ELUCIDATING THE POLYPHOSPHATE SIGNALING PATHWAY IN *DICTYOSTELIUM DISCIODEIM*

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Elucidating the Polyphosphate Signaling Pathway in *Dictyostelium discoideum*

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Polyphosphate acts as a chalone in single celled *Dictyostelium discoideum*, accumulation of the extracellular signal in the external environment causes cells to halt proliferation and proceed to the next stage of their life cycle. Not much is known about the signaling pathway that occurs within *Dictyostelium*. The Gomer Lab endeavors to elucidate the polyphosphate signaling pathway.

In past semesters, pools of *Dictyostelium* mutants were generated using Restriction Enzyme-Mediated Integration (REMI) and screened for resistance to poly-p. The genomic DNA of the mutants determined to be resistant was extracted and analyzed. BLAST was used to identify protein matches. The matches selected for verification were irlD and torA. AX2 cells were set as a negative control and gefA$^-$ and the AAA-ATPase cells were set as the positive control. Knockout mutants were grown and screened for resistance to poly-p. The results were not statistically significant and more testing is needed. It is most likely that the targets are only slightly resistant to poly-p and have other proteins that contribute to the cell's sensitivity.

DEDICATION

I would like to dedicate this thesis to my family, whose support has been paramount to my success in college.

I would also like to dedicate this thesis to the future generations of scientists. Remember that the expert was once the beginner. The only true failure in life is choosing not to try again.

ACKNOWLEDGMENTS

I would like to thank Dr. Richard H. Gomer and Dr. Shiri Raphaelli- Procaccia, for their guidance and support throughout this project.

I would also like to thank my lab partner, Rheeturaag Daas, who I worked with for the Fall 2018 and Spring 2019 semesters and contributed to the screening of the six REMI pools.

NOMENCLATURE

Poly-p	Polyphosphate
Dicty	*Dictyostelium discoideum*
Ax2	Axenic 2
REMI	Restriction Enzyme-Mediated Integration
Blast	Blasticidin
cAMP	Cyclic adenosine monophosphate
gDNA	Genomic DNA
RasGEF	Ras guanine nucleotide exchange factor
gefA	RasGEF A/aimless gene
gefA$^-$	Mutant *Dictyostelium* that lack RasGEF A gene product
irlD	Inositol requiring like protein like kinase D gene
torA	The tortoise gene A (aimless)
torA$^-$	Mutant *Dictyostelium* that lack the tortoise A gene product

CHAPTER I

INTRODUCTION

Scientists use model systems to study complex biological processes in a simpler system to gain better understanding of the process itself. In the Gomer Lab, the social amoeba *Dictyostelium discoideum* is used to study tissue size regulation and disease. *Dictyostelium* are excellent research models because of their cost-effectiveness, ability to act as professional phagocytes, similarity to animal cells, acceptance of biological and genetic changes, and other reasons (1).

In nature, *Dictyostelium* are found in soil and moist decomposing foliage. *Dictyostelium* feed on bacteria, growing and multiplying as single cells until there are not enough nutrients to continue growth. When starved, the *Dictyostelium* aggregate as a response to cyclic adenosine monophosphate (cAMP) pulses (2). They form an aggregate called a slug in which cells differentiate to produce a fruiting body consisting of a stalk and spores. This fruiting body a survival mechanism, helping the *Dictyostelium* survive and produce new generations (3).

The manner in which cells decide their fate is of interest as only those that become spores go on to germinate and possibly survive for more generations (4). *Dictyostelium* is considered an altruistic organism because the cells that become stalk cells eventually die, helping the spores to survive for another generation (4). This presents two questions: one related to cell differentiation, another to evolutionary biology (4). Firstly, how do these cells decide which among them become spores? Are there cells that take advantage of this mechanism to promote their genetics?

The answer to the first question comes from research done previously in Dr. Richard H. Gomer's Lab. *Dictyostelium,* when confronted by starvation*,* are at different stages of their growth cycle, and their stage dictates what type of cell they become. Timing and luck primarily decide which cells move on and which do not. The process, labeled by Gomer, et al. as "musical chairs", illustrates which cells were in the right phase at the right time (3). It makes sense from a competitive viewpoint that certain cells would try to take advantage of the sacrificial nature of other *Dictyostelium* cells. Evolutionary scientists investigated the existence of a development of what they call "cheater" cells (4). Single *Dictyostelium* that pushed their development as a spore cell instead of stalk cell. It was found that cells in the fruiting body were genetically identical (4). They hypothesize that other *Dictyostelium* cells that have the same genetic makeup purposely exclude or remove those dissimilar to themselves to prevent the cheater cell from progressing. Additionally, the stalk cells are now known to sacrifice themselves in order to pass on and ensure the survival of their own genetics (4).

Dictyostelium know when to halt proliferation as a response to depleting nutrients due to the synthesis of a chalone called polyphosphate (poly-p). Poly-p contains many phosphate residues that have phosphoanhydride linkages. It is synthesized in *Dictyostelium* by a kinase called DkPPK1. Developmental problems were observed in cells that lack this function, indicating that poly-p plays a role in proper growth and development (5,6). It is a highly conserved molecule in bacteria, fungi, plants, and animals, it likely plays an important evolutionary role (5,6).

While feeding and growing, *Dictyostelium* synthesize and release poly-p, which functions as an extracellular signaling molecule and interacts with cells, inhibiting further cell growth and

proliferation. Previous experiments found that cells proliferating in the presence of high amounts of poly-p cannot continue to grow, even when provided with additional nutrients. Only by removing poly-p from the cells and placing them in new media can proliferation be restarted (7). Though the function of poly-p in *Dictyostelium* is understood, little is known about the signaling pathway itself.

This focus of this project was identifying potential proteins and targets involved in the polyphosphate signaling pathway, then verifying their involvement. In order to elucidate targets of the poly-p signaling pathway, mutant *Dictyostelium* were generated using Restriction Enzyme Mutation Integration (REMI) and screened for resistance to poly-p (8). Those resistant to the effects of poly-p likely had a mutation in a target or protein involved in the poly-p signaling pathway. Once identified, potential targets were verified by knocking out or inhibiting their function. Those phenotypes that were able to proliferate in the presence of poly-p were verified for their role in the poly-p signaling pathway.

CHAPTER II

METHODS

Cell Culture

Ax2 cells and REMI mutants were cultured in HLG0102 HL5 (Formedium, Hunstanton, UK) at 21°C on a rotary shaker at 175 rpm.

Polyphosphate Preparation

In order to make a stock of polyphosphate with a concentration of 100μM, sodium polyphosphates, glassy (Spectrum Chemical MFG Corp, Gardena CA, CAS:68915-31-1) was dissolved into PMB (potassium phosphate monobasic buffer) at a concentration of 100mg/mL. The solution was filtered and sterilized using a 0.2μm syringe filter (Acrosodic, UK).

Restriction Enzyme-Mediated Integration

In order to identify targets of the poly-p signaling pathway, mutants were generated from Ax2 cells. Six distinctive mutant pools were created using REMI (8). Each REMI pool contained approximately 3,000 individual mutant clones. Out of these many mutants those most resistant to poly-p were selected.

Selection of Polyphosphate Resistant Mutants

After cells were mutated by Restriction Enzyme-Mediated Insertion they underwent a primary selection for poly-p resistance by culturing in 100% HL5 media supplemented with 10μg/mL Blasticidin for 72 hours, then in 25% HL5 (75% PBM pH 6.1) for 48 hours, and finally in 25%

HL5 and 125μM of poly-p for 48 hours. This method was repeated for nine cycles. Between cycles clones were maintained in standard HL5 media with the added Blasticidin.

After primary selection, cells were diluted, so that 50 cells were seeded on an SM/5 plate (2 g/L glucose, 2 g/L bactopeptone, 0.2 g/L yeast extract, 0.2 g/L MgSO4•7H2O, 1.9 g/L KH2PO4, 1g/L K2HPO4, 15 g/L agar) and allowed to develop colonies for 3-4 days. Several individual clones were chosen from each plate and grown in flask culture with HL5 and Blasticidin. The cells were kept at a cell density of 0.5×10 6 cells/mL to maintain logarithmic growth. For experiments, 1.5 million cells were taken from each flask and underwent a secondary selection as done above for 1 cycle in 3-4 independent experiments where cell density was recorded after the 48 hours with poly-p. If the cells were determined resistant to poly-p through statistical analysis, the cells were taken for genomic DNA extraction with phenol: chloroform-based purification.

Ligation of gDNA

In order to begin ligation of the genomic DNA (gDNA) of the mutated dicty, the cut DNA needed to be purified and concentrated. The protocol provided by Zymo Research clear and concentrator kit called for the eluted gDNA to be measured using a Nanodrop. The concentrations were recorded and the amount of gDNA needed for the reaction calculated. The use of 5ng/μL per reaction ensures proper ligation of the cut DNA. The calculation performed was: (5ng/μL*20μL total volume) =100ng of gDNA per each reaction. Next, the total gDNA needed (100ng) was divided by the concentration found via the Nanodrop, resulting in the amount required for the ligation reaction, the solution of which included gDNA, ligase, buffer, and ultra-pure water (T4 DNA Ligase and Buffer, New England BioLabs). The samples were

placed in a PCR machine with the program set to run at 16ºC for 18 hours, 65ºC for 15 minutes, and was held at 12ºC until collection.

Inverse PCR (I-PCR)

An inverse PCR reaction using Azura HS Taq 2x ready mix (Azura Genomics) used the ligated gDNA samples with primers at a final concentration of 0.2µM. The primer sets used were named "Teal" and "Yellow" (IDT Corporation). Their sequences are shown in Table 1 below. The PCR reactions were set to run at 50℃ for teal primers and 48.5℃ for yellow primers for 30 cycles. In cases where PCR products were not seen, the temperature of the reaction was dropped to at least 38℃, and the number of cycles was increased to at least 40.

Table 1. Below, the table contains the sequences for the Teal and Yellow primer set.

Primer Set Names	**Forward Sequence**	**Reverse Sequence**
Yellow	5'TGTCGTTAGAACGCGACTAC 3'	5' CGTCGATATGGTGCACTCTC 3'
Teal	5'AGGTGACACTATAGAATCACGC 3'	5' CGTATGTGTATGATACATAAGGT 3'

Purification of PCR Product from Nucleic Acid Gel

The DNA product was removed from the gel by cutting around the band and using forceps to pick it up if PCR product was found at the appropriate size on a gel (i.e., larger the primer dimer). The Thermo Scientific: GenJET Gel Extraction kit was used to extract the DNA from the gel and purify it. The eluted DNA product was then sent off for sequencing or used for subsequent experiments.

Verification of Selected Targets

Certain potential targets were chosen based on pre-established criteria. Mutant *Dictyostelium* were grown that contained knockout phenotype directed against the protein of interest. The *Dictyostelium* grown were gefA$^{-,}$ AAA-ATPase, irlD, torA^{-}, torA^{-}/torGFP rescue plasmid.

Each cell type was placed into four wells in a 24 well plate. Each well of the 24-well plates received 400,000 cells in a volume of 400µL of media, each of which contained different media. One plate contained cells resuspended in SIH media, and the other plate had cells resuspended in 25% HL5. Four different polyphosphate concentrations, 0µM, 100 µM, 125µM, and 150 µM, were added to the cells, which were allowed to grow in the wells with poly-p for 24 hours. The next day the cells were resuspended and counted using an automatic cell counter.

CHAPTER III

RESULTS

Identification of Individual Resistant Clones

A total of six pools of REMI mutants were generated. The pools underwent a primary selection for poly-p resistance. The first selection resulted in 30,000 individual cells becoming thousands of copies of the same few cells. See the supplemental material for the result of the selection of polyphosphate resistant mutants in REMI pools 1-3. After the primary selection of clones in pools, individual clones were isolated from the pools and screened for resistance to poly-p, following the workflow depicted in Figure 1.

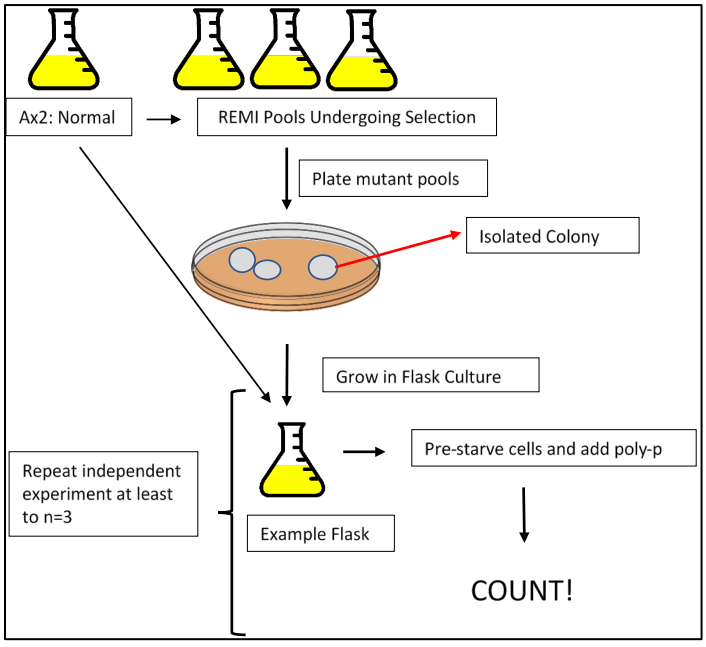

Figure 1. Work Flow Diagram Mutant cells were generated from Ax2 cells using REMI. The three pools were selected for poly-p resistant mutants. These mutants were plated on bacterial lawns and allowed to form individual colonies. Several colonies were picked and swabbed so the cells could grow in flask culture. When cells reached a density of 1.0 million cells/mL, they were moved into pre-starvation media and poly-p was added later. The cells are counted 48 hours after the addition of poly-p.

The isolated clones underwent a secondary screening for polyphosphate resistance, with the AX2 phenotype set as a negative control, and the clones then tested for at least n=3 times for resistance to polyphosphate. Figures 2 and 3 show the results of the secondary screening.

From the secondary screen, clones 1-5,1-6, 1-8, 4-5, 4-7, 5-6, 6-5, and 6-6 had proliferated significantly more than the control, as determined by a type 2 t-test. The significant clones passed the screen and moved on for genomic extraction and Inverse PCR. Clones 1-7, 1-9, 4-8, 5-5, 5-7 and 6-7 were not found to have significant proliferation, but were still taken for sequencing because their growth exceeded the control and followed the general trend of those determined significant.

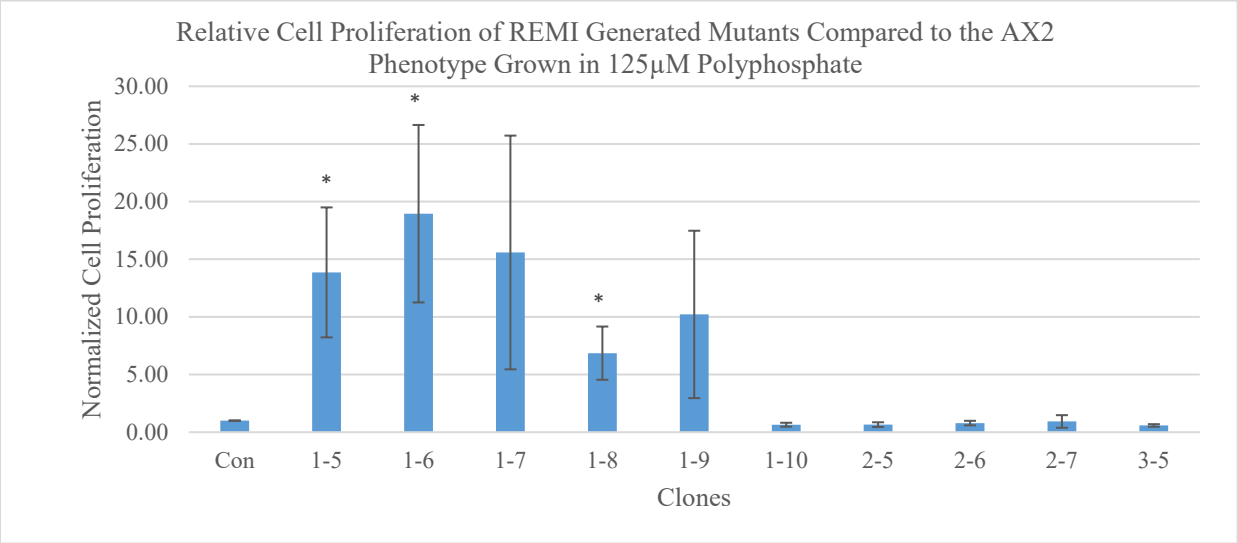

Figure 2. Graph of REMI Pools 1-3 Relative Proliferation to the Control. The figure above shows the results of the screening of clones from pool 1, 2, and 3. Their proliferation was normalized to that of the controls and was then analyzed for significance using a type 2 t-test. Clones 1-5, 1-6, and 1-8 were found to be significant. 1-7 and 1-9 though not found to have significant proliferation, were still taken for sequencing because their growth was much greater than the control.

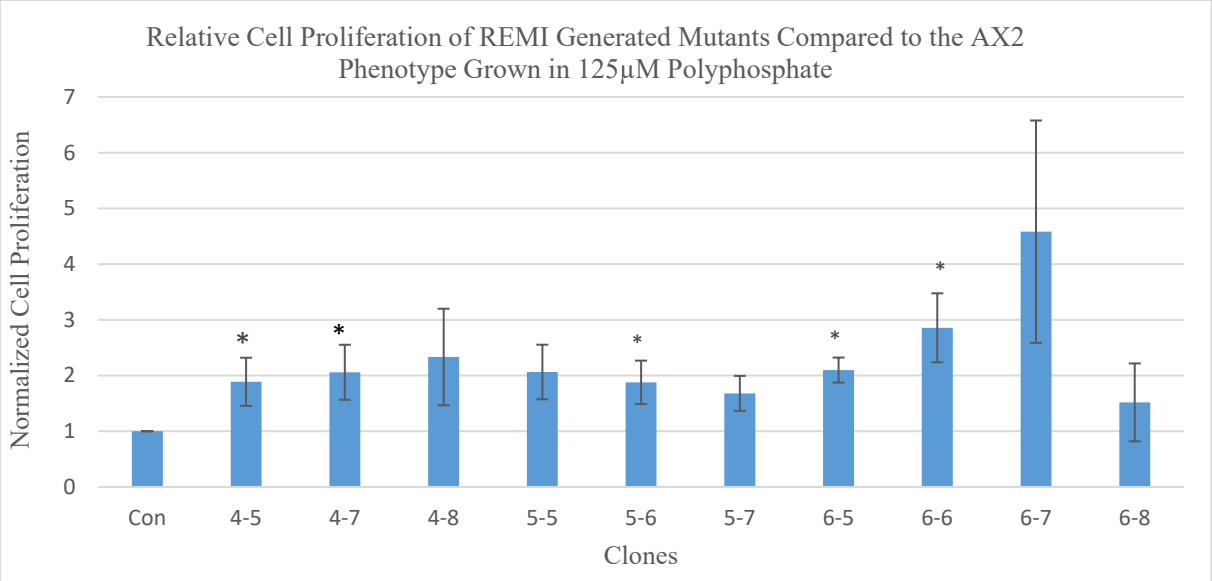

Figure 3. Graph of REMI Pools 4-6 Relative Proliferation Compared to the Control. In this figure, the proliferation of clones from pools 4,5, and 6 are shown. Their proliferation again was normalized to that of the control. Clones 4-5, 4-7, 5-6, 6-5, and 6-6 were found to be significant using a type 2 t-test. Clones 4-8, 5-5, 5-7, and 6-7 were not considered significant. They were still taken for sequencing, however, due to proliferating at a higher rate than the control.

I-PCR Gel Results

The gDNA of clones that passed the secondary screen was cut via restriction enzymes, ligated, and the product put through I-PCR. Those products were then run on a gel. Two primer sets were used for each clone. There were PCR products for clones 1-6, 1-7,1-8,1-9, 4-5, 4-7, and 4-8. Clones 1-5, 5-5, 5-6, 5-7, 6-5, 6-6, 6-7 did not have a PCR product with either primer set. The I-PCR products of clones 1-6, 1-7 1-8 and 1-9 are pictured in Figure 4 below.

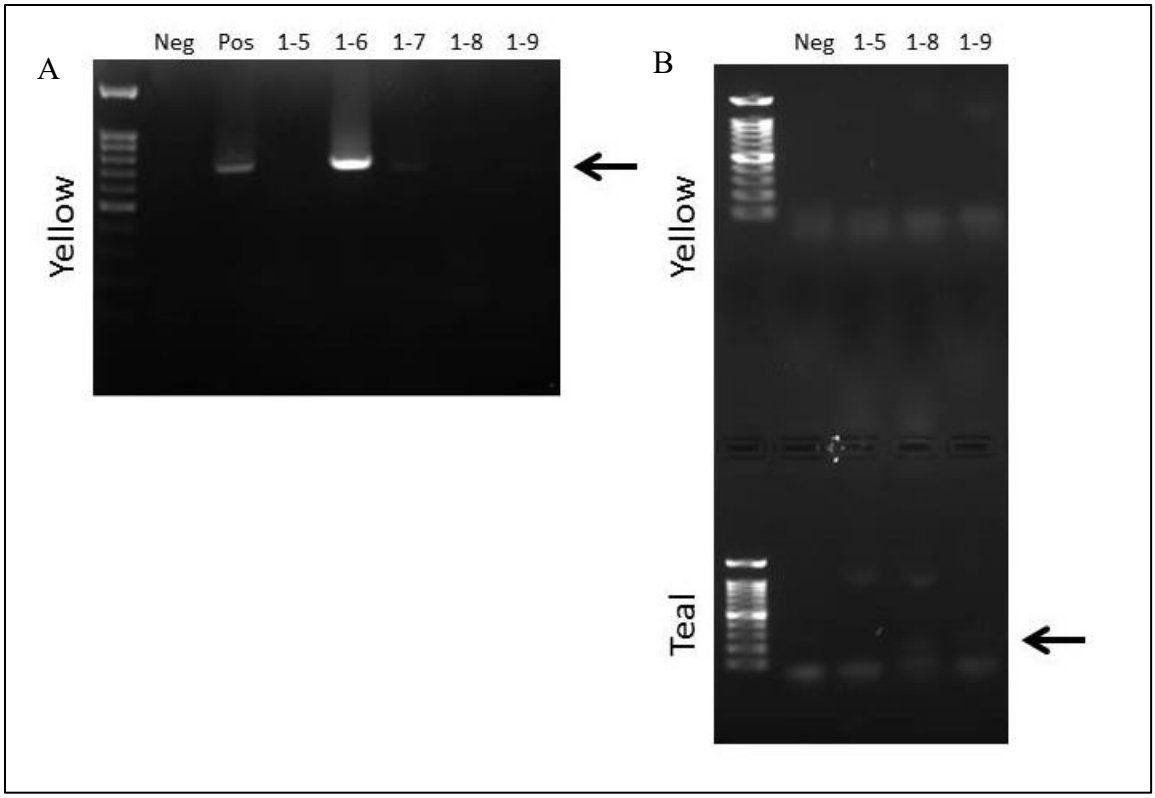

Figure 4. Inverse PCR products run on an agarose gel with two different primer sets (yellow and teal). A) Inverse PCR products run on an agarose gel with two different primer sets (yellow and teal). The small gel has clones 1-5, 1-6, 1-7, 1-8 and 1-9, along with a positive and negative control. Clones 1-6 and 1-7 have a product at the expected size of 750 base pairs. B) A gel was run with two combs; the top set of wells contained the PCR products for the yellow primer set and the bottom contained the products for the teal primer set. Clones 1-5, 1-8 and 1-9 were run with a negative control. Clones 1-8 and 1-9 have a product with at 650bp, which is expected for a teal product.

Comparison of Isolated Sequences with BLAST Database

Once sequenced, the results were searched to locate the area where the BLAST resistance cassette had been integrated into the genome and subsequently conferred resistance to polyphosphate. The cut site of the restriction enzyme AluI was searched for, as well as a DpnII cut site. Once these two sequences were identified, the sequence in the center of the two was entered into the pBLAST database. The database found either the identity of protein or homologs of the protein. BLAST returned these matches or "hits" as an output. Table 2 shows matches for clones that passed secondary screenings.

Table 2. The returned sequences were inputted into the BLAST Database to compare to known sequences. The table below shows the best potential matches for the isolated sequences based on e-values or repetition.

Clone ID	Blast Results Highlights	E-Value
4-5, 4-8	hypothetical protein (DDB_G0279499)	0.014
	hypothetical protein (DDB_G0279845)	0.05
	isopentenyl-diphosphate D-isomerase (ipi)	0.18
	protein kinase, Atypical group (tor)	0.18
	histidine kinase DhkJ (dhkJ)	0.18
4-7	FACT complex subunit SPT16 (spt16)	0.004
	hypothetical protein (DDB_G0279975)	0.015
	cGMP-specific phosphodiesterase (pde3)	0.015
	transmembrane protein (tmem170)	0.052
	FACT complex subunit SPT16 (spt16)	0.004
1-9	hypothetical protein (nek4)	0.12
	Additional Hypothetical Proteins	
1-8	hypothetical protein (DDB_G0292010)	0.031
	histidine kinase DhkK (dhkK) Hybrid signal transduction histidine kinase K	0.38
	kinesin Unc104/KIF1a homolog (Unc104)	1.3
	ABC transporter ABCC.7 (abcC7)	0.49
	glutathione-dependent formaldehyde-activating, GFA family protein	0.084
	hypothetical protein (DDB_G0275195)	0.012
	kinesin family member 12 (kif12)	0.017
	AAA+ ATPase, core domain-containing protein	0.73
1-7	hypothetical protein (irlA)	6e-04
	CigB (cigB)	0.002
	terpene synthase (TPS1)	0.002
	hypothetical protein (DDB_G0278591)	0.015
1-6	ABC transporter G family protein (abcG24)	0.001
	Ras GTPase (DDB_G0293786)	0.15
	hypothetical protein (DDB_G0267822)	0.15
	cytochrome P450 family protein (CYP518B1)	0.49

Potential protein targets were selected after analyzing all of the BLAST results. Those selected either had a strong E-value or appeared in the data set more than once. Clone 1-7 had one of the smallest e-values (indicative of a close match) from the BLAST output and matched to a

hypothetical protein, irlA. It was therefore selected for the verification process. Other potential targets were chosen because they appeared in the data set multiple times

Results from Selected Targets

After selecting putative targets, an experiment was run to see if the direct knockout of the target caused the mutant *Dictyostelium* to become resistant to polyphosphate. The cell phenotypes were gefA$^-$, AAA-ATPase, irlD (similar gene product to irlA), torA$^-$ and torA$^-$/tor GFP rescue plasmid. A cell count was taken for each *Dictyostelium* type after 24 hours in pre-starvation media with the addition of varying levels of polyphosphate. The experiment was carried out to at least n=3 for each cell type. The data was normalized to the growth of the 0µM of polyphosphate because the condition represents the normal growth of the cells. Figures 5 and 6 show the results of the verification screen. Both gefA- and AAA-ATPase were used as positive controls since they had previously been identified as polyphosphate resistant by the Gomer lab. The *Dictyostelium* used as a negative control was the AX2 phenotype, considered to be the "wildtype" cell. The phenotypes irlD, torA$^-$ and torA$^-$ /tor GFP rescue plasmid were investigated for resistance to polyphosphate. In the torA$^-$/tor GFP rescue plasmid, the cell contains a plasmid that recovers the function of the tor protein in a knockout phenotype, and was used to compare the knockout phenotype (torA$^-$).

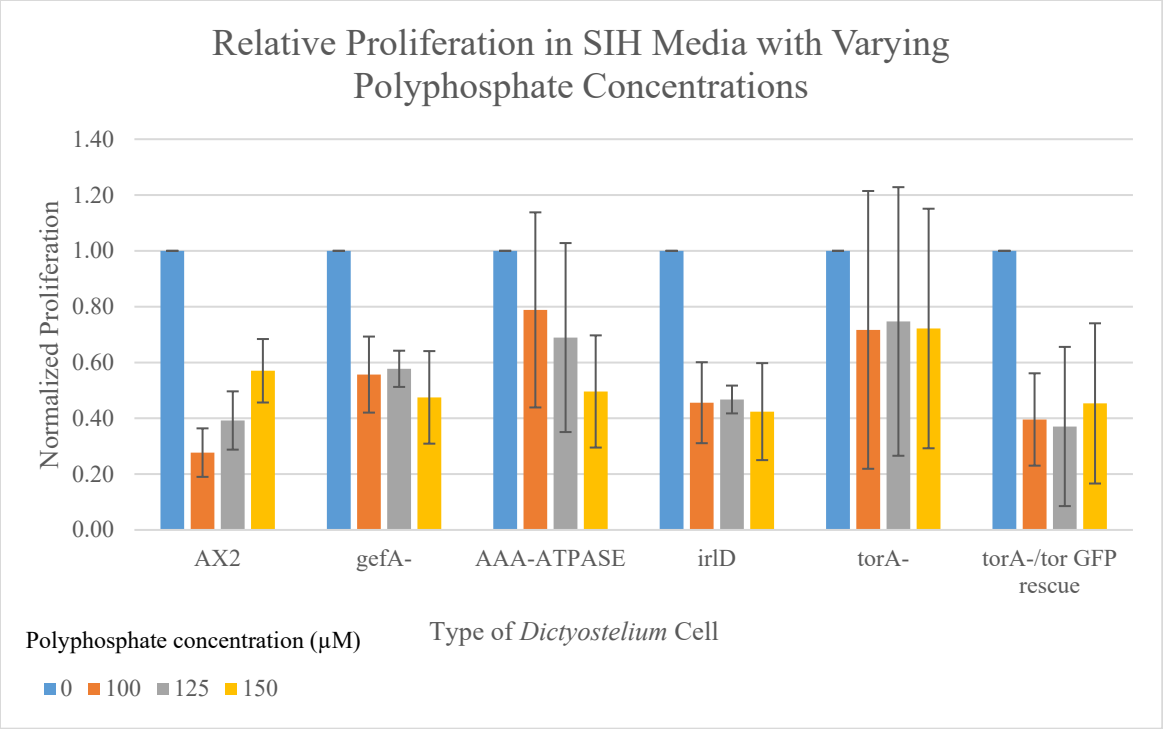

Figure 5. Graph of AX2 and Mutant *Dictyostelium* Cells were grown in SIH Media with Varying Amounts of Polyphosphate. The cell proliferation of each is normalized to the normal condition of no (0 µM) of polyphosphate added. The blue bar represents that data for 0µM addition of polyphosphate, the orange bar is data for the 100µM addition of polyphosphate, the gray represents the data for 125µM addition of polyphosphate, and the yellow bar represents the data for the 150µM polyphosphate condition.

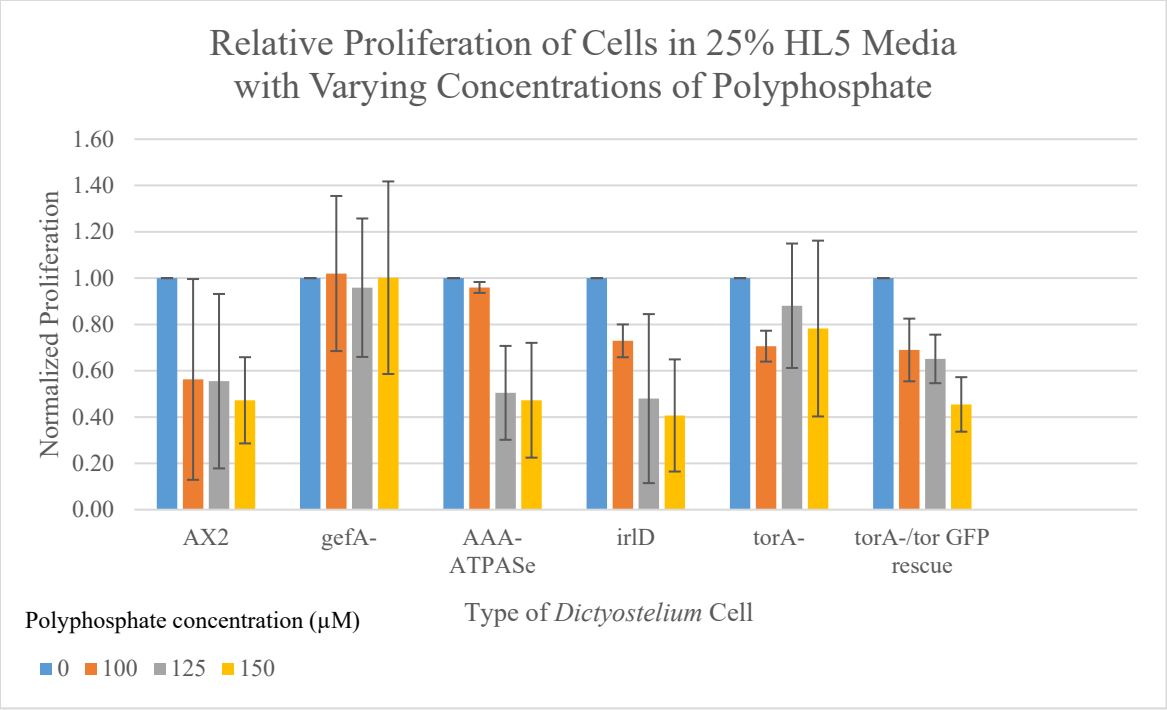

Figure 6. Graph of AX2 and Mutant *Dictyostelium* Cells were grown in 25% HL5 Media with Varying Amounts of Polyphosphate. The cell proliferation of each is normalized to the normal condition of no (0 µM) of polyphosphate added. The blue bar represents that data for 0µM addition of polyphosphate, the orange bar is data for the 100µM addition of polyphosphate, the gray represents the data for 125µM addition of polyphosphate, and the yellow bar represents the data for the 150µM polyphosphate condition

In the figures above, the 0µM poly-p condition was set as the base line for cell proliferation. Since, it is considered the normal or basal condition of cells its normalized proliferation would be the its cell count divided its cell count, setting it as the standard. The other poly-p conditions were normalized to the 0µM condition by dividing their cell count by the cell count of the zero condition. This would show if there was less, more or equivalent cell proliferation.

The two medias used in the verification screen are both medias with decreased amounts of available nutrients. By placing cells into the media, it induces nutrient stressed condition, similar to when nutrients begin to deplete in the natural environment. In past experiments, 25% HL5 was the media used to induce pre-starvation conditions. SIH was used for this experiment to

determine if there was a difference in proliferation depending on the amount of nutrients in the environment since SIH has less nutrients available than 25% HL5.

CHAPTER IV

CONCLUSION

The primary and secondary screen of the REMI clones showed that 14 clones in total were resistant to polyphosphate. From the gDNA of the 14 clones, only 8 samples produced a PCR product that could be sequenced and analyzed. After analyzing the DNA sequences in BLAST, the database generated an output of potential protein matches. Most of the matches did not have strong E-values, except for clone 1-7. Putative targets were chosen for verification either by E-value or because of a match found in other independent screens.

Clone 1-7 had the strongest E-value match with the protein irlA, a hypothetical inositol requiring like protein kinase. Because it has an E-value smaller than most of the other outputs(6E-04), the protein was selected for verification. Its corresponding mutant phenotype, irlD, is related to irlA, as they originate from the same family but are on separate genes. The two have previously been identified in a kinome study of *Dictyostelium* (9).

The generated protein sequence from clone 1-8 matched with a protein kinase called AAA-ATPase, core domain protein. The AAA-ATPase modifies proteins or other substrates through phosphorylation (10) and has been identified as a likely component of the polyphosphate pathway by the Gomer Lab. Therefore, it was selected as a positive control for resistance to polyphosphate, when its function was knocked out in *Dictyostelium*.

The other target looked at, protein tortoise, was found to play a fundamental role in chemotaxis. Data found by van Es et al. showed that *Dictyostelium* that lacked the function of the gene torA responded poorly to cAMP signals that formed a chemoattractant gradient (11). Response to cAMP is important in the life cycle of *Dictyostelium* as it helps their migration towards one another, leading to agglomeration and the start of the fruiting body formation process (2). The response to increasing levels of polyphosphate halts cell proliferation in *Dictyostelium*. Both poly-p and cyclic adenosine monophosphate are highly energetic phosphate-containing molecules that act a signal.

The other target that underwent verification was gefA^{-}, a part of the RasGEF family. RasGEF proteins interact with a Ras protein, which resides in the membranes of cells. Both play a critical role in signaling and other functions. In *Dictyostelium*, Wilkins et al. found that cells that lacked the gefA gene formed unusual slugs and had much lower proficiency in chemotaxis (12).

Target cell count results were statistically analyzed through a single factor ANOVA test. The null hypothesis for ANOVA states that no variation exists between the mean of the selected groups. The output from the test consists of an F-value, p-value and F-critical value. If the F-value of the group is greater than F critical value, the null hypothesis is rejected. If the p-value is small, the null hypothesis is again rejected. Any other result, the null hypothesis stands.

The mean proliferation of each cell type at the four poly-p concentrations were compared as a group. So, AX2 resuspended in 25% HL5 was compared to itself at the different concentrations of poly-p added and the Ax2 resuspended in SIH media was compared to itself. For this

experiment the AX2 phenotype was used as a negative control as it is known not to be resistant to polyphosphate. The knockout phenotypes of gefA and AAA-ATPase were set as positive controls because they are known to confer polyphosphate resistance. The results of the ANOVA test showed no significant resistance among the targets tested.

While some inferences may be drawn from the data of this experiment, the results are not statistically significant. The amount of deviation that occurred in the controls and disagreement in trends between the two medias cannot be overlooked. It is most likely that the cells that showed little to no variance in mean cell growth with the addition of poly-p are slightly resistant. More experiments would need to be performed in order to make definitive conclusions about the target's putative role in the polyphosphate signaling pathway. Due to the unforeseen COVID-19 Pandemic of 2020, no further evidence can be gathered at this time.

More studies need to be done in order to fully elucidate the polyphosphate signaling in *Dictyostelium discoideum*. It is likely that the pathway has multiple proteins involved, similar to a signal cascade. Target proteins, still need to be identified, investigated, and verified. It is also possible that a knockout or inhibition of just one protein will not confer complete resistance to polyphosphate. Future experiments should try inhibiting or knocking out multiple targets. Due to conserved nature of polyphosphate in all domains of life and the similarity of *Dictyostelium* to animal cells, this topic is worth investigating further. Applications of this knowledge can help with tissue size regulation, since polyphosphate acts as a chalone in *Dictyostelium*, similarly to the chalones that regulate human tissue growth. In addition, *Dictyostelium* have related functions

and characteristics similar to human macrophages and neutrophils. This makes them a valuable model organism for the study of the development of new therapeutics.

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SUPPLEMENTAL MATERIALS

Below is a figure generated by Dr. Shiri Raphaelli-Procaccia. It represents the nine-week cycle where the REMI pools were grown with polyphosphate taking the number of cells from around 3,000 individual cells to many copies of the same few cells.

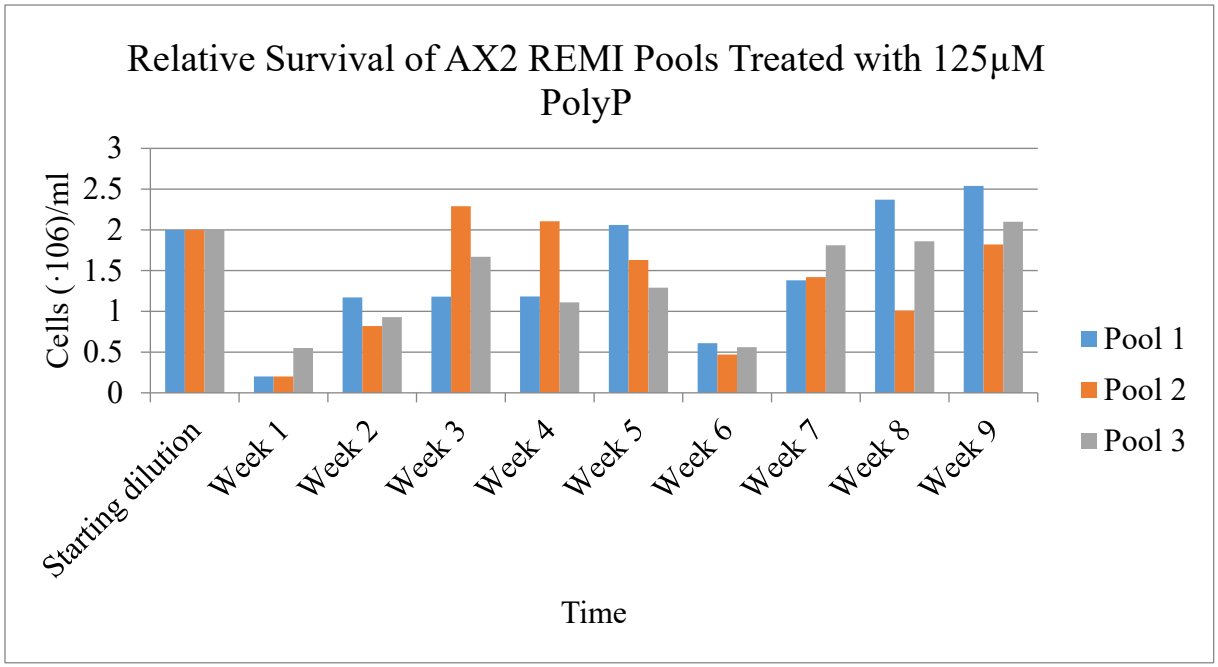

Figure 7. The results of the Primary Screening of REMI pools 1, 2 and 3 are shown above. The cells were grown for a period of nine weeks and with the addition of poly-p.