THE EFFECTS OF POLYPHOSPHATE ON SPX DOMAINS IN *DICTYOSTELIUM DISCOIDEUM*

An Undergraduate Research Scholars Thesis

by

JACOB WATSON

Submitted to the Undergraduate Research Scholars program at
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by Research Advisor: Dr. Richard Gomer

May 2017

Major: Biology

TABLE OF CONTENTS

ABSTRACT

The Effects of Polyphosphate on SPX Domains in *Dictyostelium discoideum*

Jacob Watson
Department of Biology
Texas A&M University

Research Advisor: Dr. Richard Gomer
Department of Biology
Texas A&M University

Phosphorus is an atom taken up by cells as inorganic phosphate (Pi). The mechanism behind how cells measure cellular Pi levels is not well understood. SPX domains are positively charged helical bundles located at the N-termini of phosphate transporters, PolyP polymerase vacuolar transporter chaperone proteins, and phosphate signaling proteins. SPX domains have been shown to sense inositol polyphosphate signaling molecules in plants and yeast. These proteins are conserved in eukaryotes and affect polyphosphate synthesis, phosphate transport, and phosphate starvation. The genome of the simple eukaryote *Dictyostelium discoideum* encodes five predicted uncharacterized SPX domains. Using this knowledge a homologous recombination gene knockout for SPX 2 containing a Blasticidin resistance gene as a selectable marker was created. After validating the genotype of the knockout the associated phenotypes were elucidated. The *SPX 2*$^{-}$ cells exhibited a lack of aggregation upon exposure to 150 μM PolyP. *SPX 2*$^{-}$ fruiting bodies had longer stalks, smaller spore heads, and more mounds than wild-type. *SPX 2*$^{-}$ cells also had slightly higher motility rates. As polyphosphate and SPX domains are conserved across eukaryotes, anything learned about these areas can be applied to other organisms in areas such as Pi transport and starvation responses.

DEDICATION

I dedicate this research project to my parents who have supported me throughout my undergraduate education and to pursue my interests in Molecular Biology research.

ACKNOWLEDGEMENTS

I would like to thank my Research Advisor Dr. Richard Gomer and PhD candidate Patrick Suess for their continued guidance and support throughout my research at Texas A&M. Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

NOMENCLATURE

PolyP	Polyphosphate
Dicty	*Dictyostelium discoideum*
PCR	Polymerase Chain Reaction
InsP	Inositol Polyphosphate
Pi	Inorganic Phosphate
BLAST	Basic Local Alignment Search Tool

CHAPTER I

INTRODUCTION

SPX Domains

The uptake of inorganic phosphate (Pi), transport, and storage, and their associated signaling systems in eukaryotes (1, 2),appear to be mediated by proteins containing SPX domains of unknown function (3). SPX domains have been found in eukaryotic phosphate transporters, signaling proteins, and inorganic polyphosphate polymerases. They provide a binding surface for inositol polyphosphate signaling molecules (InsPs) whose concentrations vary with Pi availability. Alteration of critical binding surface residues decreases InsP binding in vitro, Pi synthesis in yeast, and Pi transport in *Arabidopsis*. In plants, InsPs trigger the association of SPX proteins in addition to transcription factors to regulate Pi starvation responses (5). Mutations in the SPX domain of plant and human Pi exporters decreases their transport capacity and affects Pi signaling (4). These proteins are conserved in eukaryotes and have effects on polyphosphate synthesis, phosphate transport, and phosphate starvation. The mammalian SPX ortholog is XPR1, which confers susceptibility to infection with murine leukaemia viruses and is involved in phosphate homeostasis (15).

Molecular Cloning

Many techniques have been introduced for arranging new DNA sequences (6-8), and the use of restriction enzymes is one of the most commonly utilized technique in molecular cloning. Whenever compatible restriction enzyme sites are available on the insert and the vector DNA sequences, cloning can be very straightforward. If restriction sites are not compatible or not

present, the use of PCR primers in which compatible restriction enzyme sites are embedded can solve this problem and allow multistep cloning procedures (9-13). PCR cloning has been widely used in genetic engineering and follows the order of: analysis of the target gene sequence, PCR primer design, performing the PCR procedure, sequencing the resulting PCR products, checking the sequence data, and cloning the PCR product into the final vector (14).

Homologous Recombination Repair Pathway

One standard DNA cloning practice is to amplify a target DNA sequence containing oligonucleotides with 5' tails specifying endonuclease recognition sites. These allow ensuing cleavage and insertion of PCR fragments into any desired cloning vector. After preparing the desired DNA fragment to contain regions of homology to the target gene, homologous recombination may occur. The cell will recognize the regions of homology that exist, and will then use homologous recombination to repair the PCR product into the genome placing the target DNA into the host DNA. The plasmid of interest here is the pLPBLP plasmid in *Dictyostelium discoideum* that contains a blasticidin resistance gene. The blasiticidin resistance gene can be placed in-between regions of homology in the target DNA and used as a selective marker. Cells can then be grown and treated with blasticidin where only cells receiving the designed knockout fragment with the blasticidin resistance will grow and the wild type cells will not.

Polyphosphate Effects on *Dictyostelium*

Inorganic polyphosphate is secreted by growing *Dictyostelium* cells (16). When cells reach high densities that mirror starvation conditions, the associated high concentrations of extracellular polyphosphate strongly inhibit cytokinesis and slightly inhibits cell growth. This inhibition allows cells approaching starvation to conserve their resources and have as much

nutrients as possible remaining (16). Extracellular polyphosphate is also involved in eukaryotic cellular responses such as coagulation, contact pathway activation, inflammation, and proliferation (17-20). In general the intracellular signaling pathway activated by extracellular polyphosphate remains largely unknown (21-23).

CHAPTER II

METHODS

Molecular Cloning Overview

Molecular cloning reactions are comprised of two components, the DNA fragment to be replicated and the vector/plasmid backbone that contains all components for replication in the host. The DNA fragment to be replicated is created through the use of designed primers and PCR. Once this fragment is created it can be placed into the plasmid to create the first “arm” of the construct. The next fragment can then be amplified and placed onto the opposite side of the blasticidin resistance gene to create the second “arm” of the construct. The construct can then be removed from the plasmid using a restriction enzyme digest to create a designed linear piece of DNA containing two regions of homology to the target gene and the Blasticidin resistance gene in the middle. This piece of DNA can be inserted into target cells using a transformation reaction. The insert can then be validated by sequencing after amplifying the target region through PCR.

PCR Primer Design

When designing a primer for PCR it is important to consider primer length, primer melting temperature, primer annealing temperature, GC content, GC clamp, primer secondary structures, repeats, runs, 3’ end stability, template secondary structure, and cross homology. It is also important to consider Amplicon length, product position, Tm of product, optimum annealing temperature, and primer pair Tm mismatch calculation of the primer pair. The optimal design for a PCR primer is 18-22 base pairs, Tm in the range 52-58 °C, 40-60% GC content, G or C bases in the last five bases from the 3’ end of the primer, avoid hairpins, self-dimers, and cross dimers, no more than 4 dinucleotide repeats, avoid long runs of a single base, and avoid cross homology

with other sequences in the organism's genome. The website Primer3 is an effective tool to give potential primer candidates that can be worked with and adjusted to make an optimal primer. A BLAST search can also be performed in order to check for cross homology against the genome of the target organism.

PCR

To perform a PCR reaction a PCR reaction mixture must be prepared, and the mixture must be placed into the thermocycler to undergo PCR. To prepare a PCR reaction mixture adhere to the standards posted for the Polymerase used. Taq 5X Master Mix was used in this experiment and the protocol for its use can be found on the NEB website. The PCR reaction mixture should contain 0.2 µM of forward primer, 0.2 µM of reverse primer, less than 1,000 ng of template DNA, 1/5 Volume Taq 5X Master Mix, and up to the total volume with nuclease-free water. In this experiment 15 µL reactions and 50 µL PCR reactions were performed. The thermocycler conditions set for this PCR reaction are as follows: Initial denaturation (95°C) for 30 seconds, 30 cycles at (95°C) for 15-30 seconds, (45-68°C) for 15-60 seconds, (68°C) for 1 minute/kb, a final extension (68°C) for 5 minutes, and then a hold at (4°C) until removed from the machine. The thermocycler conditions can also be found on the NEB website.

Table 1

Primer	Tm (Deg C)	%GC	Length	Sequence and Reverse Sequence +RE + 4 nucleotides
1F	55.7	46	24	GATAGGTACCATTATCCCCAGAAG
1R	51.7	39	23	CAATAAGCTTGTGAACGTGATAC
2F	54.4	52	21	CAGTCTGCAGGTTCCAGATTC
2R	56.7	55	22	GACTCCGCGGCATTGGAGAATT

Creating Gene Fragments

Once the PCR product has been created it can be placed into the plasmid by performing a restriction enzyme digest and a ligation reaction. The restriction enzymes that were added to the primers are also present in the plasmid. By digesting the PCR product and the plasmid with these restriction enzymes they will now have homologous sticky ends. A ligation reaction can then join these ends into a single piece of DNA. The restriction enzymes used for the production of this gene fragment were KpnI, HindIII, PstI, and SacII. The enzyme used for the ligation in this reaction was T4 DNA Ligase. This step yields a plasmid with regions of homology inserted onto each side of the blasticidin gene as well as original target pieces of DNA as the ligation reaction can yield both products. The different products can be distinguished on an agarose gel using electrophoresis. This DNA can then be purified and used for later steps using an Omega bio-tek E.Z.N.A. Gel Extraction Kit.

Inserting Gene Fragment into *E. Coli* Cells

Once the plasmid has been made with the regions of homology on each side of the blasticidin gene, it can be transformed into competent E. coli cells. This is done by performing the transformation protocol on NEB's website. This protocol calls for thawing competent cells on ice and chilling approximately 5ng of the ligation mixture in a 1.5 ml microcentrifuge tube. Add 50 μL of competent cells to the DNA, and mix gently by pipetting up and down or flick the tube 4-5 times to mix the cells and DNA. Do not vortex. Place the mixture on ice for 30 minutes, do not mix. Heat shock at 42°C for 30 seconds, do not mix. Add 950 μL of room temperature media to the tube. Place tube at 37°C for 60 minutes, and shake vigorously (250 rpm) or rotate. Warm selection plates to 37°C. Spread 50-100 μL of the cells and ligation mixture onto the plates. Incubate overnight at 37°C.

Plasmid Preparation and Electroporation

Once the plasmid has been placed into E. coli cells a maxiprep can be performed in order to obtain large amounts of the plasmid. The maxiprep was performed with a kit from Zymopure giving large amounts of our new plasmid containing regions of homology to the target gene. This plasmid was then cute using restriction enzymes outside of the gene fragment and the DNA was purified using an Omega bio-tek E.Z.N.A. Gel Extraction Kit. This purified DNA was then inserted into *Dictyostelium* cells using electroporation. The Dicty cells were then grown on plates and treated with blasticidin where only cells receiving the inserts would survive.

Validation of Insert

To validate that the insert was located in the correct portion of the genome and not incorporated as an off target effect, PCR was carried out with new primers that lay outside of the original construct. This shows that the inserted DNA lies where the old gene was. The PCR product was sent off for sequencing and the results are then used for verification.

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, *SPX 2*- were used in the testing of extracellular polyphosphate accumulation in *Dictyostelium*. Wild Type 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.

Aggregation Assay

Mid-log cells were collected by centrifugation at 200 x g for 3 minutes, resuspended in HL5 and the centrifugation and resuspension was repeated twice. Cells were resuspended to 1.5 x 10^6/ml in PBM , and 2 ml was placed in the well of a 6-well plate containing 25% HL5 and 75% PBM. Plates were incubated at room temperature in humid boxes, then analyzed by microscopy with a 10 x phase-contrast objective on a Diaphot inverted microscope (Nikon, Garden City, NY) for aggregation and development.

Colony Expansion Assay

Serial dilutions of mid-log cells were performed in HL5 in order to get concentrations of cells down to 25-30 cells/mL. 300μL of cells were plated onto agar plates so that each plate contained approximately 10 cells. Isolated colony diameter was measured daily in cm and compared across cell types.

Fruiting Body Development Assay

To prepare a fruiting body development assay on nitrocellulose there are 3 areas to consider. First the filter paper and nitrocellulose filter must be prepared, second the cells must be prepared, and third the cells must be added to the filter paper. To prepare the filter paper and nitrocellulose filter, whatman #3 filter paper was taken and cut in half twice to give 4 slices of pie shape pieces. Be sure to handle with sterile tweezers and cut with sterile scissors. Next, 5mL

of PBM was placed into a petri dish, and one quarter piece of filter paper was placed flat into the petri dish. Another quarter piece of filter paper was placed on top and allowed to soak in PBM for 2-5 minutes. The excess PBM was then poured off into waste and a milipore filter containing nitrocellulose was obtained. The milipore filter was then gently washed with H_2O, and then placed with the darker side up on top of the white filter paper in the plate. The filters were then soaked in PBM for 2-5 minutes and then the excess PBM was poured off. At this point it is time to add cells to the milipore filter. First, the cells must be prepped by spinning down 1 x 10^7 mid log cells at 200 x g for 3 minutes and resuspended in PBM twice. Cells were resuspended in 200 µL PBM and then added dropwise in 50 µL increments around the center of the filter. Each drop should soak into the filter paper before adding the next (10-20 seconds). Keep the petri dish in a dark place in humid box with a wet paper towel under the plate. Analyze at 24 and 48 hours.

Intracellular Polyphosphate Concentration Assay

20 x 10^6 mid-log cells were collected by centrifugation at 200 x g for 3 minutes, resuspended in PBS and the centrifugation and resuspension were repeated twice. Cells were resuspended in 1mL PBS. Cell lysates were generated by passing cells through 5 micron syringe filters. Cell lysates were then treated with a solution containing 2mM MgCl2, 30mM Tris-HCl pH 8, and 50 units of Benzonase and incubated at 37C for 1 hour. Cell lysates were then treated with a solution containing 10mM EDTA and 1µL pro-k and incubated at 65C for 1 hour. A DAPI assay was performed to measure fluorescence on a synergyMx plate reader and serial dilutions of 1, ½, ¼ and 1/8 were measured. 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 and inositol phosphates.

Cell Imaging

Mid-log cells were collected by centrifugation at 200 x g for 3 minutes, resuspended in HL5 and the centrifugation and resuspension was repeated twice. Cells were resuspended to 1.5 x 10^6/ml in HL5 , and 2 ml total volume was placed in the well of a 6-well plate containing 100% HL5 and either 0 μM PolyP or 150 μM PolyP. Calculations were done so that each well contained 0.25 x 10^6 or 0.50 x 10^6 cells. Plates were incubated at room temperature in humid boxes, then analyzed by microscopy with a 10 x phase-contrast objective on a Diaphot inverted microscope (Nikon, Garden City, NY) for cellular morphology. Images of a calibration slide (SWIFT #MA663, Carlsbad, CA) were used for size bars.

Reagents and Materials

HL5 medium was from Formedium (Norfolk, UK). Polyphosphate was from Spectrum (New Brunswick, NJ). Blasticidin were from CalBioChem (Boston, MA). *Dictyostelium* cell lines were obtained from dictyBase (Northwestern University, Chicago, IL). Nylon 17 mm/ 5 μm pore size filters were from Sterlitech (Kent, WA). Tissue culture grade 6-well plates were from Thermo Fisher Scientific (Waltham, MA).

CHAPTER III

RESULTS

Treating *Dictyostelium* cells with Polyphosphate to test for aggregation

Studies with *Dictyostelium discoideum* have shown that proliferating *Dictyostelium* cells secrete polyphosphate which acts as a regulator of cell proliferation by signaling to the cell when to enter stationary phase and stop proliferating (5). Preliminary studies have also shown that polyphosphate acts as a regulator of aggregation and cell development. Additional studies have found that when wild-type *Dictyostelium* cells were cultured in the presence of polyphosphate, aggregation was enhanced (Figure 1 a-d). To test if disruption of *SPX 2*$^{-}$ affects aggregation the same assay was performed where *SPX 2*$^{-}$ cells were cultured for 48 hours in 25% HL5, a nutrient rich growth medium, and 75% PBM in the presence or absence of 150 μM polyphosphate, with pictures taken at 24 and 48 hours. This assay showed that the *SPX 2*$^{-}$ cells had an abnormal response to 150 μM polyphosphate when compared to wild type cells. Polyphosphate treated *SPX 2*$^{-}$ cells exhibited a nonspecific chemotaxis effect as well as a lack of aggregation compared to wild type cells. (Figure 1 e-h).

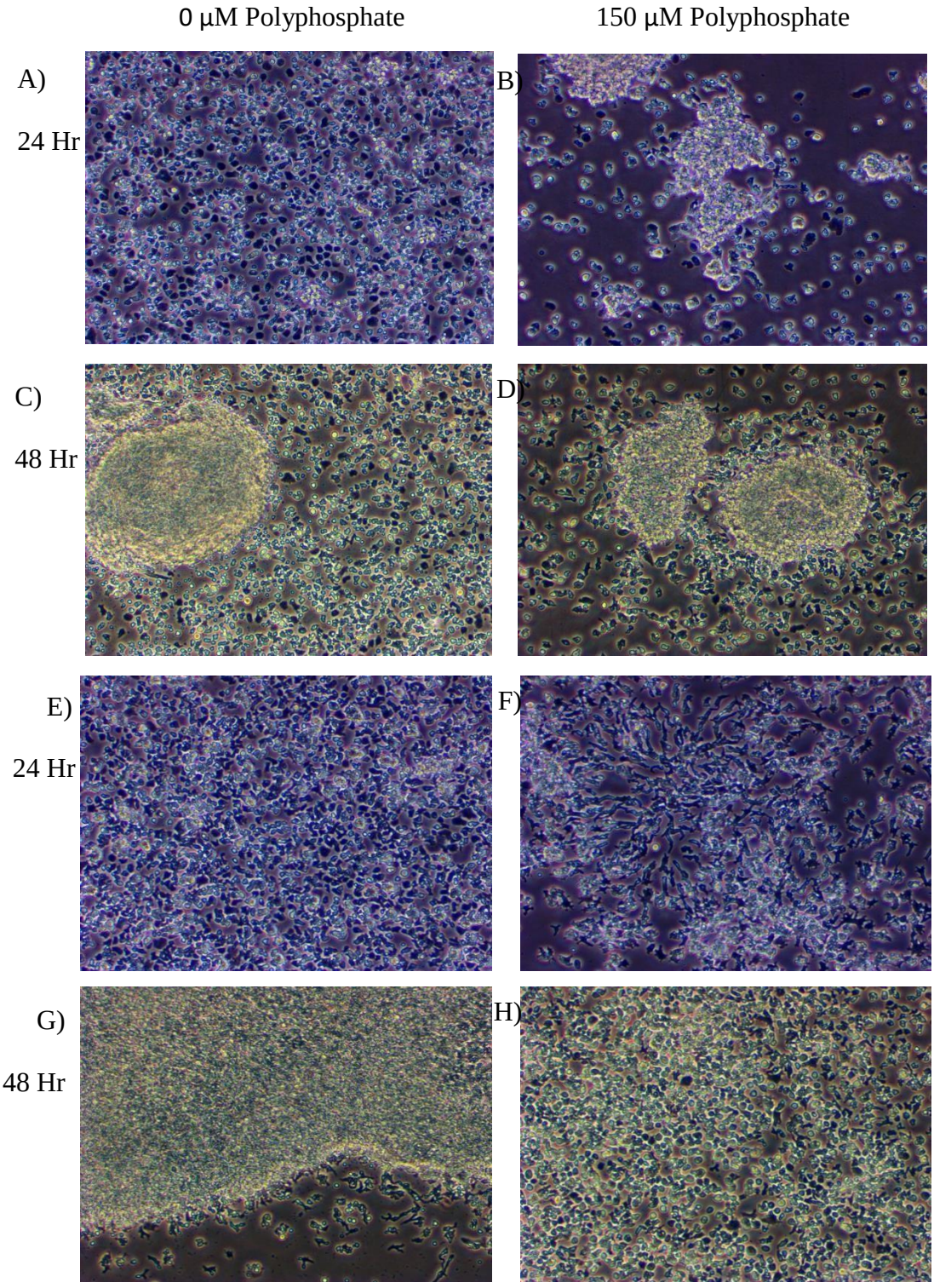

Figure 1

Figure 1: Wild type cells and SPX 2 Cell Aggregation +/- Polyphosphate treatment. Aggregation of wild type cells and *SPX2*$^{-}$ cells treated with 0 μM and 150 μM polyphosphate to show the effect of polyphosphate on cell development. Mid-log cells were cultured at $1.5x10^{6}$ cells/mL in the presence or absence of polyphosphate. Cell development was measured at 24 hours and 48 hours, and *SPX2*$^{-}$ responses (e-h) were compared to the response of wild type cells (a-d).

Polyphosphate as a growth inhibitor

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 polyphosphate inhibits the proliferation of wild-type mid-log cells (18). To identify if *SPX 2*$^{-}$ may play a role in signaling responsible for the previously observed inhibition of proliferation, wild-type and *SPX 2*$^{-}$ cells were grown in the presence of absence of 150 µM polyphosphate. The *SPX 2*$^{-}$ cells showed a proliferation similar to that of wild-type cells (Figure 2).

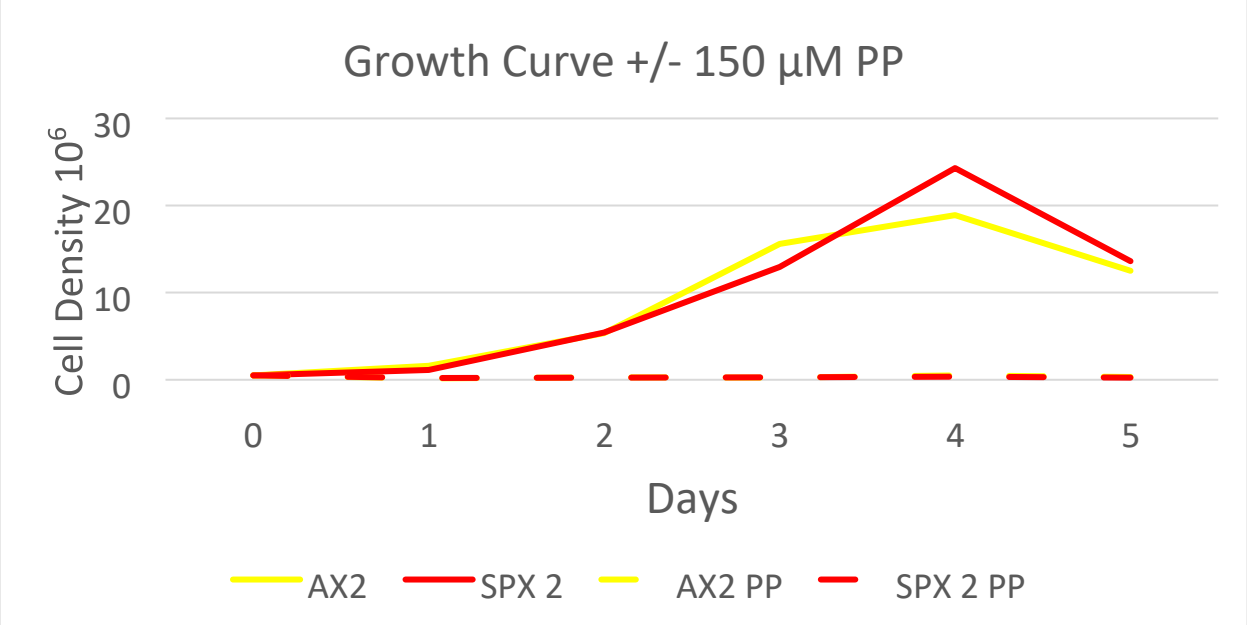

Figure 2

Figure 2: Proliferation response to polyphosphate. Growth curve of wild type and *SPX 2*$^{-}$ cells treated with 0 µM or 150 µM polyphosphate. 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.

Colony expansion to test for motility

Motility can be measured by placing 10-15 cells onto agar plates and measuring diameters of isolated colonies as they grow. The low amount of cells was obtained by performing serial dilutions of cells in HL5 media. Measureable colonies were seen after 3 days and 4 days, but after 4 days the colonies were usually overlapping not allowing measurement. While it seemed that there was little effect of disrupting *SPX 2*$^{-}$ at day 3, *SPX 2*$^{-}$ cells showed significantly larger colony diameter at day 4 (Figure 3). This suggests that *SPX 2*$^{-}$ cells are either able to clear bacteria faster than wild-type, or that motility increases over time in *SPX 2*$^{-}$ cells. An observation that was made when examining these colonies was that *SPX 2*- colonies had clearings in the middle of the colonies while wild-type cells did not. (Figure 4)

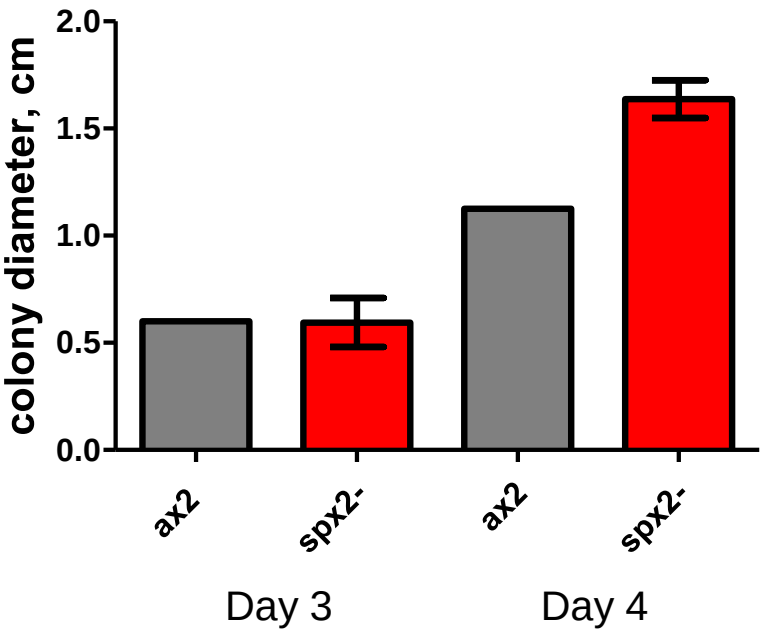

Figure 3

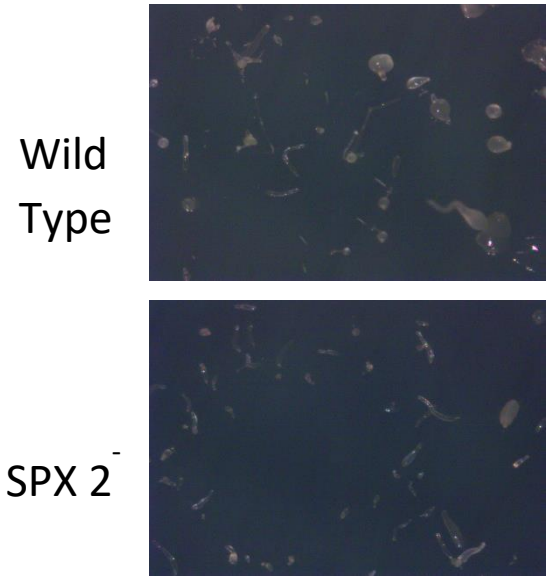

Figure 4

Figure 3: Colony diameters of wild-type and *SPX 2*$^{-}$ cells. Measurements were taken at 3 and 4 days when colonies were visible and non-overlapping. Each plate was seeded with 10-15 cells at day 1. Values are mean +/- SEM measuring the diameters of at least 5 colonies on plates from 3 independent experiments.

Figure 4: Center of colony images taken in wild-type and *SPX 2*- cells to compare density of spores at this location.

Fruiting body development

To determine if SPX 2 plays a role in fruiting body development, an assay was performed in which cells were allowed to develop on nitrocellulose membranes for 48 hours. These membranes are used to develop spores in *Dictyostelium* which can allow measurement of how quickly cells form spores or if they possess a defect in normal sporulation habits. When comparing the wild-type cells to the *SPX 2*$^-$ cells it can be seen that *SPX 2*$^-$ fruiting bodies had longer stalks and smaller spore heads than wild-type cells. The *SPX 2*$^-$ cells also had more aggregates in the mound stage of development than the wild-type cells. (Figure 5)

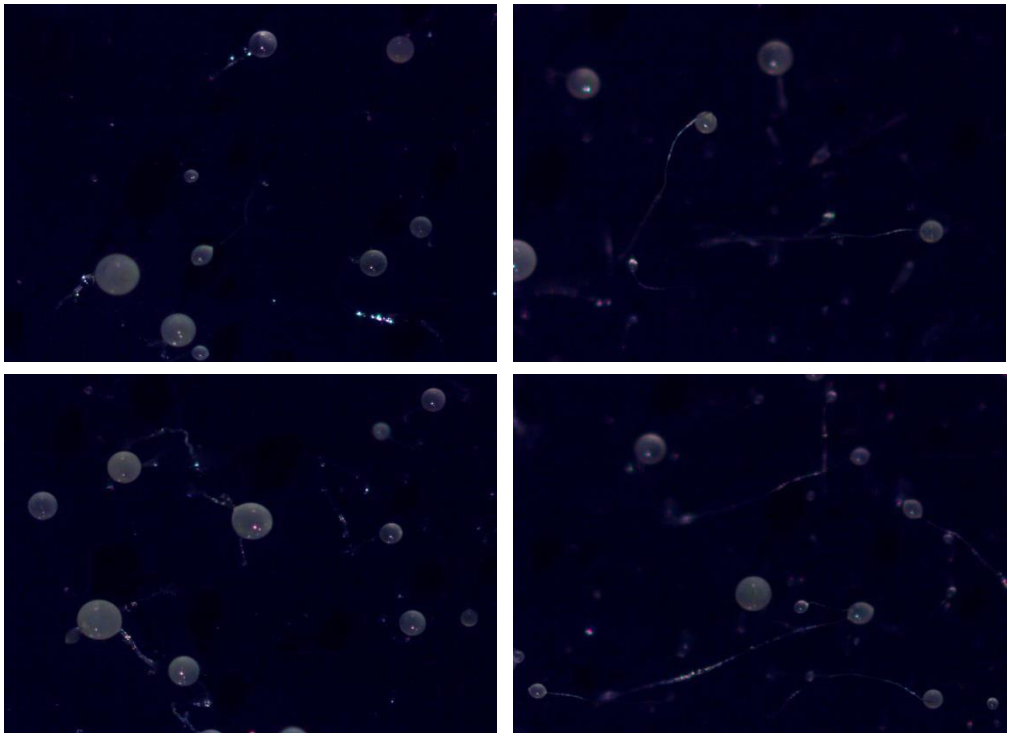

Figure 5

Figure 5: Wild-type and *SPX 2*$^-$ cells were developed on nitrocellulose membranes in a dark humid box for 48 hours before images were taken. Mid log cells were seeded at 1 x 10^7 cells/mL on the membrane.

Intracellular PolyP/InsP assay

To measure intracellular polyphosphate and inositol phosphate levels in wild-type and *SPX 2*$^-$ cells, a DAPI assay was performed on a plate reader to measure fluorescence. To ensure that the DAPI was binding to PolyP or InsP and not DNA, RNA, or other protein species, the cells were washed, lysed, and treated with Benzonase and pro-k. The wash removed extracellular contaminants and factors from the media while the lysing allowed intracellular contents to be exposed. The DNA and RNA could then be degraded by Benzonase which acts as a nuclease. Pro-k treatment was then used to digest protein, and the remaining solution was filtered using 10K filters to remove leftover enzyme and degradation products. DAPI fluorescence assays could then be performed to give concentrations of PolyP and InsP present inside of the original cells. (Figure 6)

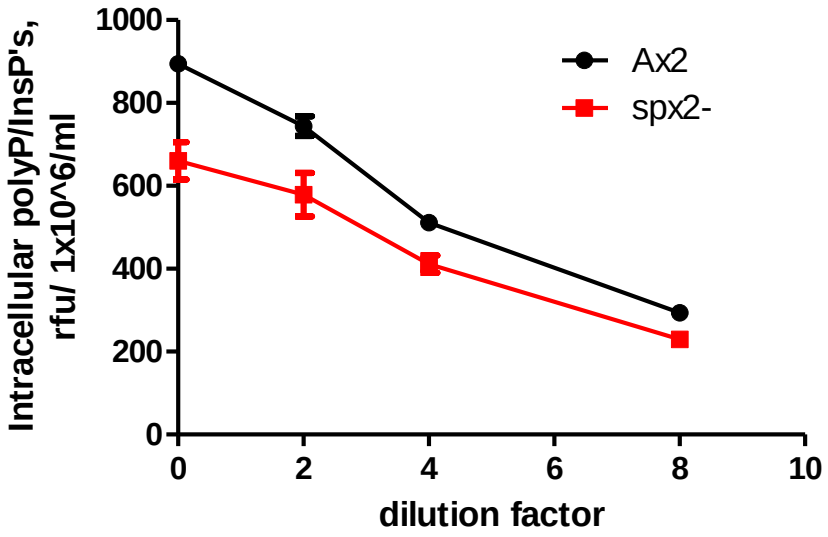

Figure 6

Figure 6: 20 x 10^6 Wild-type and *SPX 2*$^-$ cells were prepared using washes, cell lysing, Benzonase treatment, pro-k treatment, and 10K filters to isolate intracellular PolyP and InsP molecules. Serial dilutions were performed to concentrations of: 1, ½, ¼, and 1/8 and measured on a 96 well plate using DAPI as a fluorescing molecule. Fluorescence was measured on a SynergyMx plate reader using an excitation of 415 and emission of 550.

Cell morphology

Cell morphology was observed to note if there were any apparent abnormalities in the *SPX 2*$^{-}$ cells. Mid-log cells were seeded in HL5 at low density (0.25 x 10^6), medium density(0.50 x 10^6), and medium density in PolyP(0.50 x 10^6 + 150 µM PolyP) and observed at 24 and 48 hours using microscopy. There were no apparent differences between wild-type and *SPX 2*$^{-}$ cells tested in these conditions. (Figure 7)

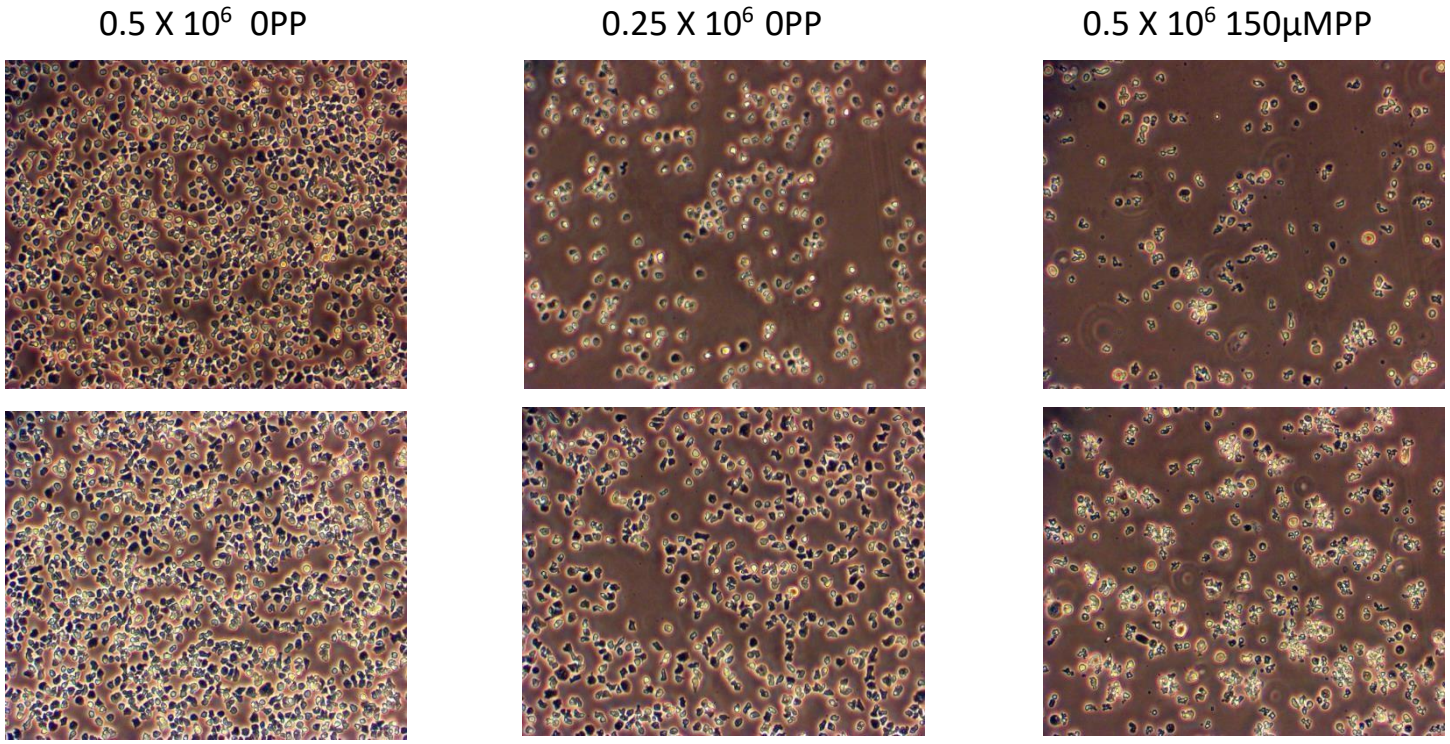

Figure 7

Figure 7: Mid-log wild-type and *SPX 2*$^{-}$ cells were taken from shaking culture and washed twice in HL5 to remove extracellular factors. Cells were then seeded at the designated concentration in HL5 media with or without PolyP and allowed to grow for 48 hours. Observations were collected at 24 and 48 hours and images were taken at 48 hours.

CHAPTER IV

CONCLUSION

In order to measure the effects of the SPX 2 gene a gene knockout had to first be made using molecular cloning techniques as this gene was uncharacterized in *Dictyostelium*. This task was accomplished through the use of restriction enzyme and homologous recombination based cloning. This involved using primers containing restriction enzymes which corresponded to restriction enzymes present in the plasmid of interest (pLPBLP). This allowed regions of SPX 2 coding sequence to be PCR amplified and then placed into the plasmid through ligation reactions of corresponding DNA sticky ends. These homologous regions of DNA were placed on either side of the blasticidin resistance gene located in the plasmid that would later be used as a selectable marker for successful transformants. Once both regions of SPX 2 DNA were intact in the plasmid large amounts could be produced by inserting the plasmid into *E. coli* cells, then purifying the plasmid from a high density culture. These plasmids could then be cut with restriction enzymes to produce DNA fragments containing homologous SPX 2 DNA on their ends and the blasticidin resistance gene in the middle. This fragment could then be transformed into *Dictyostelium* cells where it would undergo homologous recombination with the original host DNA. Validation could then be done through blasticidin treatment and then PCR amplification followed by DNA sequencing of the SPX 2 gene. Once the gene was effectively knocked out the phenotyping process could begin.

The data that is being reported in this paper is preliminary as tests are still ongoing. Ongoing investigation notes that there are no abnormalities present in the cell growth, cell

morphology, proliferation, or vegetative state of *SPX 2⁻ Dictyostelium discoideum*. Phenotypic variation is seen in different areas of the *SPX 2⁻* cells. These abnormalities have been seen in *Dictyostelium* development measured by aggregation assays that were induced by starvation and Polyphosphate (Figure 1). Abnormalities have also been seen in fruiting body development with *SPX 2⁻* cells displaying longer, thinner stalks and smaller spore heads (Figure 5). Intracellular levels of Polyphosphate and Inositol phosphates were also decreased in *SPX 2⁻* cells. Although no explanation is present for these observations yet, a possibility is that this SPX domain is involved in the starvation developmental response in *Dictyostelium* or that it may play a role in quorum sensing between cells.

SPX domains have been shown to sense Inositol Phosphates and affect Polyphosphate synthesis, phosphate transport, and phosphate starvation signaling in yeast and plants (5). These findings are what inspired the question as to if SPX domains were involved in phosphate transport and signaling in a eukaryotic system. As there are five SPX domains in *Dictyostelium* there may be multiple avenues for phosphate to be transferred and signaled, but the reduction in intracellular polyphosphate/inositol phosphate levels in *SPX 2⁻* cells is strong evidence that SPX domains may be involved in phosphate signaling or transport in eukaryotic organisms. This supports the findings in plants and yeast suggesting a conserved role for SPX domains across species.

As this project is ongoing there are still many areas left to explore. Our next focus will be to construct a rescue of the SPX 2 gene to further validate the abnormal phenotypes observed. Next, we will work to distinguish between polyphosphate and inositol phosphate in the intracellular polyphosphate/inositol phosphate concentration assay. Other tests that we would

like to perform are PolyP binding, Mass and protein determination, phagocytosis, cytokinesis, apoptosis, and cytoskeletal assays. After the phenotyping of SPX 2 we will work to create knockouts for the other SPX domains in *Dictyostelium* to gain a greater understanding of these domains and their roles in phosphate transport in eukaryotic organisms.

REFERENCES

1. M. E. Lenburg, E. K. O'Shea, *Signaling phosphate starvation.* Trends Biochem. Sci. 21, 383–387 (1996). Medline doi:10.1016/S0968-0004(96)10048-7

2. T.-J. Chiou, S.-I. Lin, *Signaling network in sensing phosphate availability in plants.* Annu. Rev. Plant Biol. 62, 185–206 (2011). Medline doi:10.1146/annurev-arplant-042110-103849

3. D. Secco, C. Wang, B. A. Arpat, Z. Wang, Y. Poirier, S. D. Tyerman, P. Wu, H. Shou, J. Whelan, *The emerging importance of the SPX domain-containing proteins in phosphate homeostasis*. New Phytol. 193, 842–851 (2012). Medline doi:10.1111/j.1469-8137.2011.04002.x

4. S. Wege, G. A. Khan, J. Y. Jung, E. Vogiatzaki, S. Pradervand, I. Aller, A. J. Meyer, Y. Poirier, *The EXS Domain of PHO1 Participates in the Response of Shoots to Phosphate Deficiency via a Root-to-Shoot Signal*. Plant Physiol. 170, 385–400 (2016). Medline doi:10.1104/pp.15.00975

5. Wild R., Gerasimaite R., Jung J.-Y., Truffault V., Pavlovic I., Schmidt A. et al. (2016) *Control of eukaryotic phosphate homeostasis by inositol polyphosphate sensor domains*. Science 352, 986–990 doi:10.1126/science.aad9858

6. Bryksin AV, Matsumura I. *Overlap extension PCR cloning: a simple and reliable way to create recombinant plasmids*. Biotechniques. 2010;48:463. doi: 10.2144/000113418.

7. Hughes RA, Miklos AE, Ellington AD. *Gene synthesis: methods and applications.* Methods Enzymol. 2011;498:277–309. doi: 10.1016/B978-0-12-3851208.00012-7.

8. Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, Smith HO. *Enzymatic assembly of DNA molecules up to several hundred kilobases*. Nat Methods. 2009;6:343–U341. doi: 10.1038/nmeth.1318.

9. Mitchell DB, Ruggli N, Tratschin JD**.** *An improved method for cloning PCR fragments.* PCR Methods Appl. 1992;2:81–2. doi: 10.1101/gr.2.1.81.

10. Radeck J, Kraft K, Bartels J, Cikovic T, Durr F, Emenegger J, et al. *The Bacillus BioBrick Box: generation and evaluation of essential genetic building blocks for standardized work with Bacillus subtilis*. J Biol Eng. 2013;7:29. doi: 10.1186/1754-16117-29.

11. Haun RS, Serventi IM, Moss J. *Rapid, reliable ligation-independent cloning of PCR products using modified plasmid vectors*. Biotechniques. 1992;13:515–8.

12. Mead DA, Pey NK, Herrnstadt C, Marcil RA, Smith LM. *A universal method for the direct cloning of PCR amplified nucleic acid.* Biotechnology. 1991;9:657–63. doi: 10.1038/nbt0791-657.

13. Holton TA, Graham MW**.** *A simple and efficient method for direct cloning of PCR products using ddT-tailed vectors.* Nucleic Acids Res. 1991;19:1156. doi: 10.1093/nar/19.5.1156.

14. Hoseini SS, Sauer MG. *Molecular cloning using polymerase chain reaction, an educational guide for cellular engineering*. *Journal of Biological Engineering*. 2015;9:2. doi:10.1186/1754-1611-9-2.

15. Battini J-L, Rasko JEJ, Miller AD. *A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: Possible role in G protein-coupled signal transduction. Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(4):1385-1390.

16. Suess, P.M. and R.H. Gomer, *Extracellular Polyphosphate Inhibits Proliferation in an Autocrine Negative Feedback Loop in Dictyostelium discoideum.* J Biol Chem, 2016. **291**(38): p. 20260-9.

17. Smith, S.A., et al., *Polyphosphate modulates blood coagulation and fibrinolysis.* Proc Natl Acad Sci U S A, 2006. **103**(4): p. 903-8.

18. Gajsiewicz, J.M., S.A. Smith, and J.H. Morrissey, *Polyphosphate and RNA Differentially Modulate the Contact Pathway of Blood Clotting.* J Biol Chem, 2017. **292**(5): p. 18081814.

19. Morrissey, J.H., S.H. Choi, and S.A. Smith, *Polyphosphate: an ancient molecule that links platelets, coagulation, and inflammation.* Blood, 2012. **119**(25): p. 5972-9.

20. Wang, L., et al., *Inorganic polyphosphate stimulates mammalian TOR, a kinase involved in the proliferation of mammary cancer cells.* Proc Natl Acad Sci U S A, 2003. **100**(20): p. 11249-54.

21. Ozeki, N., et al., *Polyphosphate induces matrix metalloproteinase-3-mediated proliferation of odontoblast-like cells derived from induced pluripotent stem cells.* Exp Cell Res, 2015. **333**(2): p. 303-15.

22. Lui, E.L., et al., *Inorganic polyphosphate triggers upregulation of interleukin 11 in human osteoblast-like SaOS-2 cells.* Biochem Biophys Res Commun, 2016. **479**(4): p. 766-771.

23. Hassanian, S.M., et al., *Inorganic polyphosphate promotes cyclin D1 synthesis through activation of mTOR/Wnt/beta-catenin signaling in endothelial cells.* J Thromb Haemost, 2016. **14**(11): p. 2261-2273.