3-19-2015

Redox Regulation of Ras Proteins in Dictyostelium discoideum

Boris Castillo Chabeco
Florida International University, bcast003@fiu.edu

DOI: 10.25148/etd.FI15032137

FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

REDOX REGULATION OF RAS PROTEINS IN *DICTYOSTELIUM DISCOIDEUM*

A dissertation submitted in partial fulfillment of

the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOLOGY

by

Boris Castillo Chabeco

2015

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

This dissertation, written by Boris Castillo Chabeco, and entitled Redox Regulation of Ras Proteins 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.

_____
Lidia Kos

_____
Ophelia Weeks

_____
Fernando Noriega

_____
Xiaotang Wang

_____
Lou W. Kim, Major Professor

Date of Defense: March 19, 2015

The dissertation of Boris Castillo Chabeco is approved.

_____
Dean Michael R. Heithaus
College of Arts and Sciences

_____
Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2015

DEDICATION

To my family and friends for their love and support.

ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Lou W. Kim, for his guidance during these years. My committee members, Dr. Lidia Kos, Dr. Xiaotang Wang and Dr. Ophelia Weeks and Dr Fernando Noriega have also been a great help with their questions and feedback. I would like to express my gratitude to my lab colleges MS Bohye Kim, Dr. Marbelys Rodriguez, MS Maria Pulido, Dr. Seon-Hee Hwang, Dr. Tong Sun, Adwait Kabra, Mujataba Sharief, Dr. Erasmo Perera, MS Beyte Barrios, and Dr. Amy Saldana for their valuable suggestions, discussions and help. I would like to thank my sources of funding: the MBRS/RISE Graduate program (NIH/NIGMS R25 Gm061347), the FIU teaching assistantship and the Graduate Professional Minority Students Fellowship.

ABSTRACT OF THE DISSERTATION

REDOX REGULATION OF RAS PROTEINS IN *DICTYOSTELIUM DISCOIDEUM*

by

Boris Castillo Chabeco

Florida International University, 2015

Miami, Florida

Professor Lou W. Kim, Major Professor

Reactive oxygen species are a normal consequence of life in an aerobic environment. However when they deviate from the narrow permissible range in cells, oxidative damage can occur. *Dictyostelium discoideum* is a model organism ideal for the study of cell signaling events such as those affected by oxidative stress. It was previously shown that Ras signaling in *Dictyostelium* is affected by genetic inactivation of the antioxidant enzyme Superoxide dismutase C (SodC) and *in vitro* data suggests that the NKCD motif of Ras is the redox target of superoxide.

The main objective of this project was to determine the mechanism of superoxide mediated Ras regulation *in vivo*. To accomplish the main objective, we cloned, and in some cases, mutated different Ras proteins and later determined their activity in wild type and *sodC*$^-$ cells. RasC and RasD showed normal activation in *sodC*$^-$ cells, however RasG and RasS displayed high Ras activity. These last two Ras proteins contain the NKC^{118}D motif inside the nucleotide binding region. A mutation of cysteine^{118} to alanine in RasG rendered the protein less active in *sodC*$^-$ than the wild type RasG protein and a mutation alanine^{118} to cysteine in RasD conferred redox sensitivity to this small GTPase. Additionally, the propensity of RasG to be targeted by superoxide was evident when the

environment of wild type cells was manipulated to induce the internal generation of superoxide through changes in the extracellular ion levels mainly magnesium. Lack of magnesium ions increased the intracellular level of superoxide and severely hampered directional cell migration. Chemotaxis of cells expressing RasG was negatively impacted by the absence of magnesium ions; however *rasG*$^-$ cells did not seem to be affected in their ability to perform chemotaxis. The last experiment implies that RasG is an important mediator of cell signaling during oxidative stress, responsible for preventing cells from continuing their developmental program. Our study suggests that the cysteine residue in the NKCD motif is essential for mediating the redox sensitivity of Ras proteins in *Dictyostelium* and that RasG is an essential mediator of the response to oxidative stress in this organism.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE PAGE

ABBREVIATIONS AND ACRONYMS

ABBREVIATION	FULL NAME
Ala	Alanine
JH10	*Dictyostelium* auxotrophic *wild type* strain
sodC$^-$	Ablation of Superoxide Discmutase C
BSA	Bovine serum albumin
cAMP	3'-5'-cyclic adenosine monophosphate
cDNA	Complementary DNA
CI	Chemotaxis index
DB	Developmental buffer
DNA	Deoxyribose nucleic acid
BES-So-AM	($C_{31}H_{19}F_4O_{13}NS$)
EDTA	Ethylenediaminetetraacetic acid
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GST	Glutathion S-tansferase
GTPase	Enzyme that hydrolyzes guanosine triphosphate
GTT	Glutathion
Hr	Hour
kD	Kilo Dalton
L	Liter

LB	Luria broth
mg	Milligram
min	Minute
ml	Milliliter
mm	Millimeter
mM	Millimolar
NADPH oxidase	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor-κB
NKCD	Asparagine, Lysine, Cysteine, Aspartic acid
nm	Nanometer
nM	Nanomolar
NOX	NADPH oxidase
OD	Optical density
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3K	Phosphatidylinositol-3-kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
Ras	Rat sarcoma
RBD	Ras binding domain
REMI	Restriction enzyme-mediated integration
rpm	Revolutions per minute
RNA	Ribonucleic acid
ROS	Reactive oxygen species

RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
s/sec	Second
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
µ	Micro-
Wt	*Wild type*
XTT	$C_{22}H_{16}N_7NaO_{13}S_2$

CHAPTER 1 INTRODUCTION

1.1 Effects of Reactive Oxygen Species and cellular defense systems

Cells living in the aerobic environments are well equipped with anti-oxidant systems not only to protect themselves from oxidative stress, but they also constitute a superimposable regulatory network that allows the use of some radical species as signal modulators (Kim et al., 2011). The use of oxygen allows cells to more efficiently produce energy compared to cells in anaerobic environments, but systems to process oxygen are often imperfect in their ability to control all the reactions that are necessary to sustain the life in this manner. That is the reason why Reactive Oxygen Species (ROS) are a normal consequence of cell functioning and fine tuning of the antioxidant machinery is vital for cell survival (Simon et al., 2000). Some enzymes and enzyme complexes produce oxygen radicals as their mayor activity or as a byproduct of it. Some examples are the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), the Xanthine oxidase and the electron transport chain (Guzik et al., 2002; Warnholtz et al., 1999). Too much or too little of these radical molecules can have the potential to change the fate of the cell (Sauer et al., 2001). If there is an overabundance, then oxidative stress ensues and macromolecules such as DNA and proteins can be affected. Aberrant depletion of ROS would also affect multiple cell behaviors (Bloomfield et al., 2003).

There are enzymatic and non-enzymatic antioxidant components at the cellular level. Among the non-enzymatic we can find for example some vitamins and glutathione (Brzezińska-Ślebodzińska et al., 1995). Some of the key anti-oxidant enzymes are

superoxide dismutase, catalase, peroxidases and reductases. These enzymes convert radical species to less reactive compounds that the cells can better manage. Antioxidant enzymes play a crucial role in cellular redox homeostasis, and their dysregualtion often times lead to the onset and development of human diseases (Marttila et al., 1988).

1.2 General effect of ROS on the regulation of small GTPase Ras

One of the proteins that are central in multiple pathological conditions and also closely related to the dysregulation of ROS is the small GTPase Ras (Lee et al., 1999; Svegliati et al., 2005). The GTPase Ras is conserved in all eukaryotes and works as a membrane bound functional switch involved in the earliest steps of multiple biochemical signaling pathways (De Luca et al., 2012). They are small monomeric GTPases involved in the regulation of very diverse cellular aspects such as motility, proliferation, differentiation and apoptosis (Campbell et al., 1998; Cox and Der, 2003). Because of their roles on these essential cell functions, Ras proteins are constantly under strict regulation. Like other members of the small GTPase proteins superfamily, Ras activity is determined by the binding of Guanosine-5'-triphosphate (GTP) or Guanosine diphosphate (GDP). Guanine nucleotide exchange factors (GEFs) have high affinity for the inactive form of their cognate Ras proteins and promote the exchange of GDP by GTP. In contrast, GTPase-Activating Proteins (GAPs) inactivate Ras proteins by increasing their normally low GTAase activity (Boguski and McCormick, 1993). Control of Ras function is so vital that even in the simple unicellular organism *Dictyostelium discoideum* there are around fourteen Ras proteins and interestingly over twenty RasGEFs (Wilkins et al., 2005) and nine GAPs.

Oxidative stress can affect Ras proteins through redox sensitive cysteine residues (Heo, 2011; Heo et al., 2006). If the affected residues are located in functional parts or domains, the result is going to be the modification of the activity towards a certain effector. One of these important regions is the NKxD motif, where x can be a few different amino acids. The NKxD motif is a part of the guanine nucleotide binding domain, which is one of the highly conserved regions in all Ras members (Heo, 201). Other well conserved regions of the of the guanine nucleotide binding domain are the Switch I (25–40) region which interacts with the nucleotide base, the ribose sugar, and the ɣ-phosphate. Also, the P-loop GXXXXGK(S/T) (10–17) and Switch II-DXXG (57–60) motifs interact with the different phosphate groups. In vitro studies demonstrated that when a Ras protein contains cysteine118 in the NKxD motif, redox sensitive modulation of the protein through cystein118 ensues changing protein activity (Heo et al., 2006). The basic molecular mechanism by which ROS lead to the dissociation of the guanine nucleotide was described previously and it is similar to the dissociation mechanism in response to nitric oxide (NO) (Heo et al., 2006). Briefly, when an NKCD containing Ras comes in contact with the ROS superoxide, the Cys118-SH which might be deprotonated even before the reaction with the radical, forms the thyl radical intermediate Ras-S118$^{\cdot}$. After this modification, the newly formed radical acquires an electron from the guanine nucleotide base leading to the generation of G$^{\cdot+}$-DP. Other residues such as the Phe28 side chain might also participate in the process by channeling the transfer of electrons. Elimination of a hydrogen from the N1 atom of the G$^{\cdot+}$-DP must lead to the G$^{\cdot}$DP form. The series of changes in the bound nucleotide and some of the residues holding it in place create a certain amount of steric hindrance that is going to ultimately lead to weakening

of the binding affinity between Ras and the nucleotide. The nucleotide can then further react with superoxide to form 5-oxo-GDP and other oxygenated derivatives that are subsequently released from the binding site (Heo and Campbell, 2005). The Ras protein is left in a state that it does not have enough affinity to bind GDP or GTP but the presence of some radical quenching agents abundant at the cellular level such as glutathione (GSH) would revive Ras to its nucleotide binding ability leading to activation because of the abundance of GTP compared to GDP in the cell.

1.3 Ras and Superoxide Dismutase C in Dictyostelium discoideum

In *Dictyostelium*, Superoxide dismutase C (SodC) heavily affects the basal activity of Ras (Veeranki et al., 2008). The SodC protein was originally isolated in an attempt to isolate upstream regulators of phosphatidylinositol(3,4,5)-trisphosphate using restriction enzyme mediated insertion (REMI) technology. The experiment in fact found a novel regulator of the pathway leading to the production of PIP3 but, upstream of that PIP3 production was Ras and the gene disrupted by REMI was *sodC* which translates into a glycosylphosphatidylinositol (GPI)-anchored membrane protein. The SodC protein contains a complete and an incomplete Cu/Zn superoxide dismutase (SOD) domains localizing to the plasma membrane and potentially other endomembrane systems. Dismutation of superoxide into hydrogen peroxide in the near vicinity of the cell membrane would protect other membrane proteins that might be sensitive to superoxide activity. *Dictyostelium* cells lacking SodC were later generated by homologous recombination, and follow up analysis confirmed that *sodC*$^-$ cells exhibit modest increase in intracellular superoxide level in addition to higher basal levels of active Ras and PIP3

(Veeranki et al., 2008). Furthermore, a decrease in the activity of Ras in the *sodC*$^-$ cells was rapidly obtained upon treating cells with the radical scavenger XTT, which indicated that the dysregualtion of Ras in *sodC*$^-$ cells is likely a result of superoxide mediated modification rather than differential expression of Ras regulatory partners. One of the misregulated Ras proteins in *sodC*$^-$ cells was RasG which participates in the control of chemotaxis in *Dictyostelium* cells (Veeranki et al., 2008). The RasG small GTPase is also one of the *Dictyostelium* Ras proteins that contain the NKCD motif. Misregulation of RasG suggested that SodC is essential for the regulation of Ras proteins that have the redox sensitive NKCD motif.

Having generated *Dictyostelium* cells lacking SodC provided us a unique position to genetically study the effects of ROS on Ras mediated events in eukaryotic cells. No other such genetic model system exists. In the present work we investigated how *Dictyostelium* Ras proteins with different NKxD motifs are regulated in *sodC*$^-$ cells in an attempt to unveil the molecular regulatory mechanism involved *in vivo*. We also determined how the presence of extracellular ions can influence Ras activity in *sodC*$^-$ cells. *Dictyostelium* is a well established and great model organism for the study of cell signaling. The *Dictyostelium* genome has been sequenced and many biochemical pathways found in this organism are conserved in higher eukaryotes. Also, it is a haploid organism with a fairly short 12 hours life cycle when grown in liquid media and thus genetic manipulations is highly feasible in this model. The Ras proteins in this protozoan are the key regulator of directional cell migration, localizing to the leading edge and controlling cytoskeleton

dynamics and other functions in response to extracellular signals such as cAMP (Sasaki et al., 2004).

1.4 Human disorders related to Ras activity

The fact that Ras proteins are so important for many different biochemical pathways at the cellular level makes them an important mediator of human disorders if they are somehow misregulated (Karapetis et al., 2008). The misregulation of Ras could result externally when an environmental agent aberrantly activates Ras upstream regulators or internally through mutations on Ras themselves or their regulators. One category of human conditions that is often associated with Ras proteins malfunction is cancer (Brose et al., 2002). The Ras proteins are vital regulators of cell proliferation which makes them responsible for initiation and progression of tumors. For example, the members of the pathway that include Ras, phosphatidylinositol-3-kinase (PI(3)K) and mTOR (mammalian target of rapamycin) often obtain gain-of-function mutations that lead to the unrestricted growth and multiplication of cells (Shaw et al., 2006). In the cases of germ line mutations of the Ras genes different conditions might result such as the Cardio-facio-cutaneous syndrome, Costello syndrome, and Autoimmune lymphoproliferative syndrome (Fernández-Medarde et al., 2011). Aberrant Ras signaling is also involved in cardiovascular disease although the mechanism is not well understood (Clerk et al., 2000). Other studies link Ras to Atherosclerosis since a number of molecules such as growth factors, peptides, and some ROS function as a mitogen by increasing Ras activity which in turn leads to vascular smooth muscle cell senescence

(Minamino et al., 2003). Research on Ras is still a priority in the attempt to understand and control a great number of human diseases.

1.5 Effects of magnesium deficiency at the cellular level

Cells experience oxidative stress not only when they gain genetic dysregulation of the anti-oxidant system genes such as *sodC*, but also from environmental fluctuation in the levels of ions such as magnesium or phosphate. It is no secret that metal ions are vital elements for cell functioning. Magnesium is one of the most abundant metals within the cell and its relative high concentration is well justified by the various cellular functions that it is involved in (Romani, 2006). Because of magnesium participation in multiple cellular processes, its regulation is also very important. One of the best known functions of magnesium is that it is used as a cofactor in a great number of cellular enzymatic reactions (Saris et al., 2000). But cells also use magnesium to regulate membrane potential and the equilibrium of ions across the cell membrane. This ion also binds to some negatively charged molecules or functional groups (Saris et al., 2000).

Magnesium deficiency is an important risk factor for many human conditions and the reason might be its importance as a redox modulator. There is evidence suggesting that magnesium deficiency may lead to oxidative stress. The lack of magnesium alters the transcriptome of certain type of cells to better equip them against radical species (Petrault et al., 2002) Examples of such genes include certain DNA repair enzymes and the ones involved in the oxidative stress response. According to another study the absence of magnesium decreases the activity of superoxide dismutase and catalase which in turn would lead to accumulation of ROS and it was observed to have accelerated cell growth

(Shivacumar et al., 1997). In plants, the effects of magnesium deficiency are clearly pronounced and lead to functional damage of the organism as a whole by inducing oxidative stress through the increase in the generation of ROS (Kumar Tewari et al., 2006). As clearly illustrated by these examples, the presence of magnesium is also essential for the cellular redox regulation.

Some of the experimental procedures that allowed us gather the data for this work were western blot, pull down assays, microscopy, chemotaxis, buffer treatments, and cloning among others. In this project we investigated changes in the activity and localization of the small GTPase Ras in cells that had the *sodC* gene ablated. The results indicate that Ras proteins that have the NKCD motif are sensitive to misregulation in *sodC*$^-$ cells of *Dictyostelium discoideum*. The study showed that the cysteine residue of the NKCD is the mechanistic element mediating the redox sensitivity of the small GTPase Ras in vivo. Also, ablation of the *sodC* gene or lack of magnesium ion in the cellular environment leads to the buildup of superoxide radicals and an enhancement on the activity level of RasG.

1.6 References

Bloomfield, G., & Pears, C. (2003). Superoxide signalling required for multicellular development of dictyostelium. *Journal of Cell Science, 116*(Pt 16), 3387-3397.

Boguski, M. S., & McCormick, F. (1993). Proteins regulating ras and its relatives. *Nature, 366*(6456), 643-654.

Brose, M. S., Volpe, P., Feldman, M., Kumar, M., Rishi, I., Gerrero, R., et al. (2002). BRAF and RAS mutations in human lung cancer and melanoma. *Cancer Research, 62*(23), 6997-7000.

Brzezińska-Ślebodzińska, E., Ślebodziński, A., Pietras, B., & Wieczorek, G. (1995). Antioxidant effect of vitamin E and glutathione on lipid peroxidation in boar semen plasma. *Biological Trace Element Research, 47*(1-3), 69-74.

Campbell, S. L., Khosravi-Far, R., Rossman, K. L., Clark, G. J., & Der, C. J. (1998). Increasing complexity of ras signaling. *Oncogene, 17*(11), 1395-1413.

Clerk, A., & Sugden, P. H. (2000). Small guanine nucleotide-binding proteins and myocardial hypertrophy. *Circulation Research, 86*(10), 1019-1023.

Cox, A. D., & Der, C. J. (2003). The dark side of ras: Regulation of apoptosis. *Oncogene, 22*(56), 8999-9006.

De Luca, A., Maiello, M. R., D'Alessio, A., Pergameno, M., & Normanno, N. (2012). The RAS/RAF/MEK/ERK and the PI3K/AKT signalling pathways: Role in cancer pathogenesis and implications for therapeutic approaches. *Expert Opinion on Therapeutic Targets, 16*(S2), S17-S27.

Fernández-Medarde, A., & Santos, E. (2011). Ras in cancer and developmental diseases. *Genes & Cancer, 2*(3), 344-358.

Guzik, T. J., Mussa, S., Gastaldi, D., Sadowski, J., Ratnatunga, C., Pillai, R., et al. (2002). Mechanisms of increased vascular superoxide production in human diabetes mellitus: Role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation, 105*(14), 1656-1662.

Heo, J. (2011). Redox control of GTPases: From molecular mechanisms to functional significance in health and disease. *Antioxidants & Redox Signaling, 14*(4), 689-724.

Heo, J., & Campbell, S. L. (2006). Ras regulation by reactive oxygen and nitrogen species. *Biochemistry, 45*(7), 2200-2210.

Karapetis, C. S., Khambata-Ford, S., Jonker, D. J., O'Callaghan, C. J., Tu, D., Tebbutt, N. C., et al. (2008). K-ras mutations and benefit from cetuximab in advanced colorectal cancer. *New England Journal of Medicine, 359*(17), 1757-1765.

Kim, L., & Castillo, B. (2011). Regulation of the small GTPase ras by reactive oxygen species. *Current Cellular Biochemistry, 1*(1), 15-19.

Kumar Tewari, R., Kumar, P., & Nand Sharma, P. (2006). Magnesium deficiency induced oxidative stress and antioxidant responses in mulberry plants. *Scientia Horticulturae, 108*(1), 7-14.

Lee, A. C., Fenster, B. E., Ito, H., Takeda, K., Bae, N. S., Hirai, T., et al. (1999). Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. *The Journal of Biological Chemistry, 274*(12), 7936-7940.

Marttila, R., Lorentz, H., & Rinne, U. (1988). Oxygen toxicity protecting enzymes in parkinson's disease: Increase of superoxide dismutase-like activity in the substantia nigra and basal nucleus. *Journal of the Neurological Sciences, 86*(2), 321-331.

Minamino, T., Yoshida, T., Tateno, K., Miyauchi, H., Zou, Y., Toko, H., et al. (2003). Ras induces vascular smooth muscle cell senescence and inflammation in human atherosclerosis. *Circulation, 108*(18), 2264-2269.

Petrault, I., Zimowska, W., Mathieu, J., Bayle, D., Rock, E., Favier, A., et al. (2002). Changes in gene expression in rat thymocytes identified by cDNA array support the occurrence of oxidative stress in early magnesium deficiency. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease, 1586*(1), 92-98.

Romani, A. (2007). Regulation of magnesium homeostasis and transport in mammalian cells. *Archives of Biochemistry and Biophysics, 458*(1), 90-102.

Saris, N. L., Mervaala, E., Karppanen, H., Khawaja, J. A., & Lewenstam, A. (2000). Magnesium: An update on physiological, clinical and analytical aspects. *Clinica Chimica Acta, 294*(1), 1-26.

Sasaki, A. T., Chun, C., Takeda, K., & Firtel, R. A. (2004). Localized ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement. *The Journal of Cell Biology, 167*(3), 505-518.

Sauer, H., Wartenberg, M., & Hescheler, J. (2001). Reactive oxygen species as intracellular messengers during cell growth and differentiation. *Cellular Physiology and Biochemistry : International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology, 11*(4), 173-186.

Shaw, R. J., & Cantley, L. C. (2006). Ras, PI (3) K and mTOR signalling controls tumour cell growth. *Nature, 441*(7092), 424-430.

Shivakumar, K., & Prakash Kumar, B. (1997). Magnesium deficiency enhances oxidative stress and collagen synthesis in vivo in the aorta of rats. *The International Journal of Biochemistry & Cell Biology, 29*(11), 1273-1278.

Simon, H., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis, 5*(5), 415-418.

Svegliati, S., Cancello, R., Sambo, P., Luchetti, M., Paroncini, P., Orlandini, G., et al. (2005). Platelet-derived growth factor and reactive oxygen species (ROS) regulate ras protein levels in primary human fibroblasts via ERK1/2. amplification of ROS and ras in systemic sclerosis fibroblasts. *The Journal of Biological Chemistry, 280*(43), 36474-36482.

Veeranki, S., Kim, B., & Kim, L. (2008). The GPI-anchored superoxide dismutase SodC is essential for regulating basal ras activity and for chemotaxis of dictyostelium discoideum. *Journal of Cell Science, 121*(Pt 18), 3099-3108.

Warnholtz, A., Nickenig, G., Schulz, E., Macharzina, R., Brasen, J. H., Skatchkov, M., et al. (1999). Increased NADH-oxidase-mediated superoxide production in the early stages of atherosclerosis: Evidence for involvement of the renin-angiotensin system. *Circulation, 99*(15), 2027-2033.

Wilkins, A., Szafranski, K., Fraser, D. J., Bakthavatsalam, D., Müller, R., Fisher, P. R., et al. (2005). The dictyostelium genome encodes numerous RasGEFs with multiple biological roles. *Genome Biology, 6*(8), R68.

CHAPTER 2 THE NKCD MOTIF IS THE TARGET OF REDOX REGULATION IN *SODC⁻ DICTYOSTELIUM DISCOIDEUM* CELLS

2.1 Introduction

Almost all eukaryotic organisms contain multiple Ras genes and the number of Ras genes generally increases as the organism becomes more complex. Considering that each Ras species exhibits both unique and redundant functions, understanding the role of Ras proteins in disease is often a challenge. For example, human genome contains 36 *ras* family gene members (Wennerberg et al., 2005), which include several proto-oncogenes. For this and other reasons, it is obviously a complicated and difficult process to determine the molecular mechanism of the Ras promoted tumorigenesis. Furthermore, some Ras genes are expressed in a tissue specific or developmental stage specific manner (Segal et al., 1986; Spiegelman et al., 2006). In contrast, there are only 14 of these small GTPases (around 20 kD) in *Dictyostelium discoideum*, which allows to more easily dissect Ras mediated cellular dysregulation mechanisms. Also, multiple Ras effectors such as PI3K are conserved in *Disctyostelium* (Sasaki et al., 2007). The degree of similarity of Ras proteins with higher eukaryotes is considerable: *Disctyostelium* RasG and RasD have ~65% identity with human H-Ras in amino acid sequence (Reymond et al., 1984; Robbins et al., 1989).

The expression of each *Dictyostelium* Ras gene is highly dependent on the life stage of the cell (Robbins et al., 1989). Under nutrient rich condition, *Dictyostelium* thrives as a haploid unicellular protozoan that lives in the soil feeding on bacteria that they locate using chemotaxis. Upon starvation, *Dictyostelium* cells initiate a

developmental program that includes chemotaxis toward cAMP (Kimmel et al., 1991). Up to one hundred thousand cells aggregate together to form a multicellular aggregate, which will further differentiate into two main cells types, the front 20% of cells will become prestalk cells and the posterior 80% of cells will become prespore cells (Wong et al., 2002). The mature spores will emerge from the prespore cell population and give rise to the next generation of cells when the environment becomes favorable (Fig 1).

Several *Dictyostelium* Ras proteins have been investigated in great detail. RasG, for example, is expressed during cell growth and is activated by chemoattractant cAMP during the start of aggregation, but its expression is reduced during aggregation itself since it is less critical for the differentiation process (Khosla et al., 1996). It is critical for cAMP chemotaxis, gene expression, cytoskeletal rearrangement, cytokinesis (when cells are grown in suspension), cell adhesion, macropinocytosis, and random motility (Tuxworth et al 1997; Lim et al., 2002; Zhang et al 1999). RasG activity is mainly regulated by RasGefR and DdNF1. RasD is highly homologous to RasG, 82 percent similarity over the full length is observed, and they have identical switch I and II effector domains (Daniel et al., 1995). The RasD protein is expressed during multicellular development and is associated with stalk cell precursors. Its main functions during development are phototaxis and thermotaxis of the multicellular structures (Wilkins et al., 2000). In vegetative cells, *rasD* expression level is maintained low by RasG (Khosla et al., 2000) and higher levels of RasD were observed in cells lacking RasG or RasG and RasC. It has been proposed that RasD exhibits some functional redundancies with RasG, such as cytokinesis and growth in suspension (Bolourani et al., 2010). RasGefE is known

to mediate RasD activation (Wilkins et al., 2005). The RasC protein is mainly expressed during cell growth and is regulated by RasGefA. It was shown that RasC is critical for chemotaxis toward a cAMP gradient and also for activating adenylyl cyclase (ACA) (Kae et al., 2007). It was also demonstrated that RasC is essential for proper TorC2 activation in response to cAMP and thus chemotaxis (Cai et al., 2010). The RasS protein is expressed during the growth stage and multicellular development, and activated possibly by RasGefB (Wilkins et al., 2000). It is involved in the processes like phagocytosis, fluid-phase endocytosis, cell growth and polarization (Lim et al., 2002). It is also a part of PI3Ks activation machinery in parallel with RasG. The control of the Ras proteins is achieved by GEFs and GAPs, but *Dictyostelium* cells lacking Superoxide dismutase C (*sodC*$^-$) display constitutive activation of Ras proteins (Veeranki et al., 2008). Also, *sodC*$^-$ cells exhibited constitutive activation of RasG, which has the redox sensitive cysteine 118 at the NKxD motif.

Previous *in vitro* study demonstrated that the NKCD motif is the target of superoxide mediated Ras activation, and thus the NKCD motif containing RasG is a likely target of superoxide in *sodC*$^-$ cells, in which modest increase of superoxide was observed (Veeranki et al., 2008). However, another interesting study showed that when the superoxide levels were decreased either by overexpressing a cytosolic superoxide dismutase or by a pharmacological superoxide scavenger, cells were incapable of exhibiting directional cell migration (Bloomfield et al., 2003). These studies suggest that cells require a defined range of superoxide levels for optimal chemotaxis. Cells lacking SodC display a modest increase in the intracellular superoxide level without severely

damaging cells, but significant enough to dysregulate intracellular signaling events such as Ras/PI3K (Veeranki et al., 2008).

In general, reactive oxygen species are considered to act often as signaling messengers in different organisms including humans (Toren Finkel., 1998). Oncogenic Ras proteins (H-Ras^{G12V} or N-Ras^{G12D}) increased the production of ROS by activating NOX, which in turn stimulated cellular proliferation, as demonstrated using human CD34^{+} hematopoietic progenitor cells (Hole et al., 2010). However, another study using Leydig cells uncovered that ROS activated Ras in response to cAMP stimulation (Tai et al., 2011). Thus, further investigation will be appreciated to determine when and where these scenarios would be applicable as well as the mechanisms behind the events.

The Ras family of small GTPases in *Dictyostelium* has a high degree of heterogeneity concerning the composition of the conserved NKXD motif. Given that *in vitro* studies suggested that the presence of cysteine 118 (NKC^{118}D) confers redox sensitivity to Ras (Heo, 2011), the sequence of each *Dictyostelium* Ras species was examined. Five of the proteins have the NKCD motif (RasS, RasG, RapC, RasB and RapA), two have NKID (RasW and RapB), two of the best known (RasC, and RasD) have NKAD, and the NKSD is present in another six (RasU, RasV, RasX, RasY, RasZ, and Rheb). We focused in some of the NKCD and NKAD containing Ras, namely RasG, RasD, RasC, and RasS to evaluate *in vivo* function of cysteine 118 in Ras signaling in normal and cells under oxidative stress.

The current study determined that superoxide anions can in fact alter Ras proteins activity *in vivo* if they possess the NKCD motif. Interestingly, not all NKCD containing

Ras proteins are affected in an identical manner under oxidative stress. Finally, we also uncovered that other conventional Ras regualtors such as RasGef and RasGAP proteins might also be a target of superoxide ions.

2.2 Materials and methods

2.2.1 Cell culture

The different strains were grown in D3T media (15.30 g Peptone #3, 7.15 g of Yeast extract, 15.4 g Glucose, 0.48 g KH_2PO_4, 0.525 g $Na_2PO_4.7H_2O$, H_2O to complete 1L). All strains were supplemented with 0.5 mg/ml thymidine (since all my cells and mutants were derived from the *JH10 wild type* strain). Additionally, 5 ng/µl blasticidin were added to all transformants in the *SodC*$^-$ background, and G418 20 µg/µl to all Ras overexpressors.

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

2.2.2 Molecular Cloning

The generation of the Ras constructs was done as described previously (Veeranki et al., 2008). Briefly, the genomic DNA sequence for *rasG*, *rasD*, *rasS* and *rasC* was obtained from Dictybase.org and the full sequence was generated by RT-PCR using *JH10* cells' RNA. Some of the DNA constructs were already in existence in the lab and others were created during this project. The primer sequences are as follows: forward primer 5'-

ATGTTTAATTTTAAATTAGTATTAGTTGG -3' and reverse5'-TTATAATAAATTACAAGATTTCTTTTTTTTAATTG-3' for rasS.

The obtained gene sequences were then cloned into the PCRII vector following the Topo TA Cloning kit (invitrogen) manufacturer's instructions. Afterwards the construct was transformed into NEB 5-alpha competent cells (cat# C2987H) for Midi-prep after about 16 hr of growth at 37 degrees C using the Quiagen Kit (cat# 12143). At this point, we would confirm the integrity of the gene by sequencing at the Sequencing Core at FIU.

Later the gene sequence was digested out of the PCRII vector using the EcoR1 enzyme (NEB), gel purified using the QIAquick gel extraction kit (cat# 28704), and ligated into the EcoR1 site of the Exp4 vector (*Dictyostelium* expression vector). The construct was transformed into *E. Coli* and DNA was obtained by Midi-prep using the Quiagen Kit. The DNA was quantified using the spectrophotometer (GENESYS 10uv) and stored as a dried pellet at -20 degrees for transfection later into *Dictyostelium* cells.

2.2.3 Site-directed mutagenesis

Site Directed mutagenesis was performed using the QuikChange Lightning Site-Directed Mutagenesis Kit from Agilent Technologies following the manufacturer's instructions. The forward primer for rasD was 5'-GATTTTGGTTGGTAATAAATGTGATTTGGATCATGAACGTCAAG-3' and the reverse primer 5'-CTTGACGTTCATGATCCAAATCACATTTATTACCAACCAAAATC-3', for RasS the forward was 5'-

CGTATTGGTAGGAAATAAAGCAGATCTCAATGAATATCGTG-3' and the reverse was 5'-CACGATATTCATTGAGATCTGCTTTATTTCCTACCAATACG-3'. The construct for RasG was already in existence. The MJ Research PTC-200 Peltier Thermal Cycler was used for this and all PCR reactions. After amplification of the mutated gene sequences, they were ligated into the PCRII Topo vector and the process continued as described in the "Molecular cloning" section.

2.2.4 Bacterial cell culture

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

2.2.5 Transfection

Five million of *Dictyostelium* exponentially growing cells were centrifuged for 5 min at 2000 rpm (4° C), washed once with 10 ml H-50 and resuspended in 100 µl of H-50. The cells were mixed with 10 µg of desired DNA, and placed in a cold 0.1 cm gap Fisher electroporation cuvette (cat# FB101). Electroporation was done at 1 kV two times with a pause of 5 sec between pulses. Cells were placed in ice for 5 minutes and then transferred into a 10 cm Petri dish with 10 ml of D-3T media, supplemented with 0.5

mg/ml thymidine. After 24 h, G418 was added as a means of selection. This method was modified from Schauer et al., (1993).

2.2.6 Cell pulsing

One hundred million of logarithmic growth-phase cells cultured in a D3T medium suspension were used for each independent pulsing experiment at a density of 20 million cells per ml of Developmental Buffer (DB medium: 2mM $MgCl_2$, 0.2 mM $CaCl_2$, 7.4 mM $NaH_2PO_4 \cdot H_2O$, 4 mM $Na_2HPO_4 \cdot 7H_2O$, pH~ 6.5). The cells were starved for one hour and then pulsed every 6 minutes with 50 nM cAMP during 4 more hours in a shaker at 150 rpm.

2.2.7 RBD binding assay

The GST-Byr2-Ras Binding Domain (RBD) and GST-Raf1-RBD recombinant proteins were expressed in *E. coli* and purified as described elsewhere (Kae et al., 2004). The RBD binding assay was has been used previously to identify active Ras in *Dictyostelium discoideum* (Sasaki et al., 2004; Veeranki et al., 2008). Basically, each sample's protein lysates were incubated with 5 µg of purified recombinant GST-RBD bound to GTT-sepharose beads (GE healthcare) and the mixture was shaken at 4° C for 90 min. Beads with the proteins attached were centrifuged and washed three times in 1x TTG plus 10 mM $MgSO_4$. The pelleted beads were mixed with LDS (lithium dodecyl sulfate) 4x sample loading buffer and the mixture was boiled for 3 min. Then the procedure continues as described in the section "Western Blot".

2.2.8 Western blot

Centrifugation was utilized to harvest the *Dictyostelium* cells and 1 ml per 25 million cells of complete TTG (1% Triton, 5% glycerol, 0.15 M NaCl, 20 mM Tris.HCl pH 7.7, 1mM EDTA, 1 mM sodium orthovanadate, 40 mM ammonium molybdate, 1% BME, 1 tablet of protease inhibitor/10 ml TTG) was used for lysis. Protein lysates were mixed with LDS sample loading buffer 4x (Invitrogen) and boiled at 100° C for 3 minutes.

An SDS–polyacrylamide gel electrophoresis (Sambrook et al., 1989) was utilized to fractionate and normalize the proteins lysates. Proteins were transferred onto nitrocellulose membranes (Millipore) once the electrophoresis was done, blocked with a 2% BSA (Amresco) for 30 min and probed overnight at 4 degrees C or 2 hours at room temperature using a suitable antibody. The bound antibody was detected by means of enhanced chemiluminescence (ECL, Cell Signaling Technology).

2.2.9 Antibodies

The primary antibodies that were used are the monoclonal anti-flag produced in mice (Sigma), and monoclonal anti-Pan Ras from Calbiochem (Ab-3). The dilution used for both antibodies was 1 : 1000. For secondary I used the Goat anti-mouse IgG-(H+L)-HRP from Southern Biotech diluted 1 : 5000.

2.2.10 Chemotaxis assay

Cells were starved for 1 hour and then pulsed during 4 hours with 50 nM of cAMP every 6 minutes in a shaker at 150 rpm. After this, cells were placed in a 35 mm tissue culture dish cover (Falcon 353001) containing 2 ml of buffer at a density of 2.5

x10^4cells/cm^2. A Schmazu micromanipulator fitted with a glass capillary needle (Eppendorf Femtotip) containing 10 μM cAMP solution was utilized for the chemotaxis experiments. Pictures were taken using the OpenLab software every 60 sec for a total of 20 minutes. Images were later superimposed to determine the cells trajectory. Speed and chemotaxis index (CI) were determined from the measurements obtained using the ImageJ software. At least 3 independent experiments were performed to generate the data used for statistical calculations. A previously published method for evaluation of *Dictyostelium* chemotaxis was used as a reference (Loovers et al., 2006).

2.2.11 Data Processing

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

2.3 Results

2.3.1 The NKCD containing RasG activity is aberrantly high in *sodC*$^-$ cells

One of the best known Ras proteins of *Dictyostelium* is RasG, which shows such high degree of homology with human K-, N-, and H-Ras that some of the key functional amino acid residues can even be found at the same position. This small GTPase is involved in a variety of cell functions such as motility, chemotaxis, and gene expression among others (Bolourani et al., 2010; Chattwood et al., 2014). This is also one of the

proteins that contain the NKCD motif that has been described as a redox target *in vitro* (Heo et al., 2006). To determine the basal as well as post-stimulatory activities of each Ras protein, we generated cDNAs encoding each Ras protein by RT-PCR. Each Ras cDNA was subcloned into *Dictyostelium* expression vector with a Flag tag, then purified and OD was measured. About 10 µg were used for transfection. *Dictyostelium wild type* (*JH10)* and *sodC*$^-$ cells expressing flag-Ras gens were isolated by G418 selection and the relative expression levels of flag-Ras genes were quantitated by western blot analysis.

Cells expressing each flag-Ras gene were grown to mid log phase (1 to 3 million cells/ml). Later they were induced to the aggregation competent stage by pulsatile cAMP stimulation for 4 hours, which simulate and expedite early aggregation stage and natural *Dictyostelium* development. Whole cell lysates were prepared using cell lysis buffer (TTG) as described in the methods section. Flag-RasG activities in *wild type* and *sodC*$^-$ cells were compared using GST-human Raf1-Ras Binding Domain (GST-RBD) assay. The input level of flag-Ras in *wild type* and *sodC*$^-$ cells was normalized by western blot of whole cell lysates using anti-flag antibody. The basal flag-RasG activity was twofold higher in *sodC*$^-$ cells than in *wild type* background as shown in figure 2.

The quantitation of the active flag-RasG band intensity was obtained from at least 3 independent experiments (Fig. 2 B). Active RasG level doubled in *sodC*$^-$ cells compared to that of *wild type*, which is largely consistent with the previous report (Veeranki et al., 2008).

2.3.2 The basal RasS activity in *sodC*$^-$ cells was increased modestly but consistently compared to that in *wild type* cells.

RasS is another of the NKCD containing Ras proteins and is involved in multiple cell functions such as cytoskeleton organization, cell growth and phagocytosis (Chubb et al., 2000). The RasS protein is unique in that it contains additional redox sensitive motif in addition to the NKCD motif. Following the phosphate binding Glycine rich motif, a redox sensitive serine residue was proposed by *in vitro* study (Heo et al., 2006). This Glycine rich region is called P-loop and it negatively affects Ras activity in response to superoxide. It is, however, unclear how superoxide affects a small GTPase when these two potentially antagonistic redox sensitive motifs coexist in a single molecule. To this end, the RasS cDNA was isolated, cloned and expressed to produce a flag-tagged protein in *wild type* and *sodC*$^-$ cells, and the basal flag-RasS activities were determined from each of the two different cell backgrounds. The basal RasS activity in *sodC*$^-$ cells was ~25% higher than that in *wild type* cells P–value = 0.0299 (Fig. 3 A). The western blot images and quantitation of band intensities were from at least three independent experiments (Fig. 3A, 3B).

2.3.3 No significant differences of basal activity of RasD and RasC in *wild type* or *sodC*$^-$ cells

The RasC and RasD proteins are the other two well characterized Ras proteins in *Dictyostelium*. RasC, for example, affects cell motility, chemotaxis, and gene expression (Bolourani et al., 2010). RasD, on the other hand, modulates phototaxis and thermotaxis (Wilkins et al., 2000). Its expression is maximal during development and introduction of

a constitutively active mutant RasD induced biased cell differentiation toward prestalk cells. These two Ras proteins contain neither NKCD motif nor the P-loop cysteine, and thus are expected to be redox insensitive. The cDNAs encoding RasC and RasD were generated by RT-PCR and subcloned into *Dictyostelium* expression vector (pEXp4(+)) with a flag tag at their amino terminus. The whole cell lysates of cells expressing either flag-RasC or flag-RasD was prepared and the basal level of active flag-RasC and flag-RasD was determined by GST-RBD assay as described above for flag-RasG. Considering that active RasC associates better with Byr2-RBD, the basal RasC activity was determined with GST-Byr2-RBD, whereas RasD was with GST-Raf1-RBD. Neither flag-RasD nor flag-RasC exhibited significant difference in the basal Ras activity in *wild type* or *sodC*$^-$ cells background, strongly suggesting that RasC and RasD proteins are redox insensitive *in vivo* as expected from *in vitro* studies (Fig. 4 A). Three independent experiments were performed and the average intensities of active Ras proteins were quantitated (Fig. 4 B).

2.3.4 Ras is independently regulated by GPCRs and redox mechanisms

The Ras proteins in general are maximally activated at 5 seconds after cAMP stimulation and then the level of activity decreases as time passes. RasG is one of the redox sensitive small GTPases in *Dictyostelium* according to the presence of the NKCD motif. RasG was expressed with a Flag tag in *wild type* and *sodC*$^-$ cells. After 4 hours of pulsing, cells were challenged with 10 μM cAMP and lysed at different time points. As expected, flag tagged RasG and the endogenous Ras proteins showed a peak in activity at 5 sec after stimulation then the activity was reduced to the pre-stimulus level in *wild type*

cells. On the other hand, *sodC*$^-$ cells showed a high basal level of activity that stayed high after cAMP stimulation (Fig. 5 A). The activity level of flag-RasG is much higher at every time point when compared to the *wild type* background, however there is a discrete increase in activity at 5 seconds and a slight decrease afterwards following cAMP stimulation. This decrease after 5 seconds suggests that the cAMP derived RasG adaption is still functioning to some extent in *sodC*$^-$ cells. (Fig. 5 A).

The RasD protein, which is not redox sensitive, was also analyzed by GST-RBD assay to determine if the pattern of activation in response to 10 uM cAMP was affected in the presence of ROS. The lysates obtained at different time points after pulsing and stimulation show that flag-RasD activity was indistinguishable between the two cellular backgrounds evaluated. Both cell types (Fig. 5 B), exhibit a distinctive peak of activity 5 seconds after stimulation and a clear significant decrease in the subsequent time points. It seems that the biochemical regulatory pathway for RasD which includes cAMP receptors, heterotrimeric G proteins, RasGEF and RasGAP proteins, is still perfectly working in the presence of superoxide.

2.3.5 RasD with a cysteine to alanine substitution mutation on the NKxD motif displayed significantly increased basal activity in *sodC*$^-$ cells.

To investigate further the *in vivo* role of cysteine 118 under oxidative stress, substitution mutantions of RasD, RasS and RasG were performed. The flag-RasD A118C protein was expressed in *wild type* and *sodC*$^-$ cells. The basal Ras activity of the flag-RasD(A118C) mutant was determined as before by using GST-RBD assay upon normalization of total flag-RasD(A118C) in whole cell lysates used for each cell type.

The basal Ras activity of flag-RasD(C118A) was twofold higher in *sodC*$^-$ cells compared to that in *wild type* cells (Fig. 6 A). This increase in activity strongly argues that the cysteine in the NKCD motif renders Ras proteins redox sensitive under the oxidative stress condition produced by the ablation of *sodC*. The experiment was repeated three times and statistical analysis showed significant differences between the two backgrounds (p-value ≤ 0.05) (Fig. 6 B).

2.3.6 RasG(C118A) *substitution mutant* displayed significantly decreased basal activity compared to the *wild type* flag-RasD in *sodC*$^-$ cells, but its activity was still higher in *sodC*$^-$ cells than in *wild type* cells

The result obtained using flag-RasG was consistent with our previous results using GFP-RasG (Veeranki et al., 2008). To further enlighten the molecular mechanism of superoxide Ras protein activation, flag-RasG(C118A) substitution mutagenesis was performed. Briefly, the cDNA was obtained and engineered with a flag tag before being subcloned into the *Dictyostelium* expression vector (pEXP4(+)) and introduced into *wild type* and *sodC*$^-$ cells. These cells were induced to be aggregation competent by stimulation with pulsatile cAMP for 4 hours and the basal activity of flag-RasG(C118A) was determined as described before using GST-RBD. As shown in figure 7 A, the basal activity of flag-RasG(C118A) was 50% higher in *sodC*$^-$ cells compared to that of *wild type* cells (Fig. 7 A, 7 B). Considering that the cysteine 118 is the main agent conferring redox sensitivity to RasG, we reasoned that there are likely other redox sensitive components in Ras regulatory circuit in *sodC*$^-$ cells. To that end, the basal activities of *wild type* flag-RasG and flag-RasG(C118) in *sodC*$^-$ cells were compared. The results

showed a clear and significant decrease in RasG activity for the mutated RasG(C118A) (Fig. 7 C and D).

2.3.7 flag-RasS(C118A) with a cysteine to alanine mutation on the NKCD motif shows a significant level of misregulation in *sodC*$^-$ cells.

As mentioned previously, RasS exhibited only modest increase in its basal activity in *sodC*$^-$ cells. To further investigate the role of the cysteine 118, flag-RasS(C118A) substitution mutant was generated. *Wild type* and the *sodC*$^-$ cells expressing flag-RasS(C118A) substitution mutant were prepared and the basal RasS(C118A) activities were determined using GST-Raf1-RBD. The basal RasS(C118A) activities in *wild type* versus *sodC*$^-$ cells displayed a modest but significant difference (Fig. 8 A, 8 B). Contrary to the flag-RasG(C118A) protein, flag-RasS(C118A) did not show higher activity in *sodC*$^-$ cells than in *wild type* cells. It is thus likely that RasG specific RasGef or RasGAP proteins are affected, but not RasS specific regulators are thought to be redox sensitive in *sodC*$^-$ cells. Alternatively, the presence of P-loop in RasS may have exerted a negatively effect on the basal activity of flag-RasS(C118A) and thus a slight decrease in basal activity was observed.

2.3.8 Mutation C118A in RasG partially restores chemotaxis in *sodC*$^-$ cells

It has previously been published that *sodC*$^-$ cells are severely defective in chemotaxis (Veeranki et al., 2008). We decided to evaluate the Chemotaxis index which is a measure of how directional is the movement of the cells and the Speed in *sodC*$^-$ cells expressing either RasG (Fig 9 A) or RasG(C118A) (Fig 9 B). The expression of RasG(C118A) partially rescues the chemotaxis defect of this cells since it helps the cells

to better polarize in the presence of a source of the chemoattractant cAMP (Fig 9 A, 9 B). The chemotaxis index improves from 0.03 cells expressing the *wild type* RasG to 0.74 in RasG(C118A) which makes them almost as directional al *wild type* cells that perform around 0.8 on this parameter (Fig 9 D). Regarding speed, the increase was not as important but it is very significant however since the cells went from 1.01 um per minute in cells expressing RasG to 3.73 um per minute in RasG(C118A) (Fig 9 C).

2.4 Figures

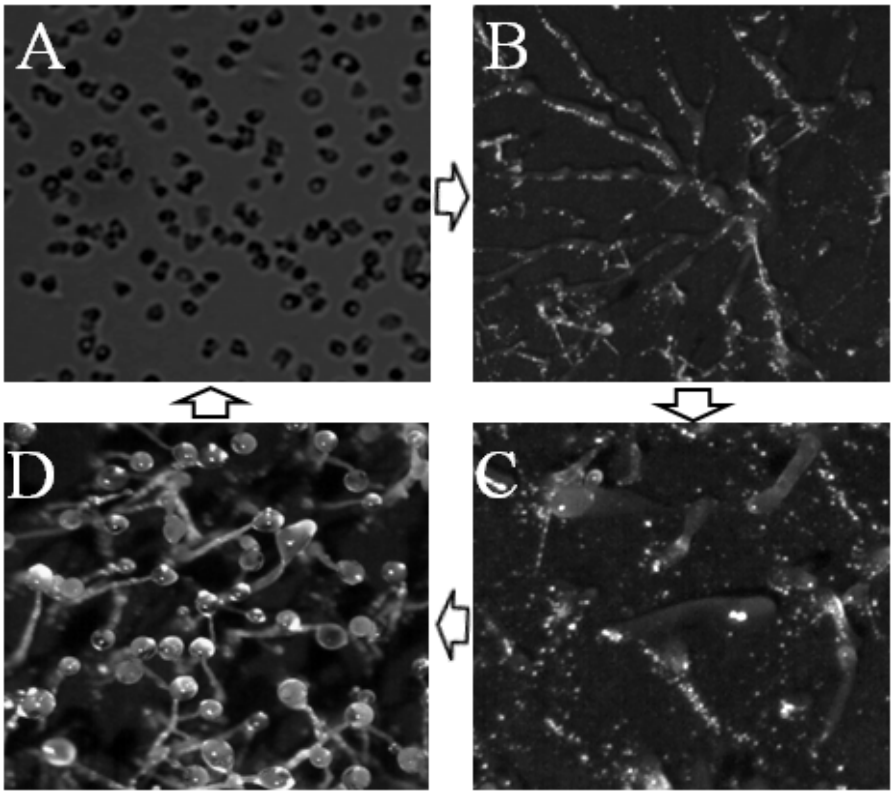


Figure 1. *Dictyostelium discoideum* life cycle.

Under favorable conditions the organism is unicellular (A). Under starvation they aggregate in response to cAMP (B). Then, they form multicellular motile structures (C).

Finally, fruiting bodies emerge that harbor the spores that will give rise to the next generation of cells (D).

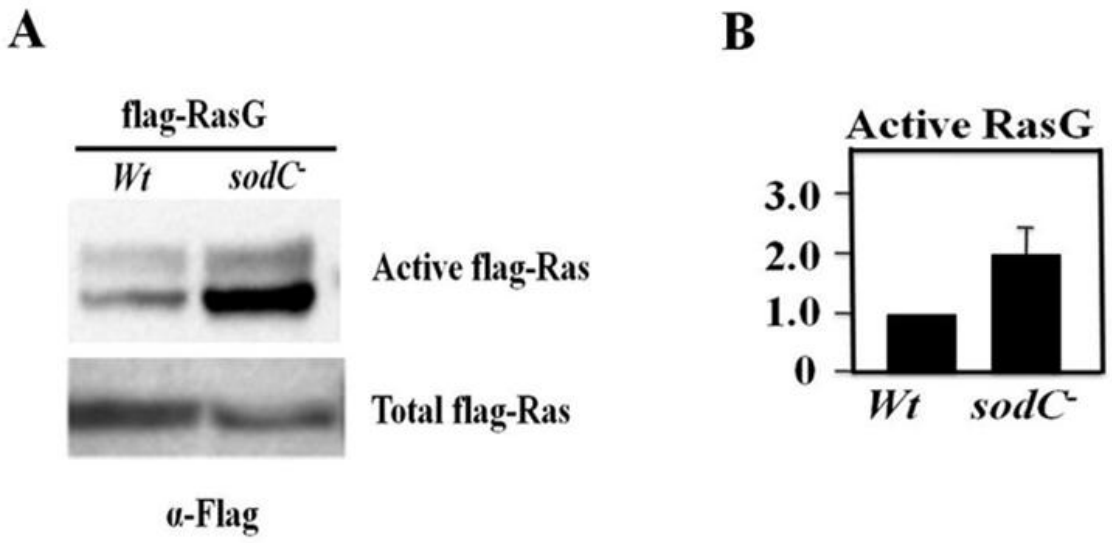


Figure 2. RasG activity in the presence of superoxide.

One hundred million cells overexpressing the NKCD containing flag-RasG in *wild type* or *sodC*$^-$ backgrounds were grown in D3T media shaking culture and then pulsed for 4 hours with 50 nM cAMP in DB media at a density of 20 million cells per ml. The cells were then lysed using complete TTG and the lysates were used for pull down by means of the human Raf1-RBD. Western blot was performed to normalize the samples (bottom) and then for pulled down proteins (top) using Pan-Ras antibody (A). The images obtained from three western blot experiments were scanned and band density was determined using the UN-Scan-It Gel software. The data obtain were used for statistical analysis (B). A graph showing the average ratio of band density and the standard deviation from three

different experiments is shown here. (B) RasG shows a significant two fold increase in activity in the *sodC*$^-$ background when compared to the *wild type* (p-value ≤ 0.05).

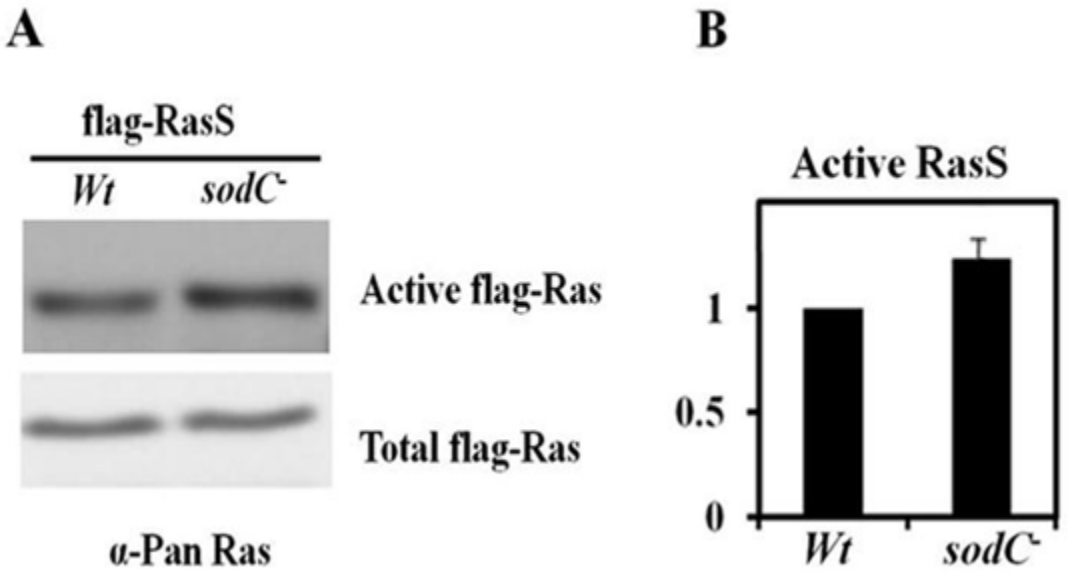


Figure 3. RasS activity comparison between *wild type* and *sodC*$^-$ cells.

Aggregation competent cells expressing flag-RasS were prepared as described in the method section and their basal RasS activities were measured. Western blot was performed to normalize the samples and then for pulled down proteins assay using Pan-Ras antibody (A). The images obtained from three western blot experiments were scanned and the active flag-RasS band intensities were quantitated using the UN-Scan-It Gel software. The data obtain were used for statistical analysis (B). A graph showing the average ratio of band density and the standard deviation from three different experiments is shown here. RasS shows a ~25% increase with standard deviation of 8% and P–value = 0.0299.

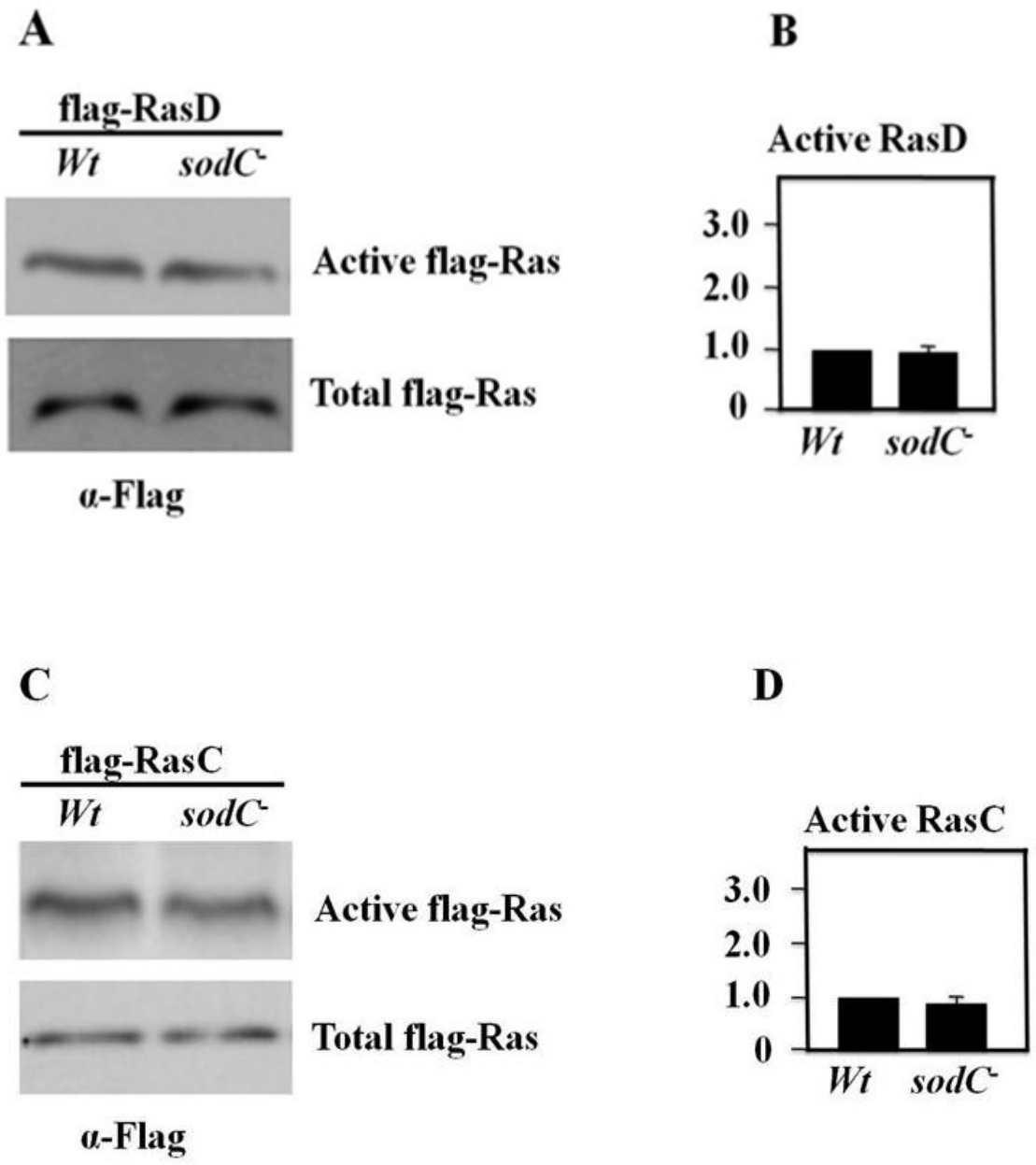


Figure 4. RasD and RasC activities in *wild type* versus *sodC*$^-$ cells.

RasD and RasC have Ala instead of Cys in the third position of the NKXD motif**.** Western blot was performed using lysates from cells grown in suspension then pulsed with cAMP and lysed afterwards. Normalization (bottom) and RBD binding images (top)

are shown on the left panel (A). Images obtained from western blot were processed using the USCAN-IT-GEL software to determine the density of the bands to be used for statistical analysis (B). This graph shows the average band density results obtained from three independent experiments using one hundred million cells each. Although RasC seems to have slightly less active in *sodC*$^{-}$ cells than that in *wild type* cells, the difference was statically insignificant. The basal RasD activities were also comparable between *wild type* and *sodC*$^{-}$ cells.

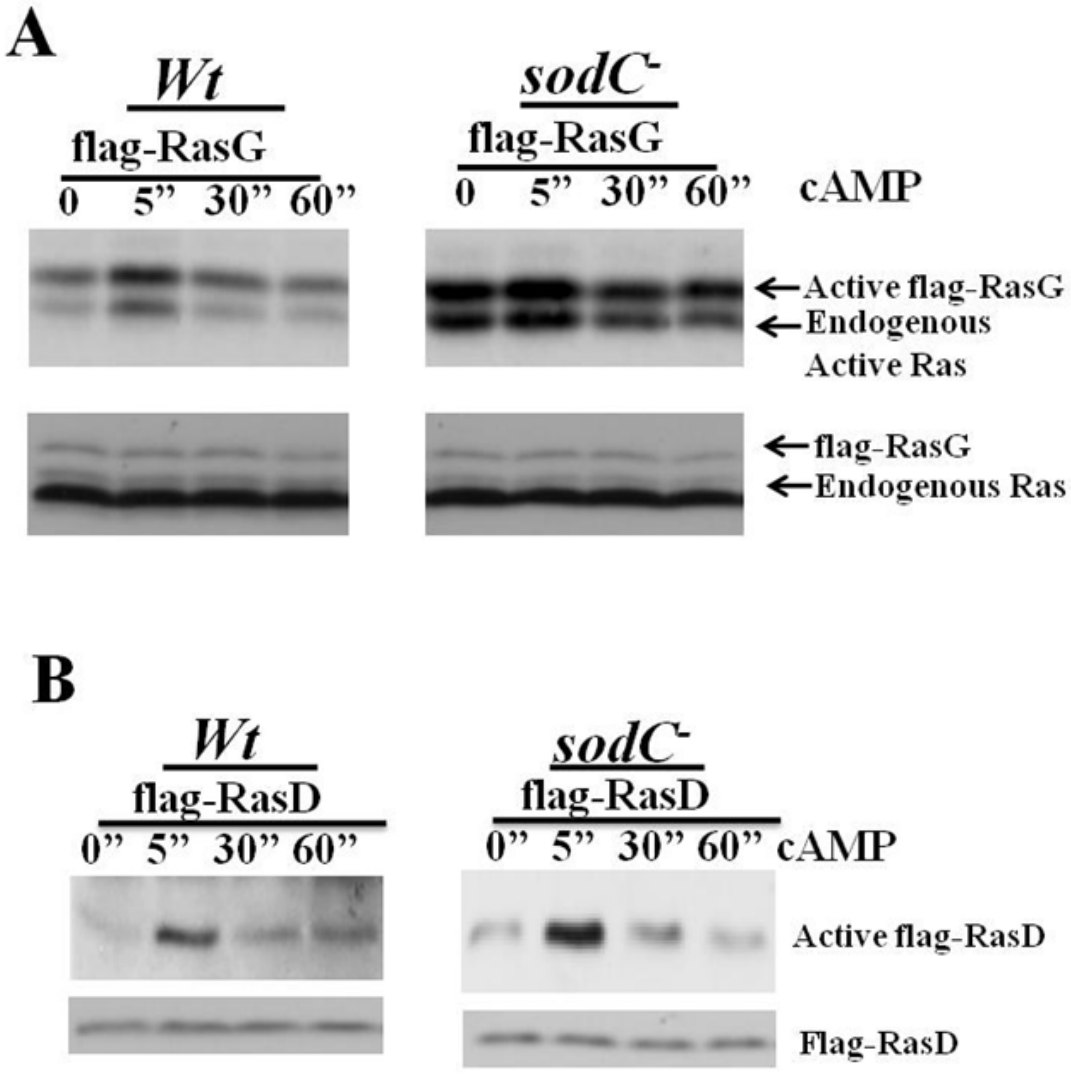


Figure 5. RasD activation dynamics not affected in *sodC*⁻ cells after being challenged with cAMP.

Flag-RasG and Flag-RasD were expressed separately in the *wild type* and *sodC*⁻ cellular backgrounds. They were grown in suspension and pulsed with cAMP during 4 hours to make them aggregation competent. The proteins activity was determined using a GST-Raf1-RBD pull down assay. (A) For RasG in *wild type* cells the pattern of activation shows an activation peak 5 seconds after cAMP addition and a gradual decrease afterwards as expected. In the *sodC*⁻ background RasG displays unusually high basal RasG activity at time 0 that increased Superoxide even more at 5 seconds and slightly decreased towards the end of the time course. (B) RasD activation dynamics did not seem to differ in the *wild type* versus *sodC*⁻ background.

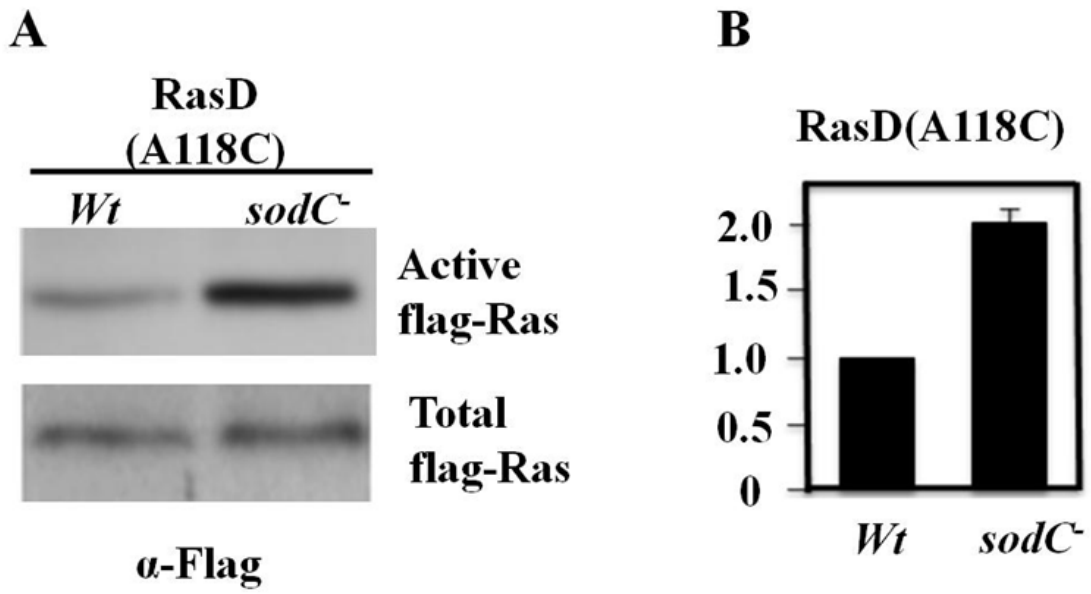
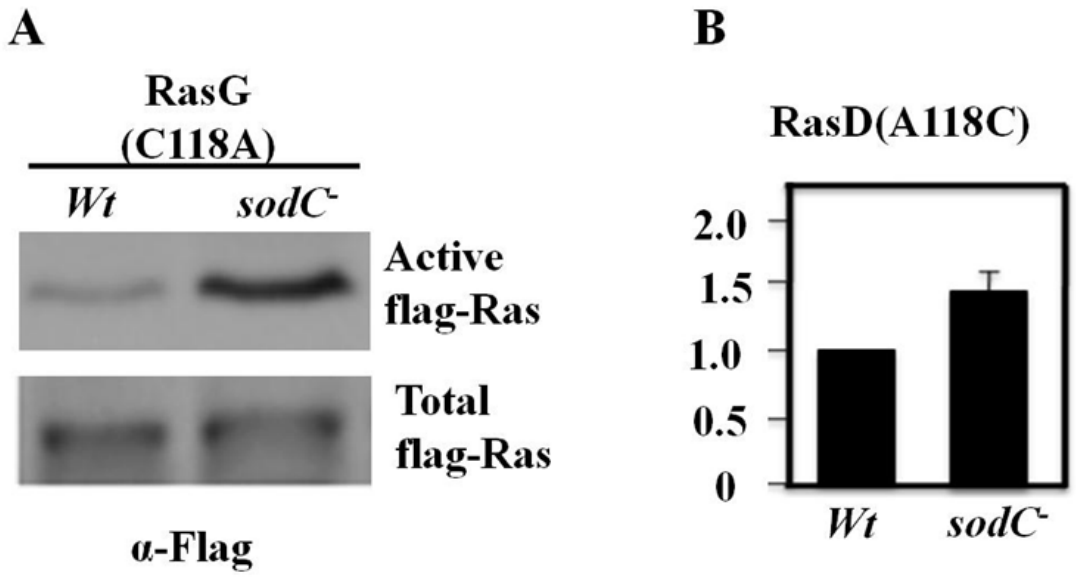


Figure 6. Activity of RasD A118C in the presence of superoxide.

(A) *Wild type* and *sodC*$^-$ cells expressing Flaf-RasD(C118A) were made aggregation competent through pulsing and then lysed using complete TTG buffer. A representative result of the western blots is shown. Lysates were normalized with anti-Flag antibody and then pull down using GST-Raf1-RBD. There is a clear increase in active RasD(A118C) in the *sodC*$^-$ background indicating that the protein has acquired redox sensitivity. (B) The western blot images were scanned and quantified using UN-SCAN-It gel imaging software and the results show a significant difference (P-value = 0.02). At least 3 separate experiments were performed to determine that the results were consistent.



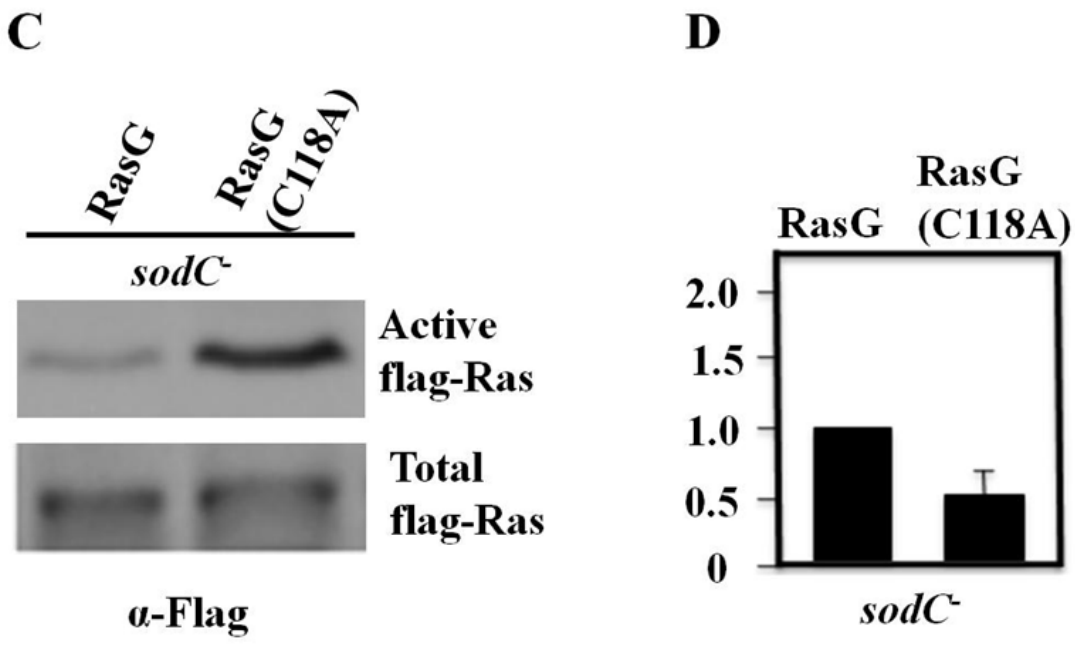


Figure 7. The C118A mutation decreases activity for RasG in the *sodC*⁻ cellular background.

RasG was mutated using site directed mutagenesis changing the cysteine into alanine at the position 118th of the nucleotide binding domain (specifically NKXD motif). The cells were grown to log phase and then pulsed and lysed. The antibody used for detection was the anti Flag. Western blot of the pulled down proteins after normalization shows that the amount of active RasG(C118A) is about 50% higher in *sodC*⁻ than in the *wild type* background (A) (p-value ≤ 0.05). However when the non mutated protein is compared to the RasG(C118A) one, both in *sodC*⁻ background, a decrease in the activity of the mutated protein is observed (C). Quantification data was obtained using the UN-SCAN-It gel software that allowed us to get band density values using the western blot results (B and D).

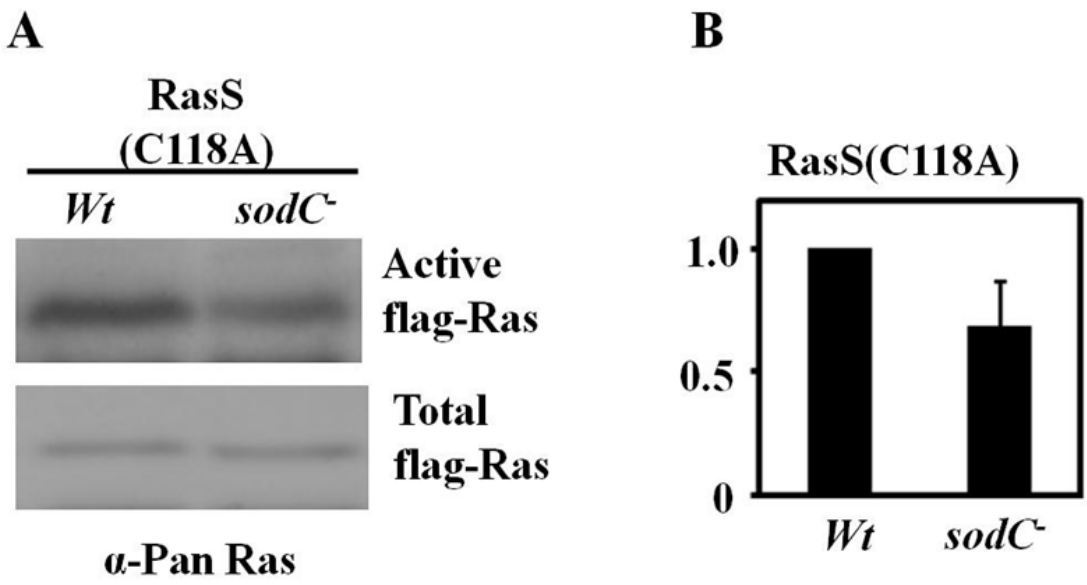


Figure 8. Activation level of the RasS decreases with the C118A mutation in *sodC*⁻ cells.

In this experiment we changed the cysteine in the NKCD motif of the RasS protein into an alanine through site directed mutagenesis using the cDNA we obtained through RT-PCR from *Dictyostelium* RNA. Both, *wild type* and *sodC*⁻ overexpressing the mutant protein with a flag tag were pulsed and lysed before the pull down and western blot assays. The results obtained show a significant decrease in RasS(C118A) activity in *sodC*⁻ cells compared to the *wild type* reference (A) (B), p-value ≤ 0.05. Experiments were repeated at least 3 times with new shaking cultures every time. (A) (B) The basal activity of flag-RasS(C118A) in *wild type* and *sodC*⁻ cells was visualized by western blot using anti-Pan-Ras antibody and the average band intensities from three independent experiments is presented.

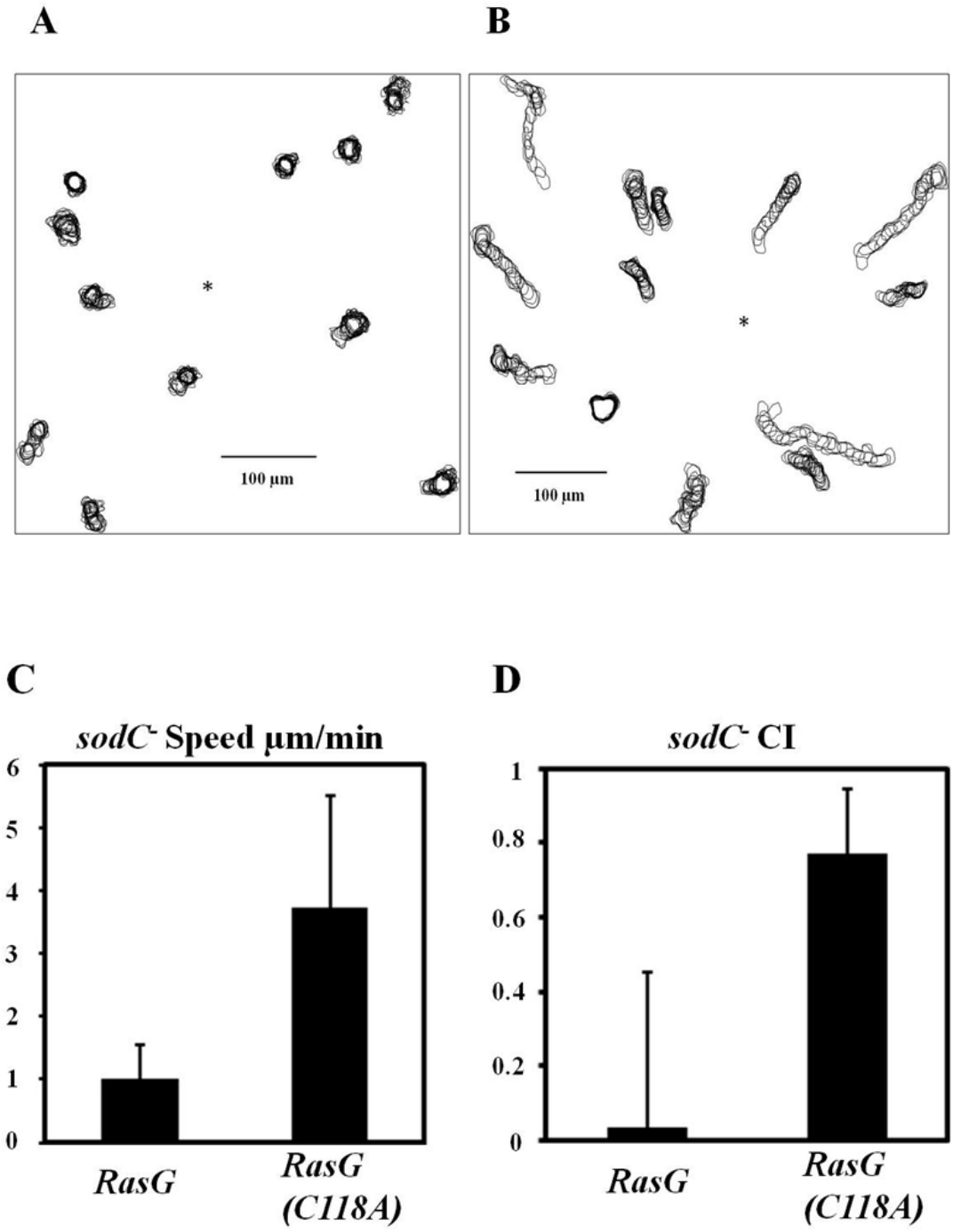


Figure 9. *sodC*⁻ cells chemotaxis is partially rescued by overexpression of RasG(C118A).

Cells were pulsed during four hours and then challenged for 20 minutes with a micropipette with 10 μM cAMP to investigate their chemotactic behavior. The cells expressing the *wild type* RasG protein (A) showed a very limited chemotaxis in response to cAMP, however, it was greatly improved in cells overexpressing RasG with the C118A mutation (B). Cells from three independent experiments were used. Graphical representations of the average difference in chemotaxis index (CI) and speed are shown(C)(D).

2.5 Discussion

2.5.1 Ras proteins with the NKCD motif are susceptible to activity misregulation in *sodC*⁻ cells

The current understanding of the effect of superoxide radicals on small GTPase Ras is based on the working hypothesis that superoxide can directly activate Ras by modifying the cysteine in the NKCD motif as shown *in vitro* (Heo et al., 2006). Previous data from our lab demonstrated that dysregualtion of superoxide metabolism by inactivating *sodC* in *Dictyostelium discoideum* resulted in a significant increase in the basal Ras activity and caused a series of defects such as lack of polarization and defective chemotaxis (Veeranki et al., 2008). Consistent with the *in vitro* studies and our previous study, cysteine 118 in RasG is essential for superoxide-mediated activation of Ras protein *in vivo*. Mutations on the Ras cysteine 118 in humans are known to cause devastating conditions such as the Costello syndrome (Wey et al., 2013).

The proteins RasG and RasS showed to be upregulated in the *sodC*⁻ background indicating that the cysteine in the NKCD motif is the target of redox regulation *in vivo*.

The degree of increase in the Ras activity level was different as expected in proteins with different structure and regulatory apparatus. Of the two, RasG showed a largest increase in activity, and thus RasG is highly likely the one that causes most aberrancies of *sodC*$^-$ cells. The RasS protein on the other hand displayed only a modest increase in *sodC*$^-$ cells. The modest increase in the basal activity of RasS is likely the result of the presence of P-loop, lack of redox sensitive RasS regulatory proteins (GEFs and GAPs), or both.

On the other hand, neither RasD nor RasC contains the cysteine residue as part of the NKXD motif but instead they have an alanine. The presence of the NKAD motif in these proteins makes them irresponsive to the presence of the radical species in question. The basal activities of RasC and RasD was not significantly different between *sodC*$^-$ and *wild type* cells, and thus RasGEF and RasGAP proteins specific for RasC and RasD are likely redox insensitive as well contrary to those of RasG.

The time course experiment for RasG and RasD indicates that the redox sensitive proteins such as RasG have both, higher basal activity and disrupted temporal regulation which could be translated as strong signals being transmitted for longer. The regulatory proteins seem to be at least partially functioning suggesting that at least some of the effect on this redox sensitive protein is through the NKCD motif, although the regulatory machinery might be a contributing element.

2.5.2 Ras mutations of the NKCD motif confirm the importance of cysteine as redox target

The RasG and RasD proteins are highly homologous proteins that have the NKxD motif in the same position, but RasD has an alanine on the 118th position instead of the cysteine seen on RasG 118th location. The single amino acid substitution suggests that the

Cys^{118} is the key residue targeted by the redox activity of superoxide on the Ras proteins studied. The mechanism by with the superoxide acts on Ras proteins through the NKCD has been suggested in previous *in vitro* studies (Heo and Campbell, 2004; Heo and Campbell, 2006). Taking into account that superoxide does not targets specific motifs or domains but rather sensitive exposed residues, the possibility of the effects being at least partially mediated by RasGefs or RasGAPs is also to be considered. This possibility of other Ras regulators being involved was evident when the NKCD motif of RasG was changed to NKAD. The mutated protein RasG(C118A) was compared to the *wild type* protein both in the *sodC*$^-$ background, there was a significant decrease in activity for the mutated protein, which is in accordance with the expected results. When the same mutated protein was expressed and compared in *wild type* versus *sodC*$^-$ cells, contrary to our expectations the mutant protein showed to be more active (~ 50%) in the *sodC*$^-$ backgroung indicating that RasG (C118A) is still receiving more activating signals on the superoxide rich environment. Binding of GTP to Ras proteins is what determines its active state and that's why the placement of Cys^{118} inside the nucleotide binding domain makes it so important as target of superoxide since the other four cysteine residues found on RasG are conserved also in the closely related but redox insensitive RasD. There is a chance that the changes observed in RasG activity as a redox consequence of the superoxide anions in *sodC*$^-$ cells might be in part through the Gef and/or GAP proteins that normally modulate the activity of this specific small GTase.

Contrary to the *wild type* RasD protein that exhibited no difference in its basal activity in *wild type* or *sodC*$^-$ cells, RasD(A118C) displayed significant increase (two

fold) in its basal activity. Considering that there seemed to be no dysregualtion of basal *wild type* RasD activity, a direct modification of the cysteine 118 of RasD by superoxide is likely the molecular mechanism of RasD(A118C) activation in *sodC*$^-$ cells. RasS(C118A) protein, however, exhibited lower basal activity in *sodC*$^-$ than *wild type* cells , implying that, unlike RasG specific GEFs and GAPs, RasS modulating GEfs and GAPs are not likely affected by superoxide in *sodC*$^-$ cells. In addition, it is tempting to speculate that the cysteine residue at the P-loop mediates an inhibitory effect on the RasS basal activity in *sodC*$^-$ cells.

Thus we propose that redox regulation of Ras can occur at two levels: one is the direct modification of the NKCD motif, and the other is to modulate RasGEF and RasGAP. The nature of superoxide mediated modulation of RasGEFs and GAPs requires further investigation but guarantees a significant advancement that will benefit a large scientific community as well as general public.

2.6 References

Bloomfield, G., & Pears, C. (2003). Superoxide signalling required for multicellular development of dictyostelium. *Journal of Cell Science, 116*(Pt 16), 3387-3397.

Bolourani, P., Spiegelman, G., & Weeks, G. (2010). Ras proteins have multiple functions in vegetative cells of dictyostelium. *Eukaryotic Cell, 9*(11), 1728-1733.

Bolourani, P., Spiegelman, G. B., & Weeks, G. (2006). Delineation of the roles played by RasG and RasC in cAMP-dependent signal transduction during the early development of dictyostelium discoideum. *Molecular Biology of the Cell, 17*(10), 4543-4550.

Cai, H., Das, S., Kamimura, Y., Long, Y., Parent, C. A., & Devreotes, P. N. (2010). Ras-mediated activation of the TORC2-PKB pathway is critical for chemotaxis. *The Journal of Cell Biology, 190*(2), 233-245.

Chattwood, A., Bolourani, P., & Weeks, G. (2014). RasG signaling is important for optimal folate chemotaxis in dictyostelium. *BMC Cell Biology, 15*(1), 13.

Chubb, J. R., Wilkins, A., Thomas, G. M., & Insall, R. H. (2000). The dictyostelium RasS protein is required for macropinocytosis, phagocytosis and the control of cell movement. *Journal of Cell Science, 113 (Pt 4)*(Pt 4), 709-719.

Daniel, J., Spiegelman, G., & Weeks, G. (1995). Dictyostelium ras genes. *Guidebook to the Small GTPases.M.Zerial and L.Huber, Editors.Oxford University Press, Oxford, UK, 100*, 104.

Finkel, T. (1998). Oxygen radicals and signaling. *Current Opinion in Cell Biology, 10*(2), 248-253.

Heo, J. (2011). Redox control of GTPases: From molecular mechanisms to functional significance in health and disease. *Antioxidants & Redox Signaling, 14*(4), 689-724.

Heo, J., & Campbell, S. L. (2004). Mechanism of p21Ras S-nitrosylation and kinetics of nitric oxide-mediated guanine nucleotide exchange. *Biochemistry, 43*(8), 2314-2322.

Heo, J., & Campbell, S. L. (2006). Ras regulation by reactive oxygen and nitrogen species. *Biochemistry, 45*(7), 2200-2210.

Hole, P. S., Pearn, L., Tonks, A. J., James, P. E., Burnett, A. K., Darley, R. L., et al. (2010). Ras-induced reactive oxygen species promote growth factor-independent proliferation in human CD34+ hematopoietic progenitor cells. *Blood, 115*(6), 1238-1246.

Kae, H., Kortholt, A., Rehmann, H., Insall, R. H., Van Haastert, P. J., Spiegelman, G. B., et al. (2007). Cyclic AMP signalling in dictyostelium: G-proteins activate separate ras pathways using specific RasGEFs. *EMBO Reports, 8*(5), 477-482.

Kae, H., Lim, C. J., Spiegelman, G. B., & Weeks, G. (2004). Chemoattractant-induced ras activation during dictyostelium aggregation. *EMBO Reports, 5*(6), 602-606.

Khosla, M., Spiegelman, G. B., Insall, R., & Weeks, G. (2000). Functional overlap of the dictyostelium RasG, RasD and RasB proteins. *Journal of Cell Science, 113 (Pt 8)*(Pt 8), 1427-1434.

Khosla, M., Spiegelman, G. B., & Weeks, G. (1996). Overexpression of an activated rasG gene during growth blocks the initiation of dictyostelium development. *Molecular and Cellular Biology, 16*(8), 4156-4162.

Kimmel, A. R., & Firtel, R. A. (1991). cAMP signal transduction pathways regulating development of< i> dictyostelium discoideum</i>. *Current Opinion in Genetics & Development, 1*(3), 383-390.

Lim, C. J., Spiegelman, G. B., & Weeks, G. (2002). Cytoskeletal regulation by dictyostelium ras subfamily proteins. *Journal of Muscle Research & Cell Motility, 23*(7-8), 729-736.

Loovers, H. M., Postma, M., Keizer-Gunnink, I., Huang, Y. E., Devreotes, P. N., & van Haastert, P. J. (2006). Distinct roles of PI(3,4,5)P3 during chemoattractant signaling in dictyostelium: A quantitative in vivo analysis by inhibition of PI3-kinase. *Molecular Biology of the Cell, 17*(4), 1503-1513.

Reymond, C. D., Gomer, R. H., Mehdy, M. C., & Firtel, R. A. (1984). Developmental regulation of a dictyostelium gene encoding a protein homologous to mammalian< i> ras</i> protein. *Cell, 39*(1), 141-148.

Robbins, S. M., Williams, J. G., Jermyn, K. A., Spiegelman, G. B., & Weeks, G. (1989). Growing and developing dictyostelium cells express different ras genes. *Proceedings of the National Academy of Sciences of the United States of America, 86*(3), 938-942.

Robbins, S. M., Williams, J. G., Jermyn, K. A., Spiegelman, G. B., & Weeks, G. (1989). Growing and developing dictyostelium cells express different ras genes. *Proceedings of the National Academy of Sciences of the United States of America, 86*(3), 938-942.

Sambrook, J., Fritsch, E., & Maniatis, T. (1989). SDS-polyacrylamide gel electrophoresis of proteins. *Molecular Cloning: A Laboratory Manual, 3*, 18.47-18.59.

Sasaki, A. T., Chun, C., Takeda, K., & Firtel, R. A. (2004). Localized ras signaling at the leading edge regulates PI3K, cell polarity, and directional cell movement. *The Journal of Cell Biology, 167*(3), 505-518.

Sasaki, A. T., Janetopoulos, C., Lee, S., Charest, P. G., Takeda, K., Sundheimer, L. W., et al. (2007). G protein-independent Ras/PI3K/F-actin circuit regulates basic cell motility. *The Journal of Cell Biology, 178*(2), 185-191.

Schauer, T. M., Nesper, M., Kehl, M., Lottspeich, F., Müller-Taubenberger, A., Gerisch, G., et al. (1993). Proteasomes from< i> dictyostelium discoideum</i>: Characterization of structure and function. *Journal of Structural Biology, 111*(2), 135-147.

Segal, D., & Shilo, B. Z. (1986). Tissue localization of drosophila melanogaster ras transcripts during development. *Molecular and Cellular Biology, 6*(6), 2241-2248.

Tai, P., & Ascoli, M. (2011). Reactive oxygen species (ROS) play a critical role in the cAMP-induced activation of ras and the phosphorylation of ERK1/2 in leydig cells. *Molecular Endocrinology, 25*(5), 885-893.

Tuxworth, R. I., Cheetham, J. L., Machesky, L. M., Spiegelmann, G. B., Weeks, G., & Insall, R. H. (1997). Dictyostelium RasG is required for normal motility and cytokinesis, but not growth. *The Journal of Cell Biology, 138*(3), 605-614.

Veeranki, S., Kim, B., & Kim, L. (2008). The GPI-anchored superoxide dismutase SodC is essential for regulating basal ras activity and for chemotaxis of dictyostelium discoideum. *Journal of Cell Science, 121*(Pt 18), 3099-3108.

Watts, D. J., & Ashworth, J. M. (1970). Growth of myxameobae of the cellular slime mould dictyostelium discoideum in axenic culture. *The Biochemical Journal, 119*(2), 171-174.

Wennerberg, K., Rossman, K. L., & Der, C. J. (2005). The ras superfamily at a glance. *Journal of Cell Science, 118*(Pt 5), 843-846.

Wey, M., Lee, J., Jeong, S. S., Kim, J., & Heo, J. (2013). Kinetic mechanisms of mutation-dependent harvey ras activation and their relevance for the development of costello syndrome. *Biochemistry, 52*(47), 8465-8479.

Wilkins, A., Chubb, J. R., & Insall, R. H. (2000). A novel Dictyostelium RasGEF is required for normal endocytosis, cell motility and multicellular development. *Current Biology, 10*(22), 1427-1437.

Wilkins, A., Szafranski, K., Fraser, D. J., Bakthavatsalam, D., Müller, R., Fisher, P. R., et al. (2005). The dictyostelium genome encodes numerous RasGEFs with multiple biological roles. *Genome Biology, 6*(8), R68.

Wilkins, A., Khosla, M., Fraser, D. J., Spiegelman, G. B., Fisher, P. R., Weeks, G., et al. (2000). Dictyostelium RasD is required for normal phototaxis, but not differentiation. *Genes & Development, 14*(11), 1407-1413.

Wong, E., Yang, C., Wang, J., Fuller, D., Loomis, W. F., & Siu, C. H. (2002). Disruption of the gene encoding the cell adhesion molecule DdCAD-1 leads to aberrant cell sorting and cell-type proportioning during dictyostelium development. *Development (Cambridge, England), 129*(16), 3839-3850.

Zhang, T., Rebstein, P., Khosla, M., Cardelli, J., Buczynski, G., Bush, J., et al. (1999). A mutation that separates the RasG signals that regulate development and cytoskeletal function in< i> Dictyostelium</i>. *Experimental Cell Research, 247*(2), 356-366.

CHAPTER 3 MAGNESIUM EFFECT ON REDOX REGULATION OF *DICTYOSTELIUM DISCOIDEUM*

3.1 Introduction

Production and management of reactive oxygen species are well-controlled processes that serve the cells to perform different functions such as defense from foreign organisms, degradation of internalized particles and signaling. Loss of the redox equilibrium at the cellular level is often characteristic of diseased tissue (Liou et al., 2010). Tumor promotion and development are active areas of research because of its impact on human health. It has been shown that there is close relationship between reactive oxygen species production and Ras mediated mitogenic activity in oncogenic H-Ras^{V12} transformed mouse fibroblasts (Irani et al., 1997). Studies showed that NADPH oxidase and reactive oxygen species generation are involved in the cellular transformation, yet the presence of oncogenic Ras activity is essential (Suh et al., 1999, Lambeth., 2004, Wu and Terada., 2009). On the other hand, several studies indicated that ROS can also affect Ras activity. A study showed that the proliferation-inducing lipid lactosylceramide (LacCer) could activate NADPH oxidase and Ras but when NADPH oxidase was inhibited pharmacologically there was no Ras activation in response to LacCer. (Bhunia et al., 1997). In addition, another set of experiments showed that activation of the small GTPase Ras and its effectors in response to PDGF was needed to activte Nox1 (Lassegue et al., 2001).

Signal induced superoxide production in intracellular vesicular compartments is often seen in phagocytic cells; however it is not an exclusive characteristic of this type of

cells. Non-phagocytic cells also have a well-defined ligand activated receptor system that directs generation of ROS at the plasma membrane and intracellular vesicles (Leborgne-Castel et al., 2008). NADPH oxidase, one of the main producers of superoxide, is not only responsible for bactericidal function in phagocytes, but also for various ROS sensitive signaling events on the redoxosome - ROS loaded intracellular vesicles (Lamb et al., 2009, Oakley et al., 2009). The Interleukin 1 receptor, type I (IL-1R1) and Tumor Necrosis Factor Receptor 1(TNFR1) pathways that lead to the activation of NF-kB are examples of ROS sensitive signaling pathway mediated on the redoxosome (Oakley et al., 2009).

In the current study we observed that the superoxide containing vesicles are also present in *Dictyostelium discoideum*. Previous studies demonstrated that superoxide production is essential for starved *Dictyostelium* cells to aggregate. In the presence of superoxide radical scavengers, starved *Dictyostelium* cells failed to properly aggregate and cells overexpressing cytoplasmic superoxide dismutase displayed significantly compromised aggregation (Bloomfield and Pears, 2003). Furthermore, cells lacking Superoxide Dismutase C exhibited severe defects in directional cell migration (Veeranki et al., 2008). Our project was aimed to elucidate some of the characteristics of superoxide distribution in the *wild type* and *sodC*$^{-}$ cells using the recently developed fluorescent probe BES-So-AM that is highly specific for superoxide, but not for hydrogen peroxide and other ROS species (Maeda et al., 2005).

Superoxide radicals produced in intracellular vesicular compartments are out of reach from the cytosolic Cu/Zn-Sod and the mitochondrial Mn-Sod. The presence of the

intact superoxide radicals in the compartments implies that unless extracellular Sod enzymes are recruited into the lumen of the vesicles, superoxide ions will build up and last longer than those in the cytosol where multiple Sod enzymes exist. It has been established previously that in endothelial cells the extracellular Sod (EC-Sod) enzymes are internalized as a result of endocytosis (Chu et al., 2006). We uncovered that *Dictyostelium* cells lacking extracellular SodC accumulate superoxide-containing vacuoles indicating that SodC is essential to suppress aberrant superoxide containing vacuole formation.

It has been previously reported that higher levels of superoxide production were observed in neutrophils and macrophages from rats under magnesium deficient diet, which suggested the involvement of magnesium in the modulation of proinflammatory signaling (Mazur et al., 2007; Weglicki, 2012). In a related experiment we have observed that in *wild type Dictyostelium* cells the response to magnesium deficiency includes generation of intracellular superoxide ions. We also discovered that RasG is one of the targets of superoxide radicals and that aberrant activation of RasG is compromising directional cell migration. The possible advantage of chemotaxis impairment in this scenario will be discussed later.

3.2 Materials and methods

Some of the methods used are described in Chapter 2.

3.2.1 Confocal microscopy

Cells were grown and pulsed as described on chapter 2. After pulsing, cells were washed two times and incubated for 10 to 30 minutes with the desired buffer treatment.

Wild type and *sodC*$^-$ cells expressing GFP-RBD were used to visualize the location of active Ras proteins *in vivo*. Images were obtained using the 60x objective of the DV Elite microscope system. A similar protocol for preparation of the cells was used to obtain the images showing the superoxide enriched regions of the cells with the difference that each one of the buffers had 100 μM of the cell permeable superoxide specific BES-So-AM probe (Wako Chemicals USA).

3.2.2 Transfection

Wild type and *sodC*$^-$ cells were transfected as described in chapter 2 using a GFP-RBD DNA (Green Fluorescent Protein plus the Ras Binding Domain of Raf1) obtained from the stock center of Dictybase. G418 was used as means of selection 24 hours after electroporation.

3.2.3 Time course assay

Cells were grown and pulsed as described in the previous chapter. After pulsing, 100 million cells were washed once with desired buffer and placed in a small cup (2 cm diameter at the bottom) at a density of 25 million cells/ml during 4 minutes shaking at 150 rpm. Cells were then stimulated with 10 uM cAMP while shaking and lysed (using the cell lysis buffer complete TTG) at 0, 5, 30, 60 and 90 seconds (0 time was taken basal). Lysates were stored at -20^{o} C for short term use and at -80 ^{o} C for long term storage.

3.3 Results

3.3.1 Intracellular superoxide level is affected by ions in the cellular environment.

According to a previous biochemical study (Veeranki et al., 2008), *sodC*$^-$ cells

exhibit modestly increased level of intracellular superoxide compared to *wild type* cells. The fluorescent probe BES-So-AM, that is highly specific to superoxide and is cell permeable (Maeda et al., 2005), was used to visualize superoxide accumulation in *wild type* and *sodC*$^-$ cells. Log phase cells were pulsed and treated with different buffers containing 100 μM BES-So-AM then images were taken using the DV Elite microscope system. In the control buffer DB, the *wild type* cells exhibited small superoxide containing vesicular structures (< 1 μm in diameter) that rarely reached moderately larger size (~2 μm (Fig. 10 A). On the other hand, *sodC*$^-$ cells showed a greater amount of superoxide all around the inside of the cells and significantly larger size vesicles up to ~5 μm in diameter (Fig. 10 B). The fluorescence was not significantly distinguishable from the background when the BES-So-AM was not added to the DB as shown in the control image.

Magnesium is a relatively abundant element inside and outside of cells. It has been shown that magnesium ions in the cell environment negatively affect the production of superoxide in macrophages and neutrophils (Mazur et al., 2007). Since magnesium is also part of the compounds commonly used in *Dictyostelium* growth media D3T and the standard nutrient deficient buffer DB, an experiment was devised to evaluate how the superoxide production is affected by the presence of magnesium ions in *Dictyostelium* cells. In a solution containing 2mM magnesium sulfate the *wild type* cells seemed to be practically unaffected showing small superoxide containing vesicles very similar to those observed in the control buffer DB. In the case of *sodC*$^-$ cells treated with 2mM magnesium (or KPO_4), the fluorescent vesicles size was restored to the *wild type*

proportions seen in the DB control used as reference. Two other buffers that did not contain magnesium ions were also used with similar results for both types of cells: when H_2O or NaCl were used, both *wild type* and *sodC*$^-$ cells exhibited significant changes in size of the superoxide containing vesicles: *wild type* cells showed vesicles up to 5 times larger than those observed in the control buffer. The *sodC*$^-$ cells had more overall signal intensity in the cytoplasm together with small to large vesicles of great superoxide content judging by the signal strength (Fig. 10 A & 10 B).

According to the outcome of the magnesium treatment assay, it seems that this ion can limit or repress the production of superoxide production in *JH10* and *sodC*$^-$ cells. In contrast, superoxide production and distribution inside the cells increased for both cellular backgrounds in the absence of magnesium. Furthermore, in this condition, *sodC*$^-$ cells that normally produce and excess of superoxide seemed to have an even stronger cytoplasmic signal as detected by BES-So-AM compared to the *sodC*$^-$ cells in DB indicating that more superoxide is leaking into the cytosol. Even though in *sodC*$^-$ cells superoxide production was decreased by 2mM $MgSO_4$, the DB buffer that contains the same concentration of magnesium ions plus other compounds did not have the same effect. The mechanism by which magnesium ions affect the production and distribution of superoxide is a very interesting question that might be addressed in future studies, however in line with the direction of this project we were not be able to cover that question as part of our work.

3.3.2 Ras activity and temporal regulation changes in response to extracellular ions

Since Ras proteins are misregulated in the presence of superoxide, a series of

experiments were devised with the purpose of observing how the presence or absence of magnesium affected superoxide mediated Ras regulation. One of the experiments was to compare flag-RasG vs flag-RasC both expressed in the *wild type* cellular background and treated with 2mM magnesium sulfate, water or DB (as control). After three independent experiments it was evident that Flag-RasG which is redox sensitive, responded to the presence or absence of magnesium by increasing or decreasing Ras activity respectively (Fig. 11 A, 11 C). These changes in activity also coincided with the magnesium linked superoxide generation changes observed using confocal microscopy. In contrast flag-RasC did not show increase in activity in the absence of magnesium ions compared to the standard condition (Fig. 11 B). A small but not truly significant decrease in RasC activity was observed in the condition lacking magnesium.

In a separate but related experiment, we looked at the temporal pattern of Ras activation in response to the chomoattractant cAMP in the presence or absence of magnesium ions. To do this, aggregation competent cells of *wild type* and *sodC*$^-$ backgrounds were treated with 15mM NaCl or 2mM magnesium sulfate and then challenged with 10 uM cAMP. The Ras activity was measured at different time points after RBD binding and western blot assays (Fig. 12 A, 12 B). The different cell types assayed under H_2O or NaCl showed an aberrant increase in Ras activation in all time points indicating lack of regulation by GEFs or GAPs. In the $MgSO_4$ containing condition however, the cells in both backgrounds displayed a restored Ras activation dynamics with a distinctive activation peak at 5 seconds after cAMP stimulation and subsequent decrease in activity.

3.3.3 The cellular pattern of Ras regulatory distribution changes in response to redox stress

With the evidence obtained through biochemistry and western blot regarding changes in Ras activity we decided to use confocal microscopy to visualize the cellular localization of active Ras in live *Dictyostelium* cells. To accomplish this, aggregation competent *wild type* and *sodC*$^-$ cells expressing a GFP-RBD fusion protein were treated with the following buffers: water, 15 mM NaCl , 2mM $MgSO_4$ and DB which was used as the control condition. W*ild type* cells when in the presence of magnesium deficient media, displayed active Ras all around the membrane and often localized to vesicular bodies inside the cells. In the DB and magnesium containing environments, *wild type* cells were often polarized and active Ras proteins were localized at the leading edge of the cells. The *sodC*$^-$ cells however, displayed a widespread aberrant distribution of the active Ras proteins throughout the plasma membrane under all the buffers used in the assay except for the one containing $MgSO_4$. Under magnesium, these cells showed a pattern of polarized Ras distribution comparable to that of *wild type* cells in DB (Fig. 12 C). This suggests that the magnesium-containing medium restores to *sodC*$^-$ cells the ability of spatially regulating the distribution of active Ras proteins.

3.3.4 RasG is vital for chemotaxis in the presence of extracellular ions.

In this set of chemotaxis experiments, *wild type* and *sodC*$^-$ cells were treated with DB, $MgSO_4$ and a magnesium deficient medium (NaCl) and then stimulated with 10uM cAMP and two different motility parameters were recorded (Fig. 13 A and 13 C). Using the control medium we observed that cells in the *sodC*$^-$ background have a considerable

deficiency in speed (~4 um/min) and chemotaxis index (0.19) and this was previously reported (Veeranki et al., 2008) and confirmed by Tong Sun unpublished results. In comparison, *wild type* cells showed a higher speed towards the chemoattractant (~10.9 um/min) and a great directionality (chemotactic index of about 7.7). Under control and magnesium media, *wild type* cells exhibited equivalent speed and chemotactic index (Fig. 13 A). *sodC*$^{-}$ cells however showed great improvement in their chemotactic ability when magnesium medium was used. Chemotaxis index improved to ~0.8, nevertheless the speed was only partially enhanced (Fig. 13 A and 13 C).

Additionally, using a magnesium deficient medium (15mM NaCl) we decided to look at the role of RasG in the chemotaxis response of the cells. *rasG*$^{-}$ cells were compared to *wild type* under DB control and magnesium deficient medium in their ability to chemotax to cAMP. *Wild type* cells showed a significant impairment in directionality towards the chemoattractant and a less severe but noticeable speed decrease in the magnesium deficient media compared to the DB condition. *rasG*$^{-}$ cells on the other had did not seem to me significantly impacted by the lack of magnesium in their medium (Fig. 13 B and 13 C).

3.5 Figures

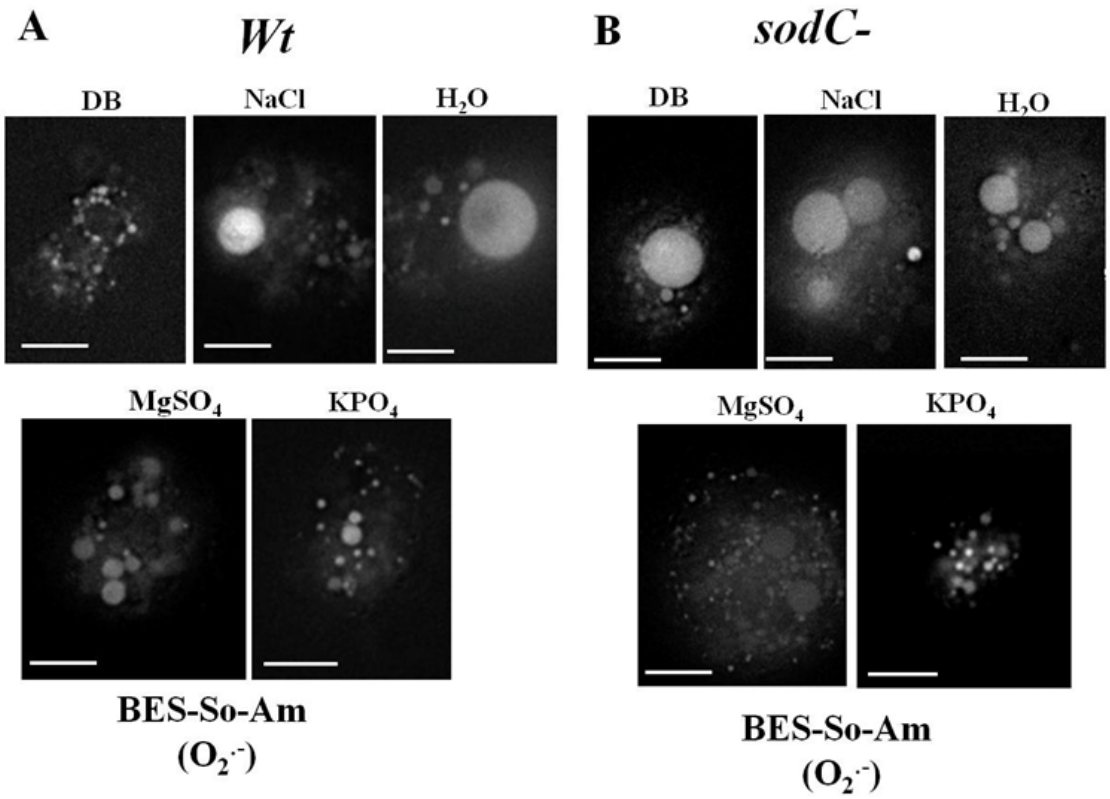


Figure 10. Production of superoxide in cellular compartments correlated with the presence of ions outside the cell.

Wild type and *sodC*$^-$ cells were grown to the log phase in shaken suspension and then pulsed for 4 hours with 50 nM cAMP. Both cell types were then separated in groups that were washed with a different buffer each (DB, H_2O, 2mM $MgSO_4$, KPO_4, or 15 mM NaCl). Finally the cells were put in the same buffer used to wash them plus 100mM of the superoxide specific florescent indicator BES-So-AM (Maeda et al., 2005). (A) Under the control buffer DB or the magnesium buffer *wild type* cells showed similar small superoxide containing vesicular bodies. These same cells treated with water or 15 mM NaCl (buffers lacking magnesium) showed the presence of significantly larger superoxide

containing vesicles. (B) In the case of *sodC*$^-$ cells, they displayed high basal superoxide signal in the cytosol and large superoxide containing vesicular compartments in the control buffer DB and also in NaCl and water. The abundant superoxide signal from the cytoplasm and the large vesicles observed in the control buffer for *sodC*$^-$ cells were greatly decreased in intensity and size respectively by the treatment with the magnesium and the KPO_4 buffers.

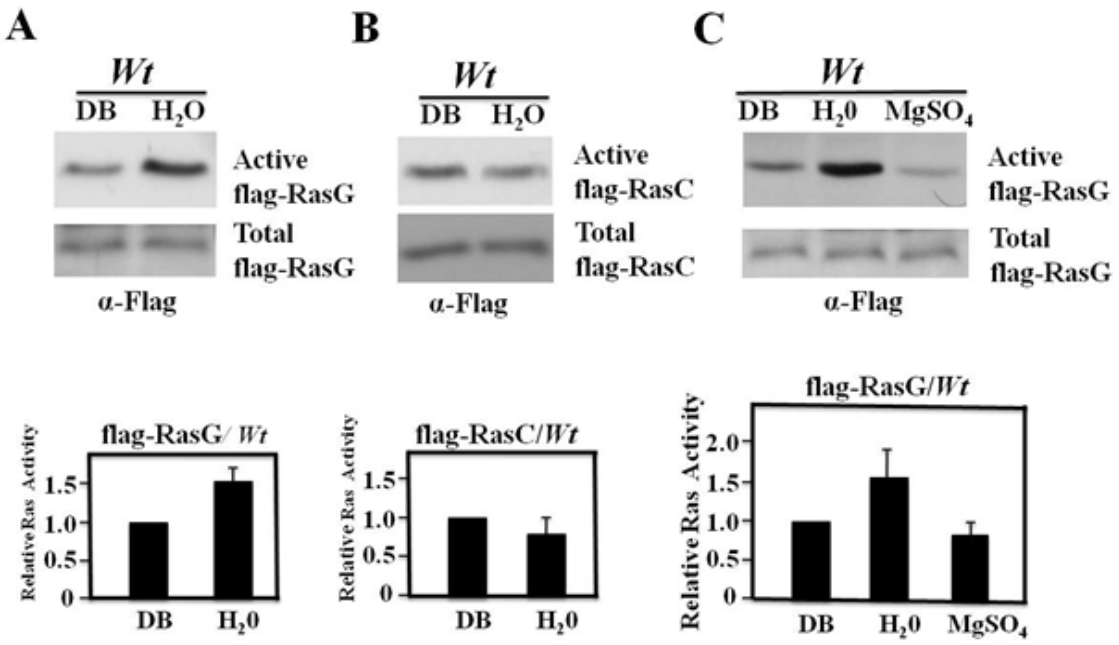
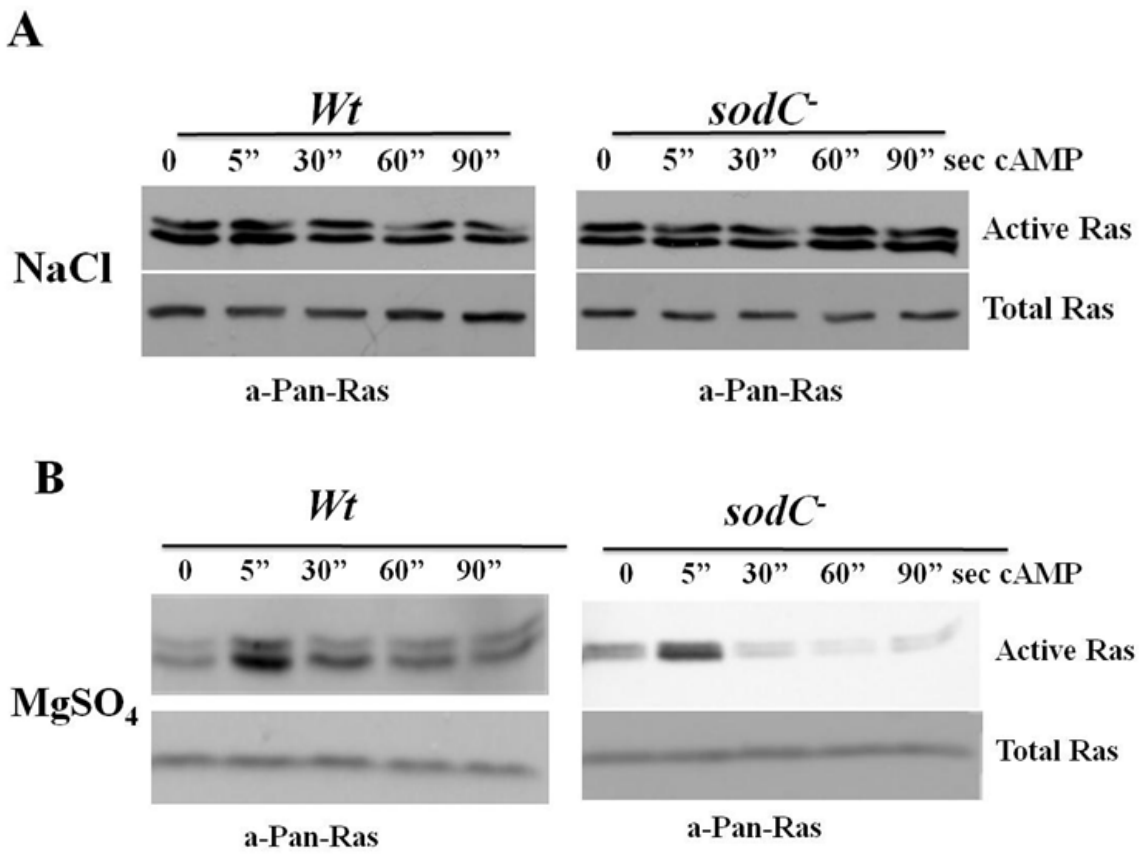


Figure 11. Only the redox sensitive RasG was aberrantly active in media lacking magnesium ions compared to RasC.

All cells were pulsed with cAMP for four hours, then lysed and protein extracts submitted to a pull down assay after normalization. The results of three separate experiments were used to establish the activity levels. (A) There was a higher level of

active RasG in magnesium lacking buffer (water) compared to the DB buffer control (p-value ≤ 0.05). However, there was no significant difference in RasC activity in the two different conditions (B). In the *wild type* cellular background the level of activity for RasG seems to be kept at a level close to what is observed in the control buffer when using 2mM $MgSO_4$.



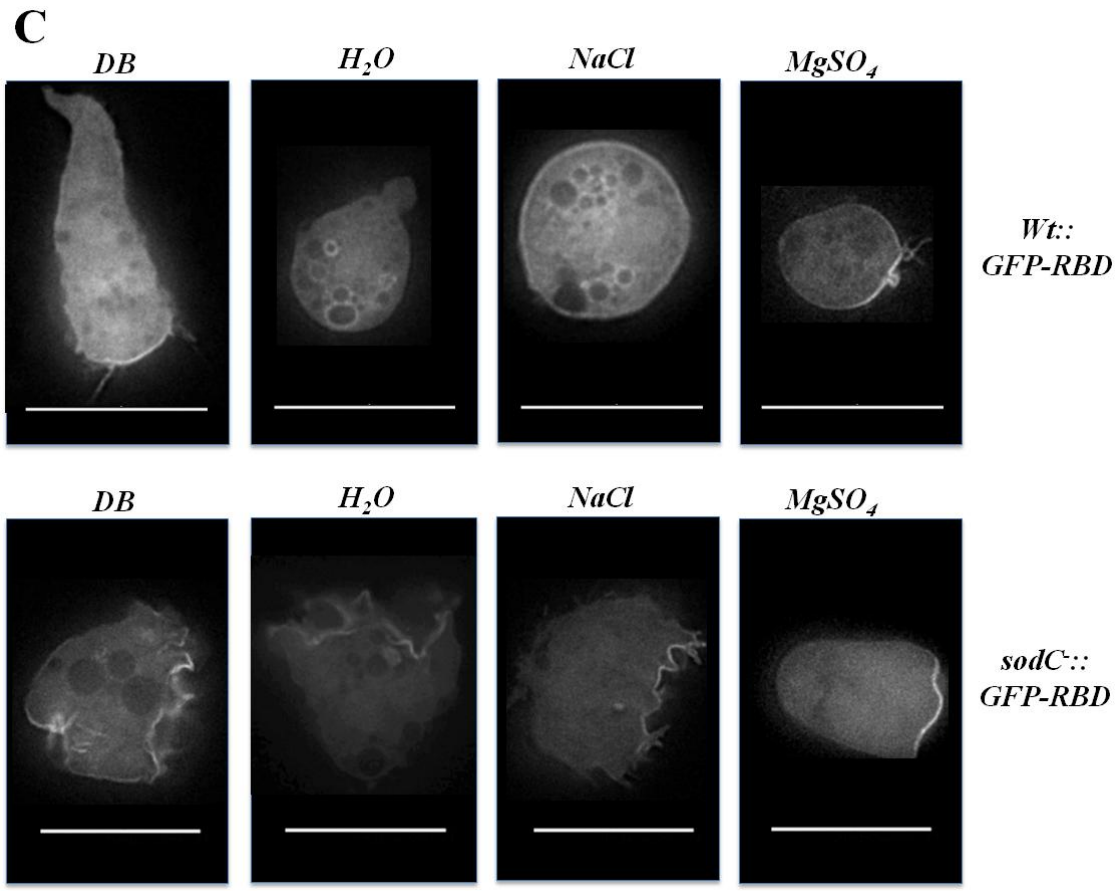
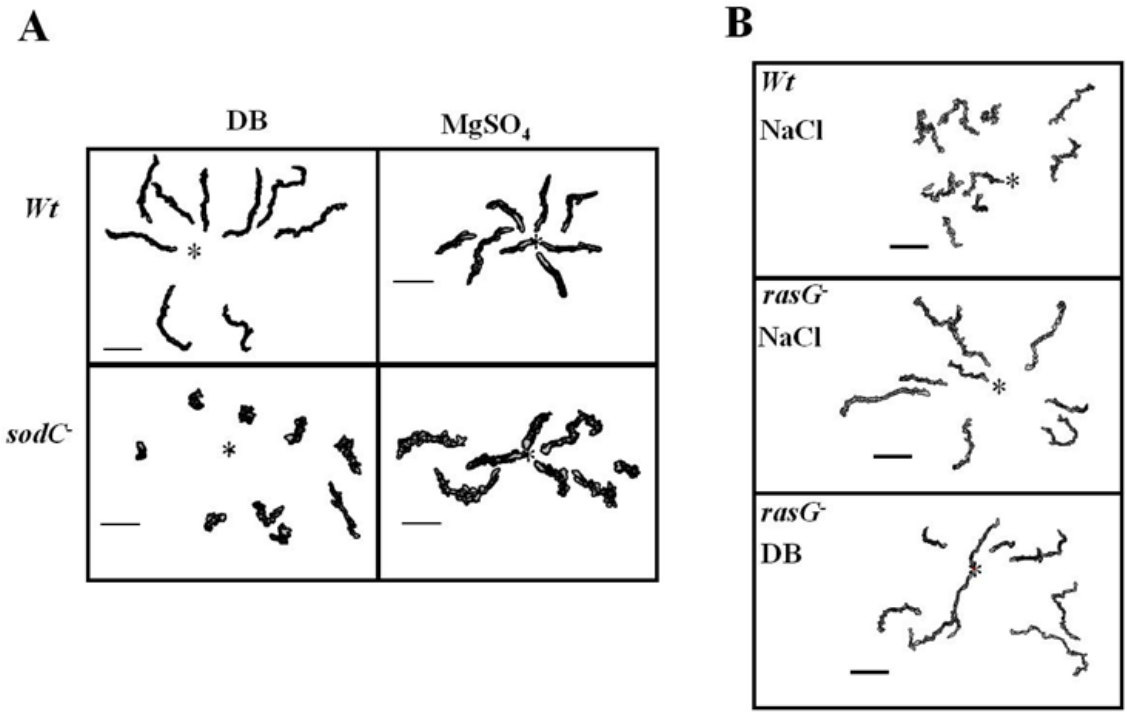


Figure 12. Ras localization and regulation dynamics are properly maintained in response to cAMP for cells in a 2 mM magnesium containing solution.

(A)The microscopy experiments to visualize the cellular localization of active Ras under different buffer conditions were performed on *wild type* and *sodC*⁻ cells overexpressing GFP-RBD using the DV Elite microscope system. In parallel to the reported GFP-Raf1-RBD results, active Ras proteins were restricted to one of the small edges of polarized *wild type* cells in DB or the magnesium containing medium. The *sodC*⁻ cells ,that are normally non-polarized, showed a strong GFP-Raf1-RBD signal all around the cells membrane in the control buffer, a fact that was also reported in the past (Veeranki et al., 2008). However, *sodC*⁻ cells in a 2 mM magnesium solution showed a great decrease in

Ras activity that was now confined to small areas of the plasma membrane of more polarized cells. (B) (C) Ras activity level was determined through a time course for aggregation ready *wild type* and *sodC*$^-$ cells in both NaCl and $MgSO_4$ buffers after 10 uM cAMP stimulation. The Ras activity pattern for both types of cells seemed to be well regulated when in the presence of 2 mM $MgSO_4$ showing a rapid increase on Ras activity at 5 seconds after cAMP stimulus. (D) Ras activity was equally disturbed showing high level at all times for the two cell types in the presence 15 mM NaCl with cAMP addition having no effect.



C

Cell Type	Condition	Chemotactic Index Mean±SD	Speed (mm/min) Mean±SD
Wt	DB	0.77 ± 0.13	10.9 ± 2.51
sodC$^-$	DB	0.19 ± 0.45	4.08 ± 2.54
Wt	$MgSO_4$	0.81 ± 0.12	9.09 ± 2.43
sodC$^-$	$MgSO_4$	0.79 ± 0.09	5.39 ± 1.88
Wt	NaCl	0.48 ± 0.30	6.78 ± 2.02
rasG$^-$	NaCl	0.75 ± 0.20	5.65 ± 2.34
rasG$^-$	DB	0.74 ± 0.17	6.51 ± 2.65

Figure 13. *Dictyostelium* directional movement is affected by magnesium.

In all conditions cells were stimulated with 10 uM cAMP and images were taken every minute for 20 minutes. The images used here represent at least 3 replicates for each one of the conditions assayed. (A)(C) In the control condition (DB), the speed and chemotaxis index of the *sodC*$^-$ cells were greatly reduced in accordance with previous findings (Veeranki et al., 2008). (A) When *sodC*$^-$ cells were placed in a 2 mM magnesium solution there was a remarkable gain in directionality toward the place of cAMP release (CI ~0.8). However the speed of these cells was not greatly increased in the magnesium media, which suggests that the signaling pathway leading to *sodC*$^-$ cells' chemotaxis is affected in other ways that cannot be restored to normal by magnesium. Treatment of *wild type* cells with the same buffer yielded results for speed and CI almost identical to

those for the cells treated with the control buffer which implies that 2 mM of magnesium ions are enough to elicit a normal chemotactic response from *wild type Dictyostelium*. (B)(C) *Wild type* cells in a 15 mM NaCl solution showed a significant decrease in chemotaxis efficiency in the direction of cAMP release. On the other hand, chemotaxis in the direction of cAMP discharge for cells without the redox susceptible RasG protein was considerably better compared to *wild type* in the magnesium deficient buffer. The chemotaxis biochemical pathway of *rasG*$^-$ cells seem to be unaffected by the absence of magnesium when compared to the standard condition, which suggests that the role of RasG in redox stress conditions might significantly differ from the one in regular conditions. The results of *wild type* cells in DB versus NaCl for chemotactic index had P value <0.001 (2.06 E-0.5, student t-test) and for *rasG*$^-$ cells it was 0.70. The speed parameter for *wild type* in DB versus NaCl had P value <0.001(9.69 E-0.9) and for *rasG*$^-$ cells were 0.18. The $MgSO_4$ treatment of *wild type* and *sodC*$^-$ cells was done by Dr. Tong Sun (unpublished).

3.6 Discussion

In addition to the ROS that can be produced at the cellular level as a result of metabolic activity, cells need to overcome the changes induced by the presence of extracellular ions to keep homeostasis (Mori et al., 2002). There is not enough information about how these ions affect the redox equilibrium of the cells and the effect on specific proteins such as Ras. This series of experiments has generated valuable data regarding the regulation of cellular functions due to the presence of superoxide or magnesium ions *in vivo*.

Using *Dictyostelium* cells as a model we observed that either ablation of the *sodC* gene or lack of magnesium in the cellular environment led to the accumulation of superoxide radicals and an increase the activity level of Ras (Veeranki et al., 2008). *Dictyostelium discoideum* is an amoeboid protozoan that lives in the soil which means that its plasma membrane is directly in contact with many natural elements found in that environment. It has been shown that natural habitats contain magnesium ions or compounds in quantities equivalent to the millimolar range (Barker et al., 2006, Lusche et al., 2010) and that weather phenomena such as rainfall leads normally to a decrease in the concentration of magnesium ions in this type of environment. Because of this, *Dictyostelium* cells might be often challenged by conditions that lead to oxidative stress situations in their ordinary habitats. In addition, experiments performed on other model systems such as rat macrophages and neutrophils have also shown that magnesium ions can act as inhibitors of superoxide production (Mazur et al., 2007).

Experiments with the mammalian equivalent of SodC, the extracellular Superoxide dismutase (EC-Sod) proteins, have shown that they are normally endocytosed in endothelial cells, and the mutation EC-Sod(R213G) that makes the protein stay always at the cell membrane, increases the risk of ischemic heart disease (Juul et al., 2004, Chu et al., 2006). Although the molecular basis of vascular protection by EC-Sod has not been elucidated, the results of this study suggest that the activity of the extracellular superoxide dismutase might change the activity level of superoxide sensitive Ras proteins through changes in the superoxide generation of internal vesicles. Taking into account that the redox sensitive Cys118 of the NKCD motif found in RasG is also present in

human Ras proteins such as H-Ras, K-Ras, and N-Ras (Heo et al., 2005; Heo, 2011), it would be interesting to find out if mammalian endothelial cells with the mutation on EC-Sod gene exhibit changes in the activity level of superoxide sensitive Ras proteins.

At the plasma membrane, superoxide is generated by the NADPH oxidase (Nox) complex towards the extracellular side of the cell membrane and also inside the luminal side of Nox containing vesicles (Lamb et al., 2009). Ras proteins on the other hand localize to the cytoplasmic side of the membrane. The results of this study indicate that even if Ras proteins and reactive oxygen species localize to different sides of the cell membrane there is a molecular pathway by which Ras activity is affected by an excess of superoxide radicals production across the cell membranes of *Dictyostelium discoideum* cells.

In our *sodC*$^-$ cells the lack of Sod activity and the Nox production of superoxide radicals at the extracellular or internal side of vesicles will cause a charge separation that will rapidly accumulate across the membrane because of the associated generation of protons at the other side of the membrane, and this should in turn discourage the electrogenic superoxide generation. Being that the case, cells should soon stop producing the superoxide radical except if the charge separation is once more equilibrated at the membrane by a different electrogenic process. It has been proposed that in cells from mammalian origin ClC-3 among other ion channels might be involved in radicals transport (Lamb et al., 2009). A possible molecular mechanism to deal with charge separation would be that ClC-3 might create a favorable environment for the superoxide to enter the plasma membrane by electrogenically encouraging the entry of one proton

towards the luminal side of vesicles and transfer of 2 chloride ions toward the cytosol. Because of the acidification of the lumen, it is possible that superoxide might be in a better situation to form hydroperoxyl radical [$HO_2^{\cdot}$]. This way the radical species loses its charge and could potentially go through the membrane and affect Ras proteins susceptible to redox activity since hydroperoxyl molecules separate once more into hydrogen and superoxide in the neutral cytoplasm.

Another possibility is that superoxide radicals could use anion channels such as ClC-3 to pass the membrane and act on cellular sensitive components (Hawkins et al., 2007). Since magnesium is very abundant in our environment, many cell components such as channels and enzymes use it at least partially for their activity which is a clear complication for studies to establish the action mechanisms related to this element. It is possible that superoxide radical production might be discouraged by the presence of magnesium ions if they are able to preserve charge separation associated with superoxide production. The results of these experiments showed that magnesium ions are very important for keeping the superoxide level and controlling the regulation of superoxide sensitive Ras and the derived pathways such as the one leading to chemotaxis in the direction of a cAMP gradient.

By comparing the *wild type* cells with the *rasG*$^{-}$ cells it was evident the importance o this small GTPase as regulator of cell activity in the presence of superoxide. The directional motility of *wild type Dictyostelium* in response to chemoattractant was considerably hindered in media that did not contain magnesium. Chemotatic index (~0.48) and speed both decreased when compared to the control buffer. In the case of

rasG^{-} though, cells were not significantly affected by the lack of magnesium showing a chemotatic index (~0.75) toward cAMP which is very close to the standard expected directionality. This indicates RasG as a sort of sensor to oxidative stress that is going to change the priorities of the cell from directional movement to an adaptive response to deal with harmful radicals.

Oxidative stress is an abnormal state that causes changes in the homeostatic equilibrium of the cells by altering signaling pathways and even possibly leading to a buildup in the number of genomic mutations (Muid et al., 2014, Cooke et al., 2003). Because of this, it could be considered to be helpful for cells to halt or deviate from the normal developmental pathway by stopping chemotaxis in an oxidative stress environment. The excess of the ROS superoxide activated RasG in the context of *Dictyostelium* development can cause profound changes on cells fate. Overactive RasG would cause most of the cells that would normally be prespore cells to rather differentiate into prestalk cells, a fact that was established more than a decade ago in experiments using the constitutively active RasG(G12T) mutant (Jaffer et al., 2001). Given that the prestalk cells will be sacrificed, RasG resulting change in cells fate will ultimately impede the possibly detrimental and random mutations produced by the oxidative stress events from being transmitted to the next generations, that way the genetic pool of social amoeba *Dictyostelium* will not be affected. In this case RasG could be considered a genetic filter that restricts the transmission of mutations created by toxic levels of ROS to the next generation. Together with the observation of *rasG*$^{-}$ cells being greatly insensitive to the magnesium lacking condition that affected *wild type* cells chemotaxis, they were

also observed to display a lesser abundance of intracellular superoxide under this condition. This seems to indicate the possibility of RasG being an upstream regulator of the signaling pathway leading to the generation of the radical species superoxide, which is similar to the case of oncogenic Ras(V12) function in the case of carcinogenesis.

Both mammalian and *Dictyostelium* cells seem to be able to regulate superoxide generation through the small GTPase Ras and keeping a low abundance of superoxide in a magnesium deficient environment seems to help cells function more efficiently. The evolutionary persistence of redox sensitive Ras might be explained by the need of cells to use some radicals as important messengers and the existence of processes such as phagocytosis in which Ras signaling and superoxide production must go together for the benefit of the cell. There is evidence from previous studies that generation of ROS might have a dual purpose, in one hand it can potentially damage cell's integrity in the case of oxidative stress but in the other hand it can also help maintain the redox equilibrium in other situations (Valko et al., 2007, Droge., 2001).

3.7 References

Bhunia, A. K., Han, H., Snowden, A., & Chatterjee, S. (1997). Redox-regulated signaling by lactosylceramide in the proliferation of human aortic smooth muscle cells. *The Journal of Biological Chemistry, 272*(25), 15642-15649.

Bloomfield, G., & Pears, C. (2003). Superoxide signalling required for multicellular development of dictyostelium. *Journal of Cell Science, 116*(Pt 16), 3387-3397.

Brown, P., Barker, A., & Pilbeam, D. (2006). Handbook of plant nutrition. *Handbook of Plant Nutrition,*

Chu, Y., Piper, R., Richardson, S., Watanabe, Y., Patel, P., & Heistad, D. D. (2006). Endocytosis of extracellular superoxide dismutase into endothelial cells: Role of the heparin-binding domain. *Arteriosclerosis, Thrombosis, and Vascular Biology, 26*(9), 1985-1990.

Cooke, M. S., Evans, M. D., Dizdaroglu, M., & Lunec, J. (2003). Oxidative DNA damage: Mechanisms, mutation, and disease. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 17*(10), 1195-1214.

Dröge, W. (2002). Aging-related changes in the thiol/disulfide redox state: Implications for the use of thiol antioxidants. *Experimental Gerontology, 37*(12), 1333-1345.

Hawkins, B. J., Madesh, M., Kirkpatrick, C. J., & Fisher, A. B. (2007). Superoxide flux in endothelial cells via the chloride channel-3 mediates intracellular signaling. *Molecular Biology of the Cell, 18*(6), 2002-2012.

Heo, J. (2011). Redox control of GTPases: From molecular mechanisms to functional significance in health and disease. *Antioxidants & Redox Signaling, 14*(4), 689-724.

Heo, J., Prutzman, K. C., Mocanu, V., & Campbell, S. L. (2005). Mechanism of free radical nitric oxide-mediated ras guanine nucleotide dissociation. *Journal of Molecular Biology, 346*(5), 1423-1440.

Irani, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J., Fearon, E. R., et al. (1997). Mitogenic signaling mediated by oxidants in ras-transformed fibroblasts. *Science (New York, N.Y.), 275*(5306), 1649-1652.

Jaffer, Z. M., Khosla, M., Spiegelman, G. B., & Weeks, G. (2001). Expression of activated ras during dictyostelium development alters cell localization and changes cell fate. *Development (Cambridge, England), 128*(6), 907-916.

Juul, K., Tybjaerg-Hansen, A., Marklund, S., Heegaard, N. H., Steffensen, R., Sillesen, H., et al. (2004). Genetically reduced antioxidative protection and increased ischemic heart disease risk: The copenhagen city heart study. *Circulation, 109*(1), 59-65.

Lamb, F. S., Moreland, J. G., & Miller Jr, F. J. (2009). Electrophysiology of reactive oxygen production in signaling endosomes. *Antioxidants & Redox Signaling, 11*(6), 1335-1347.

Lambeth, J. D. (2004). NOX enzymes and the biology of reactive oxygen. *Nature Reviews Immunology, 4*(3), 181-189.

Lassegue, B., Sorescu, D., Szocs, K., Yin, Q., Akers, M., Zhang, Y., et al. (2001). Novel gp91(phox) homologues in vascular smooth muscle cells : Nox1 mediates angiotensin II-induced superoxide formation and redox-sensitive signaling pathways. *Circulation Research, 88*(9), 888-894.

Leborgne-Castel, N., Lherminier, J., Der, C., Fromentin, J., Houot, V., & Simon-Plas, F. (2008). The plant defense elicitor cryptogein stimulates clathrin-mediated endocytosis correlated with reactive oxygen species production in bright yellow-2 tobacco cells. *Plant Physiology, 146*(3), 1255-1266.

Liou, G., & Storz, P. (2010). Reactive oxygen species in cancer. *Free Radical Research, 44*(5), 479-496.

Lusche, D. F., Wessels, D., Ryerson, D. E., & Soll, D. R. (2011). Nhe1 is essential for potassium but not calcium facilitation of cell motility and the monovalent cation requirement for chemotactic orientation in dictyostelium discoideum. *Eukaryotic Cell, 10*(3), 320-331.

Maeda, H., Yamamoto, K., Nomura, Y., Kohno, I., Hafsi, L., Ueda, N., et al. (2005). A design of fluorescent probes for superoxide based on a nonredox mechanism. *Journal of the American Chemical Society, 127*(1), 68-69.

Mazur, A., Maier, J. A., Rock, E., Gueux, E., Nowacki, W., & Rayssiguier, Y. (2007). Magnesium and the inflammatory response: Potential physiopathological implications. *Archives of Biochemistry and Biophysics, 458*(1), 48-56.

Mori, K., Miyazaki, M., Iwase, H., & Maeda, M. (2002). Temporal profile of changes in brain tissue extracellular space and extracellular ion (na , K) concentrations after cerebral ischemia and the effects of mild cerebral hypothermia. *Journal of Neurotrauma, 19*(10), 1261-1270.

Muid, K. A., Karakaya, H. Ç., & Koc, A. (2014). Absence of superoxide dismutase activity causes nuclear DNA fragmentation during the aging process. *Biochemical and Biophysical Research Communications, 444*(2), 260-263.

Oakley, F. D., Abbott, D., Li, Q., & Engelhardt, J. F. (2009). Signaling components of redox active endosomes: The redoxosomes. *Antioxidants & Redox Signaling, 11*(6), 1313-1333.

Suh, Y., Arnold, R. S., Lassegue, B., Shi, J., Xu, X., Sorescu, D., et al. (1999). Cell transformation by the superoxide-generating oxidase Mox1. *Nature, 401*(6748), 79-82.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology, 39*(1), 44-84.

Veeranki, S., Kim, B., & Kim, L. (2008). The GPI-anchored superoxide dismutase SodC is essential for regulating basal ras activity and for chemotaxis of dictyostelium discoideum. *Journal of Cell Science, 121*(Pt 18), 3099-3108.

Weglicki, W. B. (2012). Hypomagnesemia and inflammation: Clinical and basic aspects. *Annual Review of Nutrition, 32*, 55-71.

Wu, R. F., & Terada, L. S. (2009). Ras and nox: Linked signaling networks? *Free Radical Biology and Medicine, 47*(9), 1276-1281.

CHAPTER 4 DISCUSSION

The ROS production in eukaryotic cells can be linked to a number of different processes and the tight control of these molecules is an important factor of the cell's homeostasis. A number of cells use these radical species for their advantage, such as in the case of signaling or the degradation of internalized particles. When the control mechanisms, that include enzymatic and non enzymatic agents, fail to maintain the levels of radicals in a permissible narrow range, oxidative stress takes place (Finkel et al., 2000; Apel et al., 2004; Buttke et al., 1994). Proteins among other macromolecules have the potential to be the selective target of ROS when their redox sensitive residues, such as cysteine, are exposed to the radicals. It has been found *in vitro* that the small GTPase Ras is one of the proteins that can be a target of radical species (Heo et al, 2005; Heo, 2011). In this report we described that a NKCD motif containing RasG and RasS were dysregulated in *Dictyostelium discoideum* cells that lack *SodC* and the Ras dysregulation was largely rescued by the presence of extracellular magnesium ions.

4.1 Ras proteins that contain the NKCD motif are susceptible to misregulation in *sodC*$^-$ cells

Ras proteins control multiple cellular events such as growth, differentiation, movement, and apoptosis among many others (Wittinghofer et al., 2000). *Dictyostelium* cells contain multiple Ras proteins that not only share high degree of sequence homology with those of higher eukaryotes, but also multiple conserved downstream signaling pathways and functional characteristics (Robbins et al., 1989). It has been known for several decades that Ras proteins activity can be constitutively enhanced or inhibited by

single amino acid substitutions (Smit et al., 1988; Zekri et al., 1964). In addition to the classical substitution mutations, NKCD is another motif that can affect Ras activity as shown in this study. This motif is located in the guanine base binding domain of the protein and it has been shown *in vitro* to mediate activation of Ras in the presence of abundant superoxide (Heo, 2011). Little is known about the mechanism of superoxide mediated Ras activation *in vivo*. In our lab we have created a mutant strain with ablation of the *SodC* gene. These cells show several phenotypic aberrations that include motility defects, modestly increased intracellular superoxide, and constitutively increased Ras activity (Veeranki et al., 2008). RasG is one of the best characterized Ras in *Dictyostelium* cells and like a few others, contains the NKCD motif and was previously shown to be misregulated in *sodC*$^-$ cells.

We analyzed several NKCD or NKAD containing Ras proteins in *Dictyostelium* and compared their activities in *wild type JH10* cells and *sodC*$^-$ cells. RasG and RasS proteins, which contain NKCD motif, displayed high activity in the *sodC*$^-$ background. The level of misregulation was different between the two proteins with RasG showing the greatest increase and RasS showing a smaller but significant increase. Considering that RasS proteins contain not only the NKCD motif, but also the P-loop cysteine, which was suggested to play an inhibitory role upon interaction with superoxide by *in vitro* studies (Heo et al., 2005), we speculate that RasS proteins are receiving two antagonistic effects from superoxide and thus exhibit only modest effect. On the other hand, the NKAD containing RasD and RasC showed no significant activity difference in *wild type* versus *sodC*$^-$ cells.

4.2 The cysteine^{118} of NKCD confers redox sensitivity to *Dictyostelium* Ras proteins

To further uncover the mechanism of NKCD mediated redox regulation of Ras proteins we decided to generate a series of substitution mutations: NKCD to NKAD in RasG and RasS, and the NKAD into NKCD in RasD through site-directed mutagenesis at the 118th position of these proteins. The difference of the basal activities of RasG and RasG(C118A) in *sodC*$^-$ cells were clearly observed, indicating the positive role of NKCD motif in RasG regulation. However, the RasG(C118A) substitution mutant displayed still higher basal activity in *sodC*$^-$ cells than in *wild type* cells, indicating that RasG specific regulators are likely redox sensitive. RasS(C118A), in contrast, exhibited modestly decreased basal activities in *sodC*$^-$ cells compared to *wild type* cells, indicating that either the P-loop is exerting an inhibitory effect and no RasS specific regulators are redox sensitive. Additionally, *sodC*$^-$ cells expressing RasG(C118A) were able to respond to a cAMP gradient with significantly better directionality indicating that RasG downstream effector pathways are at least partially rescued. It has been shown that RasG can activate PI3K, and this leads to the generation of PtdIns(3,4,5)P3 as part of a pathway controlling cytoskeletal rearrangements and chemotaxis (Funamoto et al., 2002; Sasaki et al., 2006). The fact that RasG(C118A) is still more active in *sodC*$^-$ cells suggest that additional regulators such as GefR, a RasG specific Guanine nucleotide exchange factor, or NF1, a Ras GAP, are likely affected by oxidative stress. RasD is very close in sequence to RasG and it has been published previously to possess several overlapping functions with RasG such as growth (Khosla et al., 2000). RasD(A118C) basal activity level was significantly higher in *sodC*$^-$ cells compared to the *wild type* background strongly suggest that the NKCD motif is the main redox sensor of Ras protein *in vivo*. Among the future directions

we consider that a double knockout *sodC*$^-$/*rasG*$^-$ would be a great tool to further explore how central is RasG in the adverse phenotypic defects observed in *sodC*$^-$ cells. In addition, ablation of some of the Ras regulators such as *RasGEFR* will also generate interesting insights into the redox mediated Ras activation observed in this cellular background.

4.3 Magnesium ions are important to maintain redox integrity in *Dictyostelium discoideum*

Metal ions are very important for different functions at the cellular level and changes in their availability can be harmful to cells and tissues (Hasegawa et al., 2000; Beaven et al., 1990). Lack of magnesium in particular has been previously shown to be involved in pathological conditions such as hypertension in humans (Touyz, R M., 2003; Altura et al., 1984). In particular, at the cellular level magnesium deficiency has been implicated in the propensity of certain tissues to oxidative stress (Freedman et al., 1991). With the help of the superoxide specific probe BES-So-AM (Maeda et al., 2005) we were able to monitor superoxide accumulation in intracellular vesicular compartments. In the standard DB buffer condition, the *wild type* cells displayed ~1-2 μm size superoxide positive vesicles, but more enlarged superoxide positive vesicles and vacuoles (up to ~5 μm) were observed in *sodC*$^-$ cells. In *wild type* cells under magnesium or phosphate lacking medium such as H_2O or NaCl, enlarged superoxide positive vesicles were observed. These results imply that similar to other organism models (Hans et al., 2003), lack of magnesium encourages the generation of ROS in *Dictyostelium*.

4.4 W*ild type* under magnesium lacking medium displayed higher basal Ras activity and aberrant subcellular localization.

Observing that the magnesium deficient media stimulated vesicular accumulation of superoxide in *wild type* cells, it seemed likely that Ras proteins may be affected under these superoxide-accumulating conditions in *wild type* cells. *Wild type* cells under H_2O or NaCl, but not under magnesium media, exhibited higher basal Ras activity. Furthermore, only RasG, but not RasC, was affected by the absence of magnesium and normal cAMP induced activation of Ras proteins were observed from both *wild type* and *sodC*$^-$ cells under magnesium medium. In addition, *wild type* cells, but not *rasG*$^-$ cells, displayed significantly compromised chemotaxis under magnesium lacking condition such as H_2O or NaCl media. We propose that RasG is central in redox regulation of cellular events under oxidative stress conditions. We are confident that the findings reported in this study are invaluable to multiple scientific communities as well as the public in general considering that the oxidative conditions are one of the common phenomena accompanying multiple pathological aberrancies, whether being the cause or the effects, and that Ras proteins are one of the critical proteins that can drive multiple disease conditions.

4.5 References

Altura, B. M., Altura, B. T., Gebrewold, A., Ising, H., & Gunther, T. (1984). Magnesium deficiency and hypertension: Correlation between magnesium-deficient diets and microcirculatory changes in situ. *Science (New York, N.Y.), 223*(4642), 1315-1317.

Apel, K., & Hirt, H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. *Annu.Rev.Plant Biol., 55*, 373-399.

Beaven, G., Parmar, J., Nash, G., Bennett, P., & Gratzer, W. (1990). Effect of magnesium ions on red cell membrane properties. *The Journal of Membrane Biology, 118*(3), 251-257.

Buttke, T. M., & Sandstrom, P. A. (1994). Oxidative stress as a mediator of apoptosis. *Immunology Today, 15*(1), 7-10.

Finkel, T., & Holbrook, N. J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature, 408*(6809), 239-247.

Freedman, A. M., Cassidy, M. M., & Weglicki, W. B. (1991). Magnesium-deficient myocardium demonstrates an increased susceptibility to an in vivo oxidative stress. *Magnesium Research : Official Organ of the International Society for the Development of Research on Magnesium, 4*(3-4), 185-189.

Funamoto, S., Meili, R., Lee, S., Parry, L., & Firtel, R. A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell, 109*(5), 611-623.

Hans, C. P., Chaudhary, D. P., & Bansal, D. D. (2003). Effect of magnesium supplementation on oxidative stress in alloxanic diabetic rats. *Magnesium Research, 16*(1), 13-19.

Hasegawa, H., Suzuki, K., Suzuki, K., Nakaji, S., & Sugawara, K. (2000). Effects of zinc on the reactive oxygen species generating capacity of human neutrophils and on the serum opsonic activity in vitro. *Luminescence, 15*(5), 321-327.

Heo, J. (2011). Redox control of GTPases: From molecular mechanisms to functional significance in health and disease. *Antioxidants & Redox Signaling, 14*(4), 689-724.

Heo, J., & Campbell, S. L. (2005). Mechanism of redox-mediated guanine nucleotide exchange on redox-active rho GTPases. *The Journal of Biological Chemistry, 280*(35), 31003-31010.

Heo, J., & Campbell, S. L. (2005). Superoxide anion radical modulates the activity of ras and ras-related GTPases by a radical-based mechanism similar to that of nitric oxide. *The Journal of Biological Chemistry, 280*(13), 12438-12445.

Khosla, M., Spiegelman, G. B., Insall, R., & Weeks, G. (2000). Functional overlap of the dictyostelium RasG, RasD and RasB proteins. *Journal of Cell Science, 113 (Pt 8)*(Pt 8), 1427-1434.

Maeda, H., Yamamoto, K., Nomura, Y., Kohno, I., Hafsi, L., Ueda, N., et al. (2005). A design of fluorescent probes for superoxide based on a nonredox mechanism. *Journal of the American Chemical Society, 127*(1), 68-69.

Robbins, S. M., Williams, J. G., Jermyn, K. A., Spiegelman, G. B., & Weeks, G. (1989). Growing and developing dictyostelium cells express different ras genes. *Proceedings of the National Academy of Sciences of the United States of America, 86*(3), 938-942.

Sasaki, A. T., & Firtel, R. A. (2006). Regulation of chemotaxis by the orchestrated activation of ras, PI3K, and TOR. *European Journal of Cell Biology, 85*(9), 873-895.

Smit, V. T., Boot, A. J., Smits, A. M., Fleuren, G. J., Cornelisse, C. J., & Bos, J. L. (1988). KRAS codon 12 mutations occur very frequently in pancreatic adenocarcinomas. *Nucleic Acids Research, 16*(16), 7773-7782.

Touyz, R. (2003). Role of magnesium in the pathogenesis of hypertension. *Molecular Aspects of Medicine, 24*(1), 107-136.

Veeranki, S., Kim, B., & Kim, L. (2008). The GPI-anchored superoxide dismutase SodC is essential for regulating basal ras activity and for chemotaxis of dictyostelium discoideum. *Journal of Cell Science, 121*(Pt 18), 3099-3108.

Wittinghofer, A., & Waldmann, H. (2000). Ras—a molecular switch involved in tumor formation. *Angewandte Chemie International Edition, 39*(23), 4192-4214.

Zekri, J., Rizvi, A., Al-Maghrabi, J., & bin Sadiq, B. (1964). K-ras in colorectal cancer tumors from saudi patients: Frequency, clinco-pathological association and clinical outcome. *Differentiation, 14*(19), 74.

VITA

BORIS CASTILLO CHABECO

	Born, Santiago de Cuba, Cuba
2007-2009	B.S. Biology Florida International University Miami, Florida, USA
2009	Graduate Professional Minority Students Fellowship
2009-2010	Teaching Assistant Florida International University Miami, Florida, USA
2010-2015	MBRS RISE fellowship
2009-2015	Doctoral Candidate Florida International University Miami, Florida, USA

PUBLICATIONS AND PRESENTATIONS

Castillo Chabeco, B and Kim, L. (2011). Regulation of the Small GTPase Ras by Reactive Oxygen Species. Current Cellular Biology 1(1): 15-19.

Castillo Chabeco, B. and Kim, L. (2013). Ras activity under high levels of Reactive Oxygen Species *in vivo* . FIU MARC U*STAR & MBRS Mini-Symposium, Florida International University, Miami, FL (Oral Presentation)

Castillo Chabeco, B. and Kim, L. (2013). Activation mechanism of the small GTPase Ras in response to Reactive Oxygen Species *in vivo*. 15th Biomedical and Comparative Immunology Symposium, Florida International University, Miami, FL (Oral Presentation)

Castillo Chabeco, B. and Kim, L. (2012). Ras activity under high levels of Reactive Oxygen Species *in vivo* . FIU MARC U*STAR & MBRS Mini-Symposium, Florida International University, Miami, FL (Oral Presentation)

Castillo Chabeco, B. and Kim, L. (2012). Ras regulation by reactive oxygen species in vivo. 2012 Biology Research Symposium, Florida International University, Miami, FL (Poster Presentation)

Castillo Chabeco, B. and Kim, L. (2011). Mechanism of Reactive Oxygen Species mediated Ras regulation *in vivo*. FIU MARC U*STAR & MBRS Mini-Symposium, Florida International University, Miami, FL (Oral Presentation)

Castillo Chabeco, B. and Kim, L. (2011). Determination of the small GTPase Ras species misregulated by Reactive Oxygen Species *in vivo*. 2011 Biology Research Symposium, Florida International University, Miami, FL (Oral Presentation)

Castillo Chabeco, B. Rodriguez, M and Kim, L. (2010). Ras PP2A/B56 is essential for proper cell motility. Annual Meeting of The American Society for Cell Biology, Philadelphia, PA (Poster Presentation)

Castillo Chabeco, B. and Kim, L. (2010). Determination of the mechanism of free radical mediated Ras activation *in vivo*. FIU MARC U*STAR & MBRS Mini-Symposium, Florida International University, Miami, FL (Oral Presentation)