

**IDENTIFYING COMPONENTS OF THE CHALONE SIGNAL  
TRANSDUCTION PATHWAY IN *DICTYOSTELIUM DISCOIDEUM***

An Undergraduate Research Scholars Thesis

by

BETHANY SUMP

Submitted to the Undergraduate Research Scholars program  
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 2016

Major: Biochemistry and Genetics

# TABLE OF CONTENTS

	Page
ABSTRACT.....	1
SECTIONS	
I INTRODUCTION .....	3
II METHODS .....	7
Plasmid.....	7
Digestion of plasmid.....	7
Restriction-Enzyme-Mediated-Insertional (REMI) mutagenesis .....	7
Screening against polyphosphate.....	8
Cloning out REMI mutants.....	9
Identifying the location of the REMI insert.....	9
III RESULTS .....	11
REMI mutants show a nonresponse to inorganic polyphosphate .....	11
Plasmid insert is located by inverse PCR .....	13
Unconfirmed results on interrupted gene.....	15
IV CONCLUSION .....	16
REFERENCES .....	17
ACKNOWLEDGEMENTS.....	19

## ABSTRACT

Identifying Components of the Signal Transduction Pathway in *Dictyostelium discoideum*

Bethany Sump  
Department of Biochemistry and Biophysics  
Texas A&M University

Research Advisor: Dr. Richard Gomer  
Department of Biology

In cancer patients, primary tumors can inhibit the proliferation of distant metastasized tumor cells. The subsequent removal of the primary tumor causes the metastasized tumor cells to regain the ability to proliferate. Secreted factors that inhibit the proliferation of the cells that secrete the factor are called chalones, and while they were originally discovered as a way for tissue size to be regulated or as a method to control population densities of specific types of cells, chalones could also be used to control tumor cell proliferation and thus are important to this field of study. However, their mechanisms for limiting proliferation are still poorly understood. A new possible chalone, inorganic polyphosphate, has been identified in the model organism *Dictyostelium discoideum*, a haploid, eukaryotic social amoeba that has already been used to identify two other factors that act as chalones. To identify the components of this new chalone's signal transduction pathway, procedures such as restriction-enzyme-mediated insertional (REMI) mutagenesis, inverse PCR, and using homologous recombination to create null mutations are being implemented.

# SECTION I

## INTRODUCTION

An important observation in our understanding of cancer is that for many tumors, the primary tumor has the ability to keep metastasized tumor cells in a near-dormant state<sup>1</sup>. The removal of the primary tumor appears to have the side effect of allowing the proliferation of the previously dormant tumor cells<sup>1,2</sup>. This leads to the important questions of what is allowing these primary tumors to hold the metastasized tumor cells in this dormant state, and whether this same method could be used to stop or slow the growth of tumors as a treatment option<sup>2</sup>.

It is a widely accepted idea that some cells have a secreted factor that act as a growth inhibitor as cells reach high densities, inducing a stationary phase<sup>3,4</sup>. This negative feedback loop with the secreted factors, named chalones, then completely stops proliferation of cells once a certain threshold has been reached, such as attainment of a certain tissue size<sup>5</sup>. Examples of this negative feedback loop have already been found. A prime case would be the research that has been done on the function of myostatin as a chalone controlling the proliferation of myoblasts and ultimately the number of myocytes, or muscle cells, in mice<sup>6</sup>. Although these chalones are clearly important, the identity and the mechanisms of actions of these chalones are not known for most organisms.

The model organism *Dictyostelium discoideum* is a unicellular, eukaryotic slime mold that is ideal for further research into chalones<sup>5,7,8</sup>. There have been two secreted factors previously found that appear to act as chalones in *Dictyostelium discoideum*, AprA and CfaD<sup>7,8</sup>. AprA has

been found to slow proliferation in both wild type and *aprA*<sup>-</sup> cells without destroying the cells<sup>8</sup>. CfaD has also been found to slow proliferation in *Dictyostelium discoideum* cells without having any measurable effect on the growth of the cells<sup>5</sup>. Current conclusions lead to the idea cells have different receptors for both AprA and CfaD, and that both of these receptors must be activated in order to slow proliferation.<sup>5,7</sup> This would clearly be an advantage for the *Dictyostelium* cells to improve their success in an environment that is reaching starvation conditions. However, since AprA and CfaD only slow proliferation, and at high density proliferation is still stopped in *AprA*<sup>-</sup> and *CfaD*<sup>-</sup> cells, it stands to reason that there must be another chalone that has the ability to stop proliferation entirely in the *Dictyostelium* cells that is completely independent of those already known.

A current hypothesis for the identity of such a chalone in *Dictyostelium discoideum* is the molecule polyphosphate (polyP). PolyP is found in plants, animals, fungi, and bacteria, and is composed of long, linear chains of five to hundreds of phosphates linked with the high energy phosphoanhydride bonds similarly found in ATP<sup>9,10</sup>. Researchers have already found a number of roles that polyP plays in the cell by creating knockouts of polyP kinase (PPK), the enzyme which synthesizes polyP, in *Escherichia coli*<sup>11</sup> and many other bacteria. Some of the currently known roles of polyP in *E. coli* are its role as an energy source, and its role in assisting in the phosphorylation of alcohols<sup>9</sup>. It has been shown that polyP has a very important role to play in the survival of the cell when in stationary phase, as PPK knockout bacteria lose the ability to adapt to starvation conditions<sup>11,12</sup>. While the PPK enzymes, PPK1 and PPK2, are most known for being bacterial, both enzymes have been found to have homologs in a few eukaryotes including PPK1 being found in multiple photosynthetic eukaryotes and a few non-photosynthetic

eukaryotes including three different species of *Dictyostelium*<sup>13</sup>. Researchers have previously studied polyP in *Dictyostelium discoideum* by examining the role of the *Dictyostelium discoideum* PPK homolog *polyphosphate* kinase (DdPPK1). Mutants which lack DdPPK1 have reduced sporulation and slowed development of fruiting bodies, have altered predator-prey interactions with bacteria<sup>10</sup>.

Work in the Gomer lab found that *Dictyostelium* cells secrete increasing amounts of polyP as the density of the cells increases, and if the amount of polyP generally observed in stationary phase cells is added to cells still in mid-log growth, proliferation rates dramatically decrease, even to the point of a complete halt in proliferation (unpublished data by Patrick Sues).

Looking at polyP as a chalone in *Dictyostelium* is something that is still open to inspection, and furthermore, is something that could very well be extremely significant after examination.

Although DdPPK1 seems to regulate intracellular polyP, DdPPK1 knockout cells have completely normal stationary phase and proliferations profiles<sup>10</sup>. This would lead to the conclusion that the intracellular and the extracellular polyP are being regulated by different mechanisms. This could potentially be very telling, since the mechanism for how polyP is synthesized in higher level eukaryotes is currently unknown and the yet unknown extracellular mechanism in *Dictyostelium* could be the link that is missing.

Since the polyphosphate-sensing signal transduction pathways in *Dictyostelium* may be similar to chalone signal transduction pathways in higher organisms, once the *Dictyostelium* polyphosphate signal transduction pathway is known, it could then lead to an understanding of

the ways that proliferation in higher eukaryotes, such as a patient with a tumor, could be controlled.

## SECTION II

### METHODS

#### Plasmid

The plasmid used in the electroporation transformation was pBSR1, obtained from the *Dictyostelium* stock center. pBSR1 has a blasticidin S resistance cassette derived from the *bsr* gene of *Bacillus cereus*<sup>14</sup>.

#### Digestion of plasmid

The plasmid pBSR1 was digested using 10X Cutsmart® Buffer and the restriction enzyme BamHI-Hf (New England BioLabs, Ipswich, MA). This digestion was incubated in a 35°C incubator for approximately 24 hours. The digested plasmid was purified using a Zymoclean® Gel DNA Recovery kit (Zymo Research, Irvine, CA). A small sample of the purified pBSR1 was then tested on a 1% analytical agarose gel for confirmation of proper digestion and purification.

#### Restriction-Enzyme-Mediated Insertional (REMI) mutagenesis

*Dictyostelium discoideum* AX-2 wild type cells<sup>15</sup> were grown in shaking culture to mid-log (2-4 x10<sup>6</sup> cells/mL) in HL-5 medium (Formedium, United Kingdom) supplemented with streptomycin. For each REMI reaction, 6 x10<sup>6</sup> cells were collected by centrifugation at 400 x g for 3 minutes, and washed twice with at least half the original culture volume of H-50 transformation buffer. The recipe for H-50 is 20 mM Hepes, 50 mM KCl, 10 mM NaCl, 1 mM Mg<sub>2</sub>SO<sub>4</sub>, 5 mM NaHCO<sub>3</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, adjusted to pH 7.0. The cells were then resuspended into 178 µL ice-cold H-50 buffer per reaction. Each 200 µL REMI reaction consisted of 178 µL



of resuspended cells, 20  $\mu\text{L}$  of digested and purified pBSR1 plasmid, and 2  $\mu\text{L}$  of the enzyme DpnII (New England BioLabs). A negative control was obtained using H-50 buffer instead of the plasmid and enzyme in these reactions. These cells were then placed in 0.2 mm gap cuvettes and immediately were electroporated using a BioRad Gene Pulser set to 0.85 kV and 1  $\mu\text{F}$  with the time constants ranging from 0.08-0.2. The cuvettes were then immediately placed in ice for 5 minutes, after which 100  $\mu\text{L}$  aliquots were added dropwise onto 3.5 inch x 0.625 inch plastic petri dishes that contain 10 mL of HL-5 media supplemented with streptomycin. After approximately 24 hours at room temperature, 5  $\mu\text{L}$  of 10 mg/mL blasticidin was added to select for transformed cells. Colonies visible with an inverted microscope appeared after 4-6 days at room temperature.

### **Screening against polyphosphate**

The transformant plates were then transferred over to shaking culture by first removing the old media from the plates and then washing the cells off of the surface of the plate with 10 mL fresh HL5 media and promptly transferred to a flask where 7  $\mu\text{L}$  of 10 mg/mL blasticidin was added to select for the cells that were successfully transformed with the pBSR1 plasmid, which would confer a resistance to the drug. These new cultures were then grown to the mid-log range. The cultures were then diluted to  $1.0 \times 10^6$  cells/mL and subjected to a treatment of 125  $\mu\text{M}$  polyphosphate in HL-5, and were left to grow at room temperature on a rotary shaker. Cell densities were counted approximately every 24 hours. After 48 hours, the cells were collected by centrifugation and resuspended to  $0.5 \times 10^6$  cells/mL in HL-5 media supplemented with streptomycin, but with no polyphosphate, and left to grow for 48 hours, and cell densities were counted every 24 hours. This process of 48 hours with polyP and 48 hours without polyP is

considered one cycle, and the REMIs were subjected to 5-6 cycles to determine how they reacted to polyP as compared a similarly-treated AX-2 wild type culture.

### **Cloning out REMI mutants**

After the completion of the screening, approximately  $1 \times 10^6$  cells of the mutants were collected by centrifugation for 3 minutes at 1,500 rpm. These cells were washed in 3-5 mL of fresh HL5 media with streptomycin and resuspended into 1 mL of drug-free HL5 media. For each mutant obtained, three plates with *E. coli* bacterial lawns were obtained and labeled A-C. Different amounts of the resuspended mutant cells were spread onto each plate; the different amounts being 100  $\mu$ L, 300  $\mu$ L, and 500  $\mu$ L for plates A, B, and C respectively. It is helpful to add an extra 200  $\mu$ L of drug-free HL5 media to plate A to assist in the ease of spreading. After 2-3 days, colonies appeared and a single colony was used to make three separate streaks (labeled with 1-3 and their plate letter, i.e. B-1, B-2, B-3) onto the edges of a new plate with a bacterial lawn. After 3-4 days these clone colonies were transferred to shaking culture, allowed to grow into mid-log range, and frozen down until further study. The clones were subsequently tested with the same protocol as described before of 48 hours with polyphosphate and 48 hours without polyphosphate to insure that the cloned mutants show the same phenotypic response of proliferation in the presence of polyphosphate as the original mutant.

### **Identifying the location of the REMI insert**

The location of the REMI insert in the genome of cells that were resistant to polyphosphate was found using a process called Inverse PCR. First, the genomic DNA was extracted from the cloned cultures that did not show a proliferation change when in the presence of polyP as

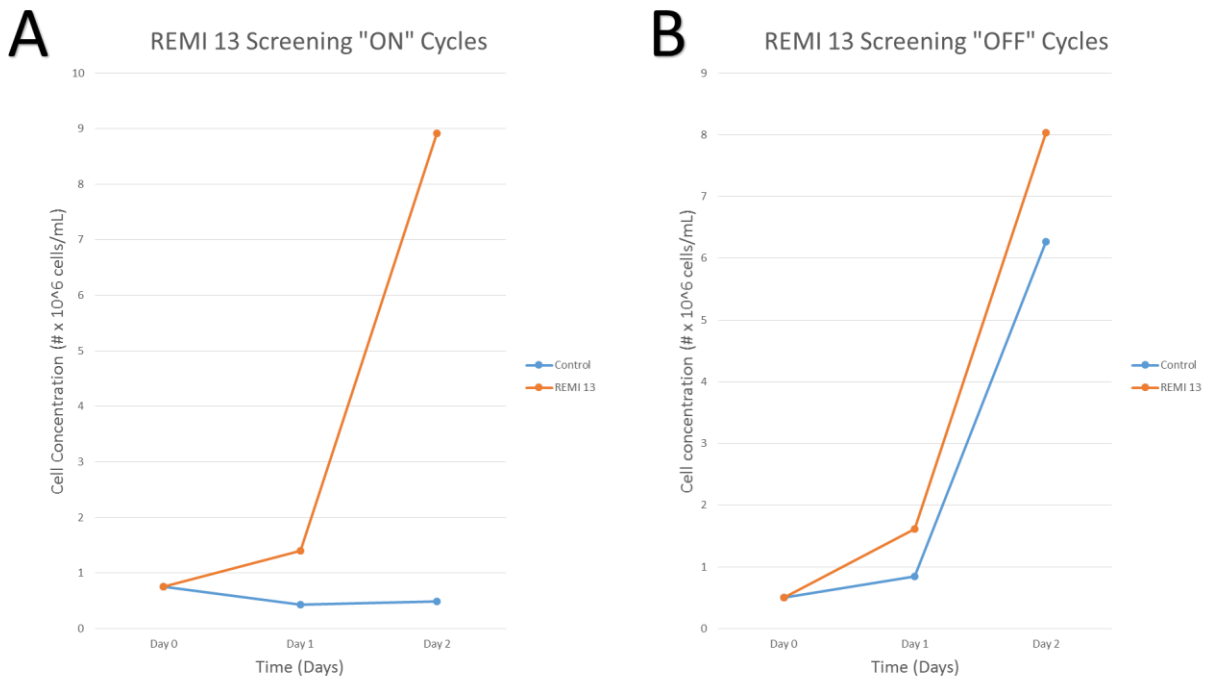
previously described.<sup>16</sup> The genomic DNA was then digested with the endonuclease Alu I, purified with a Zymoclean™ Gel DNA Recovery Kit, and ligated into circular DNA with T4 Ligase. 1 µL of the ligated DNA was used to set up a 15 µL PCR reaction for the mutants and the AX-2 control sample. These samples were run on a 1% agarose gel to insure that the mutant samples show bands and the control AX-2 samples do not. Once this was verified, three different tubes of the same 15 µL PCR reaction were prepared with the same conditions (Forward Primer – CGTCGATATGGTGCACTCTC; Reverse primer – TGTCGTTAGAACGCGGCTAC; Thermocycler on the following settings: 94°C for 30 seconds, followed by 32 cycles of 94°C for 30 seconds, 56.3°C for 30 seconds, and 65°C for 90 seconds; this is followed by a 5 minutes of 68°C and the DNA was stored at 4°C until use). The PCR product DNA from the three different tubes was purified with a Zymoclean™ Gel DNA Recovery Kit and sent off for sequencing (Lonestar Labs, Houston TX).

## SECTION III

### RESULTS

#### **REMI mutants show a non-response to inorganic polyphosphate**

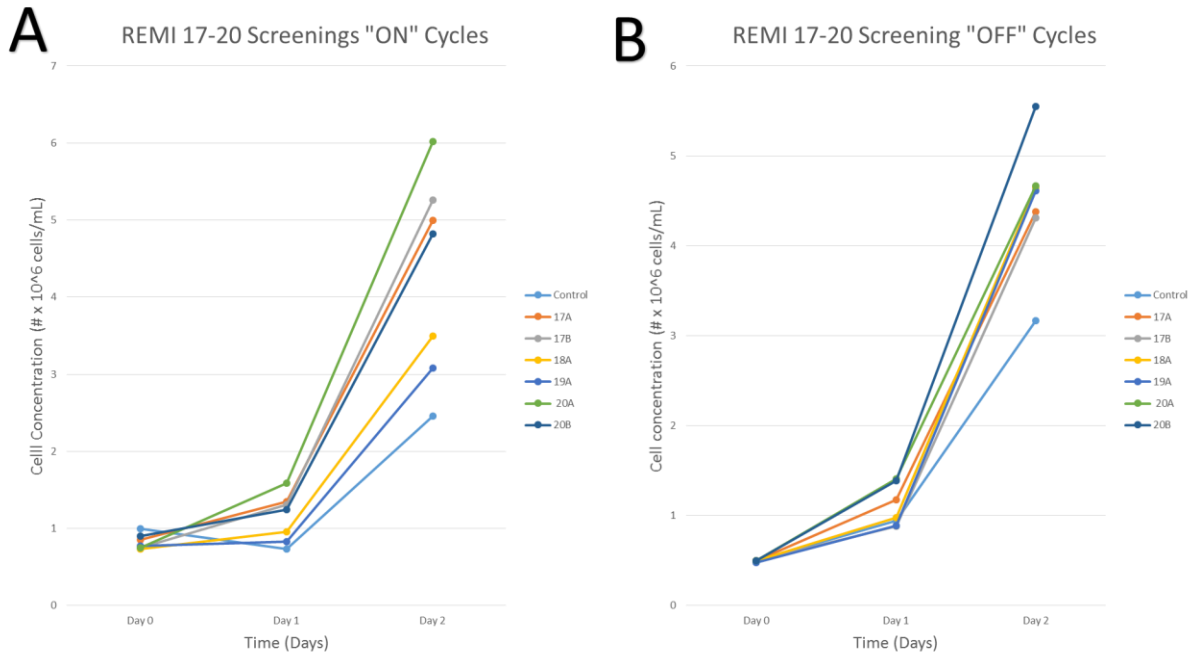
It has previously been found that inorganic polyphosphate acts as a chalone in *Dictyostelium discoideum*, by inhibiting proliferation. This has led to interest in the components of the signal transduction pathway that begins with inorganic polyphosphate as the signal and the stopping of proliferation in the late G2 phase of the cell cycle as the result. To study this, the process known as restriction enzyme-mediated insertional (REMI) mutagenesis, was used to create mutants that could be screened according to their proliferation abilities. When treated with 125  $\mu\text{M}$  polyphosphate, a number of mutants created by REMI mutagenesis show a continuation of normal proliferation as opposed to the AX-2 control cells which show almost a complete halt of proliferation (Figure 1A). This would indicate that the pBSR1 plasmid that was inserted into the genome to create these mutations was inserted in such a place as to interfere with the signal transduction pathway between the chalone and the halting of proliferation. It can likewise be seen that when in a control environment (HL-5 media with no polyphosphate), the REMI mutants show a slightly higher rate of proliferation than the control AX-2 cells (Figure 1B). This finding further backs the idea that the REMI mutants have a disrupted signal transduction pathway, because it shows that they do not respond to even the small amounts of polyphosphate that would be present in a cell culture even without the experimental conditions adding excess amounts.



**Figure 1:** REMI mutant 13 shows the phenotype of continued proliferation in the presence of inorganic polyphosphate. (A) REMI 13 mutants and AX-2 control cells were subjected to 48 hours in HL5 media supplemented with 125  $\mu$ M polyphosphate. The control cells show halted proliferation while the REMI mutants continue to rapidly proliferate. (B) REMI 13 mutants and AX-2 control cells over a 48 hour period in HL5 media. The mutant cells and the control cells proliferate at approximately the same rate, with the REMI mutants proliferating slightly faster.

Along with the mutant, REMI 13, seven other REMI mutants showed a proliferation phenotype similar to that of REMI 13 (Figure 2). Those seven others are called REMI 5, 17A, 17B, 18A, 19A, 20A, and 20B. Because all of these have similar results, we can conclude that they also have disrupted signal transduction pathways due to the insertion of the pBSR1 plasmid by the same reasoning as above. These eight mutants can all eventually be tested to discover the location of their plasmid insertions and thus, their mutations, but for the purposes of this

experiment only the mutant with the most significant result (REMI 13) has been used for further study.

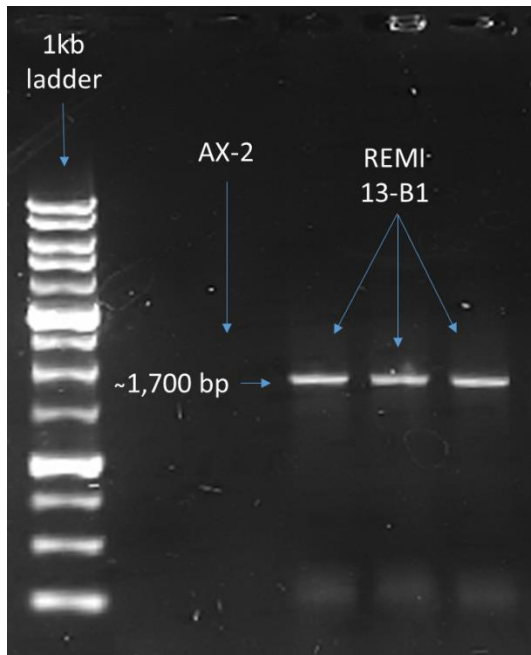


**Figure 2:** More REMI mutants show the phenotype of continued proliferation even in the presence of polyphosphate. (A) REMI mutants 17A, 17B, 18A, 19A, 20A, and 20B show greater proliferation than the control AX-2 cells over repeated 48 hour periods when grown in HL-5 media supplemented with 125  $\mu$ M polyP. (B) REMI mutants show a slightly higher rate of proliferation over repeated 48 hour periods than the control AX-2 cells in normal conditions (HL-5 media with no polyP).

### Plasmid insert is located by inverse PCR

The plasmid that was inserted into the genome to cause the mutations in the REMI cultures theoretically could have been inserted into numerous places in the genome. To find the location of this insert, we did inverse PCR. After digesting the mutant genomes and ligating the linear pieces into circles, primers were designed from the known plasmid sequence to amplify the

entire piece of circular DNA that included both the REMI vector DNA and the genomic DNA from *Dictyostelium* that had been interrupted. This was confirmed on a 1% agarose gel (Figure 3), where it is evident that the AX-2 wildtype DNA has no plasmid insertion, whereas the mutant genomes do.



**Figure 3:** Gel electrophoresis image of inverse PCR results after digestion and ligation of control (AX-2) and mutant (REMI 13-B1) genomes. The AX-2 lane has no bands which proves the lack of plasmid insert, whereas the three lanes containing REMI 13-B1 all have bands around 1,700 bp.

The bands in the REMI 13-B1 lanes appear at approximately 1,700 base pairs (Figure 3). Since the sequence of the pBSR1 plasmid is known, we can anticipate where the DpnII cut site is in relation to the primers designed and thus know that out of the 1,700 base pairs in this small, circular piece of DNA, 655 of them are from the plasmid. This leaves over 1,000 base pairs of the DNA belonging to the original *Dictyostelium* genome and showing us where the insert was located.

### **Unconfirmed results on interrupted gene**

After sending off DNA for sequencing, results indicated that the most likely gene that was interrupted by the plasmid was a gene called Mediator 20 (Med 20). Mediator 20 codes for mediator complex subunit protein. Mediators, first discovered in 1990 by Roger Kornberg<sup>17</sup>, are known to be used in support of transcriptional activation, and is known to be heavily associated with RNA Polymerase II.<sup>18</sup> However, these results have not yet been confirmed, as proper confirmation requires complete knockout of the Med 20 gene by homologous recombination, and this has yet to be completed.



## **SECTION IV**

### **CONCLUSION**

Evidence obtained leads to the conclusion that the mediator protein, Med 20, is a component in the chalone signal transduction pathway. This conclusion, however, is still uncertain as the intended phenotype of a non-response to the chalone inorganic polyphosphate, was only obtained through the interruption of the gene that codes for Med 20, not the complete removal. Further testing is necessary to prove its role in this pathway.

## REFERENCES

1. Guba, M. et al. A primary tumor promotes dormancy of solitary tumor cells before inhibiting angiogenesis. *Cancer Res* **61**, 5575-9 (2001).
2. Gomer, R.H., Jang, W. & Brazill, D. Cell density sensing and size determination. *Dev Growth Differ* **53**, 482-94 (2011).
3. Soll, D.R., Yarger, J. & Mirick, M. Stationary phase and the cell cycle of *Dictyostelium discoideum* in liquid nutrient medium. *J Cell Sci* **20**, 513-23 (1976).
4. Yarger, J., Stults, K. & Soll, D.R. Observations on the growth of *Dictyostelium discoideum* in axenic medium: evidence for an extracellular growth inhibitor synthesized by stationary phase cells. *J Cell Sci* **14**, 681-90 (1974).
5. Choe, J.M., Bakthavatsalam, D., Phillips, J.E. & Gomer, R.H. *Dictyostelium* cells bind a secreted autocrine factor that represses cell proliferation. *BMC Biochem* **10**, 4 (2009).
6. Thomas, M. et al. Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. *J Biol Chem* **275**, 40235-43 (2000).
7. Bakthavatsalam, D., Choe, J.M., Hanson, N.E. & Gomer, R.H. A *Dictyostelium* chalone uses G proteins to regulate proliferation. *BMC Biol* **7**, 44 (2009).
8. Brock, D.A. & Gomer, R.H. A secreted factor represses cell proliferation in *Dictyostelium*. *Development* **132**, 4553-62 (2005).
9. Rao, N.N., Gomez-Garcia, M.R. & Kornberg, A. Inorganic polyphosphate: essential for growth and survival. *Annu Rev Biochem* **78**, 605-47 (2009).
10. Zhang, H., Gomez-Garcia, M.R., Brown, M.R. & Kornberg, A. Inorganic polyphosphate in *Dictyostelium discoideum*: influence on development, sporulation, and predation. *Proc Natl Acad Sci U S A* **102**, 2731-5 (2005).
11. Rashid, M.H. et al. Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **97**, 9636-41 (2000).
12. Rao, N.N. & Kornberg, A. Inorganic polyphosphate supports resistance and survival of stationary-phase *Escherichia coli*. *J Bacteriol* **178**, 1394-400 (1996).
13. Whitehead, M.P., Hooley, P. & MR, W.B. Horizontal transfer of bacterial polyphosphate kinases to eukaryotes: implications for the ice age and land colonisation. *BMC Res Notes* **6**, 221 (2013).

14. Sutoh, K. A transformation vector for dictyostelium discoideum with a new selectable marker bsr. *Plasmid* **30**, 150-4 (1993).
15. Watts, D.J. & Ashworth, J.M. Growth of myxameobae of the cellular slime mould Dictyostelium discoideum in axenic culture. *Biochem J* **119**, 171-4 (1970).
16. Pilcher, K.E., Fey, P., Gaudet, P., Kowal, A.S. & Chisholm, R.L. A reliable general purpose method for extracting genomic DNA from Dictyostelium cells. *Nat Protoc* **2**, 1325-8 (2007).
17. Kelleher, R.J., 3rd, Flanagan, P.M. & Kornberg, R.D. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. *Cell* **61**, 1209-15 (1990).
18. Flanagan, P.M., Kelleher, R.J., 3rd, Sayre, M.H., Tschochner, H. & Kornberg, R.D. A mediator required for activation of RNA polymerase II transcription in vitro. *Nature* **350**, 436-8 (1991).

## **ACKNOWLEDGEMENTS**

I would like to give a special thanks to Dr. Richard Gomer and my fellow laboratory members, most especially Patrick Sues and Dr. Gomer himself as they both played a large role in directing this project. I would also like to thank the Undergraduate Research Scholars Program at Texas A&M University for the amazing opportunity this has provided me.