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Superoxide Dismutase C Modulates Macropinocytosis and Phagocytosis in Dictyostelium Discoideum

Cong Gu
cgu001@fiu.edu

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

SUPEROXIDE DISMUTASE C MODULATES MACROPINOCYTOSIS AND PHAGOCYTOSIS IN DICTYOSTELIUM DISCOIDEUM

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Cong Gu

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

This dissertation, written by Cong Gu, and entitled Superoxide Dismutase C Modulates Macropinocytosis and Phagocytosis in *Dictyostelium Discoideum*, 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.

_____
Xiaotang Wang

_____
Jessica Liberles

_____
Lidia Kos

_____
Lou W. Kim, Major Professor

Date of Defense: November 9, 2018

The dissertation of Cong Gu is approved.

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

_____
Andres G. Gill
Vice President for Research and Economic Development
and Dean of the University Graduate School

Florida International University, 2018

DEDICATION

To my Family and friends for their support

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ABSTRACT OF THE DISSERTATION

SUPEROXIDE DISMUTASE C MODULATES MACROPINOCYTOSIS AND PHAGOCYTOSIS IN DICTYOSTELIUM DISCOIDEUM

by

Cong Gu

Florida International University, 2018

Miami, Florida

Professor Lou W. Kim, Major Professor

Macropinocytosis and phagocytosis, two actin-dependent and clathrin independent events of endocytosis, enable cells like macrophages and neutrophils to either internalize pathogens and initiates the human innate immune response or serve as a direct entry route for a productive infection from pathogens. *Dictyostelium discoideum*, a soil-living amoeba, is a unicellular eukaryote that internalizes fluids or particles several fold more than that of macrophages and neutrophils. Additionally, multiple key signaling pathways are conserved between *Dictyostelium* and mammalian cells, including pathways affecting small GTPases Ras and Rac and their downstream effectors, and F-Actin remodeling. All these traits makes *Dictyostelium* an excellent model organism to study the process of macropinocytosis and phagocytosis.

Upon internalization of a food item, these macropinocytes and phagocytes are often in an environment of increased production of superoxide radicals in the food-containing vesicles,

which helps stimulate the downstream signaling pathways to digest the food inside. However, the mechanism of how superoxide regulates the process of macropinocytosis and phagocytosis is not fully understood. We previously reported that *Dictyostelium* cells lacking *Superoxide dismutase C* (*SodC*) exhibited aberrantly high level of active RasG, high basal level of Phosphatidylinositol-3,4,5-triphosphate (PIP3), and severe chemotaxis defects. Now we report that *sodC*$^{-}$ cells displayed aberrant endosomal vesicle trafficking, significantly compromised particle uptake and defective cell to substratum matrix adhesion compared to that of wild type cells. By using high resolution live imaging microscope we also show that *sodC*$^{-}$ cells have defects in F-Actin remodeling at the phagocytic rim extension and F-Actin depolymerization of the nascent phagosome. Interestingly, the introduction of overexpressing of cytoplasmic superoxide dismutase (SodA), redox insensitive RasG (C118A) or treatment of PI3K inhibitor LY294002 in *sodC*$^{-}$ cells significantly rescued the defects of endosomal vesicle trafficking, particle uptake and adhesion. This project suggests that superoxide dismutase C regulates the endosomal vesicle trafficking, phagocytosis and cell to substratum matrix adhesion through the RasG/PI3K signaling axis in *Dictyostelium* cells.

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ABBREVIATIONS AND ACRONYMS

ABBREVIATION	FULL NAME
Arp2/3	actin-related protein 2/3 complex
cAMP	3’-5’-cyclic adenosine monophosphate
CFP	Cyan fluorescent protein
CR3	Complement receptor 3
DB	Developmental buffer
DNA	Deoxyribose nucleic acid
FITC	Fluoroscene Isothiocyanate
F-actin	Filamentous actin
FcγR	Fc-gamma receptor
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GTPase	Enzyme that hydrolyzes guanosine triphosphate
Hr	Hour
IP_3	Inositol-1,4,5-Triphosphate
JH10	*Dictyostelium* auxotrophic *wild type* strain
kD	Kilo Dalton

L	Liter
LB	Luria broth
LY294002	Inhibitor of phosphoinositide 3-kinases
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor-κB
NHE	Na^+/H^+ exchanger
NOX	NADPH oxidase
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PI(4,5)P2	Phosphatidylinositol 4,5-bisphosphate
PKB	Protein Kinase B
Ras	Rat sarcoma

RasG	Ras-like protein rasG
ROS	Reactive oxygen species
SD	Standard deviation
sodC^{-}	Ablation of Superoxide Discmutase C
TorC2	Target of Rapamicin Complex 2
TRITC	Tetramethylrhodamine
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
µ	Micro
WASP	Wiskott–Aldrich syndrome protein
WASH	WASP and SCAR homolog
Wt	*Wild type*

CHAPTER 1 INTRODUCTION

1.1 Up-taking Extracellular Materials by Eukaryotic Cells

External materials can enter cells through a variety of processes. As a consequence of internalization, vesicles containing extracellular materials form, and soon start to travel through various routes (Kaksonen and Roux, 2018). The initial vesicles are either small (60-120 nm) or large (up to several micrometers). These processes cover not only the highly specific receptor-mediated processes but also rather general bulk up-taking of fluids, and thus multiple cellular processes are dependent on these processes. Examples of such events include nutrient up-taking, signaling receptor trafficking, apoptotic cell removal, and host-pathogen interaction. Processes of up-taking extracellular materials can be further subdivided into four categories: clathrin-dependent endocytosis, caveolae-dependent endocytosis, macropinocytosis, and phagocytosis.

1.2 Clathrin-dependent Endocytosis

Clathrin-dependent endocytosis is one form of endocytosis which is named after the endocytic coat protein, clathrin. The complete process can be separated into four stages (figure 1). (I). Clathrin and other accessory proteins start to be enriched at the cortical region of the inner leaflet of the plasma membrane. (II). Extracellular materials bound to their cognate receptors are then recruited through association with coating proteins which leads to the invagination of the plasma membrane. (III). Actin and proteins like dynamin that facilitate membrane fission separate the neck of the invaginating plasma membrane.

The extracellular materials containing vesicles are still coated with Clathrin. (IV). Clathrin coats then dissociate from the vesicle and form naked vesicles. These naked vesicles contain and recruit various proteins to steer their way through various vesicular trafficking routes of the cell (Kaksonen and Roux, 2018).

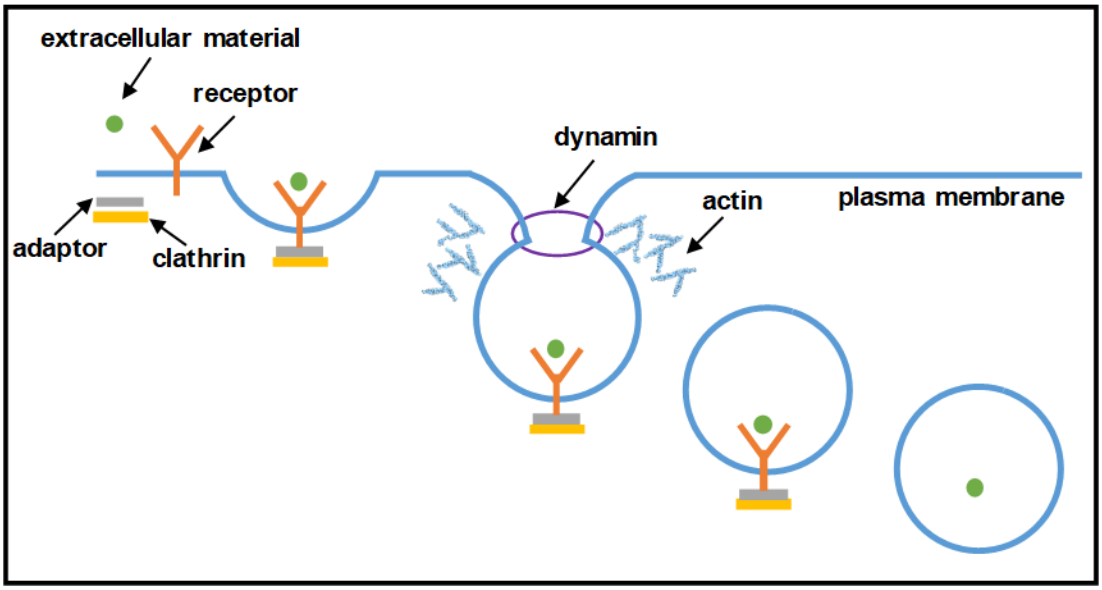


Figure 1. Stages of Clathrin-dependent Endocytosis
Cytosolic clathrin and other coating proteins aggregates at the plasma membrane to associate with extracellular materials which leads to the membrane invagination. Actin and dynamin then work cooperatively to cut the neck of invaginating plasma membrane. Clathrin and other coating proteins are then dissociated from the naked vesicles which leads to various routes of vesicle trafficking. (Modified from Kaksonen and Roux, 2018)

It has been reported that in mammalian cells, BAR domain (Bin, amphiphysin and Rvs) proteins F-BAR domain only protein 1 (FCHO1) and FCHO2 (Henne et al., 2010), the Clathrin adaptor protein AP2 complex, epidermal growth factor receptor substrate 15 (EPS15) and monomeric cargo adaptors proteins are involved in the early initiation step of the Clathrin-dependent endocytic events (figure 2). The current paradigm assumes that FCHO1/2 and AP2 proteins, which associate with the plasma membrane, recruit proteins

such as Clathrin, EPS15, and monomeric cargo adaptor proteins to the endocytic site and initiate endocytosis. Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) has also been considered as another critical regulator that affects the initiation of endocytosis and assembly of endocytic coat considering the fact that many adaptor proteins bind specifically to PI(4,5)P2 (Zoncu et al., 2007).

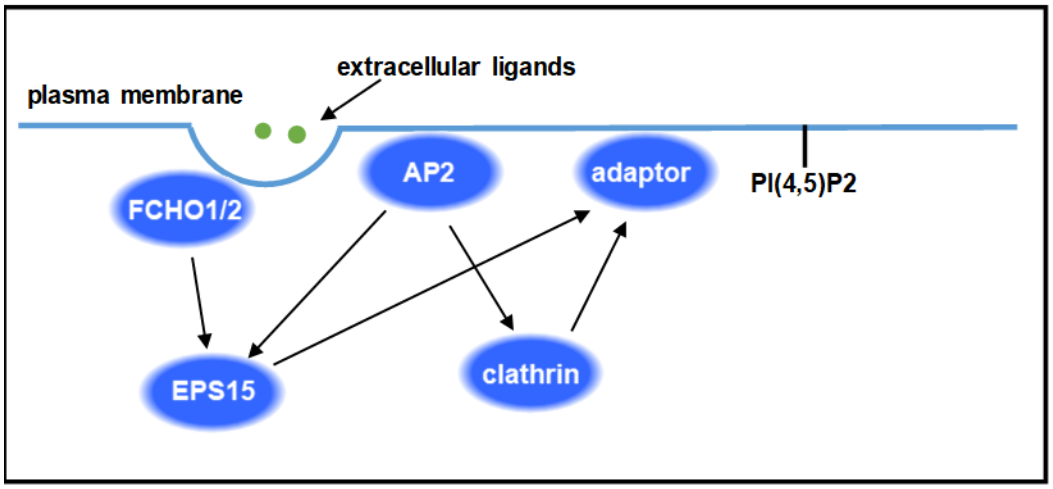


Figure 2 Early Initiation Step of the Clathrin-dependent Endocytosis
Current model assumes that activated FCHO1/2 and AP2 proteins are localized at the inner leaflet of the plasma membrane. The activation of FCHO1/2 and AP2 proteins leads to the recruitment of Clathrin, EPS15, and monomeric cargo adaptor proteins to the site of extracellular ligands and promotes clathrin-dependent endocytosis. (Modified from Kaksonen and Roux, 2018)

In yeasts, during the early period of Clathrin-dependent endocytosis, Las17 (prolin-rich protein Las17; homologue of mammalian Wiskott–Aldrich syndrome protein (WASP)) and type I myosins (Myo3/5) are reported to activate actin-related protein 2/3 complex (Arp2/3) and thus directionally promote actin polymerization at the site of membrane invagination (figure 3) (Sirotkin et al., 2005). Homologue of mammalian Huntingtin-interacting protein 1-related protein (Sla2) and epsin 1 (Ent1), two of the Clathrin adaptors,

then associate Clathrin coated invaginating structure with actin filaments and facilitate plasma membrane invagination (Sun et al., 2015).

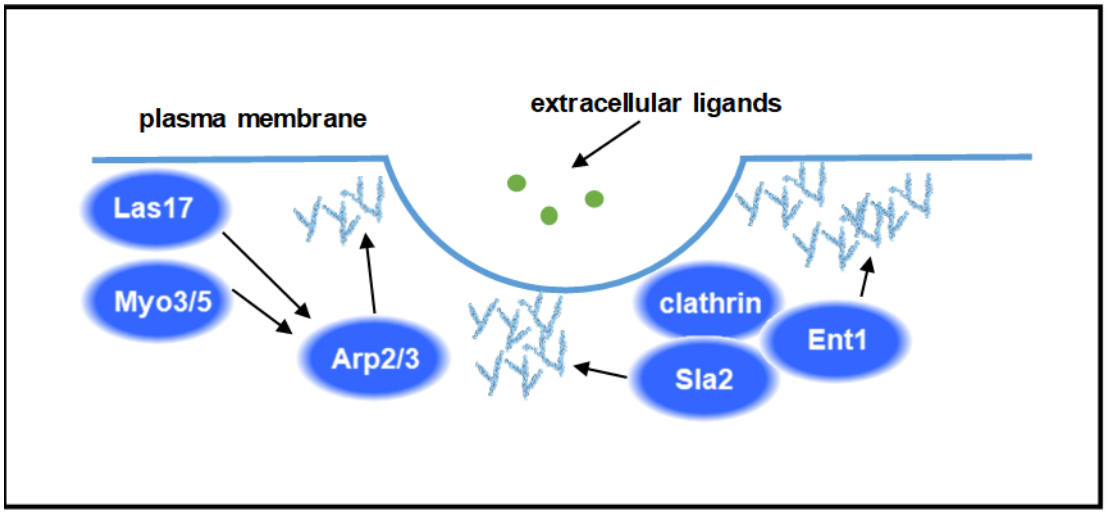


Figure 3 Actin mediated Membrane Invagination in Yeasts
In yeasts, Las17 (homologue of WASP) and type I myosins (Myo3/5) regulates the Actin dynamics at the site of membrane invagination by activating actin-related protein 2/3 complex (Arp2/3). Clathrin adaptors proteins Sla2 and Ent1 act as linkage to associate clathrin with dynamic Actin filaments to promote the membrane invagination of clathrin-dependent endocytosis. (Modified from Kaksonen and Roux, 2018)

Invagination of the membrane is followed by fission event in which at least two groups of proteins are involved. First, Formin-binding protein 17 (FBP17) and Sorting Nexin 9 and 18 (SNX9/18) are recruited to the neck of the invaginating membrane (Wu et al., 2010), and then another group of proteins such as endophilin and amphiphysin are clustered, coupled with actin, and promote the fission of the invaginating membrane (figure 4) (Takei et al., 1999). Additionally, the recruitment of dynamin-GTP to the neck of membrane invagination redistributes auxilin/ heat shock cognate 71 kDa protein (HSC70) to its location, which helps drive the fission of the membrane pit. Completion of fission is followed by the GTP hydrolysis of dynamin-GTP, which in turn relocates dynamin to

cytoplasm and interfere with its interaction with auxilin/HSC and thus drive the uncoating of clathrin (Ungewickell, 1985, Newmyer et al., 2003). At the same time, a number of adaptor proteins that bind to PI(4,5)P2 dissociate as PIP2 becomes PI4P by 5'-phosphtases such as SHP2 and OCRL (McPherson et al., 1996).

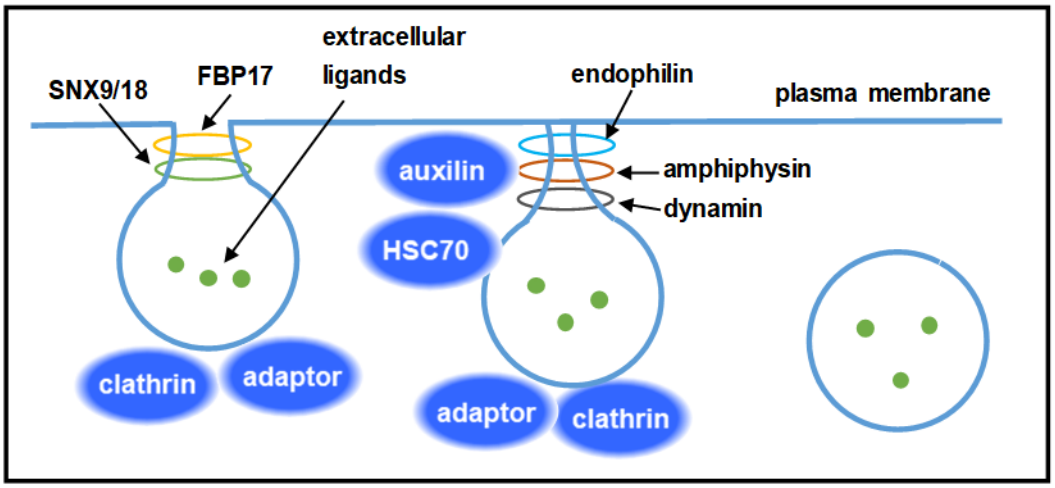


Figure 4. Fission and Uncoating Event of Clathrin-dependent Endocytosis
FBP17 and SNX9/18 are recruited to the neck of the nascent membrane invagination which is coated with Clathrin and adaptor proteins to initiate the fission of membrane. Another group of proteins including endophilin, amphiphysin, actin filament, dynamin-GTP, auxilin and HSC70 are then clustered to the neck of membrane and promotes the completion of fission event. The inactivation of dynamin then drives the dissociation of auxilin, HSC and other clathrin coating proteins concomitantly with the dissociation of adaptor proteins which bind to PI(4,5)P2 during the SHP2 and OCRL mediated de-phosphorylation of PI(4,5)P2. (Modified from Kaksonen and Roux, 2018)

1.3 Caveolae-dependent Endocytosis

Caveolae are non-clathrin-coated invaginations from plasma membrane, of which shape resemble that of a flask or letter omega and typically enriched with cholesterol and sphingolipids and have been found in multiple types of eukaryotic cells (Pelkmans and Helenius, 2002). In recent studies, Caveolae-dependent endocytosis has been reported to be a pathway of uptaking extracellular material in parallel with Clathrin-dependent

endocytosis. Caveolae are generally thought to be involved in several biological processes such as signal transduction, lipid metabolism and vesicular transport. Caveolin-1, a small integral membrane protein localized in the inner leaflet of the plasma membrane, has been suggested to be one of the main structural proteins of caveolae and responsible for the morphology of caveolae (Anderson, 1998). Furthermore, the N terminal domain of caveolin-1 has been shown to associate specifically with the hydrophobic core of cholesterol and sphingolipids (Pelkmans and Helenius, 2002). Caveolin-1 becomes tyrosine phosphorylated as extracellular materials load, which in turn initiate the internalization of caveolae. Caveolin-1 then couples with the GTPase Dynamin and actin cytoskeleton, which together execute the scission of the neck of caveolae and inward transportation of caveolae (Parton et al., 1994). After completion of Caveolae fission, motor proteins are suggested to drive the caveolae along the microtubules (Mundy, 2002). Upon internalization of caveolae, multicaveolar complexes called Caveosomes will follow different routes depending on the presence of specific trafficking cues on the Caveosomes. Certain Caveosomes will travel through the classical endocytic route towards lysosome for degradation, while Caveosomes that contain viruses often times do not fuse with lysosomes and allow viruses to escape from the digestion pathway to lysosome (Pelkmans et al., 2001). Figure 5 highlights the events that constitute the Caveolin-dependent endocytosis.

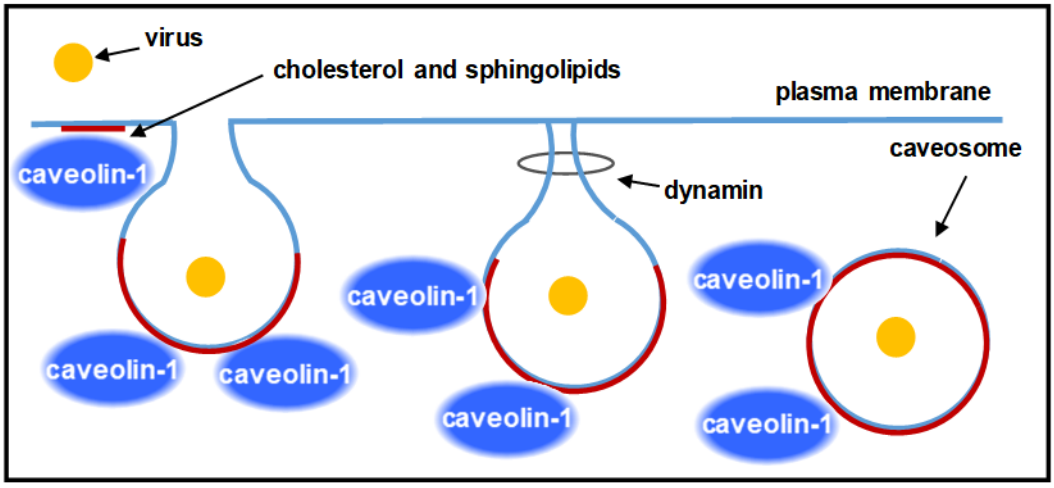


Figure 5. Caveolae-dependent Endocytosis
Caveolin-1, one of the main structural proteins of caveolae, is associated with the enriched cholesterol and sphingolipids at the inner leaflet of the plasma membrane which in turn initiates the internalization of caveolae. Multicaveolar complexes caveosomes will go through different routes depending on the trafficking cues on the caveosomes. Caveosomes containing viruses often escape the classical endocytic routes from being digested.

1.4 Macropinocytosis

Macropinocytosis is actin-dependent and clathrin-independent mechanism of internalization of bulk fluid phase. Macropinocytosis occurs both spontaneously and in an extracellular signal dependent manner. In both cases, dynamic actin driven membrane protrusions such as circular ruffles or ones that are similar to the Lamellipodia capture extracellular materials as the protruding structures close at the base of plasma membrane. Interestingly, non-dedicated phagocytes that are incapable of up-taking large particles through phagocytosis could internalize bacteria and viruses through macropinocytosis (Francis et al., 1993), which leads to the capability of uptaking numerous kinds of extracellular materials for those cells that are not the professional phagocytes. The

Lamellipodia-like protrusions arise from the base of the plasma membrane, fold back, fuse with the base of the plasma membrane, and form nascent macropinosomes (figure 6). On the other hand, multiple protrusions can exist in circular ruffles that can fuse with each other and form the macropinosome (figure 6) (Swanson, 2008). After complete closure, a macropinosome will be released from the plasma membrane and moves inward to the cytosol and undergoes macropinosome maturation.

The binding of extracellular ligand to receptor tyrosine kinase (RTKs) leads to the dimerization and activation of RTK, which in turn stimulates a signaling cascade to regulate Actin cytoskeleton rearrangement at the inner leaflet of the plasma membrane and triggers membrane protrusion (figure 7) (Maniak, 2001). After activation of RTKs, small G proteins including Rac1, Rab5 or Arf6 are activated (Bar-Sagi and Feramisco, 1986). The activation of Rac1 mediates the Actin remodeling stimulation and macropinosome closure, which is mediated by the activation of actin polymerization effector and myosin-dependent contraction regulator (Ridley et al., 1992). It has been shown that once Rab GTPase Rab 5 is activated, Rab 5 relocalizes to the circular ruffles together with Rab5 GAP/effector RN-tre and thus mediate Actin-associated membrane ruffle formation and macropinosome trafficking (Ridley et al., 1992). ADP-ribosylation factor 6 (ARF6), an ARF GTPase, has been reported to promote the formation of ruffles and macropinocytosis by localizing Rac1 to the plasma membrane in addition to its contribution to macropinosome trafficking and F-Actin remodeling (Radhakrishna et al., 1999).

Apart from small G proteins, kinase is another widely reported regulator of macropinocytosis (Mercer and Helenius, 2009). Serine/threonine-protein kinase (Pak1), also known as p21-activated kinase 1 which can be activated by Rac1. The activation of Pak 1 can further regulate the signaling pathway to promote macropinosome closure by activating carboxy-terminal-binding protein-1 (CtBP1) (Liberali et al., 2008).

Protein kinase C (PKC) also stimulates the formation of membrane ruffles and macropinosome. Interestingly, the activation of PKC by phorbol esters can induce macropinocytosis in a receptor activation-independent manner (Swanson, 1989), which can be regarded as a compensation pathway for ensuring macropinocytosis in the circumstance of receptor dysfunction. Additionally, c-Src, a non-receptor type tyrosine kinase, is activated and recruited to the site of ruffle formation at the inner leaflet of the plasma membrane to further activate the downstream proteins such as Rac1, actin related protein 2/3 complex (Arp 2/3), and PI3K to cooperatively promote the RTK mediated macropinocytosis signaling (Donepudi and Resh, 2008, Amyere et al., 2000). In addition, Na^+/H^+ exchangers, also known as NHE, have been found to be involved in macropinocytosis: the inhibition of NHE with its specific inhibitor EIPA led to the inhibition of membrane ruffles formation (West et al., 1989). However, considering that NHE could affect multiple signaling events through dysregulation of the cytoplasmic pH, the function of NHE in regulating membrane ruffles formation still requires extensive future research. It is also important to note that the Actin cytoskeleton remodeling is critical

for proper macropinocytosis given that the membrane ruffle formation heavily relies on the Nucleation Promoting Factors for F-Actin such as WAVE and Arp2/3 (Buccione et al., 2004).

Upon completion of macropinosomal membrane fission, multiple molecular events facilitate macropinopinosome maturation and trafficking. Proper regulation of phosphoinositides seems to be essential as PI3K is indispensable for the closure of macropinosome (Amyere et al., 2000). Myosin II, one of the mostly well characterized motor proteins and is mainly responsible for the contractile activity of cells, plays critical role in membrane ruffle formation and macropinosome closure (Araki, 2003). Recent studies indicates that, in addition to the modulation of CtBP-1 in macropinosomal cup, CtBP-1 also plays a role in the closure of macropinosome since the inhibition of CtBP-1 prevented the completion of macropinosome closure (Liberali et al., 2008). After complete closure, macropinosomes detach from the plasma membrane and start to traffic inwardly to various cytosolic routes. As they travel, they will undergo maturation steps which include acidification, fusion with endosome, recycling back to the plasma membrane, or fusion with lysosomes which contains digestive enzymes to help digest the cargo in macropinosome (Hewlett et al., 1994).

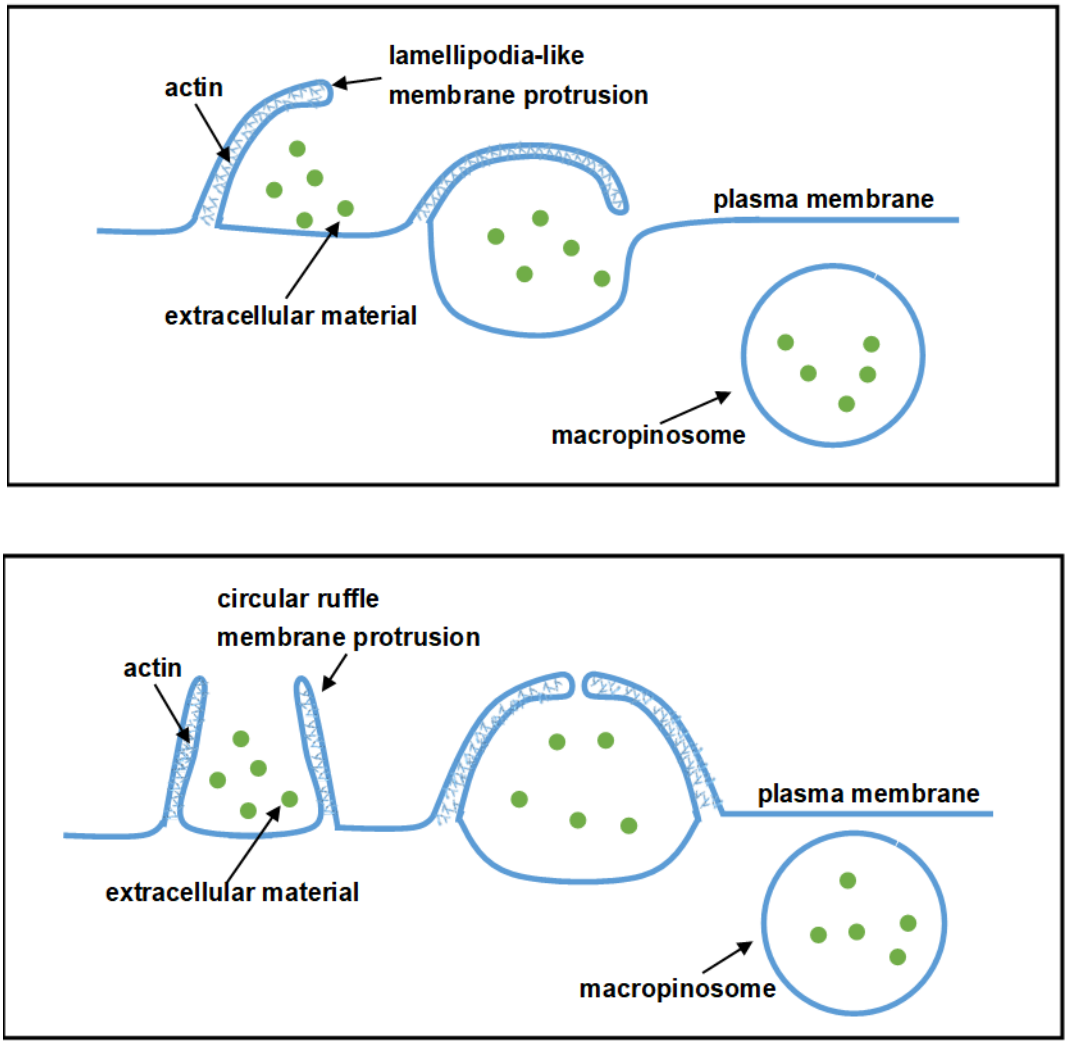


Figure 6. Actin-dependent Macropinosome Formation

The extracellular ligands stimulate the Actin cytoskeleton rearrangement at the plasma membrane, which induces the formation of membrane protrusion in the form of lamellipodia-like or circular ruffles. Lamellipodia-like protrusions arise, fold back, fuse with the base of the plasma membrane, and form nascent macropinosomes. Similarly in circular ruffles, multiple protrusions fuse with each other to form macropinosomes. Macropinosomes are then dissociated from the plasma membrane and undergo lateral maturation step. (Modified from Mercer and Helenius, 2009)

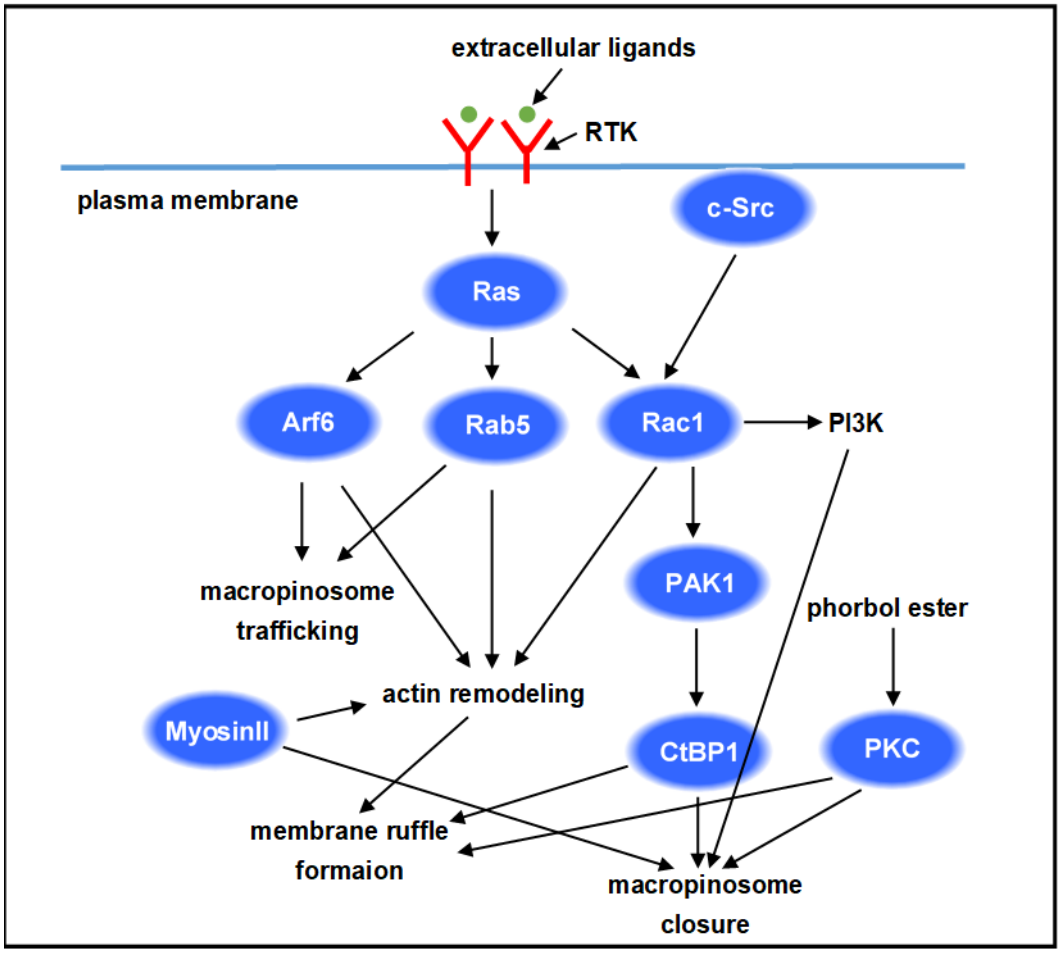


Figure 7. Signaling Pathways of RTK-dependent Macropinocytosis

The binding of extracellular ligand to receptor tyrosine kinase (RTKs) leads to the dimerization and activation of RTK, which in turn stimulates a variety of molecules such as small GTPases, kinases and PI3K to regulate downstream Actin cytoskeleton rearrangement at the inner leaflet of the plasma membrane. All of these signaling pathways working cooperatively to regulate Actin-dependent membrane protrusion which is required for macropinosome trafficking and closure. (Modified from Kaksonen and Roux, 2018)

1.5 Phagocytosis

Phagocytosis is the process by which cells bind and internalize large particle which is larger than 0.5μm in diameter. Phagocytosis could serve as a means of up-taking nutrients in protists, such as amoeba, but it could also be used as a mechanism of defense for cells dedicated for defense. Macrophages and neutrophils from higher eukaryotes are well-known examples of cells dedicated for defense, but certain amoeba such as Sentinel cells of *Dictyostelium discoideum* also actively utilize Pattern recognition Receptor mediated phagocytosis and thus operate the innate immune system. Phagocytosis thus functions not only as a means of capturing nutrients, but also as a part of innate immune system of both lower and higher eukaryotes.

Phagocytosis is a complex series of molecular events that could be organized into six stages. (I). The extracellular particle binding to the cell surface through a certain receptor. (II). Activation of signaling events that induce the phagocytic cup formation at the base of the particle binding site. (III). Elongation of the rim of the phagocytic cup. (IV). Phagocytic cup closure and actin-coat dissociation. (V). Trafficking of phagosome toward lysosome for destruction of the particle. (VI). Excretion of indigestible material through exocytosis.

The first step of phagocytosis, adhesion of the extracellular particles to cell surface receptor, is mediated by a variety of receptors tailored for the diverse types of extracellular materials.

Although diverse these phagocytic receptors can be grouped into two categories: receptors recognizing exogenous ligands and receptors targeting part of apoptosed cells. Toll-like receptors (TLRs), one of the receptors recognizing exogenous ligands, has been reported to target pathogens like bacteria and parasites. Similarly, phagocytic receptors like scavenger receptor A (CD204) can detect lipopolysaccharide and lipoteichoic acid of bacteria (Peiser et al., 2000). Another example is mannose receptor (CD206) which specifically targets yeast cells by recognizing yeast polysaccharides (Ezekowitz et al., 1990). Fc gamma receptor (FcγR) binds to the Fc region of immunoglobulin G (IgG) and CR3 detects the complement component iC3b (Anderson et al., 1990, Ross et al., 1992). Interestingly, certain regulatory protein such as small GTPase Rap1 was shown to enhance the binding affinity of CR3 to its ligand iC3b upon receiving activation signal from LPS and inflammatory signaling (Caron et al., 2000), which is generally called inside-out signaling of integrin receptors. Surprisingly, some pathogens have evolved the ability to disturb the binding of opsonins to FcγR or CR3 and thus prevent them from being phagocytosed. For instance, it has been found that some bacteria and fungi species can produce polysaccharide-based capsules which hampers the deposition of opsonin. Other bacteria species are capable of the synthesis of special surface proteins that prevent the binding of bacteria and its cognate receptor. Moreover, some pathogens like *Staphylococcus aureus*, specifically utilizes a protein called Protein A to interfere with FcγR-mediated phagocytosis, which is accomplished by high potency binding of protein A

to Fc region of IgG (Foster, 2005). Apart from the receptors which detect exogenous ligands, a variety of receptors that target endogenous ligands, such as ones on apoptosed cells, exist. In recent studies, it has been suggested that apoptotic cells usually synthesize chemoattractant molecules such as ATP, lysophosphatidylcholine, fractalkine, and sphingosine 1-phosphate during apoptosis to direct the phagocytes recruit to the sites of apoptotic cells (Chekeni and Ravichandran, 2011). To be differentiated from the healthy cells, apoptotic cells always re-localize phosphatidylserine (PS) to the outer leaflet of the plasma membrane which can then be detected by a variety of receptors such as Stabilin-2, TIM (T cell immunoglobulin mucin) family (Kobayashi et al., 2007). Among all these receptors that engage in the detection of extracellular ligand, FcγR mediated internalization of IgG coated antigen is of great importance in immunology and has been studied widely. Basically, it can be subdivided into two stages. (I). An IgG coated antigen binds to FcγR. (II). The activation of FcγR initiates a signaling cascade which drives the internalization of IgG coated antigen in an Actin-dependent manner.

The Fc receptors have always been found clustered together which is important for stimulating lateral cellular processes (Holowka et al., 2007). Immunoreceptor tyrosine-based activation motif (ITAM), which resides in the cytosolic domain of Fc receptors, is then phosphorylated by Hck, Lyn, Fgr or Src-family kinases (SFK) (Hamada et al., 1993). The activation of ITAM is required for proper signaling of phagocytosis. After activation of ITAM, spleen tyrosine kinase (Syk) is recruited to the docking site of ITAM through

specific phosphor-tyrosine - Src homology 2 (SH2) binding between the SH2 domains of Syk and phosphorylated ITAM (Johnson et al., 1995). The activation of Syk was shown to be essential for transmitting signals to several downstream adaptor proteins. For instance, transmembrane protein linker of activated T cells (LAT) was recruited to FcγR /Syk complex and phosphorylated by Syk (Zhang et al., 1998). Subsequently, Grb2 was recruited to the phosphorylated LAT through its SH2 domain, which in turn invited Grb2-associated binder (Gab2). Activated Gab2 can further activate downstream PI3K and produce phosphatidylinositol-3,4,5-trisphosphate [PI(3,4,5)P3] at the plasma membrane, which is important to induce the recruitment of other downstream proteins. Additionally, CrkII, another adaptor protein, can also bind to the phagocytic cup through its SH2 domain and help phagocytosis (Lee et al., 2007). In addition to adaptor proteins, lipid also plays an essential role in regulating phagocytosis. For example, Phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2], which uniformly localizes at the inner leaflet of the plasma membrane of resting phagocytes. However, during phagocytosis, PI(4,5)P2 was observed to transiently increase its level at the site of phagocytic cup and then quickly disappear afterwards (Botelho et al., 2000). Currently several signaling pathways regulating the disappearance of PI(4,5)P2 have been reported. For instance, phospholipase Cγ (PLCγ), which is known to be a phosphoinositide-specific phospholipase Cγ, is activated and then recruited to the site of phagocytic cup formation through the binding of SH2 domain of PLCγ and LAT (Liao et al., 1992). Activated PLCγ then converts PI(4,5)P2 to inositol-

3,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG acts as a regulator to modulate the activity of protein kinase Cε (PKCε), which promotes phaogocytosis (Larsen et al., 2002). On the other hand, PI(4,5)P2 can also be phosphorylated by class I phosphatidylinositol 3-kinase (PI3K) to produce PI(3,4,5)P3 at the inner leaflet of the plasma membrane (Marshall et al., 2001). One of the functions of PI(3,4,5)P3 is to stabilize Gab2, and thus promote FcγR mediated phagocytosis (Gu et al., 2003).

In addition to the phosphoinositides, small GTPases act as essential regulator of phagocytosis. After the activation of cell division control protein 42 (Cdc42), two other Rho family small GTPases, Rac1 and Rac2, are subsequently localized and activated at the nascent phagosome or the base of phagocytic cup, respectively (Hoppe, 2004). Macrophages or neutrophils of double-knockout (*rac1*$^{-/-}$, *rac2*$^{-/-}$) or single-knockout (*rac2*$^{-/-}$) mice showed significantly defective phagocytosis, suggesting that Rac1 and Rac2 are involved in the regulation of phagocytosis (Koh et al., 2005). The activation of Rac requires the recruitment of CrkII-mediated Dock180-ELMO1 and Syk-mediated binding of Vav proteins (Lee et al., 2007, Deckert et al., 1996).

The actin cytoskeleton rearrangement has been regarded as a driving force for the formation of phagocytic cup. The formation of actin filament requires the nucleation of actin monomers. Actin-related protein-2/3 (ARP2/3), one of the Actin nucleation factor, can specifically promotes the formation of a newly formed Actin filament from the side of an existing Actin filament (May et al., 2000). The activation of Arp2/3 requires the

activation of Wiskott-Aldrich syndrome protein (WASP) family which include WASP, N-WASP and the Scar/WAVE proteins. Small GTPase Rac, Cdc42 and PI(4,5)P2 can act as the upstream regulators of WASP family proteins. After binding of Cdc42 and PI(4,5)P2 to WASP, the VCA (verprolin homology, cofilin homology, and acidic) domain of WASP is then exposed and provide a docking site for the binding and activation of Arp2/3 complex (Higgs and Pollard, 2000). Myosin, an ATP-dependent protein, is well known for being responsible for the contraction of muscle. It is also known as Actin-associated protein which helps Actin-based membrane protrusion in a variety of motility processes such as phagocytosis in eukaryotes. Early studies indicates that Myosin X has been reported as involved in formation of phagosomes during FcγR mediated internalization of large particles in a PI3K-dependent manner (Cox et al., 2002). Signaling network of Actin Polymerization in FcγR mediated Phagocytosis is summarized in figure 8.

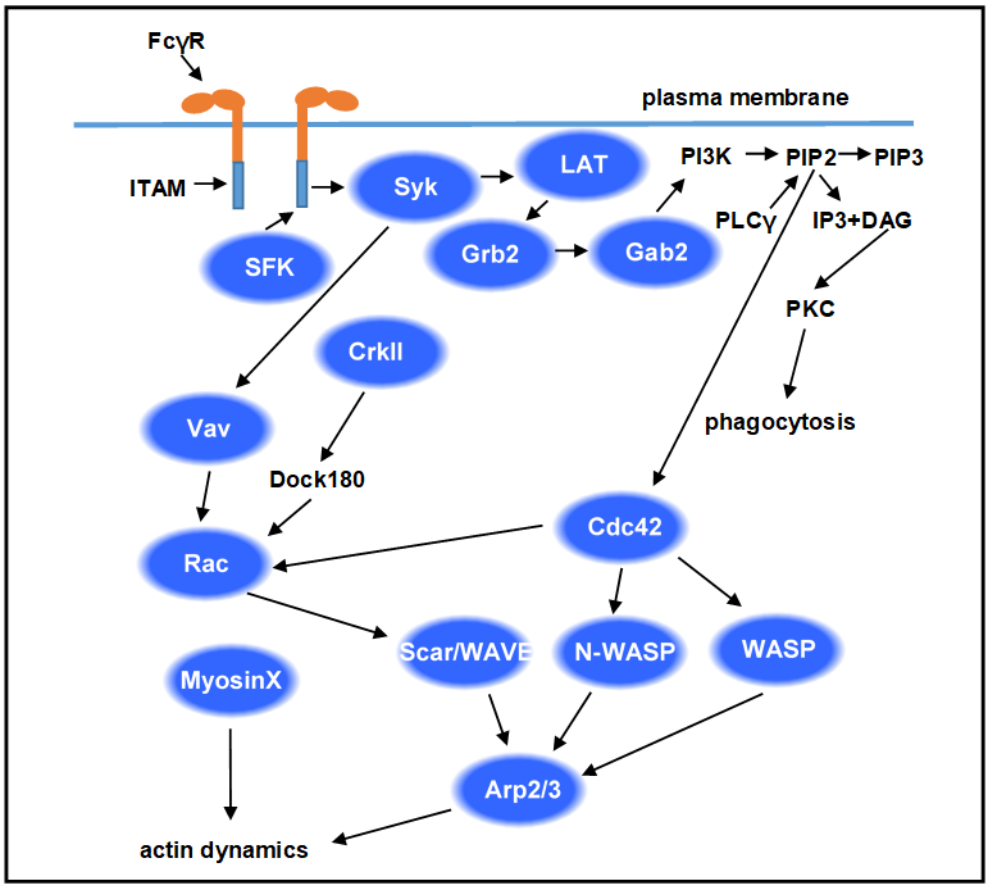


Figure 8. Signaling of Actin Polymerization in FcγR mediated Phagocytosis
The binding of extracellular ligands to clustered FcγR stimulates multiple signaling which in turn activates the Actin polymerization at the site of phagocytic cup. The molecules involved in the regulation of Actin dynamics includes kinases, adaptor proteins, lipid-regulation enzymes, small GTPases, nucleotide exchange factors and nucleation promoting factors. The vast network of Actin polymerization signaling plays an important role in FcγR meditaed phagocytosis. (Modified from Flannagan et al., 2011)

In addition to phagocytic receptor FcγR, complement receptor 3 (CR3), which is also known as Macrophage-1 antigen or $\alpha_M\beta_2$ integrin, is of great importance and has been widely studied in immunology. First of all, RhoA activates the serine/threonine kinase Rho kinase (ROCK) followed by the activation of myosin II (Olazabal et al., 2002). Activated myosin II then helps stimulates Arp 2/3 to contribute to F-Actin dynamics at the site of phagocytic cup. On the other hand, RhoA activates formin mDia1, a Rho-GTPase effector

protein, to induce F-Actin polymerization during phagocytosis (Colucci-Guyon et al., 2005). Microtubules have also been suggested to contribute to the CR3 mediated phagocytosis through the interaction of microtubule associated protein CLIP-170 and mDia1 (Lewkowicz et al., 2008). Figure 9 highlights these events.

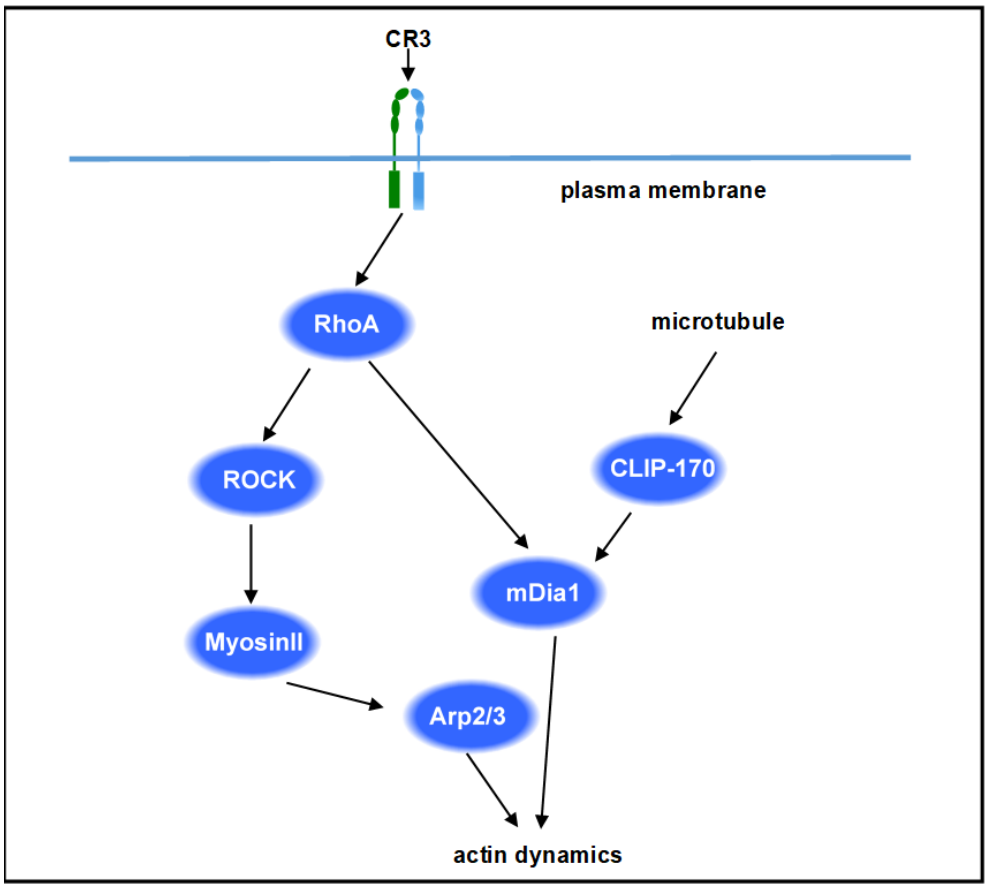


Figure 9. Signaling of Actin Polymerization in CR3 mediated Phagocytosis
The binding of complement component iC3b coated particle stimulates the activation of CR3, which in turn activates small GTPase RhoA. RhoA then stimulates two signaling pathways to regulate the Actin polymerization at the inner leaflet of the plasma membrane and thus regulate phagocytosis. One signaling is through the activation of ROCK, Actin-associated protein (myosin II) and Actin nucleation regulator (Arp2/3). The other signaling is the activation of Actin filament elongation factor formin (mDia1), which is amplified by the interaction with microtubule through microtubule associated protein (CLIP-170). (Modified from Flannagan et al., 2011)

After the internalization of particles, Rab5 promotes the fusion of the nascent phagosome with an early endosome to form an early phagosome. Additionally, Rab5 can also mediate the recruitment and activation of other effector proteins. For instance, mVps34, a class III PI3K, has been found to be recruited to the phagosome after recruitment of Rab5 (Vieira et al., 2001). Mammalian Phosphatidylinositol 3-kinase (mVps34) specifically produces PI(3)P which is required for the phagosome maturation. PI(3)P-specific PX or FYVE domain containing proteins such as EEA1 and p40 subunit of the NADPH oxidase are then accumulating on the membrane of early phagosome (Ellson et al., 2006, Fratti et al., 2001). An early study suggested that EEA1 can bind directly to Rab5, which can in turn promote the Rab5-mediated docking of a nascent phagosome with an early endosome (Simonsen et al., 1998). Early endosome antigen 1 (EEA1) concomitantly associates with the universal mediator of membrane fusion, soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) and thus facilitate the fusion with early endosome (McBride et al., 1999).

Following internalization of the nascent phagosome, a certain group of proteins must return to the plasma membrane to allow for the next round of phagocytosis. For example, some of the transferrin receptors are recycled back to the plasma membrane in a Rab4 and Rab11 mediated manner (Cox et al., 2000). Furthermore, hampering a recycling step was shown to significantly impair phagocytosis of IgG coated particles (Cox et al., 2000). Further maturation of the phagosome involves fusion with lysosomes which contain

digestive enzymes that digest the contents inside of the phagosome. Additionally, vacuolar ATPase (V-ATPase), localized on the membrane of the phagosome, acts as a proton pump to deliver H^+ into the lumen of phagosome and causes a strong acidification of lumen, which helps degradation of the contents. Furthermore, increased production of reactive oxygen species (ROS) inside the phagosome is often observed, which is believed to induce ROS-dependent killing of invading pathogens. One of the ROS compounds, superoxide, is generated by a multi-subunit Nicotinamide Adenine Dinucleotide Phosphate Hydrogen Oxidase (NADPH oxidase or NOX) complex, which contains small GTPase Rac as well as other accessory and catalytic subunits. In response to an activating signal, one of the NOX isoform subunits, NOX2, and its cytosolic components, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, Rac1, Rac2, and flavocytochrome b_{558} orchestrate transportation of an electron across the membrane of phagosome. The electron is then coupled with molecular oxygen and thus forms a superoxide radical (O_2^-). Superoxide radicals can be further converted to another ROS, hydrogen peroxide (H_2O_2) which directly targets the materials inside of phagosome (Winterbourn, 2008). These steps of phagosome maturation are summarized in figure 10.

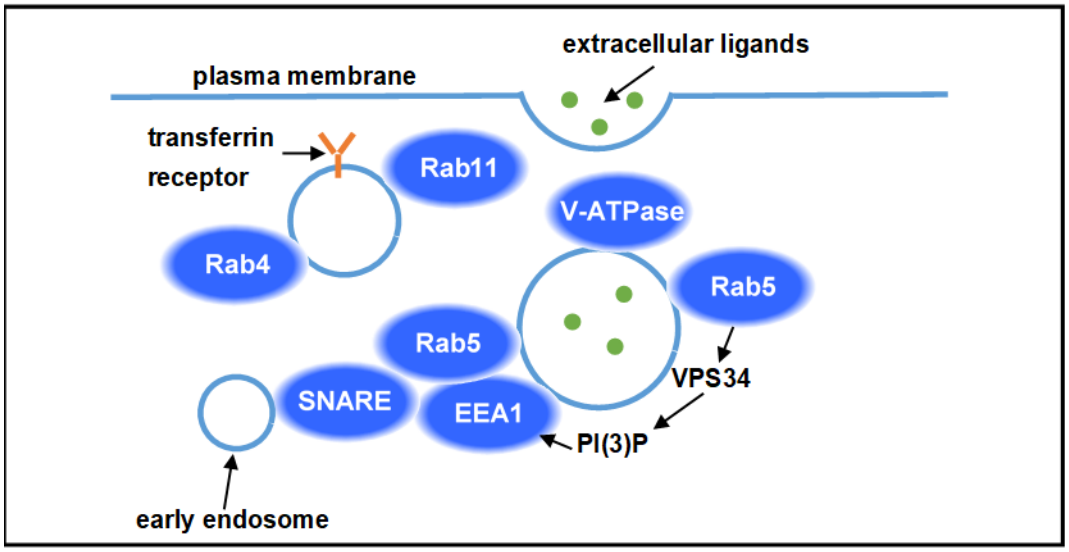


Figure 10. Stages of Phagosome Maturation
Upon internalization of particles, Rab5 is recruited to the nascent phagosome which in turn induces the recruitment and activation of Vps34 and thus produce PI(3)P. PI(3)P-specific PX or FYVE domain containing proteins EEA1 is then recruited to membrane of phagosome and cooperate with Rab5 and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) to regulate the fusion of nascent phagosome with early endosome. Rab4 and Rab11 are mainly responsible for the recycling of transferrin receptors back to plasma membrane to ensure next round of phagocytosis. Further maturation of phagosome involves vacuolar ATPase (V-ATPase) mediated acidification of phagosome lumen and production of ROS, which induces the degradation of contents of phagosome. (Modified from Flannagan et al., 2011)

1.6 Cell to Substratum Matrix Adhesion

Adhesion is the process by which cells attach to substratum matrix or other cells which is mediated by dedicated types of adhesion receptors. For example, the cell to substratum matrix adhesion is mainly through transmembrane integrin receptors, which provide linkage between extracellular ligands and intracellular signaling. Additionally, it has been reported that adhesion is of great importance and can regulate a variety of processes such as the cell cycle, cell migration, wound healing and development.

The dynamic process of cells to substratum matrix adhesion often involves several steps (figure 11). (I). The initial attachment of cells to substratum matrix via electrostatic interaction. (II). Flattening of cells in morphology concomitantly with the interaction of Integrin and substratum matrix. (III). Full spreading of cells through focal adhesion (FA) accompanied with strengthened adhesion complex (Huang et al., 2003, Hong et al., 2006).

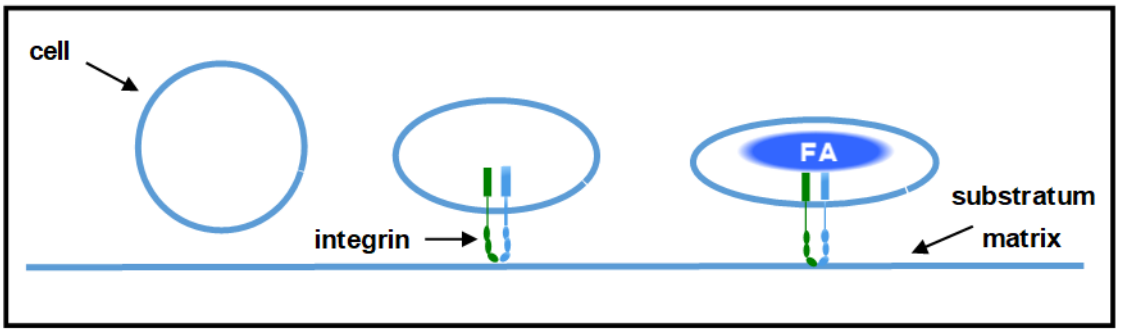


Figure 11. Stages of Cell to Substratum Focal Adhesion (FA) Complex Formation
The cell to substratum matrix adhesion starts with the initial attachment via electrostatic interaction which in turn leads to the flattening of cell in morphology through integrin bonding. Finally, cytoplasmic focal adhesion complex which is formed via integrin strengthen the adhesion between cell and substratum matrix. (Modified from Khalili and Ahmad, 2015)

Integrin, a transmembrane heterodimer protein which consists of alpha and beta subtypes, plays an essential role to anchor cells to the substratum matrix (Saif et al., 2003). Basically, there are two different patterns of signaling mediated by integrin in the process of cell-to-substratum matrix adhesion, outside-in signaling and inside-out signaling (Luo et al., 2007).

Outside-in signaling is the process by which an extracellular signal is transmitted to the cytoplasm via an integrin receptor (figure 12). Outside-in signaling starts with the binding of cell surface integrin to substratum matrix ligands, which in turn induce the

conformational change of the cytoplasmic domain of integrin and thus focal adhesion kinase (FAK) is activated (Guan, 2010). After the activation of FAK, SH2 domain-containing Src is then recruited to the site of FAK and activates the downstream p130 CRK-associated substrate (p130CAS) followed by the activation of Crk, Rac and p21-activated protein kinases (PAK) (Schaller et al., 1994, Tikhmyanova et al., 2010). The activation of PAK regulates the Actin dynamics at the inner leaflet of the plasma membrane, which is important in actin-related cellular processes such as cell migration and phagocytosis. FAK can also directly act as a scaffold protein to regulate the interaction of Rac and PAK. Additionally, FAK can regulate the activity of Arp2/3 and WASP to promote the polymerization of Actin filament. On the other hand, FAK can modulate the Actin remodeling in a Rho GTPase dependent manner. After the activation of Paxillin by FAK, PDZRhoGEF and p190RhoGEF are activated, both of which promote the replacement of GDP-RhoA with GTP-RhoA and thus stimulate the Actin cytoskeleton rearrangement (Turner et al., 1990, Iwanicki et al., 2008).

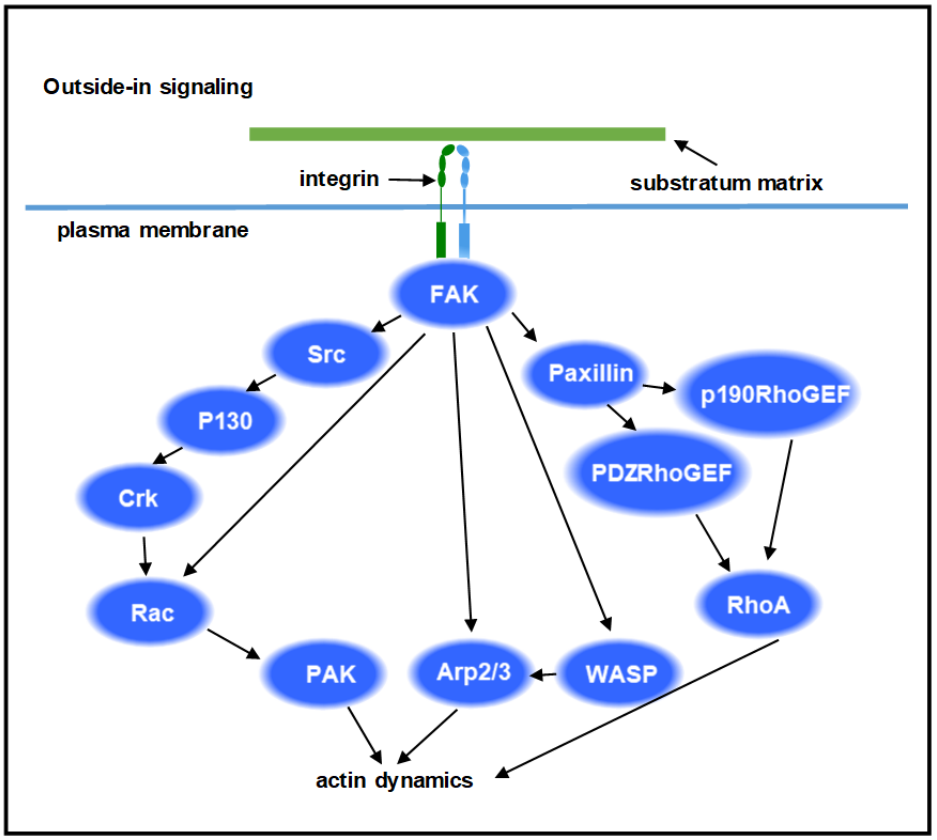


Figure 12. Integrin mediated Outside-in Signaling in Cell to Substratum Matrix Adhesion

The binding between integrin and substratum matrix stimulates a conformational change in cytosolic domain of integrin and thus focal adhesion kinase (FAK) is activated. The activation of FAK in turn induces multiples signaling to regulate the Actin polymerization at the plasma membrane, which is essential for actin-related cellular processes such as cell migration and phagocytosis. One signaling is through the activation of Src, p130, Crk, Rac and PAK. Other signaling is either through the modulation of Actin nucleation promoting factor WASP and nucleation complex Arp2/3 or through the regulation of Paxillin and RhoA. (Modified from Menter and DuBois, 2012)

Conversely, inside-out signaling is the process by which intracellular signal transmitted to integrin leading to the conformational change of cytosolic domain and thus in turn changes the binding affinity between integrin and ligands (figure 13). Inside-out signaling mainly involves the regulation of talin and kindlin (Moser et al., 2009). After the activation of G-protein-coupled receptors (GPCR), Ca^{2+} and diacylglycerol (DAG)

increase in the cytoplasm which can in turn activate GEF. The activation of GEF promotes the removal of GDP and binding of GTP to Ras-proximate-1 or Ras-related protein 1(Rap1). Rap1-GTP-interacting adaptor molecule (RIAM) is then recruited to the site of activated Rap1 and promotes the binding of C-terminus of talin to cytosolic domain of integrin and plasma membrane (Anthis and Campbell, 2011). The N-terminus of talin is attached with Actin filaments. On the other hand, the binding of kindlin to β cytosolic domain of integrin and plasma membrane helps stabilize the activated state of integrin complex which leads to higher affinity between integrin and its corresponding ligands (Meves et al., 2009).

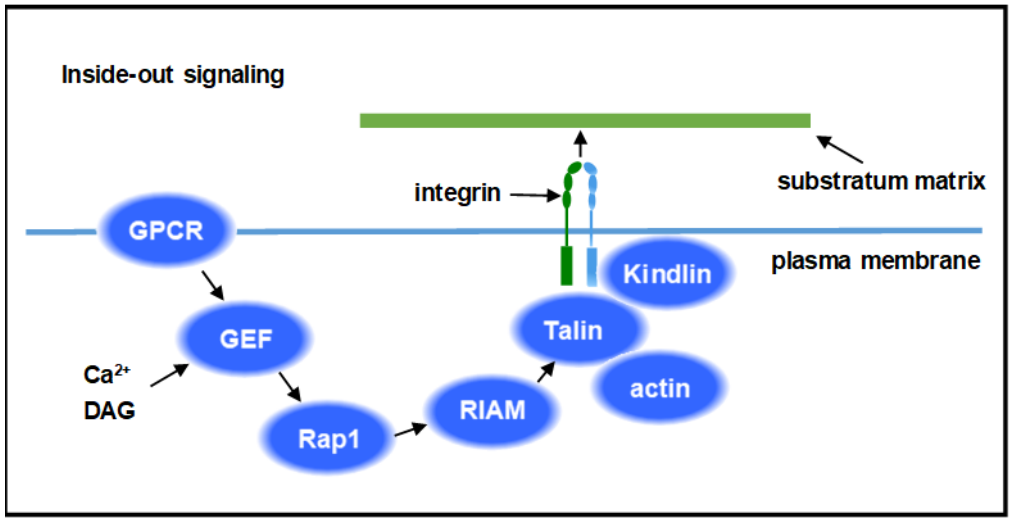


Figure 13. Integrin mediated Inside-out Signaling in Cell to Substratum Matrix Adhesion

The inside-out signaling starts with the activation of G-protein-coupled receptors (GPCR) which leads to the increase of cytoplasmic Ca^{2+} and diacylglycerol (DAG), which in turn activates GEF and thus small GTPase Rap1 is activated. Rap1-GTP-interacting adaptor molecule (RIAM) associates with Rap1 and thus promotes the interaction between C-terminus of talin and cytosolic domain of integrin. The N-terminus of talin is associated with Actin. Additionally, the interaction between kindlin and cytosolic domain of integrin stabilizes the activated state of integrin complex which leads to higher affinity between integrin and substratum matrix. (Modified from Menter and DuBois, 2012)

1.7 Cell to Cell Adhesion

In addition to cell to ECM adhesion, cell to cell adhesion is the process by which cell attached to neighboring cells via molecules on the cell surface. Basically, it can be subdivided into three different types in mammals: tight junctions, adherens junctions and desmosomes. Tight junctions, characterized by the sealing of adjacent epithelial cells, is often found associated with integral proteins such as claudins, occludin, tricellulin and junctional adhesion molecules (JAMs) (Steed et al., 2010) Tight junction is mainly responsible for acting as a diffusion barrier to control what molecules and ions are allowed to pass through the tissues. On the other hand, adherens junctions is play a fundamental role in maintaining shape and integrity of cell and tissue (Niessen et al., 2011). The adherens junction is accomplished by the interaction between transmembrane glycoprotein cadherins, cytoplasmic protein p120-catenin and β-catenin, together with the recruitment of α-catenin to β-catenin. Additionally, actin filament bridges are often formed between adjacent cells at the site of adherens junction (Harris and Tepass, 2010). For instance, in polarized epithelial cells, the actin filaments localized at the apical surface couple with cadherin family transmembrane molecules, α-actinin, vinculin and catenins to form a linkage called adhesion belts between adjacent cells (Etienne-Manneville, 2011). In desmosomes mediated cell adhesion, the extracellular domains of desmogleins and desmocollins bind to each other while the intracellular domains bind with plakoglobin or plakophilins. Plakoglobin or plakophilins can then bind to intermediate filaments (IFs)

through desmoplakin. The vast network of all these desmosomes adhesion molecules provides a linkage between adjacent cells, which is essential to provide cell stability and protect cells from high mechanical stress (Delva et al., 2009).

CHAPTER 2. THE ROLE OF SUPEROXIDE, RASG AND PI3K IN REGULATION OF MACROPINOCYTOSIS

2.1 Introduction

Macropinocytosis is the mechanism for the internalization of extracellular bulk fluid phase. It has gained great interests nowadays since recent studies have shown that macopinocytosis is not only involved in various types of cells such as human innate immune system cells, macrophages and dendritic cells, but also serves as a route of infectious agents (Racoosin and Swanson, 1989, Sallusto, 1995). *Dictyostelium discoideum*, a free-living soil amoeba, feed upon bacteria. Decades ago, in order to easily perform genetic manipulation in liquid culture, several mutants were isolated that can grow in liquid media. The uptake of rich liquid medium is largely mediated by macropinocytosis. Additionally, the rate of uptake of fluid phase in *Dictyostelium* is several fold more than that of macrophages and thus *Dictyostelium* is regarded as a dedicated macropinocyte. Furthermore, a number of regulatory proteins that affect macropinocytosis are conserved between *Dictyostelium* and mammals. All these traits make *Dictyostelium* one of the attractive models organisms to study macropinocytosis. In *Dictyostelium*, the actin-based membrane protrusions, proceed, followed by the fusion between the tips of the protrusions or between a tip and the base of the plasma membrane to trap the extracellular fluid inside, and thus generate a nascent macropinosome. The nascent macropinosomes will undergo maturation steps by which they either participate the endocytic pathway to digest the luminal contents or join the route of recycling back to the plasma membrane.

Quite a few proteins are known to affect macropinocytic cup formation in *Dictyostelium* (figure 14). For instance, Ras GTPase activating protein, which is also known as RasGAP, mainly works as a switch to inactivate bounded small G proteins through the replacement of GTP with GDP. Nelson's group (Bloomfield et al., 2015) introduced a mutation in gene coding RasGAP Neurofibromin (NF1) in *Dictyostelium* and found the loss of NF1 led to an increase in the fluid uptake, Ras and PI3K activities, and oversized macropinosomes. Additionally, NF1 was recruited to nascent macropinosomes, suggesting that NF1 works as an inhibitor to downregulate the activity of Ras which in turn restricts the size of formed macropinosome (Bloomfield et al., 2015). Another study suggested that active Ras, Rac and PIP3 were present coincidently at macropinocytic cup, which in turn recruited SCAR/WAVE and Arp2/3 to the site (Veltman et al., 2016). During the process of macropinocytosis, SCAR proteins mainly localize at the rim of the cup, but it remains unclear how they are excluded from the rest of the structure. Another actin elongation promoting factor, Formin G, a member of Diaphanous-related formin, was shown to affect the formation of the macropinocytic cup by a recent study (Junemann et al., 2016). Formin is widely knowns as being responsible for the elongation of Actin filaments from the plus end of the preexisting actin filament (Evangelista, 2003). In *Dictyostelium*, ForG, regulated by RasG and RasB, efficiently elongates the Actin filaments at the base of macropinocytic cup with the help of profilin (Junemann et al., 2016).

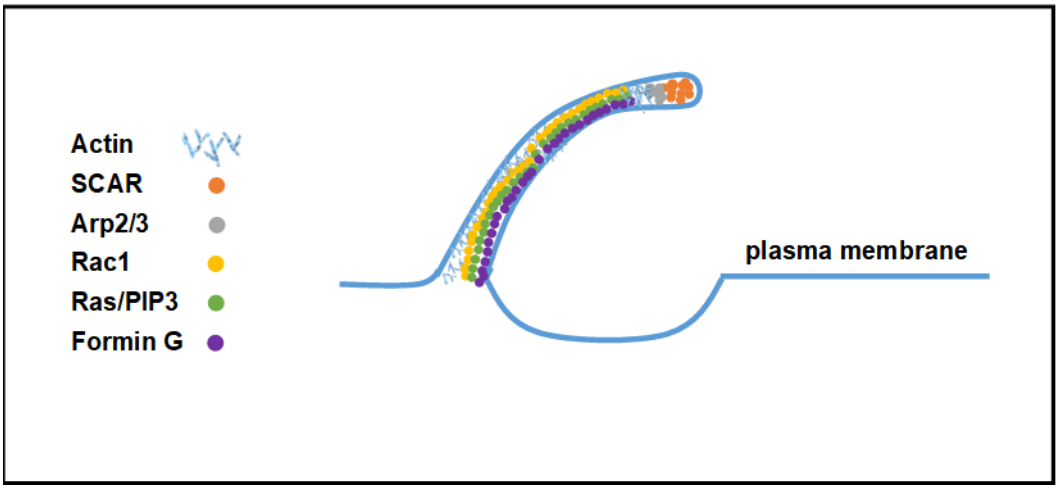


Figure 14. Actin Network-related Proteins in the Macropinocytic Cup
The vast network of actin-based membrane protrusion requires a variety of molecules. SCAR and Arp2/3 mainly regulate the Actin dynamics at the rim of the macropinocytic cup while Ras, Rac1, PIP3 and Formin G recruited to the base of cup to strengthen the formation of cup through the modulation of Actin cytoskeleton. (Modified from Veltman et al., 2016)

After the closure and fission of the macopinocytic cup, the plasma membrane proteins which are responsible for macropinocytosis are immediately recycled after the recruitment of WASH (WASP and SCAR homolog) and retromers to the macropinosome. After further investigation, it was found that WASH could mediate recycling of membrane proteins in retromer-dependent or retromer-independent manners (Buckley et al., 2016). The recycling step is vital to salvage the integral membrane proteins from being digested during the maturation steps and ensure the next round of macropinocytosis. Additionally, it has been found that after the dissociation of macropinosome, a succession of groups of proteins and phosphoinositides on the nascent macropinosome occur. First, actin, actin-associated protein coronin and PtdIns(3,4)P2 or PtdIns(3,4,5)P3, were observed, which are

then replaced by another a group of proteins such as endosomal trafficking regulating proteins, Rab7 and LmpA, and the vacuolar ATPase which induces acidification of the macropinosomal lumen within one minute after the formation of the nascent macropinosome. The mechanism of the replacement of proteins still remains to be uncovered (Temesvari et al., 2000).

In addition to proteins, other molecules are also playing an important role in modulating macropinocytosis. For instance, the dedicated macopinocyte macrophage which is from human innate immune system plays an important role in defending chronic inflammatory disease atherosclerosis. Atherosclerosis is caused from the insolubility of cholesterol in blood. Low-density lipoproteins (LDLs) bind to bad cholesterol and form a complex which can then be internalized by macrophage through the process of macropinocytosis. In a recent study, Csányi and others (2017) found that NADPH oxidase 2 (Nox2) was involved in the uptake of LDL by macropinocytosis of macrophages. Nox2 is a well known producer of superoxide radicals by transferring electron from Nox2 complex to molecular oxygen. Csányi and others (2017) further determined that Nox2 signaling activates phosphoinositide 3 kinase/Akt pathway (PI3K/Akt) and actin-binding protein (cofilin) and thus stimulates membrane ruffling which is essential in macropinocytic cup formation. Considering the fact that Nox2 is a downstream of Ras-1 and protein kinase C (PKC), Nox2 may also function cooperatively with these regulators in stimulating macropinocytosis. However, the mechanism of NOX stimulation of

macropinocytosis need to be further studied. Reactive oxygen species (ROS) in macrophage still remains unknown and requires future extensive research. Veeranki and others (Kim laboratory) previously generated *Dictyostelium discoideum* cells lacking a glycosylphosphatidylinositol (GPI)-anchored superoxide dismutase, SodC. The function of SodC is to mediate the dismutation of superoxide (O_2^-) radical into hydrogen peroxide (H_2O_2). As a result, *sodC*$^-$ cells constitutively experience higher levels of oxidative stress characterized by modestly increased intracellular superoxide level (~18% higher compared to wild type), high basal RasG activity, and higher PIP3 level (Veeranki et al., 2008). Considering the fact that both RasG and PI3K are known to affect macropinocytosis in *Dictyostelium discoideum* cells (Rupper et al., 2001, Hoeller et al., 2013, Chen and Katz, 2000). We thus hypothesized that having aberrancies in both RasG and PI3K, *sodC*$^-$ cells are likely to experience compromised macropinocytosis or later processes such as macropinosome maturation. Here we provide evidence showing that the total fluid phase uptake of *sodC*$^-$ cells was unaffected but they do have aberrant endosomal vesicle trafficking and the introduction of overexpressing of cytoplasmic superoxide dismutase (SodA), redox insensitive RasG (C118A) or treatment of PI3K inhibitor LY294002 in *sodC*$^-$ cells significantly rescued the defects of endosomal vesicle trafficking.

2.2 Materials and Methods

2.2.1 Dictyostelium Development and Growth

All *Dictyostelium discodium* cells were grown in D3T media (Dissolve 14.3 g of

Peptone No.3, 7.15 g of yeast extract, 15.4 g of Glucose, 0.48 g of KH_2PO_4 and 0.525 g of $Na_2HPO_4.7H_2O$ in Deionized water and top off to 1 L. Autoclave for 40 min, let it cool down and add antibiotics mixture to make the final D3T media has 100 μg/ml of ampicillin, 25 μg/ml of tetracycline and 25 μg/ml of chloramphenicol). In addition to D3T medium, JH10 cells will be grown with thymidine (0.5 mg/ml) and *sodC*$^-$ cells will also be grown in thymidine (0.5 mg/ml) and Blasticidin (5 μg/ml). Both types of cells were grown in shaking cultures at 19 °C at 150 rpm or in culture flasks at 19 °C for about 24 hours until they were in log phase. Cell number were monitored using a hemocytometer. Cell number for every experiment performed were between $2x10^6$ cells/ml to $2.5x10^6$cells/ml.

2.2.2 Analysis of Pinocytosis using FITC Labeled Dextran

Log phase *Dictyostelium* cells were pelleted in a table top centrifuge and re-suspended in D3T media at a concentration of 2.5×10^6 cells/ml. Let cells recover on a shaker at 19 °C at 150 rpm for 30 min. For LY 294002 treatment comparison group, cells were added with LY 294002 at 10 minute time point during recover period Fluorescein isothiocyanate–dextran (Sigma Aldrich) was added to cell suspensions at a concentration of 1mg/ml. Mixtures of *Dictyostelium* cells and FITC-dextran were incubated in shaking cultures at 19 °C at 150 rpm for 30 min, 60 min and 120 min. At different time intervals, 1 ml of the sample was withdrawn and washed with two times of 1 ml of ice cold Sorensen buffer. The pellets were then re-suspended with 1 ml of TTG lysis buffer and 50 ul of the lysates were taken to determine protein concentration by performing the BCA assay

described earlier. The rest of the lysates were re-suspended with 2.05 ml of TTG lysis buffer and fluorescence was measured using spectrofluorimeter (Schimadzu RF-1501, excitation 470 nm; emission 515 nm). Fluorescence data were divided by protein concentration and plotted into relative percentage by setting the data of wild type cell at 2 hour time point as 100 percent. All of the assays were repeated at least three times to get statistical analysis by performing unpaired *t* test. Internalized FITC-dextran images were taken using 100x oil lens on a Leica DM IRB inverted epifluorescence microscope equipped with CoolSNAP digital camera and OpenLab imaging software. Quantitation of mean number of vacuoles and mean size of vacuoles of 90 cells was performed followed by unpaired *t* test to get statistical analysis.

2.3 Results

2.3.1 *sodC*$^-$ Cells have Defects in Endosomal Vesicle Trafficking while the Total Amount of Fluid Phase Uptake was Unaffected

Dictyostelium cells lacking *Superoxide dismutase C* (*sodC*$^-$) have been reported to have defects in chemotaxis, the process by which cells move towards high chemical gradients. During chemotaxis, the aberrant regulation of superoxide in *sodC*$^-$ cells led to high basal RasG activity and high PIP3 level, which in turn hamper PKB activity and F-Actin remodeling at the leading front of *sodC*$^-$ cells (Veeranki et al., 2008). Given that both macropinocytosis and chemotaxis involve F-Actin cytoskeleton rearrangement at the plasma membrane, it will be significant to determine if macropinocytosis is also hampered in *sodC*$^-$ cells. Cells lacking *SodC* showed comparable level of relative internalized FITC-

dextran at a 2 hour time point compared to that wild of type cells (Fig. 1A). However, when looking at the fluorescence images, the vesicles in *sodC*$^-$ cells are bigger in size but reduced in numbers compared to that of wild type cells (Fig. 1B). Statistical analysis as described in the Materials and Methods also showed the mean number of vesicles was significantly lower and mean size of vesicles was significantly bigger in *sodC*$^-$ cells compared to that of wild type cells (Fig. 1C), which indicates the endosomal vesicle trafficking is defective in *sodC*$^-$ cells.

2.3.2 *sodC*$^-$*::SodA* cells Partially Rescued the Aberrant Endosomal Vesicle Trafficking of *sodC*$^-$ Cells

To further corroborate the defects of endosomal vesicle trafficking in *sodC*$^-$ cells is because of the aberrant regulation of superoxide, a cytoplasmic superoxide dismutase (SodA) was introduced into *sodC*$^-$ cells. The fate of endosomal vesicle trafficking of *sodC*$^-$*::SodA* cells was then determined as described in Materials and Methods. Statistical analysis indicates that the mean number of endosomal vesicles is significantly higher and the mean size of vesicles is significantly smaller in *sodC*$^-$*::SodA* cells compared to that of *sodC*$^-$ cells, indicating that the endosomal vesicle trafficking in *sodC*$^-$*::SodA* cells was significantly improved (Fig. 2A & B). These together suggest that superoxide is involved in the regulation of endosomal vesicle trafficking.

2.3.3 *sodC*$^-$*::*RasG(C118A) Cells Partially Rescued the Aberrant Endosomal Vesicle Trafficking of *sodC*$^-$ Cells

Considering that RasG is one of the major targets of the excessive superoxide in *sodC*$^-$ cell, an introduction of superoxide insensitive RasG mutant RasG(C118A) into *sodC*$^-$

cells would alleviate aberrant vesicle trafficking. To this end, fed with FITC-dextran and the endosomal vesicles were monitored. Interestingly, the mean number of FITC-dextran positive vesicles was significantly higher and the mean size of vesicles was significantly smaller in *sodC⁻:: RasG(C118A)* cells compared to those of *sodC⁻* cells (Fig. 3A & B), showing that the endosomal vesicle trafficking in *sodC⁻:: RasG(C118A)* cells was significantly improved. These together suggest that redox sensitive RasG is involved in endosomal vesicle trafficking and RasG/PI3K signaling axis is affecting the endosomal vesicle trafficking in a superoxide sensitive manner.

2.3.4 PI3K Inhibitor LY 294002 Treatment Partially Rescued the Aberrant Endosomal Vesicle Trafficking of *sodC⁻* Cells

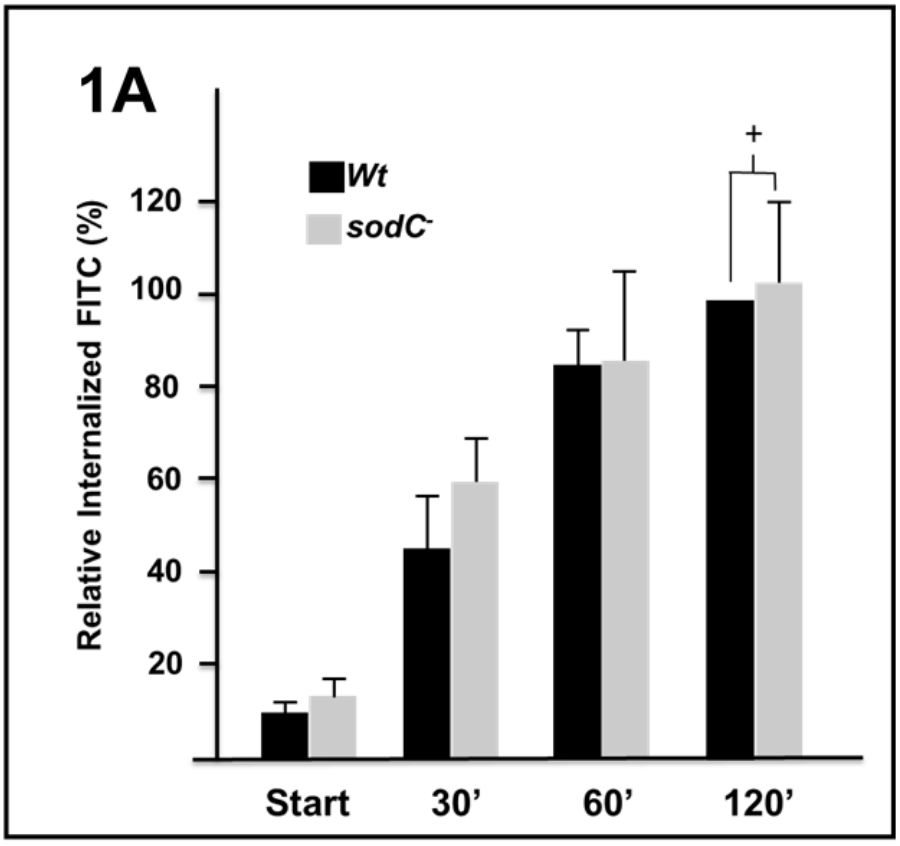
Given that PI3K inhibitor LY 294002 treatment partially restored the defects of chemotaxis in *sodC⁻* cells (Veeranki et al., 2008), we hypothesize that LY 294002 could improve the endosomal vesicle trafficking. The fate of the endosomal vesicle trafficking was then determined after cells were treated with 50 μM of LY 294002 for 20 minutes. The treatment of 50 μM of LY 294002 significantly increase the mean number of vesicles and decrease the mean size of vesicles (Fig. 4A & B), indicating the endosomal vesicle trafficking was significantly improved. Thus, excessive PI3K activity in cells like *sodC⁻* is inhibitory to proper endosomal vesicle trafficking.

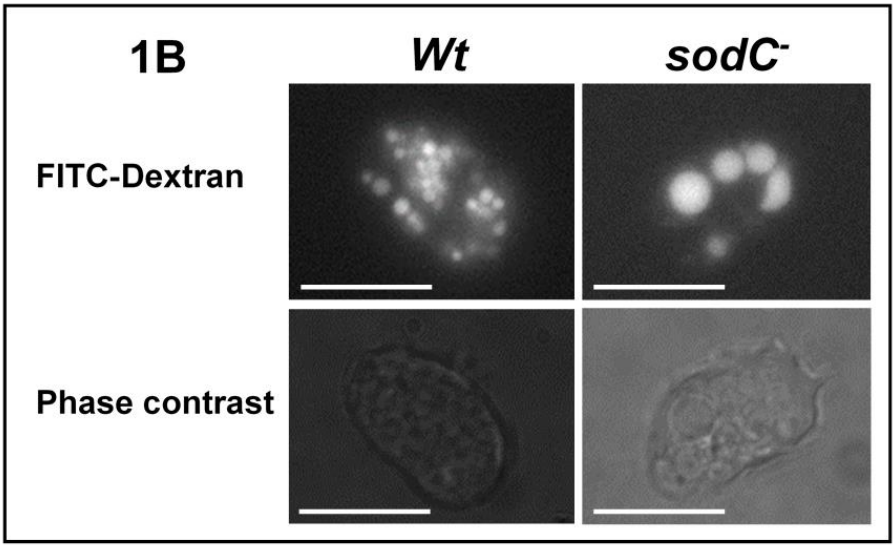
2.3.5 PI3K inhibitor LY 294002 Treatment Inhibited Macropinocytosis of both *Wt* and *sodC⁻* Cells

Previous study has reported that PI3K can affect the process of macropinocytosis (Hoeller et al., 2013). To further confirm the role of PI3K in the regulation of

macropinocytosis in *sodC*$^{-}$ cells, the total fluid phase uptake was determined after both wild type and *sodC*$^{-}$ cells were treated with 50 μM of PI3K inhibitor LY 294002 for 20min. Interestingly, both of wild type and *sodC*$^{-}$ cells showed significantly decreased level of FITC-dextran uptake compared to that of non-treated control cells (Figure 4C). Furthermore, the fluid phase uptake in LY294002 treated *sodC*$^{-}$ cells was comparable to those of the LY294002 treated wild type cells (Figure 4C). All these together suggests that PI3K can affect the process of macropinocytosis, which is consistent with the previous study (Hoeller et al., 2013). Additionally, wild type and *sodC*$^{-}$ cells are comparable in their capacity of internalizing fluid phase in a LY294002 sensitive manner.

2.4 Figures


1A
Wt
sodC-
Relative Internalized FITC (%)
120
100
80
60
40
20
+
Start
30'
60'
120'


1B
Wt
sodC-
FITC-Dextran
Phase contrast

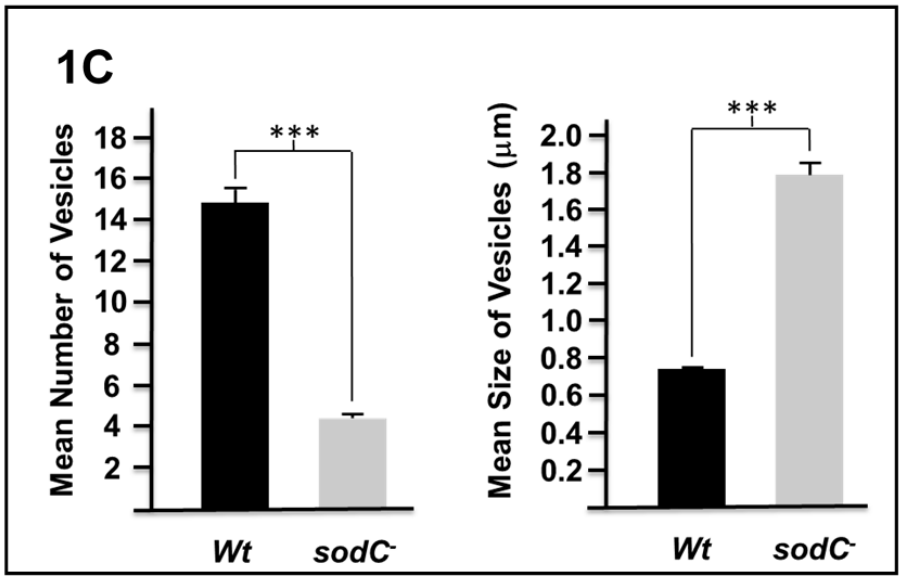


Figure 1 *sodC*⁻ Cells have Defects in Endosomal Vesicle Trafficking

(A) Relative internalized FITC-dextran of *Wt* and *sodC*⁻ cells were determined by quantitating fluorescence to protein concentration ratio. *sodC*⁻ cells showed comparable level of relative FITC-dextran uptake with *Wt* cells as the difference between *Wt* and *sodC*⁻ cells was statistically insignificant. (B) Internalized FITC-dextran images of *Wt* and *sodC*⁻ cells at 1 hour time point were taken using inverted epifluorescence microscope. Phase contrast (left) and FITC-dextran fluorescence images (right) are shown (size bar: 10 μm). Obviously, *sodC*⁻ cells showed less number of endosomal vesicles but larger size of endosomal vesicles compared to that of *Wt* cells. (C) 90 images of each *Wt* and *sodC*⁻ cells were taken to get statistically analysis of endosomal vesicle trafficking. *sodC*⁻ cells showed significantly decrease in mean number of endosomal vesicles but increase in mean size of endosomal vesicles.

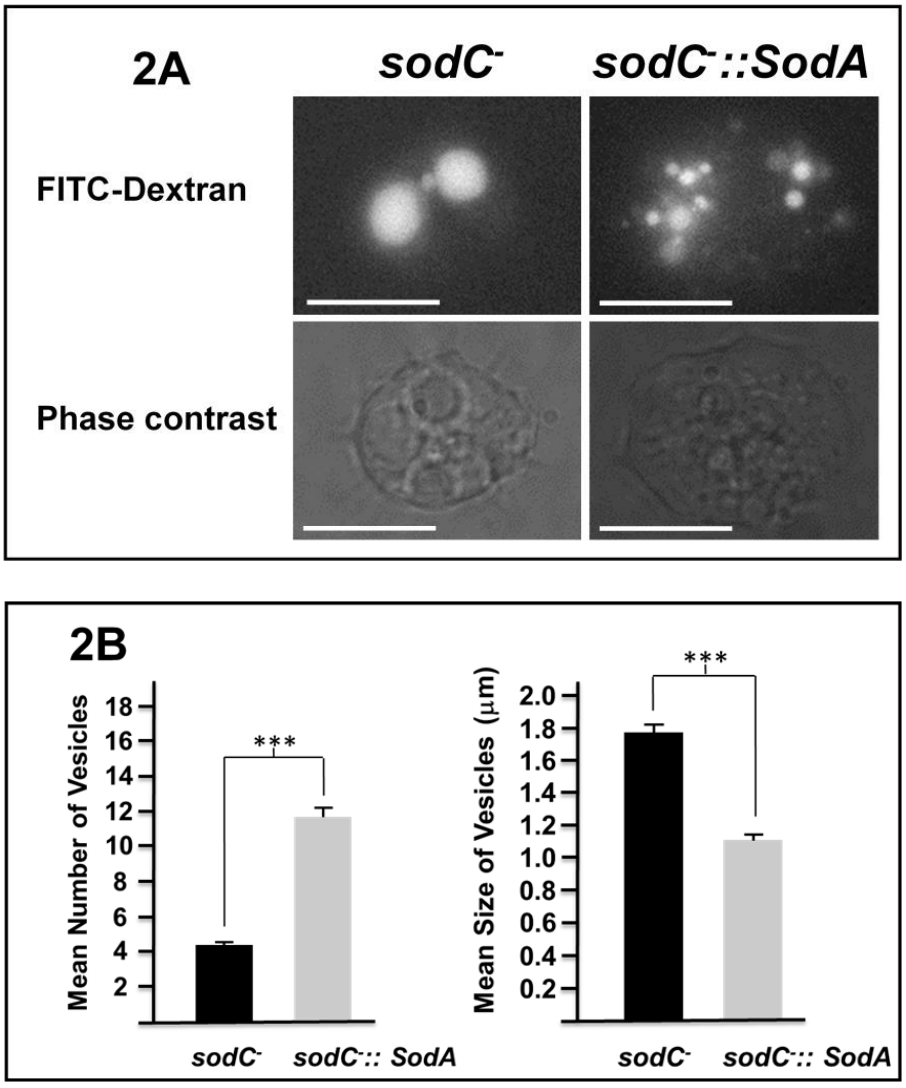


Figure 2 *sodC⁻::SodA* Cells Partially Rescued the Aberrant Endosomal Vesicle Trafficking of *sodC⁻* Cells

(A) Internalized FITC-dextran images of *sodC⁻* and *sodC⁻::SodA* cells at 1 hour time point were taken using inverted epifluorescence microscope. Phase contrast (left) and FITC-dextran fluorescence images (right) are shown (size bar: 10 μm). *sodC⁻::SodA* cells showed improved endosomal vesicle trafficking. (B) 90 images of *sodC⁻::SodA* cells were taken to get statistically analysis of endosomal vesicle trafficking. *sodC⁻::SodA* cells showed significantly rescued in terms of mean number of endosomal vesicles and mean size of endosomal vesicles compared to that of *sodC⁻* cells.

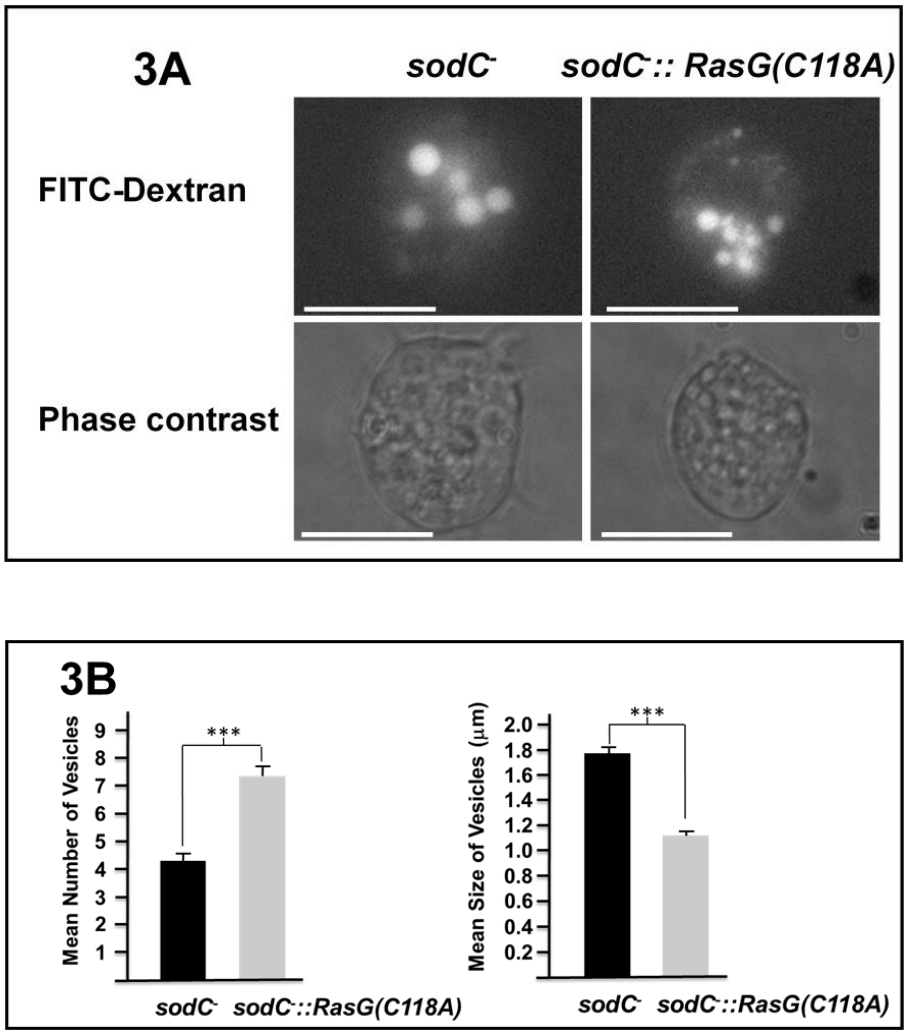
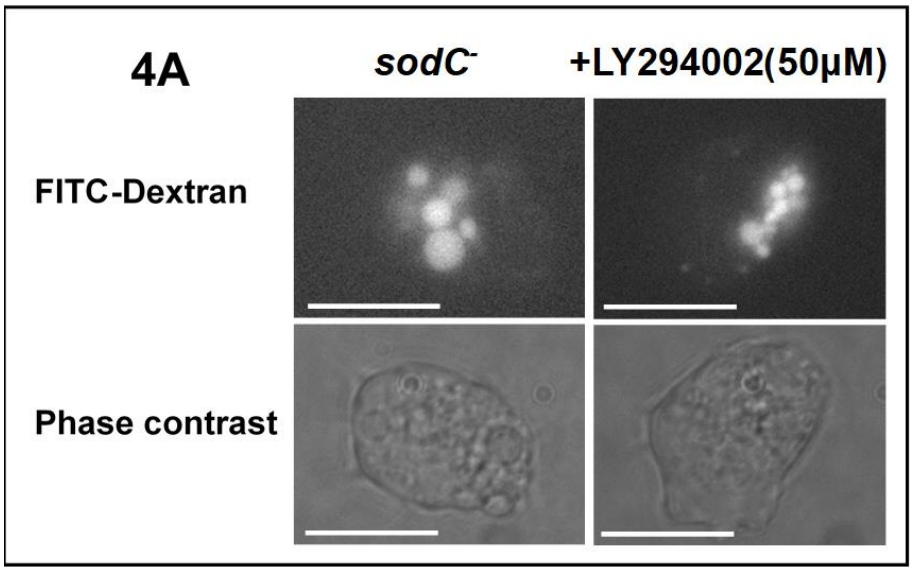
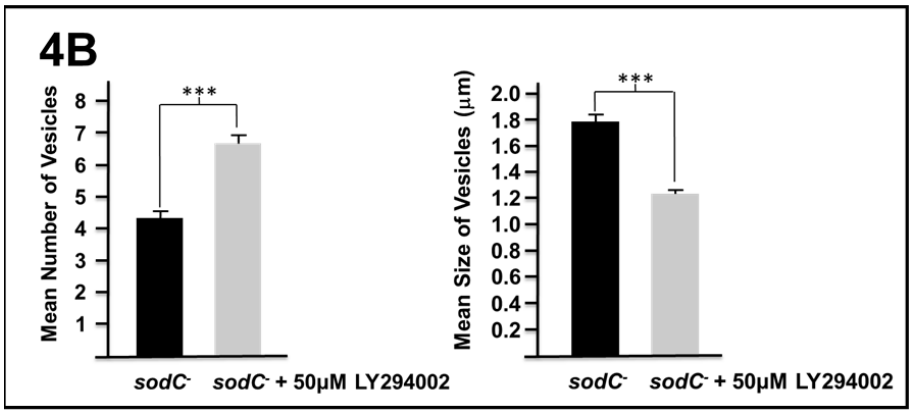


Figure 3 *sodC-:: RasG(C118A)* Cells Partially Rescued the Aberrant Endosomal Vesicle Trafficking of *sodC-* Cells

(A) Internalized FITC-dextran images of *sodC-* and *sodC-:: RasG(C118A)* cells at 1 hour time point were taken using inverted epifluorescence microscope. Phase contrast (left) and FITC-dextran fluorescence images (right) are shown (size bar: 10 μm). *sodC-:: RasG(C118A)* cells showed rescued in endosomal vesicle trafficking. (B) 90 images of *sodC-:: RasG(C118A)* cells were taken to get statistically analysis of endosomal vesicle trafficking. *sodC-:: RasG(C118A)* cells showed significantly rescued in terms of mean number of endosomal vesicles and mean size of endosomal vesicle compared to that of *sodC-* cells.


4A
sodC-
+LY294002(50μM)
FITC-Dextran
Phase contrast


4B
Mean Number of Vesicles
8
7
6
5
4
3
2
1
***
sodC-
sodC- + 50μM LY294002
Mean Size of Vesicles (μm)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
***
sodC-
sodC- + 50μM LY294002

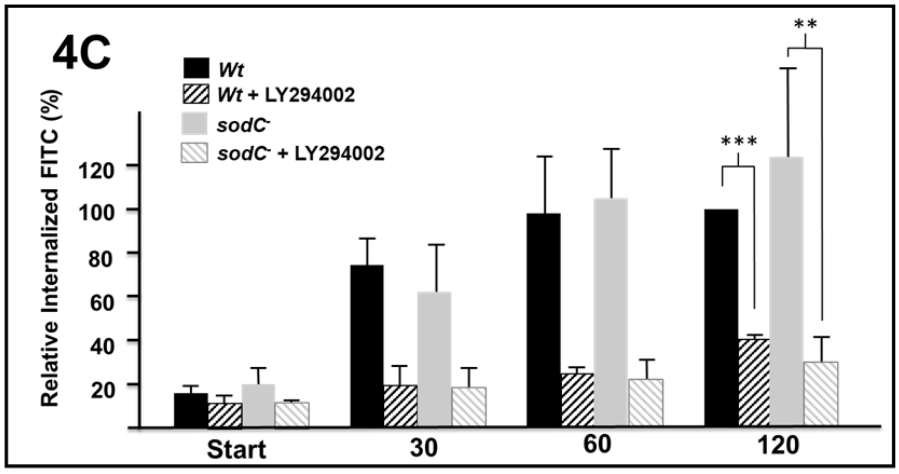


Figure 4 PI3K Inhibitor LY 294002 Treatment Partially Rescued the Aberrant Endosomal Vesicle Trafficking of *sodC*$^-$ Cells

(A) *sodC*$^-$ cells were treated with 50 μM of PI3K inhibitor LY294002 for 20min as described in Materials and Methods and then internalized FITC-dextran images were taken at 1 hour time point using inverted epifluorescence microscope. Phase contrast (left) and FITC-dextran fluorescence images (right) are shown (size bar: 10 μm). *sodC*$^-$ cells after LY294002 treatment showed rescued in endosomal vesicle trafficking. (B) 90 images of *sodC*$^-$ cells after LY294002 treatment were taken to get statistically analysis of endosomal vesicle trafficking. *sodC*$^-$ cells after LY294002 treatment showed significantly rescued in terms of mean number of endosomal vesicles and mean size of endosomal vesicles compared to that of *sodC*$^-$ cells. (C) Relative internalized FITC-dextran of *Wt* and *sodC*$^-$ cells in the absence and presence of 50 μM LY294002 were determined by quantitating fluorescence to protein concentration ratio. Both of *Wt* and *sodC*$^-$ cells showed significantly decrease in relative uptake of FITC-dextran compared to that of non-treated cells.

CHAPTER 3. THE ROLE OF SUPEROXIDE, RASG AND PI3K IN REGULATION OF PHAGOCYTOSIS

3.1 Introduction

Phagocytosis is the process by which cells attach and internalize solid particles from extracellular environment. The organism *Dictyostelium discoideum* is a unicellular eukaryote that could professionally phagocytose bacteria several folds more than neutrophils (Bozzaro et al., 2008). In addition, multiple key signaling pathways are conserved between *Dictyostelium* and mammalian cells, including pathways affecting small GTPases Ras and Rac and their downstream effectors, and F-Actin remodeling. Furthermore, *Dictyostelium* cells are highly amenable for genetic manipulation such as protein overexpression, gene knockout and gene knockdown for investigating the function of a specific protein. Recent advances in various *in vivo* imaging technologies have been indispensable for studying phagocytosis. All these traits qualifies *Dictyostelium* cell an excellent model organism to study the molecular mechanisms of phagocytosis (Maniak, 2002, Annesley and Fisher, 2009).

The overall process of phagocytosis in *Dictyostelium* is similar to that of mammalian cells. The presence of extracellular particles leads to the movement of *Dictyostelium* cell to the site of particles through chemotaxis, which is followed by the attachment of cells to particles via corresponding membrane receptors. The activation of membrane receptors stimulates a signaling cascade which regulates Actin cytoskeleton remodeling at cortical region of plasma membrane and then phagocytic cup is formed.

Phagocytic cup elongates its rim which in turn leads to the cup closure and thus form the nascent phagosome. After the dissociation of Actin-coat from the nascent phagosome, naked phagosome will undergo classical endocytic route for digestion of particles and lateral excretion of indigestible particles via exocytosis.

Various types of membrane receptor have been reported for detecting different extracellular particles. An early study showed that *Dictyostelium* cell used a lectin-type receptor to specifically target the terminal glucose on the *E.coli* lipopolysaccharide and a "nonspecific" receptor which was responsible for the internalization of hydrophilic latex beads. Another study showed that Wheat Germ Agglutinin-binding membrane receptors interact with yeast and regulate the uptake of yeast in *Dictyostelium* cell (Vogel et al., 1980). Recent study suggested that another phagocytic receptor SibA, a member of Sib (similar to integrin-β) family, regulated the phagocytosis of fluorescent latex beads through its cell adhesion function (Cornillon et al., 2006). However, the uptake of bacteria in SibA mutant was not affected which suggests that SibA might be one of the nonspecific receptors for internalizing latex beads (Cornillon et al., 2006). After the binding of extracellular particle to its receptor, heterotrimeric G protein (G4αGβγ) is activated which induces the downstream signaling to regulate Actin cytoskeleton remodeling, which in turn promotes the formation of phagocytic cup (Pan et al., 2016). It has been widely accepted that Actin cytoskeleton dynamics is the key driving force to initiate phagocytosis. For examples, in early study, after the treatment of mouse macrophage with cytochalasin B, a toxin which

blocks actin polymerization, the uptake of IgG-coated erythrocytes was then hampered (KAPLAN, 1977). Dynamic Actin cytoskeleton requires the participation of nucleation promoting factors (NPF) Scar/Wave, WASP, and ENA-VASP and formin. Upon activation of certain NPFs (Scar, WASP, and ENA-VASP), Arp2/3 plays a critical role in the growth of a new Actin filament from the base of an existing filament and thus form the network of Actin filament (May et al., 2000). Formin, an Actin elongation factor, is responsible for the elongation of the plus end of F-actin (Evangelista, 2003). Further investigation of formin from *Dictyostelium* cell indicates that Diaphanous-related formin G (ForG), activated by RasG and RasB, is recruited to the site of phagocytosis and works as actin polymerase of Arp2/3-nucleated filaments which in turn drives the formation of phagocytic cup.

In addition to Actin nucleation and elongation factors, other Actin binding or associated proteins are also involved in the regulation of dynamic Actin cytoskeleton at the phagocytic cup. For example, ABP-120, an actin-crosslinking protein which belongs to the filamin group of actin binding proteins (Noegel et al., 1989). Interestingly, ABP-120 null cells showed decreased rate of up-taking particles when compared with control cells, suggesting that ABP-120 mediated F-actin cross-linking affects phagocytosis. Coronin, another actin binding protein, is localized in the dynamic F-actin rich area of *Dictyostelium*. It has been suggested to be participating in the phagocytosis because of the inefficient phagocytosis of coronin-null cells (Maniak et al., 1995). Myosin is widely known as regulating muscle contraction, but it is also an actin-associated protein functioning as a

motor protein that regulates contraction, cell migration and phagocytosis. For instance, the conventional Myosin II is involved in the regulation of chemotaxis as well as phagocytosis (Bosgraaf and van Haastert, 2006), Myosin VII participates in the regulation of phagocytosis in *Dictyostelium* cells (Titus, 1999). Additionally, cofilin, the actin-destabilizing protein, is also suggested to regulate the Actin dynamics at phagocytic cup due to the finding that disruption of cofilin-regulator protein Aip1 leads to hampered phagocytosis in *Dictyostelium* cells (Konzok et al., 1999).

Phosphatidylinositol phosphates are another group of critical molecules regulating phagocytic cup formation. For instance, phosphatidylinositol (4,5)-bisphosphate PI(4,5)P_2 is responsible for the recruitment and activation of actin-related proteins and thus form phagocytic cup. Furthermore, the de-phosphorylation of PI(3,4,5)P3 into PI(3,4)P2 which is mediated by *Dictyostelium* homolog of the phosphatase OCRL (Dd5P4) plays a crucial role in closing of the phagocytic cup (Loovers et al., 2007).

After the closure of phagosome in *Dictyostelium* cells, Rab5 is recruited to the nascent phagosome and soon after that Rab5 is replaced with Rab7 which stimulates the fusion of phagosome with lysosome (Gutierrez, 2013, Vieira et al., 2003). A recent study uncovered that one of the NPF member, WASH (WASP and SCAR homolog), is essential for proper recycling of phagocytic receptor back to the plasma membrane (King et al., 2013). In this way, the integral membrane proteins are saved and ready for the next round of phagocytosis. Furthermore, a proton pump vacuolar ATPase (V-ATPase) is recruited to

the early phagosome and thus create an acidification lumen of phagosome, which is important for the degradation of particles through digestive enzymes in lysosome (Clarke et al., 2002). WASH NPF was also shown to be essential in the retrieval of V-ATPase and digestive enzymes. The indigestible material is then expelled by the cell through exocytosis.

Cells of the mammalian immune system such as neutrophils and macrophages destroy invading microbial pathogens by phagocytosis, which is preceded by chemotaxis. Upon arriving the target of chemotaxis, mammalian phagocytes are often in an environment of inflammatory response. Inflammation is a complex cellular response, but increased production of reactive oxygen species such as superoxide is often included. Thus it will be significant to understand the effects of superoxide on cellular particle uptake. The release of reactive oxygen species (ROS) occurs normally as a result of oxygen metabolism in the cells. Conversely, the excessive accumulation of reactive oxygen species (ROS) in the cells can be detrimental. The well-studied harmful effects caused by the excessive accumulation of reactive oxygen species (ROS) include: damage to the DNA, lipid peroxidation, oxidation of amino acids and the inactivation of specific enzymes (Brown and Griendling, 2009). One of the functions of ROS that demands further understanding is its role in defense against pathogens in mammalian hosts. A study showed that increased levels of ROS facilitated the signaling to activate interferon regulatory factors three and seven (IRF-3 and IRF-7), and nuclear factor – kappa B (NF_kB) that result in an antiviral state (West et al., 2011). A more recent study also showed that the production of ROS could

be responsible for the secretions of interferon γ to control infection by the influenza A virus (Kim et al., 2013).

One of the main reactive oxygen species produced in the cell is superoxide. Normally, cells can defend against superoxide by the family of enzymes of superoxide dismutases. Sod proteins convert superoxide into hydrogen peroxide, which eventually will be converted into oxygen and water. Although the properties of superoxide have been studied *in vitro*, understanding its role *in vivo* has been proven to be difficult because of the high reactivity and the short half-life of the superoxide radical. In order to further uncover the roles of superoxide in phagocytosis, we used *Dictyostelium* cells that lack *sodC* as a model system. We hypothesized that having aberrancies in both RasG and PI3K, *sodC*$^-$ cells are likely to experience hampered phagocytosis. Here we provide the evidence which shows that *sodC*$^-$ cells are indeed compromised in up-taking TRITC labeled yeast particles. The introduction of overexpressing of cytoplasmic superoxide dismutase (SodA), redox insensitive RasG (C118A) or treatment of PI3K inhibitor LY294002 in *sodC*$^-$ cells significantly rescued the defects of particle uptake. Additionally, we show that *sodC*$^-$ cells have defects in F-Actin remodeling at the phagocytic rim extension and F-Actin depolymerization of the nascent phagosome.

3.2 Materials and Methods

3.2.1 Dictyostelium Development and Growth

All *Dictyostelium discodium* cells were grown in D3T media (Dissolve 14.3 g of Peptone No.3, 7.15 g of yeast extract, 15.4 g of Glucose, 0.48 g of KH_2PO_4 and 0.525 g of $Na_2HPO_4.7H_2O$ in Deionized water and top off to 1 L. Autoclave for 40 min, let it cool down and add antibiotics mixture to make the final D3T media has 100 μg/ml of ampicillin, 25 μg/ml of tetracycline and 25 μg/ml of chloramphenicol). In addition to D3T medium, JH10 cells will be grown with thymidine (0.5 mg/ml) and *sodC*$^-$ cells will also be grown in thymidine (0.5 mg/ml) and Blasticidin (5 μg/ml). Both types of cells were grown in shaking cultures at 19 °C at 150 rpm or in culture flasks at 19 °C for about 24 hours until they were in log phase. Cell number were monitored using a hemocytometer. Cell number for every experiment performed were between $2x10^6$ cells/ml to $2.5x10^6$cells/ml.

3.2.2 Bacterial Cell Culture

The E. coli bacterial cells which contained plasmids of interest were grown in 50 ml of LB medium and 100 μg/ml ampicillin on shaker at 37 °C for 18 hours. Once optimal growth density of E. coli was reached, the cell suspensions were centrifuged at 4000 rpm, at 4 °C for 25 min (Precision, durafuge 200R). The plasmid DNA was purified from the pellets by using midi kit (Qiagen) and DNA concentration was measured with spectrophotometer (Thermo, Genesys 10 UV). DNA tubes were stored in -20 °C frige for future use.

3.2.3 Transfection

A total of 10 million cells *of Dictyostelium discoideum* growing in the log phase were centrifuged (2000 rpm, 4 °C, 5 min), The pellets were washed in 10 ml of ice cold H-50 buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4) and re-suspended with 70 µl of H-50 buffer. The DNA tube was re-suspended with 30 µl of H-50 buffer and mixed with the pre-mixed cell solution above. Then the mixtures of DNA and cell solutions were transferred to electroporation cuvette (Fisherbrand) and perform electroporation (1000 V, 5 sec intervals between two times of pulses, BIO-RAD GenePulser Xcell). Electroporation cuvette was incubated on ice for 5 min and electroporated solutions were then transfer to a 10 cm petri dish which containing 15 ml of D3T media plus 0.5 mg/ml thymidine. A selection marker G418 was added at a concentration of 20 µg/ml after 24 hours

3.2.4 Analysis of Phagocytosis using TRITC Labeled Yeasts

Heat-inactivated yeast cells were labeled following the previous publication (Rivero and Maniak, 2006). Briefly, heat-inactivated yeasts were re−suspended with Sorensen buffer (2 mM Na_2HPO_4, 14.6 mM KH_2PO_4, pH 6.0) at $1x10^9$ yeast particles/ml. 20 ml of yeast particles were incubated with TRITC containing labeling buffer (0.1 mg/ml TRITC (T5646, Sigma), 50 mM Na2HPO4, pH 9.2) for 30 min at 30 ^oC and washed twice with labeling buffer without TRITC. Log phase *Dictyostelium* cells were pelleted in a table top centrifuge and re-suspended in D3T media at a concentration of 2 x 10^6 cells/ml. Let

cells recover on a shaker at 19 °C at 150 rpm for 30 min. For LY 294002 treatment comparison group, cells were added with LY 294002 at 10 minute time point during recover period. 0.5 ml of cell suspension was then taken after shaking for BCA assay. BCA assay samples were centrifuged, washed with 1ml of Sorensen buffer and lysed with 400 µl of TTG lysis buffer (50 mM Na_2HPO_4, pH 9.4, 0.2% triton X-100). 50 µl of the lysates were then taken and protein concentration was determined by performing Pierce BCA protein Assay (Thermo Scientific). TRITC-yeasts were then added at a concentration six fold to that of the rest *Dictyostelium* cells suspension. Mixtures of *Dictyostelium* cells and TRITC-yeasts were incubated in shaking cultures at 19 °C at 150 rpm for 30 min, 60 min and 120 min. At different time intervals, 1 ml of the sample was withdrawn, added to 100 µL of tryphan blue and incubated for 3 min. The samples were then centrifuged, re-suspended in 3 ml of Sorensen buffer and the fluorescence was measured using spectrofluorimeter (Schimadzu RF-1501, excitation 544 nm; emission 574 nm). Fluorescence data was divided by protein concentration and plotted into relative percentage by setting the data of wild type cell at 2 hour time point as 100 percent. All of the assays were repeated at least three times to get statistical analysis by performing unpaired *t* test. Internalized yeasts images were taken using 100x oil lens on a Leica DM IRB inverted epifluorescence microscope equipped with CoolSNAP digital camera and OpenLab imaging software.

3.2.5 Analysis of Phagocytosis using Microscope

A total of 0.2 million cells *of Dictyostelium discoideum* growing in the log phase taken directly from shaking culture were added to #1.5 German borosilicate coverglass (Nalge Nunc International) and allowed to settle down for 5 minutes. The media on the top was then discarded and cells were re-suspended with 0.5 ml of Development Buffer (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 6.5) and let it settle down for another 5 minutes. A total of one million TRITC-yeasts were then added directly into coverglass and mixed well. The live imaging of phagocytosis of TRITC-yeasts was taken using 60x oil lens on a DeltaVision Elite Deconvolution/TIRF microscope (GE Healthcare Life Sciences) equipped with Softworx imaging software (one frame per second, 15 seconds time intervals between each frame).

3.3 Results

3.3.1 *sodC*$^-$ Cells have Defects in Phagocytosis

sodC$^-$ cells constitutively experience oxidative stress as a result of modestly increased intracellular superoxide level (~18% higher compared to wild type), which in turn induce high basal RasG activity and higher PIP3 level (Veeranki et al., 2008). Interestingly, both RasG and PI3K are known to affect phagocytosis in *Dictyostelium discoideum* cells (Chen and Katz, 2000, Hoeller et al., 2013). We thus hypothesized that having aberrancies in the regulation of both RasG and PI3K, *sodC*$^-$ cells are likely to experience severely compromised particle uptake. Growth phase *Dictyostelium* cells were

fed with TRITC-yeast for different time duration. The relative internalization of TRITC-yeasts in *Dictyostelium* cells were determined as described in the Materials and Methods. Compared to wild type cells, *sodC*$^{-}$ cells displayed significantly decreased yeast uptaking, which indicates that phagocytosis of *sodC*$^{-}$ cells is significantly compromised (Fig. 5A). Fluorescence images also support that particle uptake of *sodC*$^{-}$ cells is hampered: a representative image of *sodC*$^{-}$ cells shows three yeasts compared to five yeasts in wild type cells (Fig. 5B).

3.3.2 *sodC*$^{-}$*::SodA* Cells Partially Rescued the Defects of Phagocytosis of *sodC*$^{-}$ Cells

To further corroborate that the excessive superoxides in *sodC*$^{-}$ cells is linked with aberrant phagocytosis, a cytoplasmic superoxide dismutase (SodA) was introduced into *sodC*$^{-}$ cells. Statistical analysis showed that there was significant increase in the relative internalization of TRITC-yeast in *sodC*$^{-}$*::SodA* cells when compared to *sodC*$^{-}$ cells, which indicates that the defects of phagocytosis was partially rescued (Fig. 6A). Representative images also supported the observation that an introduction of SodA into *sodC*$^{-}$ cells partially rescued the defects in phagocytosis of *sodC*$^{-}$ cells (Fig. 6B). These together support the view that superoxide affects the process of phagocytosis.

3.3.3 *sodC*$^{-}$*:: RasG(C118A)* Cells Partially Rescued the Defects of Phagocytosis of *sodC*$^{-}$ Cells

As mentioned earlier, RasG is one of the major targets that are affected in *sodC*$^{-}$ cell, an introduction of a superoxide insensitive RasG mutant would improve defective phagocytosis of *sodC*$^{-}$ cells. Therefore, the uptake of TRITC-yeast was determined in redox

insensitive *sodC⁻:: RasG(C118A)* cells. Statistical analysis indicated that there was significantly increase in the relative TRITC-yeast internalization in *sodC⁻:: RasG(C118A)* cells compared to *sodC⁻* cells (Fig. 7A). Representative images also supported the observation that the introduction of redox insensitive *RasG(C118A)* partially rescued the defects of phagocytosis of *sodC⁻* cells (Fig. 7B). Therefore, RasG can affect the process of phagocytosis in a superoxide sensitive manner.

3.3.4 PI3K Inhibitor LY 294002 Treatment Partially Rescued the Defects of Phagocytosis of *sodC⁻* Cells

Considering PI3K is one of the major targets of RasG and aberrantly high PIP3 level was reported in *sodC⁻* cells (Veeranki et al., 2008), it is plausible that the defects of phagocytosis of *sodC⁻* cells may be due to the dysregulation of PIP3 level. To this end, the rate of uptaking TRITC-yeasts was determined in *sodC⁻* cells after treatment with PI3K inhibitor LY 294002. A recent study indicated that inactivation of TOR complex 2 (TORC2) would increase the rate and efficiency of uptaking yeast particles (Rosel et al., 2012). Another study showed that LY294002 could also inhibit TORC2 at higher concentrations. Thus, we determined the level of LY294002 treatment to ensure it does not affect the rate of TRITC-yeast uptaking in wild type cells. Both of wild type and *sodC⁻* cells grew in log phase were fed with TRITC-yeasts in the absence and presence of 15μM of LY294002. Statistical analysis showed that treatment of 15μM of LY294002 didn't affect the relative internalization of TRITC-yeasts in wild type cells but significantly increased that of *sodC⁻* cells (Fig. 8A), which indicates that treatment of 15μM of LY294002 partially rescued the

defects of phagocytosis of *sodC*$^{-}$ cells. Representative images were consistent with the observation that defects of phagocytosis of *sodC*$^{-}$ cell was attentuated (Fig. 8B). Thus, properly regulation of PI3K is necessary for effective phagocytosis.

3.3.5 *sodC*$^{-}$ Cells have Defects in F-Actin Remodeling during Phagocytosis

During chemotaxis, Ras/PI3K signaling module activates PKB, which in turn mediates chemoattractant induced F-Actin remodeling at the leading front of a cell (Cai et al., 2010) Likewise, cells uptaking particles display Ras/PI3K activation with concomitant F-actin remodeling events at the site of phagocytosis. As previously reported or shown earlier in this dissertation, *sodC*$^{-}$ cells have aberrant RasG/PI3K signaling, F-Actin remodeling, and defective chemotaxis and phagocytosis. To further gain an insight on the function of SodC during the process of phagocytosis, growth phase cells expressing CFP-Coronin were fed with TRITC-Yeast and the live imaging of phagocytosis of TRITC-yeasts of wild type and *sodC*$^{-}$ cells was monitored using a DeltaVision Elite Deconvolution/TIRF microscope as described in Materials and Methods. CFP-Coronin (green), a marker of F-Actin, was enriched at the initial phagocytic cup in wild type cells. Then the phagocytic cup entered into the elongating step and then to the closing step (Fig. 9A). Phagocytic cup was observed to complete closure around 75 sec. Once internalized, F-Actin depolymerization ensued at 90 sec and depolymerization of F-Actin was completed around 145 sec. While in *sodC*$^{-}$ cells, instead of elongation and closure of phagocytic cup, F-Actin was observed to re-enrich at the base of phagocytic cup for 5 minutes leading to incomplete

internalization of TRITC-yeast, which suggested that the elongation of the rim of the phagocytic cup was defective in *sodC*$^-$ cells (Fig. 9B). Another live imaging of *sodC*$^-$ cells showed that, unlike complete depolymerization of F-Actin after internalization of TRITC-yeast in wild type cells, F-Actin was only partially depolymerized at 15 sec but re-polymerized from 30 sec and 120 sec resulting in defective phagosome processing (Fig. 9C). These together suggest that SodC is affecting F-Actin remodeling at the phagocytic rim extension and F-Actin depolymerization of the nascent phagosome.

3.3.6 Sodium/Hydrogen Exchanger 1 Inhibitor EIPA Treatment didn't affect Phagocytosis of *Wt* and *sodC*$^-$ Cells

We had previously demonstrated that 25 μM of sodium/hydrogen exchanger 1 inhibitor EIPA treated *sodC*$^-$ cells displayed restored Ras regulation and significantly improved chemotaxis toward cAMP gradient. Considering that both phagocytosis and chemotaxis involve RasG and F-Actin cytoskeleton rearrangement, it was significant to determine if EIPA treated *sodC*$^-$ cells display improved phagocytosis. Quantitative analysis indicated that there were no significant changes in phagocytosis of both *Wt* and *sodC*$^-$ cells after 25μM of EIPA treatment (Fig. 10A & B). The level of attenuation of the aberrantly high levels of RasG in EIPA treated *sodC*$^-$ cells was sufficient for chemotaxis, but it seems insufficient for uptaking yeast particles. EIPA may affect not only RasG activity, but also other targets involved in phagocytosis.

3.4 Figures

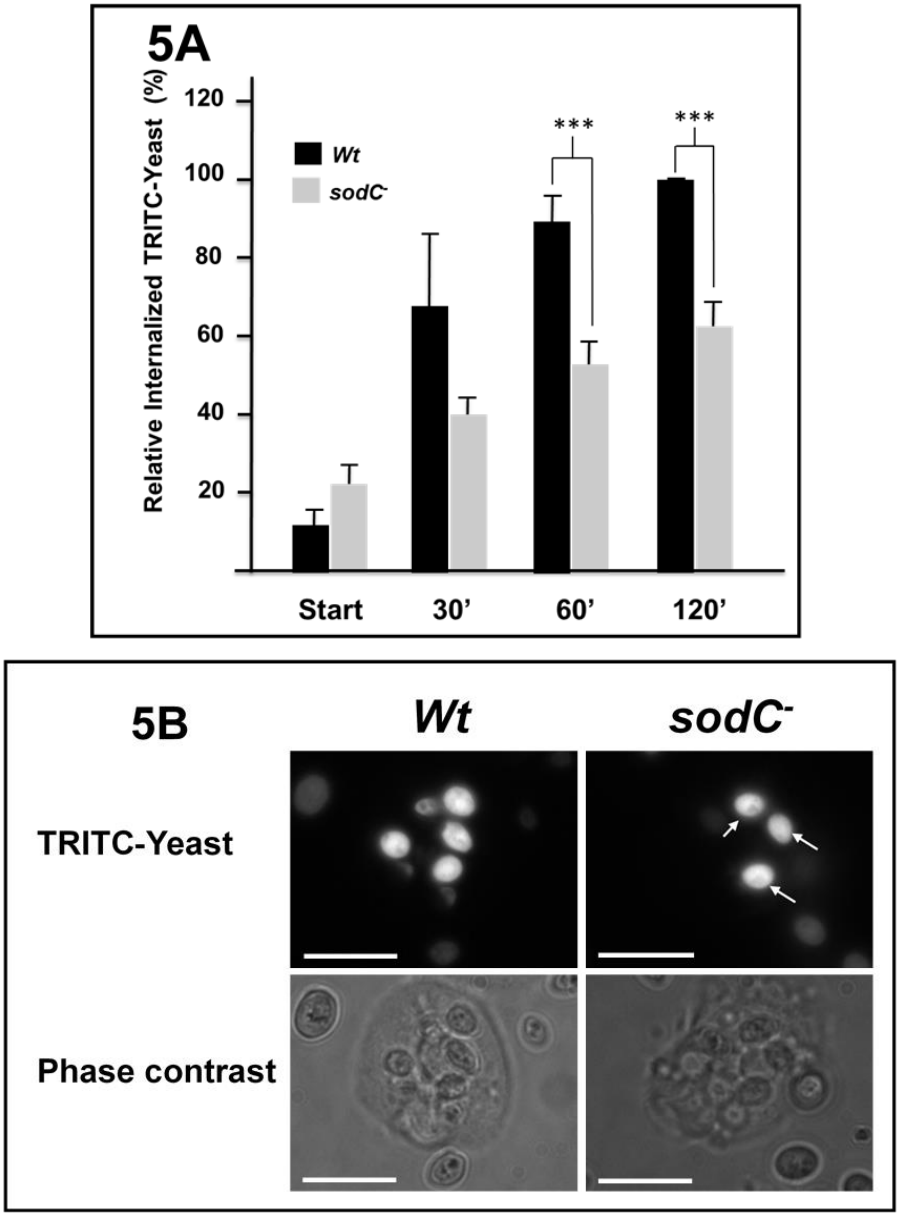


Figure 5 *sodC*$^-$ Cells have Defects in Phagocytosis

(A) Relative internalized TRITC-yeast of *Wt* and *sodC*$^-$ cells were determined by quantitating fluorescence to protein concentration ratio. *sodC*$^-$ cells showed significantly defects in total particle uptake compared to that of *Wt* cells. (B) Internalized TRITC-yeast images of *Wt* and *sodC*$^-$ cells at 30 min time point were taken using inverted epifluorescence microscope. Phase contrast (left) and TRITC-yeast fluorescence images (right) are shown (size bar: 10 μm). *sodC*$^-$ cells showed less uptake of TRITC-yeast compared to that of *Wt* cells.

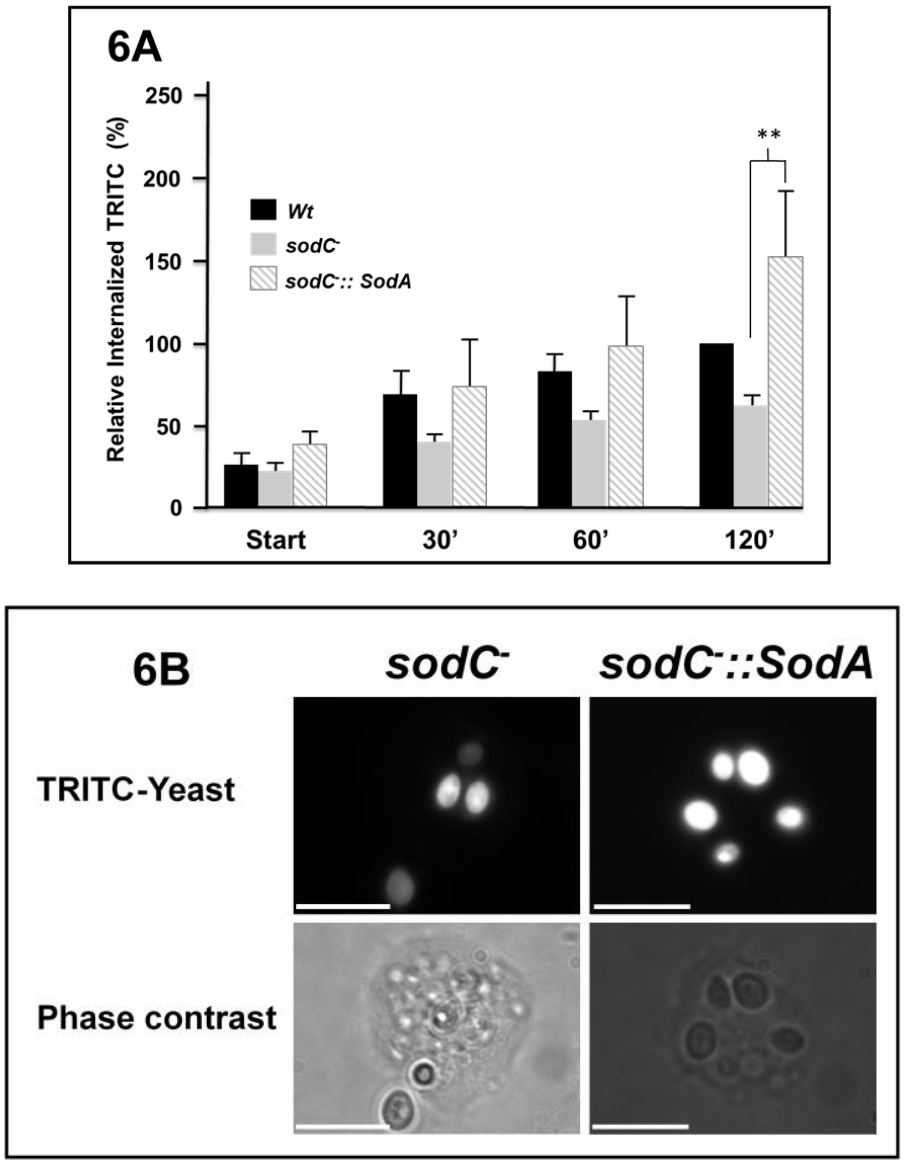


Figure 6 *sodC*$^{-}$*::SodA* Cells Partially Rescued the Defects of Phagocytosis of *sodC*$^{-}$ Cells

(A) Relative internalized TRITC-yeast of *Wt*, *sodC*$^{-}$ and *sodC*$^{-}$*::SodA* cells were determined by quantitating fluorescence to protein concentration ratio. *sodC*$^{-}$*::SodA* cells showed significantly increase in particle uptake compared to that of *sodC*$^{-}$ cells. (B) Internalized TRITC-yeast images of *sodC*$^{-}$ and *sodC*$^{-}$*::SodA* cells at 30 min time point were taken using inverted epifluorescence microscope. Phase contrast (left) and TRITC-yeast fluorescence images (right) are shown (size bar: 10 μm). Obviously, *sodC*$^{-}$*::SodA* cells showed rescued in total uptake of TRITC-yeast.

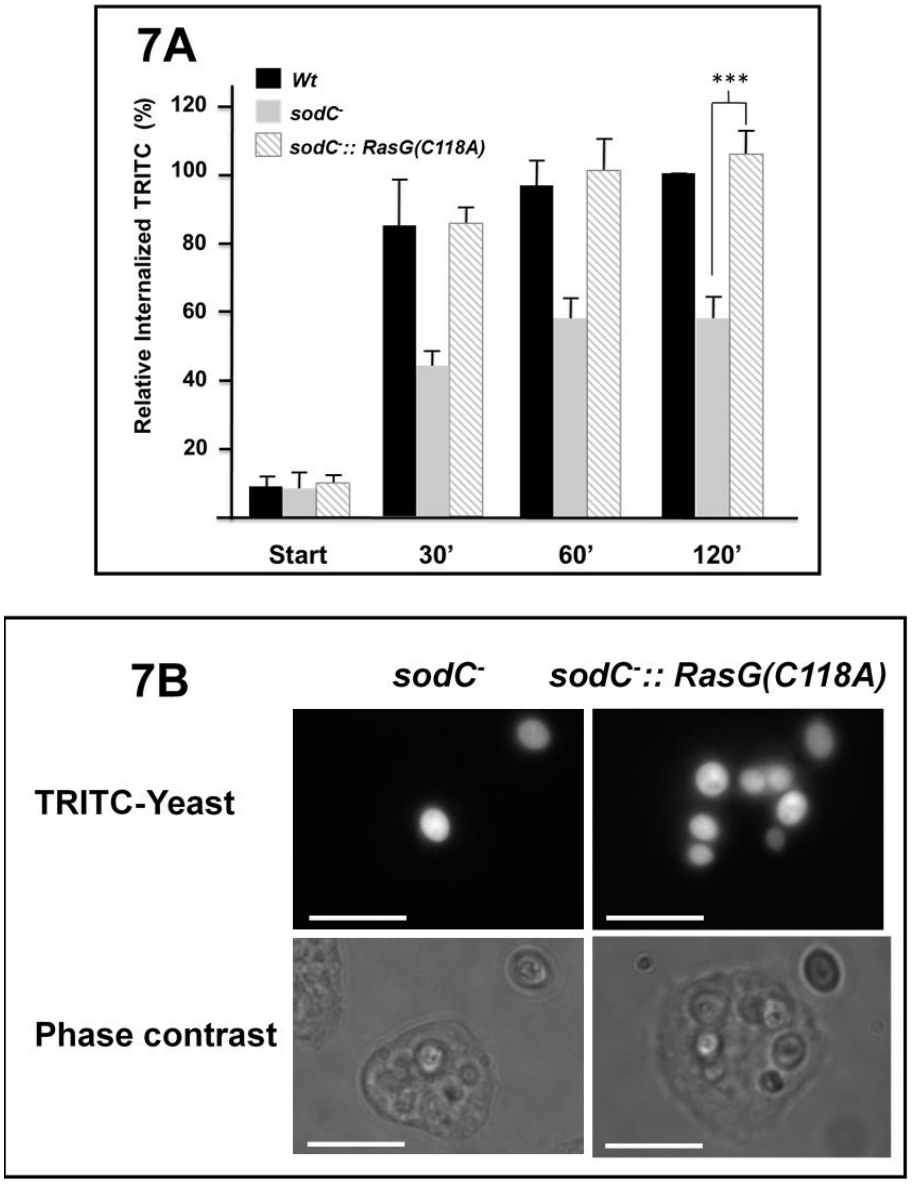


Figure 7 *sodC⁻:: RasG(C118A)* Cells Partially Rescued the Defects of Phagocytosis of *sodC⁻* Cells

(A) Relative internalized TRITC-yeast of *Wt*, *sodC⁻* and *sodC⁻:: RasG(C118A)* cells were determined by quantitating fluorescence to protein concentration ratio. *sodC⁻:: RasG(C118A)* cells showed significantly increase in particle uptake compared to that of *sodC⁻* cells. (B) Internalized TRITC-yeast images of *sodC⁻* and *sodC⁻:: RasG(C118A)* cells at 30 min time point were taken using inverted epifluorescence microscope. Phase contrast (left) and TRITC-yeast fluorescence images (right) are shown (size bar: 10 μm). Obviously, *sodC⁻:: RasG(C118A)* cells showed rescued in total uptake of TRITC-yeast.

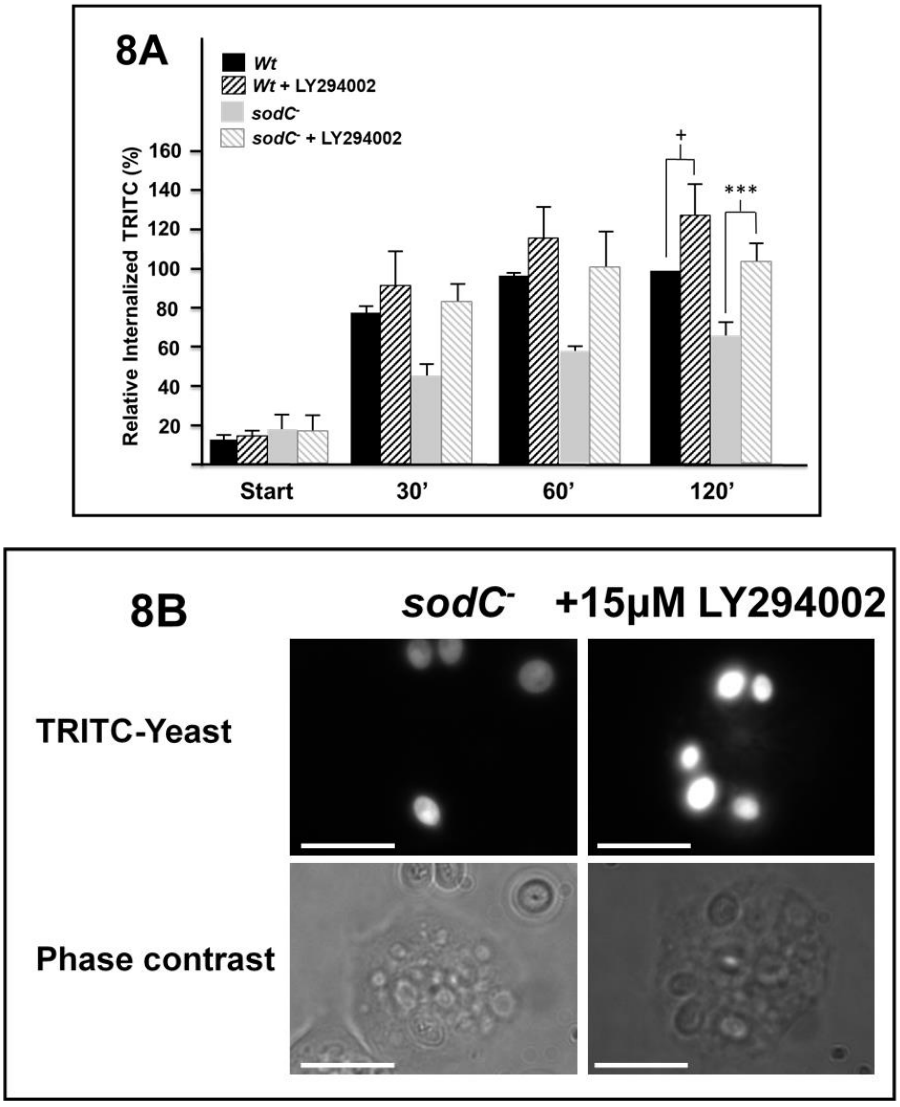
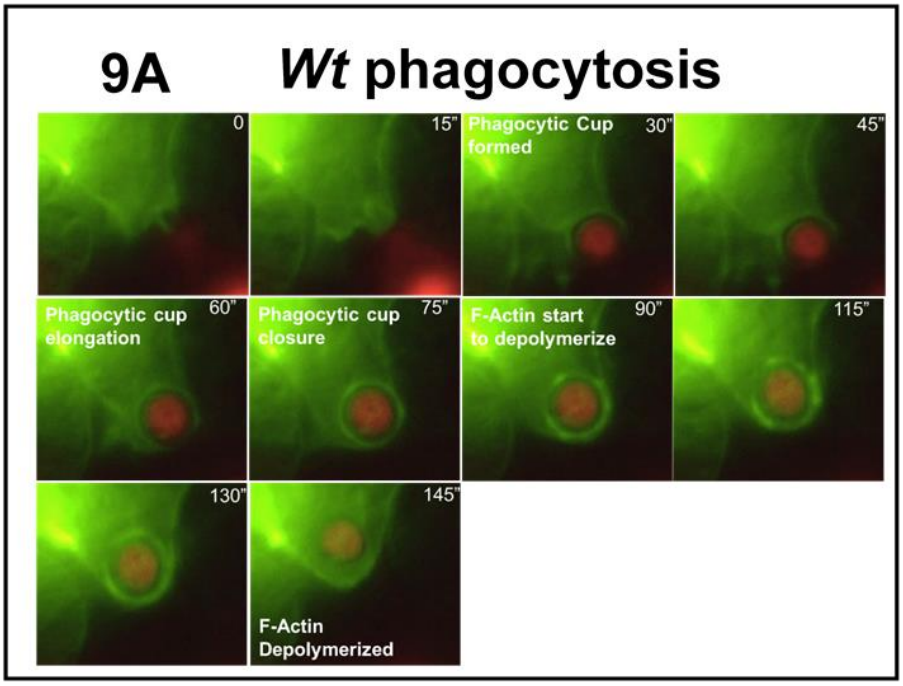


Figure 8 PI3K Inhibitor LY 294002 Treatment Partially Rescued the Defects of Phagocytosis of *sodC*$^-$ Cells

(A) Relative internalized TRITC-yeast of *Wt* and *sodC*$^-$ cells in the absence and presence of 20min of 15 μM of PI3K inhibitor LY294002 were determined by quantitating fluorescence to protein concentration ratio. *Wt* cells after LY294002 treatment showed insignificant difference when compared with *Wt* cells only. Conversely, *sodC*$^-$ cells after LY294002 treatment showed significant increase in particle uptake compared to that of non-treated *sodC*$^-$ cells. (B) Internalized TRITC-yeast images of *sodC*$^-$ cells in the absence and presence of LY294002 treatment at 30 min time point were taken using inverted epifluorescence microscope. Phase contrast (left) and TRITC-yeast fluorescence images (right) are shown (size bar: 10 μm). Obviously, *sodC*$^-$ cells after LY294002 treatment showed rescued in total uptake of TRITC-yeast.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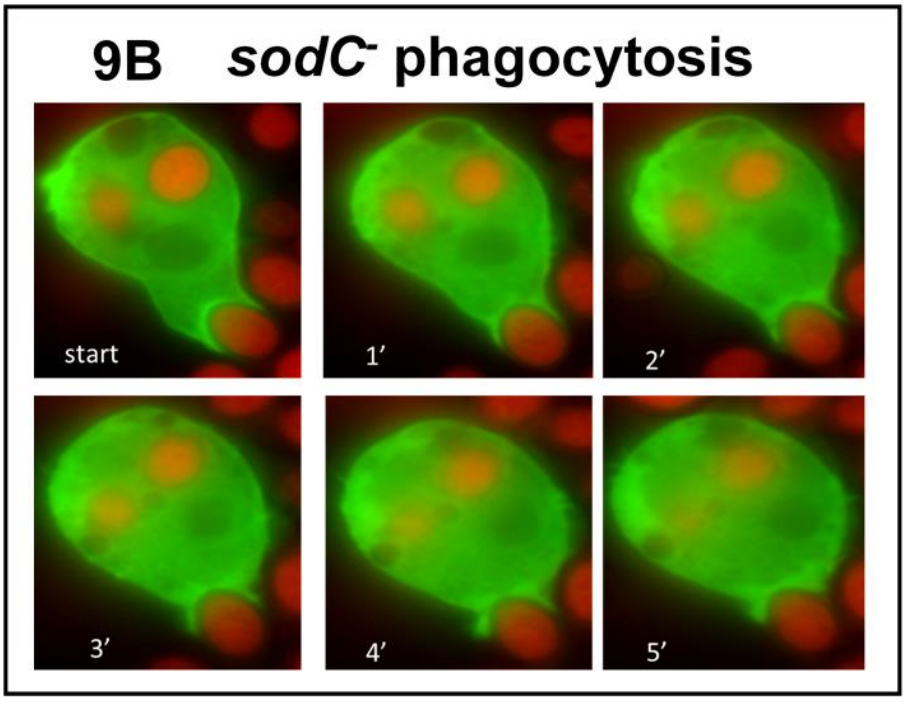
Wt phagocytosis
0
15"
Phagocytic Cup formed
30"
45"
Phagocytic cup elongation
60"
Phagocytic cup closure
75"
F-Actin start to depolymerize
90"
115"
130"
145"
F-Actin Depolymerized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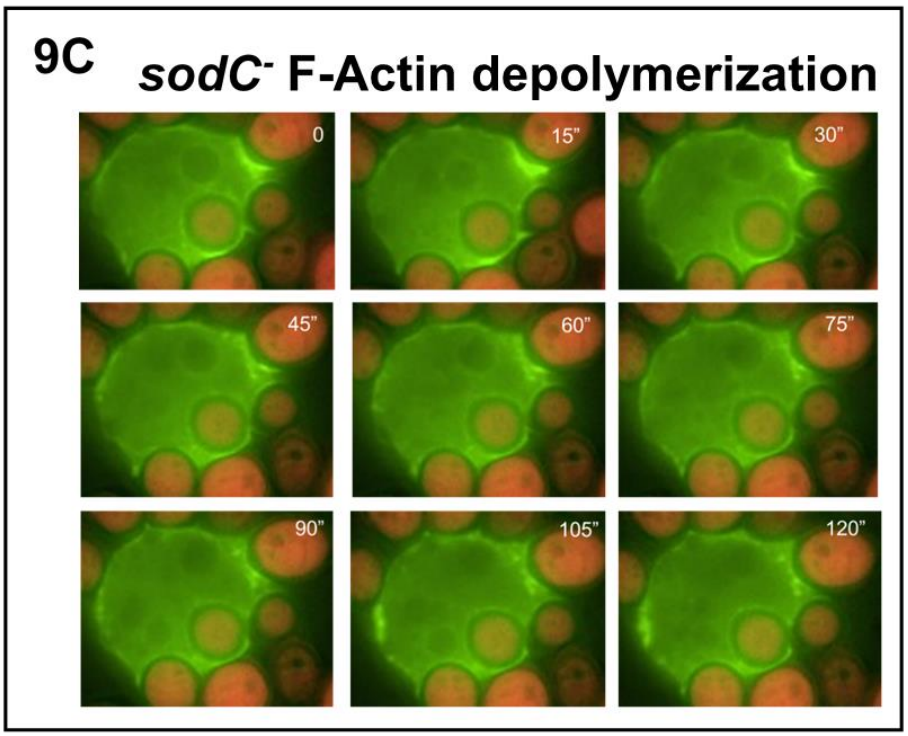


Figure 9 *sodC*$^{-}$ Cells have Defects in F-Actin Remodeling during Phagocytosis
The live imaging of phagocytosis of TRITC-yeasts of *Wt* and *sodC*$^{-}$ cells was taken using a DeltaVision Elite Deconvolution/TIRF microscope as described in Materials and Methods. (A) CFP-Coronin (green) was enriched at the phagocytic cup and *Wt* cell started to engulf the TRITC-yeast (red). The complete phagocytosis of *Wt* cells lasted for about 130 sec. (B) incomplete internalization of TRITC-yeast lasting for 5 min was observed in *sodC*$^{-}$ cells which suggest the defects of phagocytosis. (C) F-actin was observed partially depolymerized at 15 sec but re-polymerized from 30 sec to 120 sec in *sodC*$^{-}$ cells resulting in defective phagocytosis.

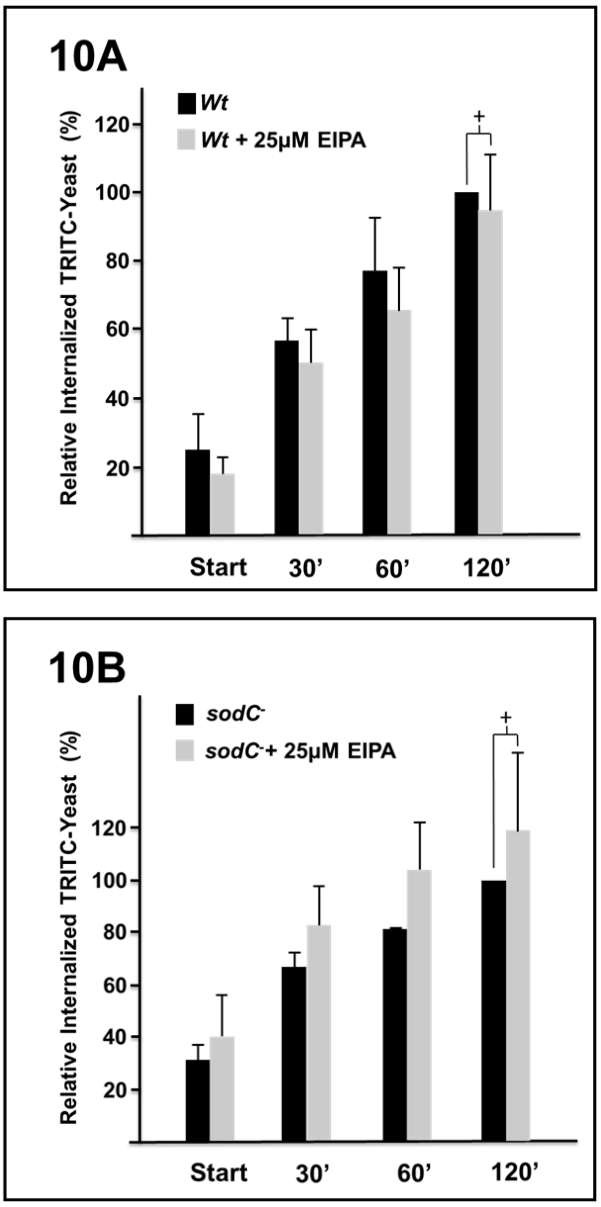


Figure 10 Sodium/Hydrogen Exchanger 1 Inhibitor EIPA Treatment didn't affect Phagocytosis of *Wt* and *sodC*$^-$ Cells

Relative internalized TRITC-yeast of *Wt* and *sodC*$^-$ cells in the absence and presence of 25 μM of NHE1 inhibitor EIPA were determined by quantitating fluorescence to protein concentration ratio. (A) *Wt* cells after EIPA treatment showed comparable level of relative TRITC-yeast uptake compared to that of non-treated *Wt* cells. (B) *sodC*$^-$ cells after EIPA treatment showed comparable level of particle uptake compared to that of non-treated *sodC*$^-$ cells.

CHAPTER 4. THE ROLE OF SUPEROXIDE, RASG AND PI3K IN REGULATION OF ADHESION

4.1 Introduction

Adhesion is the process by which cells attach to substratum matrix or its adjacent cell through specialized protein complexes that include adhesion receptors. The overall process of integrin mediated cell to substratum adhesion by *Dictyostelium* cell involves several phases. The initial attachment of cells to substratum matrix via electrostatic interaction followed by the interaction between extracellular domain of integrin and substratum matrix. Cell to substratum matrix adhesion is then strengthened by the formation of focal adhesion (FA) complex.

Integrin family proteins, as classical cell to substratum matrix adhesion receptors, play an essential role in mammalian cells by providing linkage between extracellular ligands and intracellular signaling and orchestrating multiple signaling events such as cell differentiation, cell cycle and actin remodeling related events such as cell migration. In *Dictyostelium* cells, a number of adhesion receptors and adhesion molecules have been discovered. For instance, similar to Integrin Beta protein A (SibA), is regarded as a membrane protein that affects phagocytosis. After detailed structural studies by Cornillon and others (2006) suggested that *Dictyostelium* SibA is likely an ortholog of integrin β chains of higher animals based on the highly conserved domain structures. For example, both SibA and integrin β chains of higher animals contain the von Willebrand factor type A domain (VWA). Furthermore, *sibA*$^{-}$ cells showed defective cell to substratum matrix

adhesion, which was suggested to be the consequence of the lack of SibA association with talin. Considering that Talin is the key regulatory protein that controls Integrin function in mammalian cells, it was strongly suggested that SibA is Integrin orthologue of *Dictyostelium* cells (Cornillon et al., 2006),

As just mentioned before, talin is one of the most critical regulators that affects the cell to substratum adhesion in mammals (Das et al., 2014). *Dictyostelium* cells have two talin homologues, talA and talB. The disruption of talA and/or talB caused reduced cell to substratum matrix adhesion. Moreover, overexpression of talA into *talB*$^{-}$ cells or talB into *talA*$^{-}$ cells empowered the cells to better adhere to the substratum. Talin was also shown to be essential for the formation of paxillin-rich adhesion complexes, suggesting that Talin is a part of adhesion complex supporting integrin receptor proteins (Tsujioka et al., 2008). A more recent study by Katarzyna and others (2016) suggested that small GTPase Rap1 of *Dictyostelium* cell plays a crucial role in activating talB through the interaction between Rap1 and Ras association (RA) domain of talB and thus regulates cell to substratum matrix adhesion. This interaction is similar to that of mammalian cells characterized by the binding of Rap1 and talin mediated by scaffold protein RIAM (Plak et al., 2016). Another small GTPase RasG was also suggested to affect cell to substratum adhesion considering the fact that the disruption of *rasG* led to flattening of Dictyostelium cells in morphology together with stronger adhesiveness of substratum matrix (Tuxworth et al., 1997). Adhesion–deficient protein A (SadA) is another adhesion receptor given that the disruption

of *sadA* led to the complete detachment of mutant cell from substratum matrix in growth medium (Fey et al., 2002).

A few adhesion receptors affecting cell-to-cell adhesion have been identified in *Dictyostelium* cells. During the early stage of cell development, a glycoprotein DdCad-1 (gp24), which is similar to vertebrate cadherins, plays an essential role in cell to cell adhesion (Brar and Siu, 1993).

One of the obvious downstream target of Ras proteins during phagocytosis is NPF Formin. It is, however, unclear how Ras affect cell-substratum adhesion of Dictyostelium cells. As a downstream effector of Ras, PKB may affect cell-substratum adhesion, given that talin is one of the well characterized PKB substrates. *sodC*$^{-}$ cells have aberrantly high level of PKBs activity (Castillo, et al., 2017), and thus have aberrantly high level of PKB-mediated phosphorylation of several proteins that likely include adhesion molecule Talin, Pak, RasGEF and RacGAP proteins. Therefore we hypothesize that the defects of total particle uptake of *sodC*$^{-}$ cells could be partially due to the aberrant adhesion which may be caused by aberrant RasG/PKB/Talin signaling axis. Here we provide evidence showing that *sodC*$^{-}$ cells have defective cell to substratum matrix adhesion. Additionally, the introduction of overexpressing of cytoplasmic superoxide dismutase (SodA), redox insensitive RasG (C118A) or treatment of PI3K inhibitor LY294002 in *sodC*$^{-}$ cells significantly rescued the defects of cell to substratum matrix adhesion.

4.2 Materials and Methods

4.2.1 *Dictyostelium* Development and Growth

All *Dictyostelium discodium* cells were grown in D3T media (Dissolve 14.3 g of Peptone No.3, 7.15 g of yeast extract, 15.4 g of Glucose, 0.48 g of KH_2PO_4 and 0.525 g of $Na_2HPO_4.7H_2O$ in Deionized water and top off to 1 L. Autoclave for 40 min, let it cool down and add antibiotics mixture to make the final D3T media has 100 μg/ml of ampicillin, 25 μg/ml of tetracycline and 25 μg/ml of chloramphenicol). In addition to D3T medium, JH10 cells will be grown with thymidine (0.5 mg/ml) and *sodC*$^-$ cells will also be grown in thymidine (0.5 mg/ml) and Blasticidin (5 μg/ml). Both types of cells were grown in shaking cultures at 19 °C at 150 rpm or in culture flasks at 19 °C for about 24 hours until they were in log phase. Cell number were monitored using a hemocytometer. Cell number for every experiment performed were between $2x10^6$ cells/ml to $2.5x10^6$cells/ml.

4.2.2 Cell Adhesion Assay

Adhesion of cells to a solid substratum was measured as described elsewhere (Khurana et al., 2005). Briefly, 2 x 10^5 of log-phase cells were plated on 3 cm tissue culture petri dishes in 2ml of D3T media and allowed to settle for 2 hours. For LY 294002 treatment comparison group, cells were added with LY 294002 at 1 hour 40 minute time point during settlement period. The dishes were then shaken for 60 minutes at 50 rpm, 70 rpm and 100 rpm. 2 ml of top media was taken and spun down. The cells pellets were then re-suspended with 100ul of Development Buffer (5 mM Na_2HPO_4, 5 mM KH_2PO_4,

1 mM $CaCl_2$, 2 mM $MgCl_2$, pH 6.5). Cells dissociated from the substratum were counted using a Hemocytometer**.** Cell adhesion assays were repeated at least three times to get statistical analysis by performing unpaired *t* test.

4.3 Results

4.3.1 *sodC*$^-$ Cells have Defects in Cell-to-Matrix Adhesion

A previous study (Chen and Katz, 2000) showed that the cells expressing constitutive active RasG(G12T) exhibited enhanced cell-substratum adhesion with increased tyrosine phosphorylation of Actin. It is intuitive to perceive that the adhesion is necessary for particle uptake. One of the major targets of RasG in the context of chemotaxis is PI3K/PKB singling network, which functions through phosphorylating a group of proteins that are substrates of PKB. Upon phosphorylation, these proteins, which include adhesion molecule TalinB, were likely to exhibit differential activity to accommodate spatio-temporal remodeling of F-Actin at the leading edge of a chemotaxing cell. Given that *sodC*$^-$ cells have aberrant regulation of RasG/PI3K activity and aberrant PKB substrate phosphorylation under the context of chemotaxis. It is plausible that phagocytosis was affected at least in a partially redundant manner. Thus, we hypothesize that persistent RasG/PI3K activation in *sodC*$^-$ cells may hamper phagocytosis through aberrant adhesion. Adhesion of cells to a solid substratum was measured as described in Materials and Methods. Briefly, log-phase cells were plated on tissue culture petri dishes and then shaken for 60 minutes at 50 rpm, 70 rpm and 100 rpm. Cells dissociated from the substratum were

counted using a Hemocytometer as shown in figure 11A. Quantitative analysis indicated that *sodC*$^{-}$ cells showed significantly increase in relative detached cells at 2 hour time point when compared to wild type cells which suggested *sodC*$^{-}$ cells have defects in cell-to-matrix adhesion.

4.3.2 *sodC*$^{-}$*::SodA* Cells Partially Rescued the Defects in Cell-to-Matrix Adhesion

We hypothesize the defects in cell-to-matrix adhesion of *sodC*$^{-}$ cells is due to aberrant regulation of superoxide level. Thus, the adhesion of *sodC*$^{-}$*::SodA* cells was determined. Quantitative analysis indicated that *sodC*$^{-}$*::SodA* cells showed significantly decrease in the relative levels of detached cells at 2 hour time point when compared to those of *sodC*$^{-}$ cells (Fig. 11B), suggesting *sodC*$^{-}$*::SodA* cells partially rescued the defects in cell-to-matrix adhesion. Considering *sodC*$^{-}$*::SodA* cells also partially rescued the defects of phagocytosis, thus we speculate that superoxide level can affect phagocytosis through adhesion at least partially.

4.3.3 *sodC*$^{-}$*::RasG(C118A)* Cells Partially Rescued the Defects in Cell-to-Matrix Adhesion

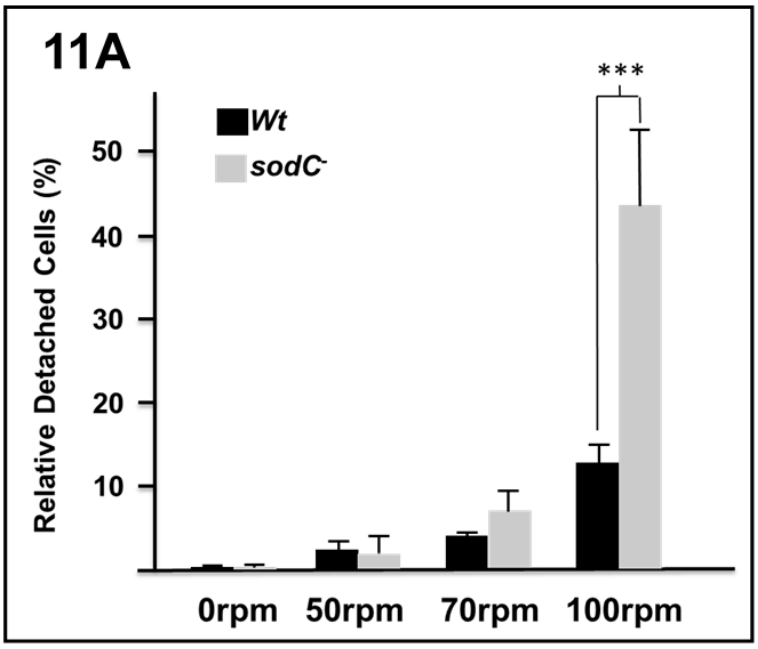
To determine if the defects in adhesion of *sodC*$^{-}$ cells is because of dysregulation of redox sensitive RasG activity, the cell-to-matrix adhesion of *sodC*$^{-}$*:: RasG(C118A)* cells was determined. Quantitative analysis showed that there was significantly decrease in the relative levels of detached *sodC*$^{-}$*:: RasG(C118A)* cells compared to those of *sodC*$^{-}$ cells (Fig. 11C), which suggested *sodC*$^{-}$*:: RasG(C118A)* cells partially rescued the defects in cell-to-matrix adhesion. Together with the finding that *sodC*$^{-}$*:: RasG(C118A)* cells also

displayed improved yeast particle uptake, redox sensitive RasG seems to affect phagocytosis through adhesion at least partially. .

4.3.4 PI3K Inhibitor LY 294002 Treatment Partially Rescued the Defects in Cell-to-Matrix Adhesion of *sodC*$^-$ Cells

Considering that aberrant high PI3K activity in *sodC*$^-$ cells (Veeranki et al., 2008), we hypothesize that the defects of adhesion in *sodC*$^-$ cells is due to the dysregulation of PI3K activity. Therefore, the adhesion of *sodC*$^-$ cells were determined in the absence and presence of 15 μM of PI3K inhibitor LY 294002. Quantitative analysis showed that there was significant decrease in the relative levels of detached *sodC*$^-$ cells after 15 μM of LY294002 treatment when compared to those of *sodC*$^-$ cells (Fig. 11D), indicating that PI3K inhibitor LY 294002 treatment partially rescued the defects of cell-to-matrix adhesion of *sodC*$^-$ cells. Given that 15 μM of LY294002 treatment also attenuated the defects of phagocytosis of *sodC*$^-$ cells, it is plausible that PI3K activity can affect phagocytosis through adhesion, but it doesn't exclude other phagocytic steps that are potentially sensitive to PI3K activity.

4.4 Figures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***
Wt
sodC-
Relative Detached Cells (%)
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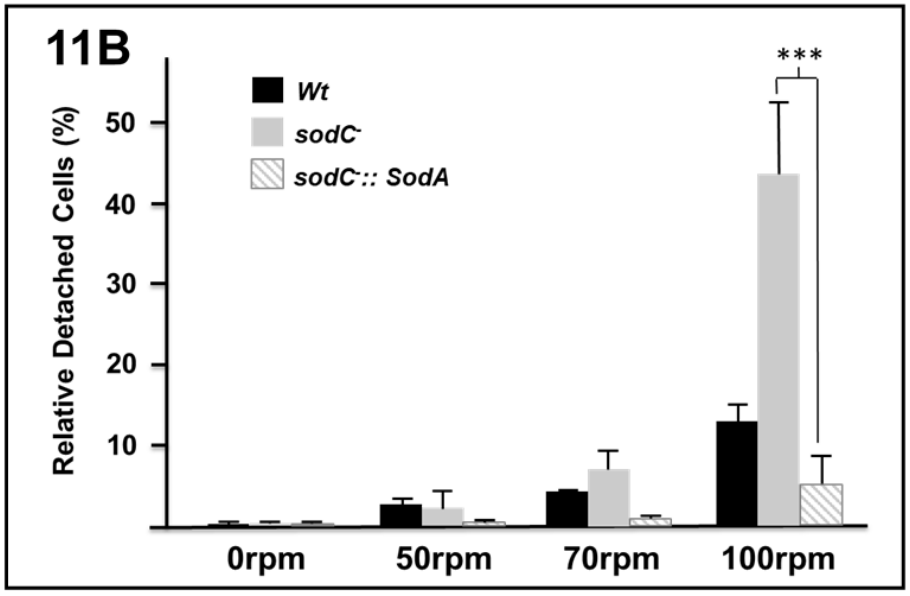
40
30
20
10
0rpm
50rpm
70rpm
100rpm


11B
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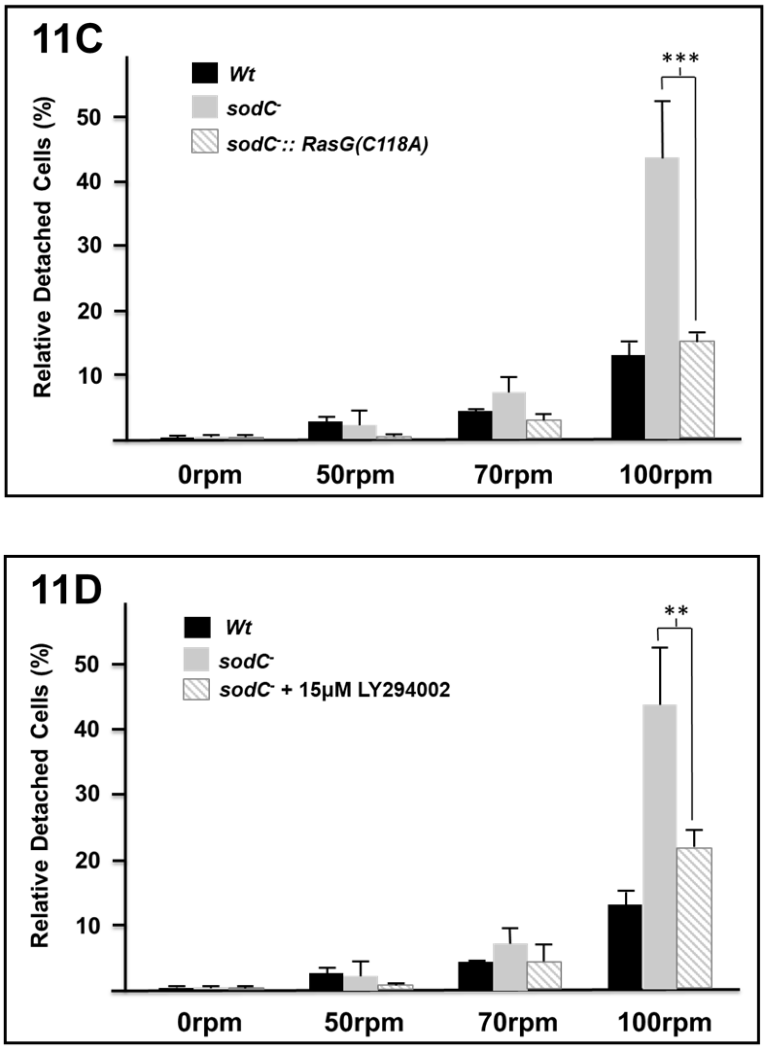

Figure 11 The Role of SodC in regulation of Cell to Substratum Matrix Adhesion
(A) Adhesion of cells to a solid substratum was measured using hemocytometer. *sodC*$^{-}$ cells showed significant increase in relative detached cells compared to that of *Wt* cells suggesting its adhesion defects. (B) *sodC*$^{-}$*::SodA* cells showed significant rescued in adhesion compared to that of *sodC*$^{-}$ cells. (C) *sodC*$^{-}$*:: RasG(C118A)* cells showed significant rescued in adhesion compared to that of *sodC*$^{-}$ cells. (D) *sodC*$^{-}$ cells treated with 15 μM of PI3K inhibitor LY294002 showed significant rescued in adhesion compared to that of *sodC*$^{-}$ cells.

CHAPTER 5. DISCUSSION AND FUTURE DIRECTION

5.1 Potential Roles of SodC in Fluid Phase Up-taking and Trafficking

The laboratory strains of *Dictyostelium discoideum* were selected toward having enhanced capacity of total fluid phase up-taking as a means of securing their food source. As discussed in the introduction, cells achieve this through multiple routes that include clathrin dependent, caveolin mediated, and macropinocytosis. *Dictyostelium* cells lacking SodC was previously shown to exhibit severe mis-regulation of redox-sensitive small GTPases such as RasG (Veeranki et al., 2008, Castillo et al., 2017). *sodC*$^{-}$ cells suffer from constitutively enhanced level of active RasG protein, which in turn induces persistent activation of its downstream signaling targets such as PI3K and PKB. Persistently increased level of PI3K product PIP3 and persistent phosphorylation of PKB substrate are suggested to negatively affect efficient chemotaxis of *sodC*$^{-}$ cells toward chemoattractant cAMP gradient.

Decades of extensive studies on not only the mechanism of chemotaxis but also of pinocytosis and phagocytosis strongly suggest that the proper regulation of phosphoinositides and small GTPase RasG are crucial for executing these events for the survival of *Dictyostelium* cells. Considering that *sodC*$^{-}$ cells not only displayed defective chemotaxis, but also aberrant regulation of phosphoinositide metabolism and RasG, *sodC*$^{-}$ cells are likely experiencing defective fluid phase uptake and phagocytosis. The current study uncovered that *sodC*$^{-}$ cells are defective in phagocytosis, but not in the total level of

fluid phase uptake. Interestingly, the trafficking of internalized fluid was significantly altered, culminating as enlarged vesicles.

Accumulating enlarged vesicles in *sodC*$^{-}$ cells could arise either through enhanced fusion of small vesicles of clathrin- and/or caveolin- mediated endocytosis or enhanced activity of macropinocytosis with concomitant suppression of the above-mentioned endocytosis, or both. To determine if the macropinocytosis activity is altered in *sodC*$^{-}$ cells, *sodC*$^{-}$ cells were treated with a pharmacological inhibitor that can inhibit macropinocytosis. One of such inhibitors is a PI3K inhibitor, LY294002. Intriguingly, LY294002 treated *sodC*$^{-}$ cells exhibited significantly reduced level of the total fluid phase uptake compared to that of control *sodC*$^{-}$ cells (Figure 4C). Furthermore, the levels of total fluid phase in LY294002 treated *sodC*$^{-}$ cells were comparable to those of the LY294002 treated wild type cells (Figure 4C). These together suggest that wild type and *sodC*$^{-}$ cells are comparable in their capacity of importing extracellular fluid in a LY294002 sensitive manner.

Several recent studies unveiled a new mode of cell death called Methuosis. The hallmark of Methuosis is the accumulation of enlarged vesicles without the typical phenotypes of apoptosis. Extensive mechanistic studies demonstrated that these enlarged vesicles originated from macropinosomes (clathrin independent endosomes (CIE)), of which generation depended on the presence of active Ras and disturbed fusion with early endosomes (Maltese and Overmeyer, 2014, Bloomfield and Kay, 2016). Current understanding of mammalian Methuosis suggests that persistent Ras activation and

subsequent Rac activation induce the facilitated fusion of CIEs with decreased targeting toward endosomal entities (Bhanot et al., 2010), but how these aberrancies lead to the above described symptoms of Methuosis needs to be further explored.

Consistent with the involvement of aberrant Ras activation in the development of Methuosis, *sodC*$^{-}$ cells have enlarged vesicles filled with extracellular fluid which was alleviated by the introduction of redox-insensitive RasG(C118A) mutant (Figure 3A & B). Our previous study also reported that *sodC*$^{-}$ cells expressing RasG(C118A) protein displayed significantly improved directional cell migration toward the chemoattractant cAMP gradient (Castillo et al., 2017). At the molecular level, *sodC*$^{-}$ cells display significantly enhanced level of PKB protein kinases and exaggerated phosphorylation of PKB substrates proteins. Introduction of RasG(C118A) induced significantly alleviated level of PKBs substrates phosphorylation. It will be significant to determine if PKB signaling affect macropinosome trafficking as a downstream target of Ras in *Dictyostelium* cells. Understanding the mechanism of Ras mediated CIE trafficking in *Dictyostelium* cells may provide invaluable insight to the understanding of the mechanism of mammalian Methuosis.

We have also determined that *sodC*$^{-}$ cells expressing a cytoplasmic superoxide dismutase (SodA) also exhibited an alleviated phenotype of enlarged vesicles containing extracellular fluids (Fig. 2A & B). Unlike SodC protein, which encodes GPI anchoring signal and functions on the outer leaflet of membrane, SodA is a cytoplasmic protein. We

have confirmed that *sodC*$^{-}$ cells expressing SodA cells alleviated the level of active Ras proteins (unpublished data, Kabra and Kim, 2018). Thus alleviating Ras activity through three independent ways, PI3K inhibition, expression of Redox insensitive Ras mutant RasG(C118A), and introduction of additional cytoplasmic superoxide dismutase, consistently reduced the size of extracellular fluid filled vesicles and increased the number of such vesicles.

Considering that misregulation of superoxide is often associated with various types of cellular diseases, we speculate that an increased level of superoxide may also facilitate mammalian Methuosis through activating redox sensitive Ras protein in mammalian cells.

5.2 Potential Roles of SodC in Particle Up-taking

Having aberrantly high level of RasG activity as well as PIP3, it was likely that *sodC*$^{-}$ cells show defective fluid phase up-taking during macropinocytosis. However, as described earlier in this study, *sodC*$^{-}$ cells displayed aberrant trafficking of macropinosome into enlarged vacuoles, but no overt defects in up-taking extracellular fluid. Interestingly, another Actin-driven event that mediate extracellular particle up-take also involves spatio-temporal regulation of Ras and PIP3. Figure 5A and 5B show that *sodC*$^{-}$ cells are seriously compromised in up-taking heat-killed yeast particles.

It is clear that PI3K product PIP3 accumulate at the rim of the elongating pseudopod that encompassing the particle. However, cells treated with PI3K inhibitor LY294002 displayed diverse effects on up-taking particles of different sizes and properties (Schlam et

al., 2015). It has been debated for a while, and some of the consensus were that different level of LY294002 have distinct effects on phagocytosis and multiple receptors and signaling pathways exist and can be utilized in genetically distinct cell types and distinct types of particles that are being up-taken.

Previous studies and the earlier part of this study demonstrated that LY294002 treated *sodC*$^{-}$ cells displayed significantly improved directional cell migration toward chemoattractant cAMP (Veeranki et al., 2008) and improved macropinosomal trafficking (Figure 4A & B). The current study also showed that LY294002 treated *sodC*$^{-}$ cells internalized significantly more yeast particles than the control *sodC*$^{-}$ cells (Figure 8).

Extensive previous studies showed that PIP3 could affect F-actin remodeling through three distinct effectors: SCAR/WAVE actin nucleation promoting factors, PH domain containing unconventional Myosin family members, and Akt and its substrates that encompass Talin, Pak, RasGEF, and RacGAP proteins (Veltman et al., 2016, Chen and Iijima, 2012, Kamimura et al., 2009). Thus, it is plausible that the constitutively increased level of PIP3 in *sodC*$^{-}$ cells could affect some of these PIP3 effectors and thus hamper efficient phagocytosis. It will be significant to determine if SCAR/WAVE and the unconventional Myosin proteins are aberrantly regulated in *sodC*$^{-}$ cells. In addition, we have previously reported that Akt kinases are hyperactivated and subsequently their substrates are phosphorylated higher than normal in *sodC*$^{-}$ cells (Castillo et al., 2017). It

will be interesting to determine if PKB mediated phosphorylation of proteins that could affect F-actin remodeling are dysregulated in cells lacking SodC.

Upon up-taking particles, professional phagocytes recruits and activate superoxide generating NOX protein complex on the phagosome. The resulting superoxide radicals supposed to damage particles in the phagosome, but it remains to be further studied that how cells protect their own proteins. In addition, if they need to protect themselves from the rising superoxide radicals, the identity of proteins that are ought to be protected need to be discovered. We speculate here that redox sensitive Ras proteins need to be protected from the superoxide which could leak out from the phagosome through some anion channels such as ClC3 (Hawkins et al., 2007). Figure 6A demonstrated that an expression of cytoplasmic superoxide dismutase (SodA) in *sodC*$^{-}$ cells improved yeast up-taking activity even higher than that of wild type cells. One of the possibilities of the superoxide depletion mediated elevation of particle up-taking is that a small GTPase which is negatively regulated by superoxide may function in parallel with Ras-mediated phagocytosis. As previously shown in other studies, superoxide radicals can target the cysteine in the NKCD motif of small GTPase in a positive manner, but some small GTPases that contain the cysteine residue right after the P loop region are negatively regulated by superoxide (Wey et al., 2014). Thus depleting superoxide with SodA not only restore RasG, but may also restore other yet to be identified type of small GTPases which could affect phagocytosis.

Figure 7 showed that the redox insensitive RasG(C118A) mutant expressing *sodC*$^{-}$ cells were up-taking yeast particle to the level of wild type cells, indicating that aberrantly high level of active RasG proteins is the major factor that causes compromised particle up-taking in *sodC*$^{-}$ cells. Aberrantly and constitutively activated RasG proteins may aberrantly activate several of its downstream effector. One of the RasG effector is TorC2, which was recently shown to suppress phagocytosis of *Dictyostelium* cells. Reliving TorC2 mediated inhibition may restore phagocytic capacity of *sodC*$^{-}$ cells. Another effector of RasG is one of the nucleation promoting factor Formin G. ForminG was shown to be involved in the formation of phagocytic cup, and thus dysregulation of ForG activity by aberrant RasG activity from the signals from the particle adhesion would hamper efficient nucleation of F-Actin at the initiation site of particle uptake.

5.3 Time-lapse Analysis of Particle Up-taking of *sodC*$^{-}$ Cells

Figure 9A demonstrates efficient yeast particle up-take by a wild type cell. An early phagocytic cup was observed as early as 30 second and complete internalization was observed at 75 second, which was in turn followed with depolymerization of actin-coat of the nascent phagosome. After 145 second post-internalization, no more actin coat was observable. *sodC*$^{-}$ cells in contrast displayed significantly inefficient phagocytic cup elongation as shown in figure 9B. This compromised elongation of the rim of the phagocytic cup could have been the consequence of several possibilities. Persistent and global presence of PIP3 in *sodC*$^{-}$ cells may perturb spatio-temporal activation of

SCAR/WAVE necessary for the cup elongation. Aberrant regulation of the PH domain containing unconventional myosin proteins could negatively affect the cup elongation. Global RasG activation may hamper proper Formin activation at the base of the cup, and thus discourage cup elongation.

Another defect of *sodC*$^-$ cells was the slow depolymerization of actin coat after the phagosome closure (Figure 9C).

It has been suggested that the hydrolysis of PI(4,5)P2 is the critical regulatory point for actin disassembly from the phagosome. The level of PI(4,5)P2 on the phagosome may decrease through the action of PLC, PI3K, and 5'-Phophatases (Peracino et al., 2010). Loover and others (2007) showed that *Dictyostelium* cells lacking 5'-phosphatase OCRL-1 displayed high level of PI(4,5)P2 with delayed actin-coat disassembly and defective phagosome closure and Rupper and other (2001) also showed that cells treated with LY294002 or cells lacking PI3K1 and 2, or Akt exhibited similar phenotypes. *sodC*$^-$ cells display increased level of PIP3, but conspicuously normal PTEN binding which indicates normal presence of PI(4,5)P2 (Veeranki et al., 2008). It will be interestingly to determine if *sodC*$^-$ cells suffer from the persistent presence of PI(4,5)P2 or PIP3 on the phagosome.

5.4 Potential Roles of SodC in Cell-Substratum Adhesion

Figure 11A addressed adhesiveness of *sodC*$^-$ cells toward substratum with increasing shear force. When cells attached on the substratum was agitated with rotation of 100 rpm, 10 % of the wild type cells detached from the substratum, while 40% of *sodC*$^-$

cells were detached from the substratum. Currently what aspect of aberrancies of *sodC*$^-$ cells directly causes the decrease in the strength of cell-matrix adhesion, but there are two plausible scenarios. As a typical cell to matrix adhesion receptor, integrin family proteins are central in orchestrating signaling events that not only enhancing the strength of the adhesion, but also affecting vast range of cellular responses such as cell cycle and various actin-remodeling events. The small GTPase Rap and integrin beta subunit binding protein Talin are essential for mediating cellular signals that increase the strength of adhesion. *Dictyostelium sodC*$^-$ cells have aberrantly high level of PKBs activity (Castillo et al., 2017), and thus have aberrantly high level of PKB-mediated phosphorylation of several proteins that likely include Talin, Pak, RasGEF and RacGAP proteins. Considering that Talin plays a key role in inside-out signaling that induces activation of integrin adhesion receptors in diverse eukaryotic cells, aberrant PKB-mediated phosphorylation of Talin in *sodC*$^-$ cells may cause hyperphosphorylation of Talin and thus decreased adhesions. It will be significant to determine if Talin phosphorylation has a role in adhesion and phagocytosis. Another possibility is that excessive PKB activity may cause a feedback inhibition of RasC/TorC2 signaling axis (Charest et al., 2010). It was shown that Tor activity is essential for mammalian cell adhesion (Chen et al., 2015), but not for *Dictyostelium* cells (Rosel et al., 2012).

Previous studies showed that cells lacking RasG displayed significantly stronger adhesion to the substratum than wild type cells (Tuxworth et al., 1997). Another study,

however, showed that cells overexpressing constitutively active RasG(G12T) mutant induced stronger cell-substratum adhesion (Chen and Katz, 2000). The current study showed that active RasG is inhibitory to cell-substratum adhesion, which is consistent with the report of Tuxworth and others (1997). It will be significant to determine the levels of PKB-mediated Talin phosphorylation in cells lacking *RasG*, cells overexpressing RasG(G12T), and cells lacking *SodC*.

5.5 Future Direction

As the member of human innate immune system cells, macrophages have been playing an essential role in protecting human body by phagocytosis, which is characterized by internalizing substances such as cellular debris, microbes, and cancer cells. The dysfunction of macrophage can cause frequent infections and diseases like chronic granulomatous disease (CGD). The main reason for causing CGD is the failure of immune cells which including macrophages to produce reactive oxygen species such as superoxide via NADPH oxidase. However, excessive superoxides can also lead to a variety of side effects such as DNA damage, lipid peroxidation, oxidation of amino acids, and inactivation of specific enzymes (Brown and Griendling, 2009). Fortunately, the existence of superoxide dismutase in nearly all living cells exposed to oxygen allows cells to keep a proper level of superoxide and thus enable cells to utilize superoxide as a killing agent for invading pathogens. Previous study has reported that extracellular superoxide dismutase (EC-SOD) stimulates the phagocytosis and the killing of *Escherichia coli* in mice

macrophage considering that macrophages lacing EC-SOD showed significantly compromised phagocytosis and clearance of bacteria (Manni et al., 2011). However, how superoxide dismutase affects phagocytosis of macrophage and other phagocytic cells are not well understood. According to the results of early part of this dissertation and previous study, superoxide directly targets cysteine in NKCD motif of RasG and in turn induces aberrantly high level of active RasG (Castillo et al., 2017), which is the major factor driving the defective phagocytosis in *Dictyostelium sodC*$^{-}$ cells. Additionally, the aberrantly high level of RasG may well hamper the activity of its downstream target Formin G and thus hamper the phagocytic cup elongation (Evangelista, 2003). Furthermore, persistent high level of PIP3 may also perturb its' downstream effectors such as SCAR/WAVE, myosin, PKB and PKB substrates, and then causes defective F-Actin remodeling (Veltman et al., 2016, Chen and Iijima, 2012, Kamimura et al., 2009). Similarly, a previous study on the phagocytosis of macrophage suggested that Ras homolog gene family member A (RhoA) is activated after CR3 binds to ic3b-opsonized particle. The activation of RhoA then in turn would activate its downstream target mammalian formin mDia and affect the F-Actin dynamics (Colucci-Guyon et al., 2005). Another study indicated that RhoA is activated after the internalization of IgG-opsonized zymosan through phagocytosis by macrophage, which in turn induces the stable binding of RhoA-GTP to Rho guanosine nucleotide dissociation inhibitor (RhoGDI) and thus leads to the dissociation of inactive Rac1 from Rac1-RhoGDI complex. Rac1 is then activated by guanine nucleotide exchange factor

(Vav2) and then stimulates the activation of NADPH-oxidase and thus produce superoxide (Li et al., 2012). It is thus of interest to determine if superoxide dismutases are involved in the modulation of superoxide mediated effects such as bactericidal activity in the phagosome or may play a role in the upstream signaling events as suggested in this dissertation.

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VITA

CONG GU

Born, Nanjing, Jiangsu Province, China

2008-2012	Bachelor of Agronomy
	Yangzhou University
	Yangzhou, Jiangsu, P. R. China
2013-2018	Teaching Assistant
	Florida International University
	Miami, Florida, USA
2013-2018	Doctoral Candidate
	Florida International University
	Miami, Florida, USA

PUBLICATIONS AND PRESENTATIONS

Cong Gu and Lou Kim. 2018. Superoxide dismutase C modulates macropinocytosis and phagocytosis in *Dictyostelium discoideum*. (Under Revision)

Mujataba Sharief, Cong Gu, and Lou W. Kim. PP2A/B56 and LKB1 affect PKBs substrates phosphorylation and phagocytosis in *Dictyostelium*. (Under Revision)

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