EXCTRACELLULAR POLYPHOSPHATE ACCUMULATION INHIBITS PROLIFERATION IN *DICTYOSTELIUM DISCOIDUEM*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Extracellular Polyphosphate Accumulation Diminishes Proliferation in *Dictyostelium Discoideum*

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Cancer is a collection of diseases that display abnormal cell proliferation with the potential to spread throughout the body. Much remains to be understood about the regulation of cell proliferation. Regulation of proliferation is difficult to study in complex mammalian systems, but can be studied using a simpler system such as the model organism *Dictyostelium discoideum*. Using *Dictyostelium* we have discovered that inorganic polyphosphate is acting as a signal to help regulate cell proliferation. This research project focuses on regulation of cell proliferation in *Dictyostelium* and consists of examining the phenotypes of mutants that we have identified as having abnormal responses to polyphosphate, measuring the extracellular polyphosphate accumulation of existing mutants to see if polyphosphate is being synthesized by these mutants, and assaying identified mutants for binding to polyphosphate using biotinylated polyphosphate. My work will elucidate the mechanism whereby polyphosphate regulates cell proliferation in *Dictyostelium*. This research will then hopefully shed light on how cell proliferation is regulated in mammalian systems. It was found that tested mutants reacted as either hypersensitive, normal response, or exhibited resistance to polyphosphates inhibitory proliferation signal. Cells lacking PPK1, an enzyme responsible for the production of intracellular polyphosphate, exhibited complete resistance to inhibition suggesting that intracellular polyphosphate is needed for the inhibition of proliferation by extracellular polyphosphate. Binding assays provided evidence that

hypersensitive mutants are becoming saturated with polyphosphate at lower concentrations suggesting that these mutants may play a role in receptor regulation or the intracellular regulation mechanism. Extracellular polyphosphate synthesis concentrations were recorded and this data suggested that initial exposure to increasing concentrations of endogenous polyphosphate decreases polyphosphate production from *Dictyostelium*. Through testing the identification of mutants as well as binding and synthesis of polyphosphate in *Dictyostelium*, the effects on the regulation of cell proliferation in addition to the mechanism behind this regulation in mammalian systems may be learned and applied to areas such as cancer treatment.

DEDICATION

I dedicate this research project to my parents. Without their support this opportunity would not have been possible.

ACKNOWLEDGMENTS

I would like to thank Dr. Richard Gomer and graduate student Patrick Suess for their support. Their teaching and assistance has been imperative in the production of this thesis and to my continued education as a student and individual.

NOMENCLATURE

PolyP	Polyphosphate
Dicty	*Dictyostelium Discoideum*
DAPI	4', 6-diamidino-2-phenylindole
PPK1	Polyphosphate Kinase 1
Alexa	alexa 647-streptavidin conjugated
PBM	Polyphosphate Buffered Media
GPCR	G Protein Coupled Receptor
HTGP	Heterotrimeric G protein
S/T Kinase	Serine/Threonine Kinase
TORC2	Tor Complex 2
PH domain protein	Pleckstrin homology domain protein
TF	Transcription Factor

CHAPTER I

INTRODUCTION

Polyphosphate as a regulator of cell proliferation

Regulation of cell proliferation is an area of research that can have tremendous impact into the treatment of diseases such as cancer. If a means to regulate cell proliferation were discovered, then diseases that are characterized by uncontrollable cell proliferation may be slowed or stopped altogether. An intriguing possible way to slow or stop abnormal cell proliferation would be to treat a patient with an autocrine factor that represses cell proliferation. Such autocrine proliferation-inhibiting factors are called chalones (1). Despite evidence for their existence in mammalian systems, chalones have generally not been identified. The term chalone was first used about fifty years ago to represent autocrine growth inhibiting factors with a tissue specific and reversible effect (9). Some chalones that have been discovered and worked with in recent years are AprA and CfaD in *Dictyostelium*; cells without either AprA or CfaD would proliferate faster than wild type cells while AprA or CfaD overexpressors would proliferate at a slower rate. These factors secreted by the proliferating cells themselves could function in a negative feedback loop to regulate the density of cells in a tissue or the size of a group of cells (10, 11, 12).

Structure and testing of Polyphosphate

The Gomer lab recently found that polyP also appears to be a chalone in *Dictyostelium*. PolyP consists of a linear chain of tens or hundreds of orthophosphate (P_i) residues joined by highenergy phosphoanhydride bonds such as the ones observed in ATP (2). Much research has been completed concerning the function of PolyP in prokaryotes, but in recent years PolyP properties and mechanisms in eukaryotes have attracted the interest of many researchers. This

new interest has helped lead to many discoveries such as the function of Polyphosphate Kinase 1 (PPK1) in eukaryotes. PPK1 homologs are found in only a few eukaryotes, one being the PPK1 homolog in the model organism *Dictyostelium* (3,4). While several enzymes have been found to contribute to the synthesis of PolyP, PPK1 catalyzes the change of the terminal phosphate in ATP into a PolyP chain that takes place in a reversible and an immensely processive reaction (5). PPK is found in bacteria as well as *Dictyostelium* that feed on bacteria; this suggests the possibility that *Dictyostelium* may have acquired a copy of the bacterial PPK gene through the process of horizontal gene transfer (6). Extracellular levels of PolyP can be readily measured using a fluorometric assay and the dye DAPI. DAPI staining coupled with flow cytometry analysis has been used to measure intracellular inorganic polyphosphate in mammalian cell populations (7,8). PolyP binding can be measured by mixing *Dictyostelium* with biotinylated PolyP, washing, taking the cell pellet, and examining the level of biotin remaining. This procedure allows the levels of PolyP bound to cells to be measured (13). My work characterizing mutants will provide insight into the function of chalones in eukaryotic organisms which will hopefully lead to discoveries of signals involving the regulation of cell proliferation.

Applications of polyphosphate research

As mentioned, the Gomer lab has evidence for polyphosphate acting as a chalone in Dicty; my research would provide new insight into not only the function of inorganic polyphosphate, the effects of inorganic polyphosphate accumulating in Dicty cells, but also provide additional insight into the function of chalones in eukaryotic organisms. Chalones are an important element in understanding the regulation of cell proliferation, and ultimately, a possible treatment for cancer. Any new information that could be discovered in this area of research would constitute an enormous impact in the scientific and medical domains.

CHAPTER II

METHODS

Cell culture and polyphosphate response by mutants.

Multiple mutant strains of *Dictyostelium Discoideum* were used in determining the effect of polyphosphate on cell proliferation. The strains AX2 wild-type*, crlA, gα4-, gα8-, bzpN-, sgcA/gcA-, piaA-, rasC-/G-, gα1-, akt1-/2-, pkbA-, lst8-, dag A-,* and *qkgA-* were used in the testing of extracellular polyphosphate accumulation in *Dictyostelium*. All strains were obtained from dictybase.org. Frozen stocks of cells were grown on lawns of bacteria, then transferred to liquid shaking cultures in the standard growth media HL5 (Formedium). Fresh cultures were started every 3-4 weeks. In measuring the effect of polyphosphate on cell proliferation the mutants were tested with concentrations (0μM-150μM) to determine the response that different levels of polyphosphate would have on cell proliferation. Mid-log cells were cultured at $1x10^6$ cells/mL in the presence or absence of polyphoshate. Cell density was measured daily using a hemocytometer over a five-day span. Mutant responses to polyphosphate were compared to the response of wild type cells.

Conditioned media samples and DAPI assay.

To measure polyphosphate degradation, conditioned media samples were taken daily over a 5 day period and DAPI assays were performed using the Gen5 2.05 program and a Biotek SynergyMx plate reader. Mid-log cells were seeded at 1×10^6 cells/mL in SIH media (Formedium) in the presence or absence of polyphosphate. Conditioned media (700 μL) was harvested by centrifugation at 9500 X G for 10 minutes at 4°C in a micro centrifuge. 500 μL of

supernatant was saved at 4°C. These collection steps were repeated daily for days 1-5. To remove proteins and large molecules samples were filtered using Amicon Ultra 10kDa size exclusion filters and spun down at 14,000 X G for 15 minutes at 4°C and the filtrate was saved. 180 µL of each filtered supernatant and 20 µL (0.25µg/mL) of DAPI were incubated in 96 well black plates for 5 minutes. Fluorescence was measured using an excitation wave length of 415nM and emission wavelength of 550nM, shown to be specific for polyphosphate. The amount of polyphosphate remaining in the extracellular environment was determined over a 5 day period, allowing for measurement of extracellular polyphosphate over time.

Binding assays and flow cytometry

To measure levels of polyphosphate bound to *Dictyostelium* cells, binding assays using biotinylated polyphosphate, alexa 647-streptavidin conjugated (alexa), and mutant cells were analyzed on a flow cytometer. 2.5×10^6 mid-log cells were collected by centrifugation at 500 X G for 3 minutes. The cell pellet was washed 2x in ice-cold PBM, then resuspneded in 1ml of ice cold PBM. Samples containing cells, biotinylated polyphosphate, and alexafluor 647 were incubated in 200µL volumes (brought up to volume using ice-cold PBM) and were incubated on ice for 2-5 minutes. 1mL of ice cold PBM was then added. The cells with the bound polyphosphate were then collected by centrifugation at 1,000 X G for 3 minutes. The cell pellet was re-suspended in 1mL of ice cold PBM and mixed well. This solution was then analyzed on the flow cytometer to measure binding of biotinylated polyphosphate to mutants and determine if the effect on cell proliferation is due to failure to degrade polyphosphate or due to an inability to sense it.

CHAPTER III

RESULTS

Identifying mutants responsible for inhibition

Preliminary studies with *Dictyostelium Discoideum* have shown that proliferating *Dictyostelium* cells secrete polyphosphate which acts as a regulator of cell proliferation signaling to the cell when to enter stationary phase and stop proliferating. Additional studies have found that when wild-type Mid-log cells were cultured in the presence of polyphosphate strong inhibition of proliferation was exhibited (Figure 1). To identify the signaling components responsible for the previously observed inhibition of proliferation, gene knockouts were performed on *Dictyostelium* cells to create mutants. These mutants were tested with varied concentrations of polyphosphate (0μM-150μM) and placed into one of three categories based on their responses: hypersensitive, normal response, or resistant (Table 1). Hypersensitive mutants experienced intense inhibition when exposed to polyphosphate and achieved lower densities than the wild-type control. Normal response mutants showed growth and inhibition that mirrored or was close to the wild-type control. Resistant mutants' growth was not affected by the inhibition mechanism or was affected in a lesser amount than that of the wild-type control (Figure 1). Of the twenty mutants tested *PPK1*$^{-}$ was the only mutant to show full resistance to the inhibition mechanism that is enacted on *Dictyostelium* cells upon exposure to extracellular polyphosphate (Table 1).

Figure 1

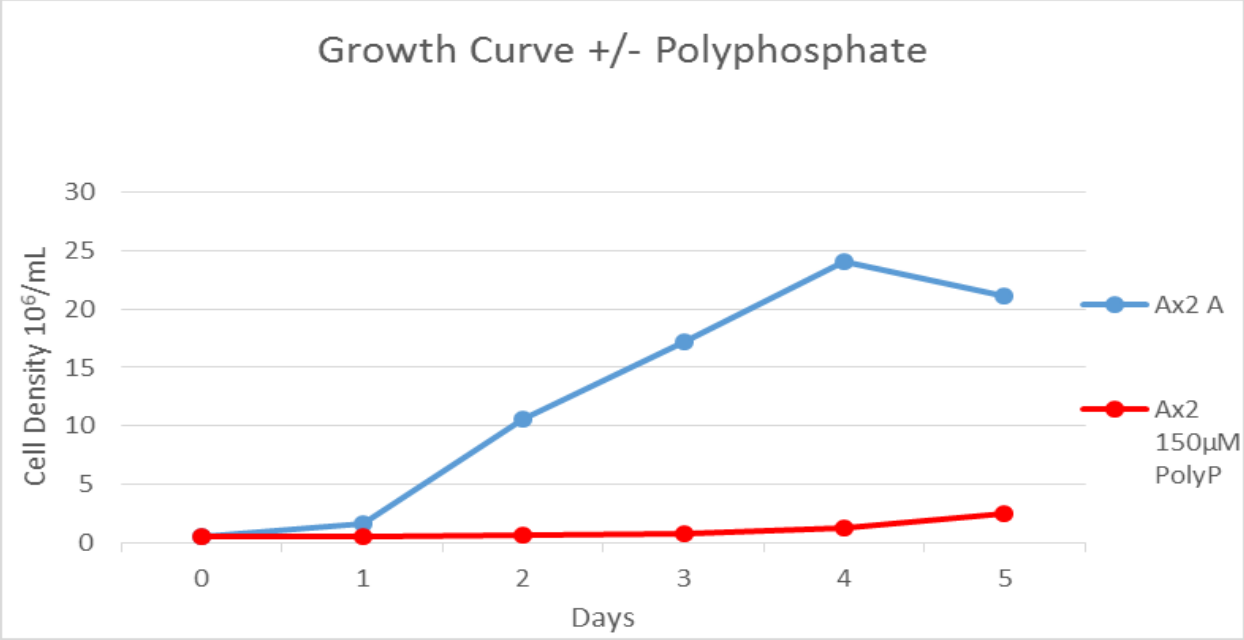

Figure 1: Wild type cells responding to polyphosphate. Growth Curve of wild type cells treated with 0 µM and 150 µM polyphosphate to show the effect on proliferation. Mid-log cells were cultured at $1x10^6$ cells/mL in the presence or absence of polyphosphate. Cell density was measured daily using a hemocytometer over a five-day span. Mutant responses to polyphosphate were compared to the response of wild type cells.

Table 1

Dictyostelium Mutant Responses to Polyphosphate		
Mutant	Description	Response to polyphosphate
crlA$^-$	GPCR	Hypersensitive
akt1$^-$/*2*$^-$	S/T Kinase	Hypersensitive
pkbA$^-$	S/T Kinase	Hypersensitive
lst8$^-$	TORC2	Hypersensitive
GrH$^-$	GPCR	Hypersensitive
FSL B$^-$	GPCR	Hypersensitive

dagA^{-}	PH Domain Protein	Slight Hypersensitive
gα4^{-}	HTGP	Normal
gα8^{-}	HTGP	Normal
bzpN^{-}	TF	Normal
gα1^{-}	HTGP	Normal
GrB^{-}	GPCR	Normal
GrD^{-}	GPCR	Normal
GrE^{-}	GPCR	Normal
FSL K^{-}	GPCR	Normal
qkgA^{-}	Roco Kinase	Slight Resistance
rasC^{-}*/ rasG*$^{-}$,	Ras Protein	Resistance
piaA^{-}	TORC2	Resistance
PPk1^{-}	Kinase	Complete Resistance

Table 1: Polyphosphate effects proliferation in different mutants. All strains were obtained from dictybase.org. Frozen stocks of cells were grown on lawns of bacteria, then transferred to liquid shaking cultures in the standard growth media HL5 (Formedium). In measuring the effect of polyphosphate on cell proliferation the mutants were tested with concentrations (0μM150μM). Mid-log cells were cultured at $1x10^6$ cells/mL in the presence or absence of polyphosphate. Descriptions of mutants being tested were found on dictybase.org.

Concentration of Polyphosphate Binding to Cell

The molecule polyphosphate inhibits cell proliferation. The 2 mutants selected for analysis have been previously tested and determined to be hypersensitive mutants in regards to inhibiting their proliferation rates in the presence of polyphosphate. (Table 1) This gave rise to the question as to what made these mutants unique and this was attempted to be resolved by measuring the binding of biotinylated polyphosphate to the cells to examine concentrations that were bound. Wild type cells (AX2) were also measured alongside these mutants to use as a control. In measuring the binding ability of the two selected mutants to polyphosphate, the mutants were tested with concentrations (0μM-150μM) of biotinylated polyphosphate to allow for fluorescence to be measured using a flow cytometer. Polyphosphate binds to the cell with a biotin attached to it, we then incubate with a fluorescent molecule that has streptavidin attached. Biotin and streptavidin bind each other very strongly. Fluorescence will only be seen if the PolyP-biotin-streptavidinfluorophore has bound to the cell. These values can then be compared between the wild-type and hyper sensitive mutants as well as across a concentration gradient of added polyphosphate. Between wild-type and hypersensitive mutants a pattern was observed that held true at all concentrations. This pattern is that hypersensitive mutants need to bind less polyphosphate than wild-type in order to become saturated. Although concentrations differed each strain reached its highest fluorescence at 100μM of added polyphosphate suggesting that *Dictyostelium* becomes saturated by polyphosphate at this concentration (Figure 2). This data raised questions as hypersensitive mutants required less polyphosphate, but displayed a stronger inhibition response when tested on a growth curve (Table 1)

Figure 2

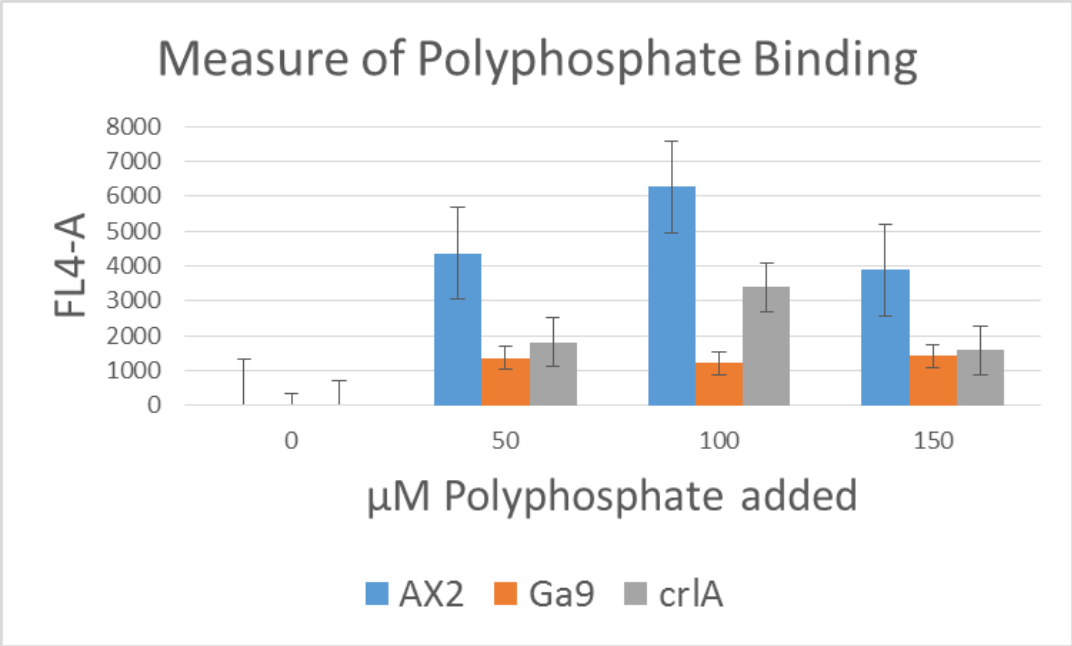

Figure 2: Measure of polyphosphate bound to *Dictyostelium* using fluorescence. Cells of the indicated genotype in SIH media were used to prepare samples using biotinylated polyphosphate (0µM-150µM), and alexafluor 647. This solution was then analyzed on the flow cytometer to measure binding of biotinylated polyphosphate. Fluorescence was measured as FL4-A on a flow cytometer and this data was then grouped by concentration and mutant to compare. Initial fluorescence at 0µM was set to 0 and the corresponding amount was removed from each strains fluorescence measurements at 50µM, 100 µM, and 150 µM to account for other fluorescing molecules that were present.

Measuring cellular production and degradation of polyphosphate. Preliminary studies have found that *Dictyostleium Discoideum* produce polyphosphate as a chalone as they proliferate and increase in density. The mutants *gα1*$^{-}$, *gα9*$^{-}$, and *CrlA*$^{-}$ as well as wild-type cells were tested for extracellular polyphosphate levels using DAPI assays. These tests were performed in order to see if adding initial concentrations of polyphosphate (0μM-150μM) would affect the rate of extracellular polyphosphate production in *Dictyostelium.* It was observed that as concentration of polyphosphate added to *Dictyostelium* increased, extracellular polyphosphate production by the cells slowed, halted (Figure 3B), and at high concentrations of polyphosphate converted metabolism of polyphosphate into degradation of polyphosphate (Figure 3C). In regards to the data compared between mutants, *gα1*$^{-}$ showed lower levels of polyphosphate metabolism at 0μM added polyphosphate while other samples grew in accordance with wild-type (Figure 3A). At 115μM added polyphosphate, mutants exhibited similar responses being a halt in polyphosphate metabolism with a near constant level present in the samples taken over three days (Figure 3B). At 150 μM added polyphosphate, metabolism was no longer present and all samples demonstrated degradation of extracellular polyphosphate with *gα9*$^{-}$ showing the strongest degradation (Figure 3C).

Figure 3A

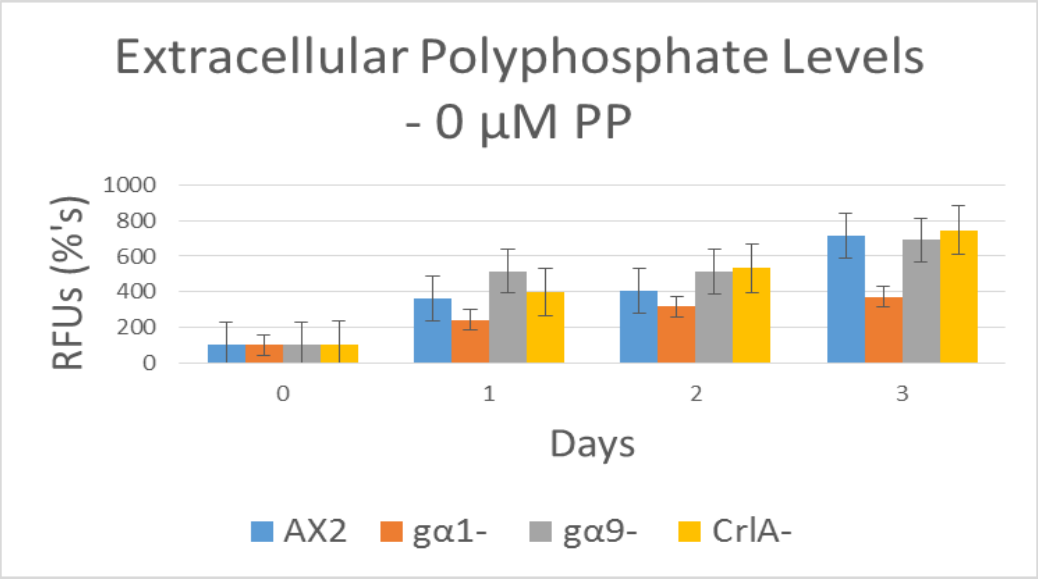

Figure 3B

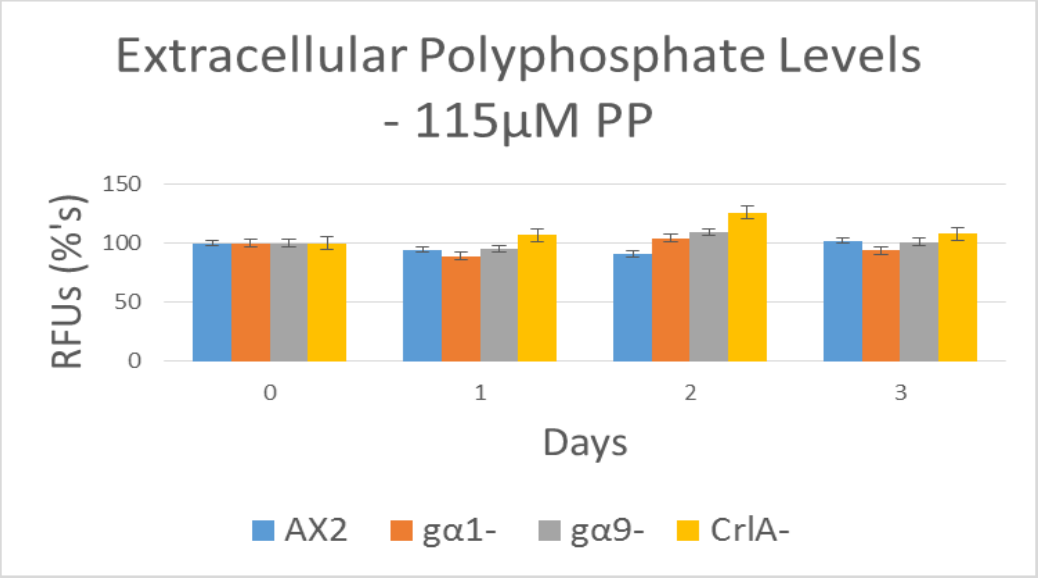

Figure 3C

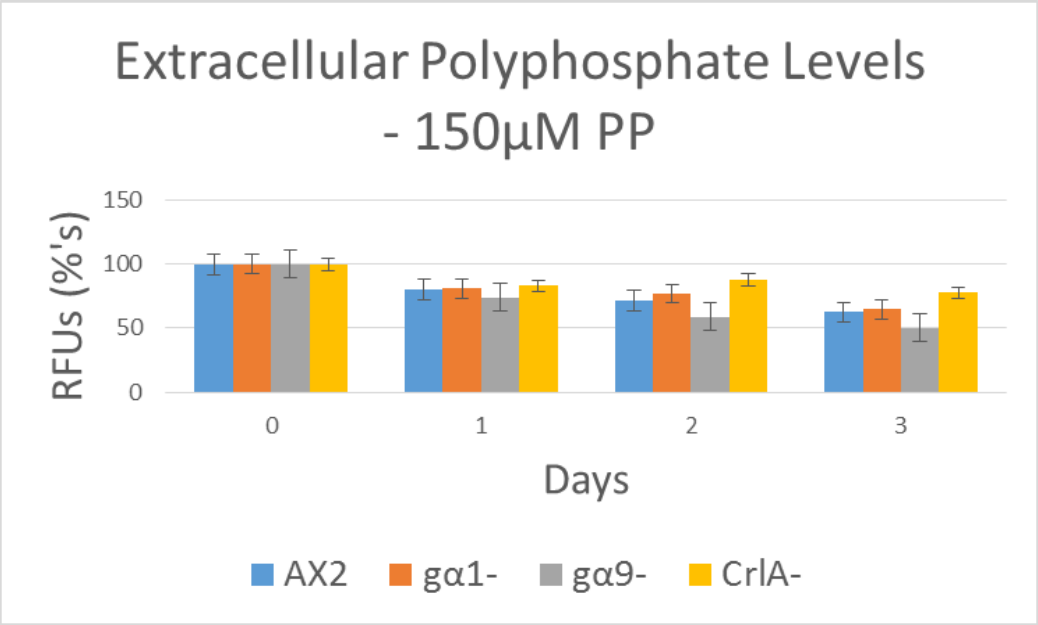

Figure 3. Degradation of extracellular polyphosphate production. Mid-log cells of the indicated genotyped were seeded in SIH media (Formedium) in the presence of absence of polyphosphate. Conditioned media was harvested by centrifugation and stored over 5 days. These samples were then filtered and tested for fluorescence using a DAPI assay on SynergyMx plate reader using the Gen5 2.05 program. DAPI fluoresces and binds to polyphosphate allowing a mechanism for extracellular polyphosphate concentrations to be measured. **(A)** 0µM of polyphosphate was added to each sample and measured over five days **(B)** 115µM **(C)** 150µM .

CHAPTER IV

CONCLUSIONS

The results presented support the function of inorganic polyphosphate as a proliferation inhibitor. Additionally, the findings presented for identifying mutants that affect the response to polyphosphate show that the kinase PPK1 is necessary in the polyphosphate proliferation inhibition signaling pathway. Furthermore, these results show that proteins CrlA, Akt1/2, PkbA, Lst8, GrH, FSL B, DagA, Gα4, Gα8, BzpN, Gα1, Rnsc/G, GrB, GrD, GrE, and FSL K are not necessary for polyphosphate's inhibitory effect while QkgA, RasC/ RasG, and PiaA may be involved in lesser degrees. The involvement of the kinase PPK1 in the proliferation inhibition signaling pathway was unexpected, as PPK1's function is to produce intracellular polyphosphate (3,4) and previous data suggested that the inhibition pathway involves only extracellular polyphosphate. These new data suggest that there is an intracellular component of polyphosphate to the proliferation inhibition pathway.

The findings from determining polyphosphate binding to cells, using different *Dictyostelium* strains, show that the two hypersensitive mutants tested bind less polyphosphate than wild type cells. As polyphosphate provides an inhibitory effect on proliferation, this suggested that the hypersensitive mutants, for unknown reasons, could be binding less polyphosphate due to a reduction in the number of receptors present on the cell surface.

The findings from measuring cellular production and degradation of extracellular polyphosphate, using DAPI assays, show that as extracellular polyphosphate levels increase, polyphosphate

synthesis slows, stops, and switches to degradation at high concentrations (150 μM). When samples were exposed to 0 μM of extracellular polyphosphate initially, the extracellular levels increased up to 700% over 3 days indicating that synthesis of polyphosphate was being carried out by the cell. When samples were exposed to 115 μM of extracellular polyphosphate, the extracellular levels stayed constant over 3 days, indicating no net synthesis or degradation of polyphosphate. When samples were exposed to 150 μM of extracellular polyphosphate, the extracellular levels decreased from 100% to as low as 50% over 3 days, suggesting that this high concentration of polyphosphate induced polyphosphate degradation or uptake by cells. Together, these results suggest that *Dictyostelium* cells can sense extracellular polyphosphate levels in their environment and adjust their own output accordingly.

The data uncovered through the culmination of the three experiments run for this project integrates to form new inferences about polyphosphate's effects and the mechanism behind them in regards to inhibiting proliferation in *Dictyostelium* cells. The first is that the mechanism behind the inhibition of proliferation due to polyphosphate involves intracellular polyphosphate instead of just extracellular polyphosphate. Next is that there may be an interplay present between extracellular polyphosphate levels and intracellular polyphosphate levels. Since intracellular polyphosphate has been indicated to be involved in inhibition, and high extracellular polyphosphate levels induce stationary phase in cells and inhibit growth, it could be suspected that as extracellular polyphosphate levels increase they may induce an upregulation in production of intracellular polyphosphate that contribute to the inhibition effect. Lastly, as extracellular polyphosphate is degraded over time, as seen in the conditioned media DAPI assays, this may contribute to not only the synthesis of intracellular polyphosphate, but it may also be taken up

and used as components for this synthesis. Additional tests such as intracellular polyphosphate extractions are being performed in order to gain a better understanding of this new model.

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