady state extracellular monovalent cations concentration and regulate the same intracellularly as a previous study demonstrated that increase in the concentration of extracellular monovalent cation results in an increase in the cytosolic concentration of that cation (Salyman, et al., 1964). Previous study reported that in differentiated *Dictyostelium* cells, cAMP induces an increase in the intracellular pH, which is required to perform chemotaxis (Van Duijn and Inouye., 1991). In a recent study, it was reported that *Dictyostelium* NHE1 is responsible for regulating the kinetics of F-actin

polymerization and production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to attain a polarized shape during chemotaxis (Patel and Barber, 2005). In the same study, it was found that cells lacking *Dictyostelium nheI*⁻, displayed defects in leading edge actin polymerization and persistent elevation of PIP3 in response to extracellular cAMP stimulation.

The actin cytoskeletal arrangements in *Dictyostelium* are also controlled by members of Ras family of GTPase, mainly RasG and RacC.. RasG lies upstream to the PI3K, which is responsible for the conversion of PIP2 to PIP3 (Whitman et al., 1988), which localizes at the membrane and activates WASP (Wiscott Aldrich Syndrome protein) which is responsible for F-actin polymerization (Kölsch, et al., 2008). Previous studies have demonstrated that a precise level of RasG is required to regulate developmental and cytoskeletal functions by direct interaction with more than one downstream effectors (Weeks and Spiegelman, 2003) as expressing constitutively active RasG in *Dictyostelium* cells, resulted in defects in aggregation because of reduced capacity of the cells to generate cAMP (Khosla et al., 1996) while *rasG*⁻ cells exhibited defects in chemotaxis and have compromised cytokinesis (Fukui et al., 1990). RacC on the other hand regulates WASP mediated F-actin polymerization (Han, Leeper, Rivero, & Chung, 2006) at the anterior as well as Myosin II assembly, by activating PAKa, a serine threonine kinase, which is responsible for proper retraction of the posterior part of cells performing chemotaxis (Pascale et al., 2007; Chung & Firtel., 1999). Previous studies have demonstrated that overexpression of RacC in the cells resulted in increased F-actin polymerization, in a PI3-Kinase dependent manner and increased rate of phagocytosis was also observed (Seastone et al., 1998).

Dictyostelium Myosin II, also known as conventional Myosin II, comprises of two heavy chain (MHC), two light chains (MLC) and two regulatory light chains (RLC) (Chisholm et al., 1988; Tafuri et al., 1989; Warrick et al., 1986). Myosin filaments slide over the actin filaments thus hydrolyzing the ATP in the process (Yumura et al., 2005). There are two important function of Myosin in the cells; First is to stabilize the actin filaments by forming a group of 10-20 hexamers and second is to provide the cell with contraction ability which is achieved by ATP hydrolysis (Bosgraaf & van Haastert, 2006). The filament assembly of Myosin is regulated by the activity of Myosin Heavy Chain Kinase (MHCK), which phosphorylate the Myosin tail causing a bend which further results in blocking of the assembly domains to form a filament (De La Roche et al., 2002). Refer to Figure 9 for the following text. PAKa promotes the Myosin filament assembly by inhibiting the activity of MHCK (Chung and Firtel., 1999) as previous reports suggest that disrupting the gene for PAKa resulted in disassembly of Myosin filaments while cells expressing constitutively active PAKa displayed hyper assembly of Myosin (Firtel and Chung., 2000). Previous studies have reported that RasB and Rap1 proteins also affect the assembly of Myosin II by activating MHCK through an unknown mechanism. It was suggested that Rap1 and RasB are localized at the leading edge of the cell and thus prevent the assembly of Myosin II (Jeon, et al., 2007a; Jeon, et al., 2007b; Kortholt, et al., 2006; Mondal, et al., 2008).

Rho specific Guanine Dissociation Inhibitors, also known as Rho-GDI, block the dissociation of GDP from the Rho-GTPase rendering them to inactive state (Fukumoto et al., 1990). Rho-GDIs have also been described in various organism, including *C. elegans*, yeast, plants and even in mammals, where they have specific activity towards the Rho proteins (Olofsson, 1999). Rho-GDI1 binds to different Rac members such as Rac1a/b/c,

RacB, RacC and RacE but no activity was shown towards RasG, which suggested that Rho-GDI specifically binds to the GTPase of Rho family (Rivero, et al., 2002). A recent study suggested role of Rho-GDI in CV bladder morphogenesis as the cells lacking Rho-GDI displayed enlarged CV bladders (Rivero, et al., 2002). In the same study, it was reported that the behavior and function of the contractile vacuole was not hampered. Another previous study reported that *Dictyostelium* Rac-GAP1 (DRG) protein regulates the GTPase activity of Rho/Rac GTPase and Rab GTPase (Knetsch, et al., 2001). In the same study, it was found that RabD, a *Dictyostelium* Rab14 homologue, which is responsible for communicating between the contractile vacuole system and endosomal pathways (Harris and Cardelli, 2002), was misregulated and displayed a 30-fold increase in its basal activity. *Dictyostelium* cells lacking DRG displayed deformed CV system and displayed defects in the morphology of contractile vacuole bladders, which was similar to the phenotype observed in cells expressing dominant negative RabD(N121I) (Harris and Cardelli, 2002).

Previous studies suggest that inhibition of NHE by a pharmacological inhibitor, EIPA (5-(N-Ethyl-N-isopropyl) amiloride), decreased the active RasG levels and rescues the defect of chemotaxis in *sodC*⁻ cells but has no effect on RacC (Kabra et al., 2017 (under review)). In another study it was demonstrated that *Dictyostelium* NHE1 is essential for cell polarization mediated by the conventional Myosin II and cells lacking NHE1 have defective localization of Myosin II (Lusche, et al., 2011). The mechanisms of Myosin II regulation by NHE are poorly understood.

Interestingly, Myosin II is the one which is well characterized CV component that can convey contractility to the CV system by creating the necessary cytosolic tensions, the events of which are regulated by the conventional protein kinase, VwK (Betapudi &

Egelhoff, 2009). It was found that cells lacking VwkA displayed excessive number of CV which were defective in undergoing proper fusion with the plasma membrane as well as expression levels of Myosin II were also elevated in *vwkA*⁻ cells. In a more recent study it was found that MyoJ, a type V Myosin, is involved in regulating the movement of contractile vacuole membrane along the cortex during the discharge phase of CV cycle (Jung, et al., 2009). The study suggests that MyoJ localizes to the CV membrane and drives the CV membrane along the actin rich cortex. It is also responsible for the movement of CV tubules along cortical actin filaments which are generated after the bladder collapse. Previous studies report that heavy chain region of Myosin II and MyoJ proteins are related as they have a lot of similar repeats in their sequences (Peterson, et al., 1996).

In this chapter, we described the role of NHE inhibitor, EIPA on the contractile vacuole morphogenesis and function by treating the *Wt* and *sodC*⁻ cells. We also discovered that *sodC*⁻ cells, which show multiple vacuole phenotype and aberrancies in contractile vacuole function were partially rescued on treatment of cells with EIPA. We also observed that cells lacking myosin heavy chain, *mhcA*⁻ showed abnormal CV phenotype where cells showed multiple vacuole which were abnormally large and their function was compromised too. Furthermore, when we compared the size of the cells, *mhcA*⁻ cells were 3 times bigger than the average size of the cells.

Figure 9 Schematic representations of signaling pathways leading to cytoskeleton arrangements in *Dictyostelium* cells.

3.2 Materials and Methods

3.2.1 Cell Culture

The different strains were grown in D3-T media (15.3 g Peptone #3, 7.15 g Yeast Extract, 15.4 g Glucose, 0.525 g Na₂HPO₄ · 7H₂O, 0.48 g KH₂PO₄, 0.53 g Na₂HPO₄ · 7H₂O to 1L of water, pH 6.5–6.9). Thymidine was complemented to the media at the concentration of 0.5 mg/ml of thymidine for cells derived from JH10 wild type strain. In addition, 5 ng/μl of Blasticidin was added to the cells from *sodC*⁻ background and for all the other overexpressors 20 μg/ml of G418 was supplemented to select the transformants. Cells were kept in Nunc stationary culture flasks (Thermo scientific cat# 136196) at 20 degrees Celsius.

3.2.2 Western Blot

To prepare the cell lysates, log phase growing *Dictyostelium* cells were harvested and subjected to complete lysis buffer TTG (1% Triton, 5% glycerol, 0.15 M NaCl, 20 mM Tris.HCl pH 7.7, 1mM EDTA, 1 mM sodium orthovanadate, 40 mM ammonium molybdate, 1% BME, 1 tablet of protease inhibitor/10 mL TTG). Protein lysates were mixed with 4x sample loading buffer and boiled at 100°C for 3 min. SDS–polyacrylamide gel electrophoresis (Sambrook, Fritsch, & Maniatis, 1989) was utilized to fractionate and normalize the proteins lysates. After electrophoresis, Proteins were transferred onto nitrocellulose membranes (Millipore) and blocked with a 2% Bovine serum albumin (BSA) for 25 min. A suitable primary antibody was probed overnight at 4 degrees C or 2 hours at room temperature following a treatment with the enzyme linked secondary antibody

specific to primary antibody for 3 hours. The bound antibody was detected by means of enhanced chemiluminescence (ECL, Cell Signaling Technology).

3.2.3 Antibodies

The primary antibodies that were used are the monoclonal anti-PanRas produced in mice (Sigma), and monoclonal anti-CFP (Covance). The dilution used for both antibodies will be 1:1000. For secondary we used the Goat anti-mouse IgG-(H+L)-HRP from Southern Biotech.

3.2.4 Bacterial cell culture

E.coli Cells were grown overnight in 100ml of LB media with 50ug/mL ampicillin for about 16 hours at 37 degrees C and 200 rpm. Cell stock for the different plasmids were made in final 30% glycerol solution and stored at -80 degrees C for future use. After that, cells were pelleted using a IEC CL30R centrifuge at 4000 rpm for 25 minutes. The DNA was extracted using the Midi-Prep kit from Qiagen.

3.2.5 Transfection

Log phase growing *Dictyostelium* cells were centrifuged for 5 min at 2000 rpm (4° C), washed once with H-50 and resuspended in 100 µl of H-50. The cells were mixed with 10 µg of desired DNA, and placed in a cold 0.1 cm Fisher electroporation cuvette (cat# FB101). Electroporation was done as described in the previous chapter. Cells were placed on ice for 5 minutes and then transferred into a 10 cm Petri dish with 10 ml of D-3T media,

supplemented with 0.5 mg/mL thymidine. After 24 h, G418 was added as a means of selection. This method was modified from Schauer et al., 1993.

3.2.6 RBD and GBD binding assay

The GST-Raf1-RBD recombinant protein was expressed in *E. coli* and purified as described (Kae et al., 2004). The RBD binding assay has been used previously to identify active Ras in *Dictyostelium discoideum* (Sasaki et al., 2004; Veeranki et al., 2008). Basically, each sample's protein lysates were incubated with 5 µg of purified recombinant GST-RBD bound to GTT-sepharose beads (GE healthcare) and the mixture was shaken at 4° C for 1:30 hr. Beads with the proteins attached were centrifuged and washed three times in 1x TTG. The pelleted beads were mixed with LDS (lithium dodecyl sulfate) 4x sample loading buffer and the mixture was boiled for 3 min. Then the procedure continues as described in the section "Western Blot".

3.2.7 Microscopy

Log phase growing cells in D3-T media were plated in a plastic chamber for 10 min to allow them to settle at the bottom. The media was then replaced with hypotonic condition (water). The contractile vacuole were visualized by styryl dye FM 4-64 (Invitrogen), used at a final concentration of 1µg/ml and the images were taken between 2 and 18min post staining. To visualize the complete CV system, Dajumin-GFP expressing cells were harvested at log phase and plated as described above. Images were recorded from DV ELITE microscope at 100X magnification. Data processing of the images was accomplished by using Softworks.

3.2.8 EIPA treatment

Log phase growing *Dictyostelium* cells were harvested and plated at a concentration of 5×10^5 cells/ml as described above. The cells were treated with 4mM EIPA for a period of 30min. Media was then replaced with water and the images were then recorded using the DV ELITE microscope.

3.2.9 Data Processing

The films with the images from western blot were scanned and saved as JPEG images. Band density of the protein bands was determined using the “UN-SCAN-IT gel” software version 5.3 (Silk Scientific Corporation). The numerical values obtained this way were used for statistical analysis of the results of the western blots. For the statistical analysis we used $\alpha = 0.05$. Each experiment was performed at least 3 times with different cell cultures and lysates every time.

3.3 Results

3.3.1 RasG but not RacC may be involved in the regulation of CV morphogenesis.

Although superoxide anions are generated to the extracellular space by NADPH Oxidase (NOX) proteins, they can be imported into the cytoplasmic side either through anion channels such as chloride channels (ClC3) (Hawkins et al., 2007), or through diffusion upon neutralization by protonation (Korshunov and Imlay, 2002). One of the proteins that could facilitate the neutralization of extracellular superoxide is Na-H exchanger. In response to chemoattractant stimulation, *Dictyostelium* cells activate heterotrimeric G proteins that in turn activate Ras proteins, and promote significant protein efflux. Thus we reasoned that inhibition of Na-H exchanger with amiloride (5-(N,N-Dimethyl) amiloride hydrochloride (EIPA)) might attenuate *sodC*⁻ CV phenotypes. Wild type and *sodC*⁻ cells were treated with 25μM EIPA and active Ras proteins were compared. As shown in figure 10A, EIPA treatment decreased the level of basal Ras activity in both wild type and *sodC*⁻ cells. When treated with EIPA and then stimulated with cAMP, wild type cells were largely unable to activate Ras proteins, but *sodC*⁻ cells displayed robust activation of Ras proteins (Fig. 10B).

A series of *in vitro* studies of superoxide mediated regulation of small GTPases have shown that not only Ras, but other small GTPases such as Rac and Rab proteins are susceptible (reviewed by Heo, 2011). A previous study showed that several Rac proteins such as RacC might affect CV system through interaction with Rho GDP-dissociation inhibitors (RhoGDI) (Rivero et al., 2002). Considering that RacC proteins are likely superoxide sensitive due to the presence of at least two types of superoxide sensitive motifs (the P loop motif and NKCD motif), RacC activity in wild type and *sodC*⁻ cells were

compared. CFP-RacC was expressed in wild type and *sodC*⁻ cells and active RacC proteins were isolated with GST-WASP-GBD protein. As shown in figure 10C, RacC activity in *sodC*⁻ cells was significantly lower than that of the wild type cells. The effect of EIPA on RacC activity was also determined in wild type and *sodC*⁻ cells expressing CFP-RacC. In response to EIPA treatment, *sodC*⁻ cells persistently showed significantly low RacC activity compared to that of wild type cells (Figure 10D) indicating that EIPA does not affect RacC activity. The figure 10E is collaborating work with Dr. Heo at University of Texas, Arlington. To examine the effect of redox reagents on the activity of RacC in *in vitro* conditions, we purified GST-RacC and subjected to Rac GDP dissociation assay. We found that RacC was well activated by superoxide in the redox environment created by incubation with Xanthine oxide and the GDP dissociation was nearly equal to the Human Rac1 control. The GDP dissociation activity was also measured in presence of SOD. These *in vitro* results were different from our *in vivo* data which suggest that *in vitro* global increase in the level of superoxide does not represent dysregulation of superoxide induced by the loss of superoxide dismutase C in *Dictyostelium* cells.

3.3.2 Effect of EIPA on CV morphogenesis and function in *sodC*⁻ cells.

Previous studies suggested that cells lacking NHE1 showed persistent elevation of PIP3 in response to the extracellular cAMP stimulation (Patel and Barber, 2005). Similar phenotype was observed in *sodC*⁻ cells, which display aberrant activation of Ras and random PIP3 localization (Veeranki, et al., 2008). Since PI3K is downstream to Ras and is responsible for the conversion of PIP2 to PIP3, we hypothesized that elevated levels of PIP3 in *nhe1*⁻ cells is a result of elevated Ras levels. *sodC*⁻ cells displayed elevated levels

of Ras (Veeranki et al., 2008). As discussed previously, *Wt* and *sodC*⁻ cells displayed reduced Ras activity when treated with pharmacological inhibitor of NHE1, 5'-(N-ethyl-N-isopropyl) amiloride (EIPA). To gain further insight on the possible role of NHE on CV morphogenesis, direct or indirect, we treated the *wild type* and *sodC*⁻ cells with 25uM EIPA and then placed the cells under water to observe the contractile vacuole morphogenesis. We found that the average number of CV bladders were reduced to 50% in *sodC*⁻ cells after treatment with EIPA (Figure 11A & B) while the average size of the CV bladders increased and were now comparable to the *wild type* cells. Interestingly, we found a partial rescue of CV function in *sodC*⁻ cells treated with EIPA as the CV bladders completed one cycle of charging and discharging in 105 sec which is half the time required by *sodC*⁻ cells without treatment (Figure 11C). These data together strongly indicate that NHE might be regulating CV morphogenesis and function indirectly by modulating RasG activity.

3.3.3 Myosin heavy chain is important in regulating CV morphogenesis and function.

Recent work done by Betapudi & Egelhoff, 2009 proposed that contractile vacuole system use Myosin II for the periodic contraction which is regulated by the unconventional protein kinase, VwK and cells lacking VwK show excessive number of CV which were defective in undergoing proper fusion with the plasma membrane. Myosin II comprises of 2 heavy chains MHC A and MHC B. To observe the CV phenotype in *mhcA*⁻ null cells, we expressed the complete CV marker, Dajumin-GFP in *mhcA*⁻ cells. The results indicated a multiple CV phenotype in *mhcA*⁻ cells (Figure 12A & B). We observed that the average number of CV in *mhcA*⁻ cells ranged between 5-6 contractile vacuole per cell which was quite similar to the CV phenotype observed in *sodC*⁻ cells. Surprisingly, the average size

of the contractile vacuole bladder was quite big in *mhcA*⁻ cells. We found that the average number of contractile vacuoles were 50% more in *mhcA*⁻ cells while their average size was also 1.5 times bigger as compared to the *wild type* cells. An interesting observation for the *mhcA*⁻ cells was that the size of the cells was twice as big as compared to any other cells, *Wt* or *sodC*⁻, which is consistent with previous reports suggesting Myosin is responsible for providing structural integrity to the cells as well as help in contraction with actin.

To explore the effect of Myosin II on the function of contractile vacuole, Dajumin-GFP expressing *mhcA*⁻ cells were kept in water to analyze the time required by the cells to complete one CV cycle. We found that *mhcA*⁻ cells took approximately 225 secs to complete one CV cycle which is thrice more than the time taken by *Wt* cells under the same condition (Figure 12C). Interestingly, bladders and tubules in *mhcA*⁻ cells were still connected to each other, although we have a multiple bladder phenotype.

3.4 Figures

Figure 10. Effect of superoxide on RacC activity. (A) Active Ras levels in *Wt* and *sodC*⁻ cells with and without EIPA treatment were determined with GST-RBD assay. As previously reported (Veeranki et al., 2008, Castillo et al., 2016), *sodC*⁻ cells displayed significantly higher level of active Ras proteins. In response to EIPA treatment, the basal Ras activity decreased in both wild type and *sodC*⁻ cells. (B) To determine if the EIPA treated cells can elicit Ras activation in response to GPCR mediated signal transduction, EIPA treated cells were stimulated with cAMP and Ras activities were determined at the

indicated time points. EIPA treated wild type cells failed to elicit measurable Ras activation, while *sodC*⁻ cells were able to induce a robust Ras activation C) Wild type and *sodC*⁻ cells overexpressing CFP-RacC were pulsed with 50nM cAMP for 4 hours and treated with EIPA. The total RacC protein was normalized using the α -CFP and the basal levels of active RacC proteins were quantified with GST-WASP-GBD pull-down assay. The level of active RacC proteins in *sodC*⁻ cells was significantly lower than that of the wild type. D) EIPA treated *sodC*⁻ cells showed no significant change in the level of active RacC level. The images obtained from western blot were scanned and quantified using UN-Scan-IT gel software. E) This figure is collaborating work with Dr. Heo at UT, Arlington. Effect of redox agents on the RacC GDP dissociation. Xanthine Oxidase was pre-incubated with xanthine (10 mM) and O₂ in the presence and absence of Superoxide Dismutase (SOD, 5,000 units) for 1 h. The assay was initiated by an addition of RacC (1 μ M) loaded with [³H]GDP to the assay solution, and aliquots of the assay sample were then withdrawn with a syringe at specific times and spotted onto nitrocellulose membrane filters, then washed three times with an assay buffer. The radioactivity of the nitrocellulose membrane filters was measured with a scintillation counter (Beckman). Rac1 was used as a control.

Fig. 10

C

***Wt* *sodC*⁻**

**Active RacC
(GST-WASP-GBD)**

Total RacC

D

EIPA

***Wt* *sodC*⁻**

**Active RacC
(GST-WASP-GBD)**

Total RacC

E

Figure 11. EIPA treatment significantly improved CV morphogenesis, and functional CV cycle of *sodC*⁻ cells. A) The CV images of Wt and *sodC*⁻ cells treated with 25μM EIPA are shown. The EIPA treated *sodC*⁻ cells mostly displayed a single bladder. (B) The number and size of the EIPA treated *sodC*⁻ cells were comparable to those of wild type cells. The average number of bladders was reduced by 40% while the average size was increased by 30% after treatment with EIPA. These values were from three independent experiments and the p-value was smaller than 0.05. (C) *sodC*⁻ cells treated with EIPA displayed a single fully charged bladder that discharged around 100 seconds, which is significantly faster than that of *sodC*⁻ cells.

Fig. 11

B

C

sodC-::GFP-Dajumin + EIPA

Figure 12. Aberrant phenotype of contractile vacuole in *mhcA*⁻ cells. A) Wild type and *mhcA*⁻ cells were transformed with the GFP-Dajumin to examine CV system. B) The representative images show the average number of CV bladders were 3 times more in *mhcA*⁻ cells as well as their average size was 1.5 times more. The results obtained from 3 independent experiments with p-value less than 0.001. C) *mhcA*⁻ cells displayed extended time taken to complete the CV cycle, more than 3 minutes. (Scale=10μm).

Fig. 12

B

C

3.5 Discussion

Small GTPase such as Ras and Rac play key role in integrating signaling pathways essential for cellular functions such as chemotaxis and cell migration (Insall et al., 1996; Wilkins et al., 2000). Previous *in vitro* studies have suggested that these small GTPase have redox sensitive motifs, NKCD or P-loop, which are the direct target of reactive oxygen species causing their dysregulation (Heo, 2011). Since *sodC*⁻ cells displayed higher levels of active Ras (Veeranki, et al., 2008), we examined the active RacC levels in *sodC*⁻ cells and found that compared to wild type cells, *sodC*⁻ cells had five times lower active RacC. On the contrary, RacC activity *in vitro*, tested by GDP dissociation assay revealed that RacC dissociated from GDP and was activated in redox environments. The molecular mechanism of RacC dysregulation is currently unknown, but it is plausible that the P loop cysteine might have dominating negative effect over the NKCD motif under superoxide-enriched conditions.

The mechanism of PI3K mediated CV bladder fusion with the plasma membrane is currently unknown, but PI3K could organize the cortical F-Actin remodeling to allow bladder juxtaposition to the target region of the plasma membrane. Depleting PIP2 by converting PIP3 may decrease the cortical F-Actin densities. Optimizing the cortical structure of the target region for the fusion may also involve remodeling the cortical region that includes Myosin II. A study showed that proper regulation of Myosin II is essential for CV bladder function (Betapudi and Egelhoff et al., 2009). Other studies previously showed that Von Willebrand factor Kinase A (VwkA) modulate number and size of CV bladders and dischargement of the bladder with the plasma membrane in Myosin II dependent

manner (Betapudi, et al., 2005), although VwkA does not directly phosphorylate Myosin II (Crawley and Côté 2008).

Considering that superoxide could generally target small GTPases that contain NKCD motif, it is plausible that RasB and Rap1 proteins, both of which contain the NKCD motif, are constitutively activated in *sodC⁻* cells (Pino, et al., 2015). If these small GTPases were persistently activated in *sodC⁻* cells, Myosin II activity would be decreased as well, which in turn will lead to the aberrant CV morphogenesis and function. One of the proteins that modulate Myosin II assembly in *Dictyostelium* cell is NHE1. Myosin II preferentially localize to the posterior end of a migrating *Dictyostelium* cells. Cells lacking NHE1 displayed lack of polarized Myosin II assembly (Lusche, et al., 2011), suggesting that EIPA treatment would have similar effect on the Myosin II assembly in the context of aggregation competent chemotaxing *Dictyostelium* cells.

Previous experiments conducted in our lab to study the effect of EIPA on chemotaxis by modulation of Ras and Rac proteins suggest that EIPA treated wild type cells displayed comparable levels of directional migration (chemotaxis index) but hampered motility. Considering that RasC is redox insensitive (Castillo, et al., 2016), it is likely that the attenuated RasG activity would only partially interfere chemotaxis. In contrast, EIPA treated *sodC⁻* cells exhibited significantly improved chemotaxis both in directional migration and motility. Furthermore, although both Ras and RacC were aberrantly regulated in *sodC⁻* cell, only the Ras regulation was restored by EIPA treatment. The full elucidation of the molecular mechanism of EIPA mediated CV modulation requires further investigation, but it is plausible that EIPA mediated restoration of Ras proteins may not only include RasG, an upstream regulator of PI3K, but may also include

RasB and/or Rap1, regulators of Myosin II assembly. It will be important to determine in the future that if the EIPA treatment indeed have effects on the Myosin II assembly in vegetative cells under hypotonic environment.

As discussed previously, conventional Myosin II play key role in regulating the cellular processes pertaining to cytokinesis, cell development and chemotaxis (Shelden and Knecht, 1996, De la Roche and Côté, 2001). As the binding of Myosin filaments to F-actin is required for myosin to act as molecular motor, it can be argued that due to impaired F-actin polymerization in *sodC* cells, myosin II filaments are unable to function and provide the cells with necessary cortical tension required for the proper CV discharge. In support of the hypothesis, we found that cells lacking *MhcA* also displayed poor CV function.

The study to elucidate the molecular mechanism of CV function is still poorly understood. More study needs to be carried out to understand the signaling pathways and molecular mechanism integrating to the final CV discharge process at the plasma membrane. It is quite possible that because of oxidative stress multiple signaling pathways are affected which produce a parallel effect on the other organelle such as contractile vacuole system.

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CHAPTER 4. CONCLUSIONS.

4.1 Conclusions

Contractile vacuole is an osmoregulatory organelle which is mainly utilized by protists such as *Dictyostelium*, *Paramecium*, *Amoeba*, *Trypanosomes* and to a lesser extent in green algae *Chlamydomonas* (McKanna, 1976). During evolution, this organelle disappeared in multicellular eukaryotes but still exist in unicellular stages of various fungi and sponges (Brauer and McKanna, 1978). Since this organelle is utilized as means of survival by many parasites, proteins regulating its morphogenesis and function have been of great interest. *Dictyostelium discoideum* has been widely used as a model organism to study the contractile vacuole system as large number of proteins regulating CV morphogenesis and function have been conserved among all the metazoans (Maniak, 2003). Furthermore, it provides experimental advantages as it is easier to create gene knockout and knockdown to study the role of specific genes and their interacting counterparts. Since *Dictyostelium* is a soil dwelling amoeba which inhabits variable osmotic environment, it has a well-developed and strong contractile vacuole system.

Contractile vacuole system is a network of bladders and interconnected tubules which collect the excess water entering the cell by osmosis and expel it through an intricate mechanism (Allen and Naitoh, 2002). This process is tightly regulated by many proteins and their effector molecules which provide the required competence for the cell to survive under hypoosmotic stress. As previously discussed in Chapter 1, contractile vacuole is a post Golgi organelle (Gabriel, et al., 1999) and proteins associated with the plasma

membrane are also localized on CV membrane. Number of contractile vacuole bladders may differ from one organism to other but they all share the common function (McKanna, 1976).

In this study, we report that SodC affects CV morphogenesis and function as *sodC*- cells displayed multiple CV bladders which were poorly interconnected resulting in defects of contractile vacuole function too. Furthermore, *sodC*- cells were defective against hypoosmotic stress as the survival efficiency of *sodC*- cells was indigent as compared to the *wild type* cells under same conditions. The aberrant phenotype of contractile vacuole bladders in *sodC*- cells was rescued upon treatment with superoxide scavenger, XTT, but there was only a modest effect on the CV function which suggest that there might be a direct or indirect interaction between the superoxide and the proteins related to CV morphogenesis.

Ras proteins control multiple cellular events such as growth, differentiation, movement, and apoptosis among many others (Wittinghofer, et al., 2000). *Dictyostelium* cells contain multiple Ras proteins that not only share high degree of sequence homology with those of higher eukaryotes, but also multiple conserved downstream signaling pathways and functional characteristics (Robbins et al., 1989). In a recent study, it was reported that hypoosmotic environment results in generation of superoxide (Castillo, et al., 2016) which can target small GTPases and cause their misregulation (Heo, 2005). *Wild type* cells under oxidative stress promote the localization of active RasG onto the vesicular compartments which is mediated by the superoxide dismutase C (SodC) (Castillo, et al., 2016) but cells lacking SodC displayed constant RasG activation at the plasma membrane. In our study, we observed the CV morphogenesis in redox insensitive mutant,

RasG(C118A)/*sodC*⁻ cells and found that the defects of CV biogenesis were rescued. These results were coherent with the hypothesis that redox sensitive NKCD motif is the direct target of superoxide and is misregulated under oxidative stress. We also found that overexpression of cytoplasmic SodA eliminated the multiple CV phenotype of *sodC*⁻ cells as well as decreased the active RasG levels in *sodC*⁻ cells. Together these results suggest a prominent role of RasG in contractile vacuole morphogenesis but a direct role of RasG cannot be established. In addition to Ras, we studied the downstream targets of Ras, PI3K, as *sodC*⁻ cells displayed dysregulation of PIP3 (Veeranki, et al., 2008). *sodC*⁻ cells treated with LY294002, displayed rescued phenotype for the CV morphogenesis but there was no effect on CV function. The above results strongly indicate that superoxide mediated misregulation of RasG is responsible for the aberrant CV morphogenesis and its defective function but the mechanisms regulating the interaction of RasG with the CV components are yet to be determined.

Dictyostelium cells perform chemotaxis for growth and development by sensing the extracellular environment (Lee, et al., 2005). The process of chemotaxis depends on localization of PIP3 at the membrane, followed by F-actin polymerization at the leading edge and Myosin II assembly in the posterior part of the cell (Kolsch, et al., 2008). Efflux of proton outside the cell has been established as a critical event during chemotaxis which is mediated by the action of NHE, localized at the plasma membrane (Patel and Barber, 2005). Inhibition of NHE by its pharmacological inhibitor, EIPA resulted in reduction of active RasG levels in *sodC*⁻ cells (Kabra, et al., 2017, (Under review)) as well as rescue of CV morphogenesis and its function which was coherent with our hypothesis that RasG

might be involved in regulating CV morphogenesis but there was no effect of EIPA on the active RacC levels.

Previous studies have demonstrated that RasG and RacC actively take part in integrating the regulatory mechanism for cytoskeleton assembly (Chung and Firtel, 1999). In our study, we found that *sodC*⁻ cells have lower active RacC as compared to wild type cells and there was no significant effect of EIPA on RacC activity. Although, the results from our *in vitro* GDP dissociation assay suggest RacC-GDP dissociation under redox environment. Considering the observation that NHE inhibitor, EIPA not only rescued defective Ras and chemotaxis, but also CV biogenesis and function, it is plausible that NHE proteins and SodC constitute an inherent regulatory circuit that controls extracellular superoxide mediated intracellular small GTPases.

The plausible theory for lower active RacC can be explained due to the presence of NKCD as well as P-loop which might be inhibiting the superoxide mediated RacC activation. Together our observation suggests that RasG is the major regulator of CV morphogenesis and function as our results suggest that there is no effect of EIPA on activity of RacC but still the CV defects were rescued. There are other Ras members such as RasB and Rap1, which indirectly regulate the activity of Myosin II assembly by activating MHCK (Jeon, et al., 2007, Mondal, et al., 2008). Both these members have redox sensitive motifs which might be misregulated under oxidative stress and could result in aberrant CV phenotype. Further study is necessary to elucidate the complete mechanism of Ras involvement in CV morphogenesis and function. Also, CV morphogenesis and function is also regulated by Rab GTPase which also have redox sensitive motif. It would be

interesting to study the misregulation of Rab members under oxidative stress and their effect on contractile vacuole.

Since contractile vacuole is an essential part of survival strategy utilized by a large number of pathogens such as *Trypanosoma*, which can survive immense osmotic pressure during the infection in mammals (Docampo, et al., 2013). Elucidating the regulatory circuits governing the morphogenesis and function of CV will allow us to create drugs aiming at disruption of this osmoregulatory organelle. Since most of the proteins involved in the CV network are conserved among all the protists, *Dictyostelium* could serve as a model organism to dissect the mechanism of this system. The natural habitat of *Dictyostelium* is soil where it encounters severe hypoosmotic and hyperosmotic environments and thus has a strong CV system. Since most of the proteins in *Dictyostelium* have a human orthologue, study of proteins in *Dictyostelium* gives us a better chance of understanding the evolutionary mechanism and conservation of function from lower eukaryotes to higher eukaryotes. Considering that all the regulatory proteins studied here are conserved in most of the eukaryotes, the current study provides a new paradigm to uncover the mechanisms of CV biogenesis and function in many other organisms.

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