CHALONE SIGNAL TRANSDUCTION PATHWAY IN *DICTYOSTELIUM DISCOIDEUM*: A POSSIBLE SIGNALING MECHANISM FOR CELL PROLIFERATION INHIBTION

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Chalone Signal Transduction Pathway in *Dictyostelium discoideum*: A Possible Signaling Mechanism for Cell Proliferation Inhibition. (May 2015)

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Cancer is a group of diseases characterized by uncontrolled cell proliferation. Cancer is responsible for nearly 1 of every 4 deaths, and ranks as the second highest cause of death worldwide, and costs the American population more than $216 billion annually. The fundamental mechanism that controls cell density regulation in cancer and in healthy tissues is not well understood. Some healthy cells appear to secrete a diffusible, extracellular factor called a chalone. The chalone is involved in a signal transduction pathway, which inhibits cell proliferation once cells sense that a high enough chalone concentration has been collectively secreted signaling the desired cell population density has reached. However, currently there is not a solid understanding of the genetic components and proteins involved in this pathway.

Dictyostelium discoideum, a slime mold, was used as the model organism. *Dictyostelium discoideum* is eukaryote and has many genes that are homologous to human genes. This makes it a valuable model organism applicable to human cancer research.

Dictyostelium discoideum is a eukaryote model organism, which uses a chalone mechanism to control cell density. *Dictyostelium discoideum* will be used as a model organism to elucidate the

components involved in the chalone pathway by comparing differences in the rate of proliferation, maximum population size reached, and length of cell survival between wild type cells and cells missing a specific genetic component when exposed to various chalone concentrations. Understanding this pathway could lead to possible therapeutics for inhibiting cell proliferation in cancer. The results of this research will be important to the biomedical field and useful in future scientific research on cell density regulation via the chalone mechanism.

DEDICATION

This thesis is lovingly dedicated to my family. I would like to acknowledge the unconditional love and support that my mother, Cynthia Boyle Allen, has provided that inspires and drives me each day and got me through the year of firsts without my father. I would also like to thank my little brother, Brian Edwards Allen, for being so encouraging to me in my endeavors and for being my best friend. I honestly would have not been able to do any of this without family and friends' support, including that of my dear friend, Danielle Parks.

Ultimately, I would like to illuminate my primary motivator in this specific endeavor — my Dad. I dedicate this work in loving memory of my beloved late father, Dr. Donald Anderson Allen. We miss you everyday. You were on my mind each day that I worked on this project. I carry your love close to my heart, and your comforting voice offering words of wisdom still rings loudly in my life guiding my steps. Your unwavering integrity, devotion, and resilient compassion for others above all that had pervaded in your life and career has acted as a beacon illuminating my path towards who I am working to be.

Foremost, I would like to thank our Heavenly Father for provision of joys, challenges, strength to overcome those challenges, grace for growth, and the opportunity to explore His beautiful design of His creation throughout the exploration and production of this thesis.

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I would like to thank Dr. Richard Gomer for providing me with the opportunity to work in his lab and for introducing me to the field of research. His teaching has been paramount to the production of this thesis and my improvement as a student and person. His patience with me over the past two years encouraged me to persevere with this project. Thank you for having confidence in me to put me on this project.

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I would also like to acknowledge the Undergraduate Scholars Research Program and its amazing staff including Tammis Sherman, Austin Ford, and Annabelle Aymond. Their dedication to undergraduate research encouraged me to experience research early on in my undergraduate career and, more importantly, to continue each semester to the completion of this project.

NOMENCLATURE

D. discoideum (Dicty)	*Dictyostelium discoideum*
IPCR	Inverse polymerase chain reaction
KA	Klebsiella aerogenes
kDa	Kilodaltons
Mb	Map-based
ppk	Polyphosphate kinase
REMI	Restriction Enzyme Mediated Insertion

CHAPTER I

INTRODUCTION

Manifestation of cancer

Cancer is a group of diseases characterized in part by uncontrolled cell proliferation [3]. Cancer is responsible for nearly 1 of every 4 deaths, and ranks as the second highest cause of death worldwide, and costs the American population more than $216 billion annually [1,2]. Despite many types of FDA-approved cancer treatments (i.e. surgery, chemotherapy, radiation therapy), the ability to combat cancer effectively is limited to treatments with harmful and invasive side effects since there is not a solid understanding about how to control cell proliferation [1]. A thorough understanding of the necessary components of the chalone mechanism could influence the way cancer is treated; for instance, noninvasive treatment techniques to fight cancer might be developed by mimicking the pathway responsible for tumor dormancy. Tumor dormancy is a stage in cancer when the cells halt proliferation but survive in an inactive form until the surrounding environment reverts to suitable conditions for growth and cell division resumes [8].

Tissue size regulation

Tissue size regulation is not only important in cancer research, but is also a fundamental question in organ size determination within healthy tissues. Understanding the chalone mechanism's role in cell proliferation regulation may also give insight into the mechanics of tissue size determination of vital organs which would have medical applications with regards to organ transplants [Figure 1]. For example, this could give health care professionals a way to grow replacement organs *in vitro*. Therefore, exploring endogenous cell proliferation regulation

mechanisms is of high importance, because the information gained could not only save lives of cancer patients and those in need of organ transplants, but also prevent the expenditure of billions of dollars within the health care system [9].

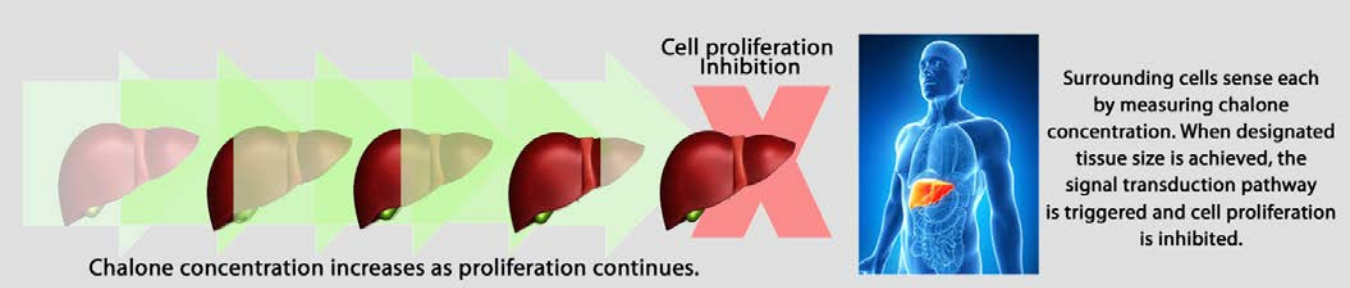

Figure 1: Example of how a chalone could contribute to proper tissue size regulation in the human body. Credit: Christina Allen

Chalone mechanism

It is hypothesized that cell proliferation is regulated by a chalone mechanism [2]. A chalone is a signaling molecule that is secreted by a cell [3]. It is suspected that each proliferated cell secretes an equivalent unit of a chalone. As proliferation continues and cell density increases, the concentration of the chalone increases. Thus, cells can determine the surrounding cell density by sensing the concentration of the chalone in their immediate environment [4] [Figure 2]. Once proper tissue size is reached and the chalone concentration is high enough to indicate an intended cell density, then the chalone will trigger a signal transduction pathway, which will inhibit the cells' physiological activity, thus regulating cell proliferation [4-6]. The chalone mechanism operates by a negative feedback loop system as demonstrated by *Figure 3*. The chalone will become more concentrated as cell density increases, and the chalone will activate a signal transduction pathway to inhibit cell proliferation [7]. It has been observed, firsthand, that the chalone concentration increases simultaneously as cell density increases. Our goal is to identify

the chalone and to determine the validity of the hypothesized chalone mechanism role in cell proliferation regulation.

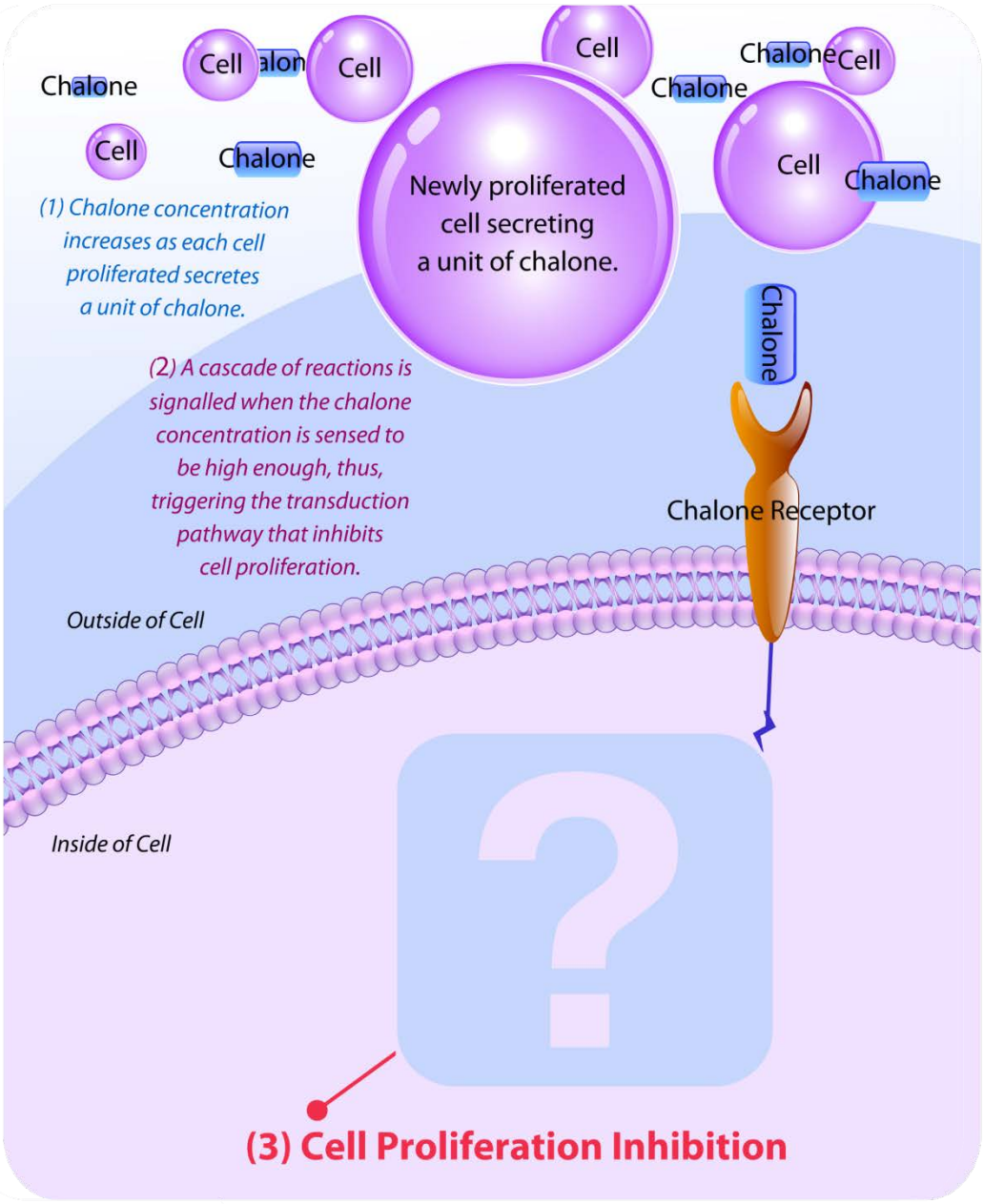

Figure 2: Illustration summarizing a possible chalone signal transduction pathway. Credit: Christina Allen

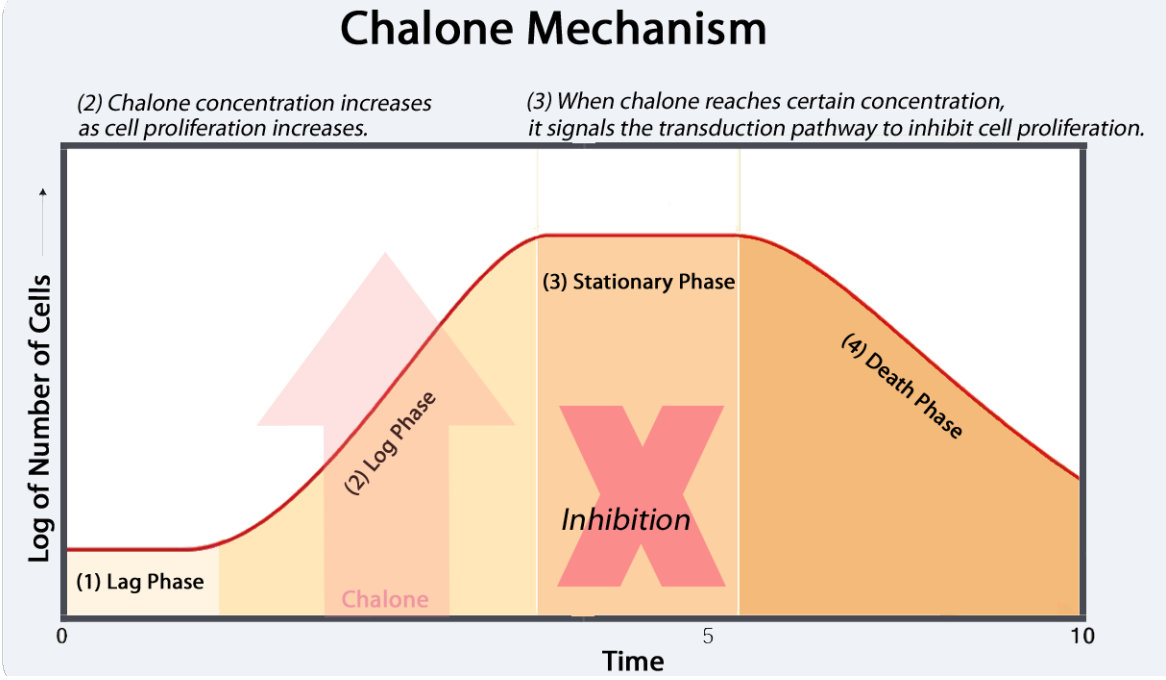

Figure 3: Diagram describing how the chalone mechanism operates via a negative feedback loop system to regulate cell proliferation. Credit: Christina Allen

Examples of chalone mechanism in humans

The existence of chalones, autocrine factors that negatively regulate the growth and/or proliferation of secreting cells, is supported by many observations in science [2]. Myostatin is a prime example of a chalone. Myostatin is a polypeptide secreted by myoblasts that acts as a signaling molecule. Myoblasts can sense the density of surrounding cells by sensing the concentration of myostatin, which increases as skeletal muscle mass increases. When myostatin concentration reaches high enough levels indicating that muscle tissue size is appropriate, then myostatin will trigger a signal transduction pathway that halts myoblast proliferation in order to regulate human muscle tissue size [10]. Myostatin is responsible for inhibiting skeletal muscle mass when appropriate size is achieved [11, 12]. Consequently, as skeletal muscle mass increases, more myostatin is secreted by myoblast [10]. When the chalone reaches a high enough

concentration indicating the muscle tissue has reached appropriate size, then the chalone acts as a negative regulator of muscle growth and operates by inhibiting myoblast proliferation [13].

Myostatin binds to a cell bound receptor (activin type II receptor), which triggers a negative feedback loop mechanism to regulate muscle size differentiation. Myostatin inhibits myoblasts proliferation by halting growth during the G_1- to S-phase of the cell cycle, thus signaling cell proliferation inhibition when a certain concentration of myostatin is reached [14]. A result of having a deficiency or no myostatin would be a lack of regulation of myoblast proliferation, which would lead to abnormally large muscle tissue [12]. Mutations of myostatin or a disruption of a necessary component active in the signal transduction pathway controlling muscle tissue size regulation has been observed to be consequential in impediment of healthy skeletal muscle tissue size regulation causing abnormally large muscles [12].

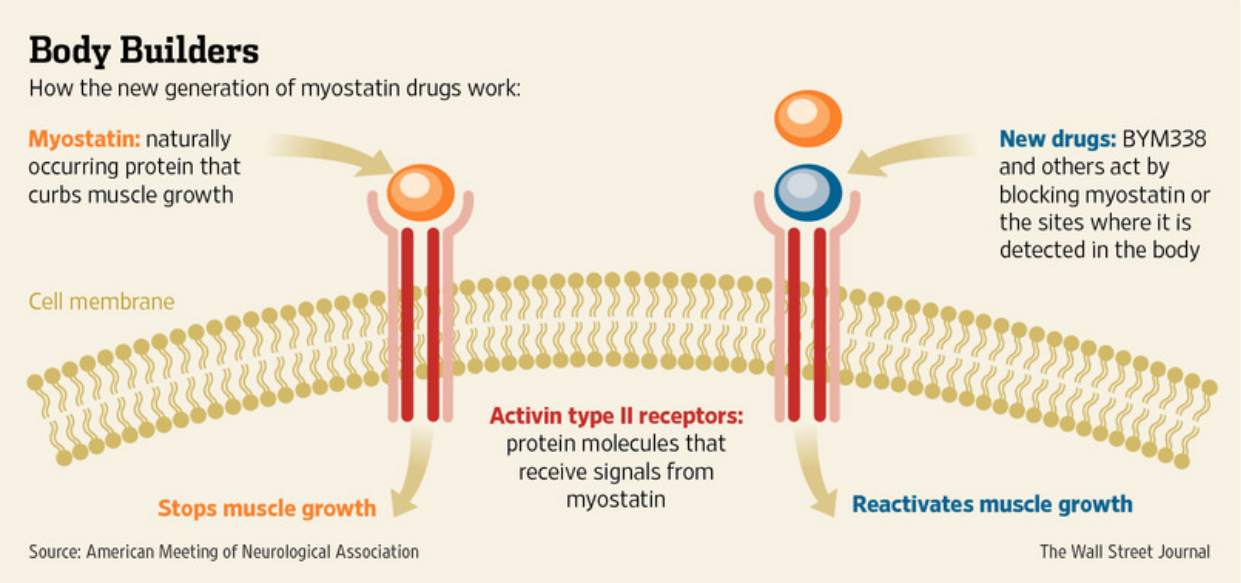

Figure 4: Diagram of how myostatin concentration regulates skeletal muscle tissue cell proliferation inhibition serves as an example of a chalone mechanism [12]. Credit: *American Meeting of Neurological Association*

Overview of *Dictyostelium discoideum*

Dictyostelium discoideum (also referred to as "Dicty" or *"D. discoideum"*), a slime mold, is used as the model organism in this project. *Dicty* is an eukaryotic model organism with a 34 Mb genome [15]. The National Institutes of Health chose this organism to be declared a model organism in the Model Organism Initiative because *D. discoideum* has a high degree of sequence similarity to genes in vertebrate species [29]. Many *D. discoideum* genes are homologous to human genes. This makes it a valuable model organism applicable to human cancer research. In addition, *D. discoideum* has many suitable characteristics to make it a suitable human model organism including having a relative short lifetime, a unique asexual lifecycle, and life cycle involves four developmental stages (vegetative, aggregation, migration, and culmination) that can be easily identified under a microscope by observing the morphology of *D. discoideum* [Figure 5]. This allows observation of unicellular behavior (vegetative stage in shaking culture) or multicellular level (colonies grown on agar plates).

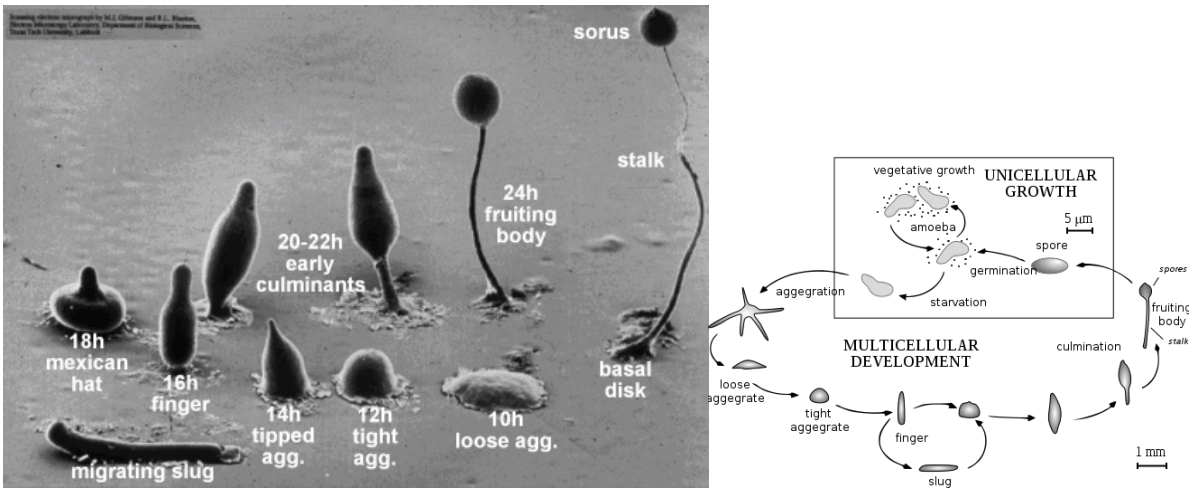

Figure 5: Image (left): Representation of *Dictyostelium discoideum* life cycle stages. Credit: Photo taken by M.J. Grimson and R.L. Blantay, *dictyBase* [8]; Image (right): *Dictyostelium* life cycle. Credit: Diagram drawn by Hideshi and authored by Tijmen Stam, Wikimedia Commons

Maintenance of Dictyostelium discoideum

The vegetative stage is observed when *D. discoideum* is grown in shaking flask culture. *D. discoideum* were grown in shaking flasks and were allowed to grow in log phase for two to three days depending on the rate of proliferation and generation time [15]. The cultures genomic integrity was maintained by not allowing the cells to reach a high density for too long and was supplied with fresh media prior to experimentation to prevent increased chances of mutations. Mutant cell lines were kept frozen in a -140 freezer prior to being used [15]. *D. discoideum* will be used to explore a chalone mechanism regulating cell proliferation [16].

D. discoideum, a social slime that forms fruiting bodies with spores when in non-vegetative cell form, can be observed on a multicellular level when grown on plates on SM/5 agar plates [21]. Observing *D. discoideum* on a multicellular level will allow movement, chemical signaling, and growth development to be analyzed [Figure 5]. This technique will also allow chemotaxis patterns to be observed if present. Using *D. discoideum* as a model organism has many advantages due to its simplicity, and *D. discoideum* research applications may allow findings to be discovered that might be applicable to human research.

Growth kinetics of Dictyostelium discoideum

Some cells secrete a diffusible factor called a chalone [2]. When the cells attain the programmed suitable tissue size, the cells become saturated with the chalone, which signals a transduction pathway inhibiting proliferation and bringing the population to stationary phase [17]. The stationary phase is initiated during the transitioning period between the Gap phase 2 (G2) to the mitosis phase (M) in the cell cycle [18]. This process is enzymatically regulated by Cyclin β and

Cyclin-dependent kinase 2 (Cdc2) [4]. Currently, additional upstream or downstream genetic components in the signal transduction pathway responsible for halting cell proliferation are not well understood.

Vital genetic components to the chalone mechanism

Evidence suggests that a chalone mechanism responsible for cell proliferation regulation triggers a signal transduction pathway. Comprising this signal transduction pathway, the upstream and downstream genetic components that are vital to chalone function are unknown. Identifying potential vital and non-vital genetic components through "wreck and check" assays will help characterize the signal transduction pathway responsible for cell proliferation regulation. This could not only reveal what is controlling cell proliferation regulation and give insight into missing or mutated components causing a lack of cell proliferation regulation in cancerous cells, but could also give insight into other genetic components to target for cancer therapeutics.

Size of the chalone

In preliminary experiments, conditioned media was collected from the Axenic (Ax2) cells (wild type cells), which had been allowed to reach stationary phase indicating the presence of the chalone. Then the conditioned media containing the chalone was filtrated with a 2kDa and a 10kDa filter. Then this conditioned media was exposed to axenic cells and growth curves were recorded and analyzed. In our preliminary research, if the cells still exhibited a normal stationary phase as observed in screens of axenic cells using non-filtrated conditioned media, then we can conclude the size of the chalone must by smaller than the filtrate size (2kDa or 10kDa). Results

had shown that the chalone is smaller than 2kDa. So the conditioned media containing the chalone in these assays all have been 2kDa filtrated.

Overview of polyphosphate

Inorganic polyphosphate is a ubiquitous stable polymer made of repeating phosphate residues. Polyphosphate can range in length from a few phosphate residues up to hundreds of residues. Polyphosphate is present in prokaryotes, simple eukaryotes such as *D. discoideum*, and even higher eukaryotes such as mammals, and in all cell types and organisms [19]. Inorganic polyphosphate plays important biological roles in the cell. The cellular roles of polyphosphate vary between organisms, and polyphosphate's role is dependent upon several factors such as its location within the cell [19]. Polyphosphate also plays critical roles in animal cells, because it affects the cells' development, cellular proliferation and the differentiation of regulatory processes [9]. In bacteria, polyphosphate participates in the induction of rpoS [20]. RpoS is an RNA-polymerase subunit, which is known to regulate the expression of genes that function to implement adaptation to the stationary growth phase, and external stress responses that serve to increase survival [20].

Polyphosphate found to be present in the 2kDa filtrated conditioned media and enzyme responsible is unidentified

In preliminary experiments, in addition to purifying the chalone, the team also took steps to purify the different components in the 2kDa filtrated chalone conditioned media and found polyphosphate to be present which favors the hypothesis of polyphosphate potentially being involved in the chalone mechanism. Polyphosphate is known to be a molecule smaller that 2kDa.

In the beginning, we had thought the polyphosphate kinase gene (*ppk* gene) to be responsible for extracellular polyphosphate regulation that seemed to be correlating the chalone mechanism in *D. discoideum* cells [27]. However, the team's experiments attempting to recreate Kornberg's DdPPK1 gene (also known as the *ppk* gene) knockout had failed, but instead had generated another knockout that also lacked extracellular polyphosphate. This is a significant finding. It revealed that another unidentified gene is responsible for extracellular polyphosphate regulation. Identifying this gene and protein could give hints to an enzyme homolog in humans.

Polyphosphate presence in Dictyostelium discoideum and humans

Extracellular polyphosphate is known to be present in *D. discoideum* and in mammalian cells. In fact, it is estimated that polyphosphate is found at levels up to 20 percent in all subcellular organelles of the cell dry weight in mammalian eukaryotes [21, 28]. However, the mammalian enzyme that produces polyphosphate in mammalian organisms is unknown despite the acknowledged presence of polyphosphate. The polyphosphate kinase gene (*ppk* gene) is known to be one regulator of extracellular polyphosphate in *D. discoideum* [27]; however, humans do not have a *ppk* gene. We have found that there is another unidentified gene or genes that is regulating extracellular polyphosphate in *D. discoideum*. This gene could potentially have a homolog in the human genome.

Hypothesis of polyphosphate as a potential chalone

Observations in preliminary research and known characteristics of indicate that polyphosphate meets the criteria of being an involved vital component in the chalone mechanism the many ways aforementioned. These findings inspired the hypothesis of polyphosphate as a potential

chalone that triggers the signal transduction pathway to inhibit cell proliferation ultimately responsible for cell proliferation regulation in *D. discoideum*. Assays will be purposed to test this hypothesis and to characterize chalone mechanism and the genetic components involved in signal transduction pathway that it triggers to regulate cell proliferation.

The purpose will be to examine polyphosphate and to determine if it fits the criteria to be potentially the chalone. The following questions will be ask to explore the hypothesis:

1. What is the size of the chalone?
2. Which genetic components are vital to the chalone mechanism?
3. Is polyphosphate present in the conditioned media?
4. Would a mutant lacking polyphosphate have chalone function?
5. How does polyphosphate affect unicellular development?
6. How does polyphosphate affect cells on a multicellular level?

CHAPTER II

METHODS

Prior to beginning my investigation, I completed proper training for Texas A&M University Biosafety Level 1 (BSL1) and Biosafety Level 2 (BSL2) requirements in addition to specialized safety training, which is required to participate in Dr. Gomer's laboratory. Techniques from cell biology, microbiology, biochemistry, and genetics will be employed during my experiments.

What is the size of the chalone?

Preliminary research was lead by Dr. Gomer and graduate student, Patrick Suess. Vegetative cells grown in shaking culture were exposed to filtrated conditioned media containing the chalone collected from media of cells at stationary phase with a 2kDa filter to determine chalone size. If cells exposed to the 2kDa filtrate still exhibited normal proliferation inhibition enzymatic activity, then the chalone would be smaller than 2kDa. The chalone was found to be smaller than 2kDa.

Which genetic components are vital to the chalone mechanism?

"Wreck and check assay" were used to determine if certain genetic components are vital to chalone function, possibly directly or indirectly interacts with the chalone function, or is not vital at all to the chalone function. True knockout cell lines were exposed to three different conditions: 0%, 25%, and 50% conditioned media containing the chalone. Vegetative cells were grown in shaking culture and standard growth curves were recorded. If a knockout cell line shows sensitivity to the chalone and does not significantly deviate from the control's normal

proliferation pattern, then the known missing gene does not affect the function of the chalone mechanism and is not a necessary component for signal transduction pathway.

The purpose of these assays was to determine the components necessary for the chalone's signal transduction pathway to function and will involve multiple steps. Step one will be to cultivate and maintain knockout strains of *D. discoideum* each missing a different genetic component. Step two will be to isolate and purify that chalone from axenic cell media collected from cell culture that has been allowed to reach stationary phase to create the conditioned media. Step three will be to screen these knockout strain cultivated in step one with the conditioned media containing the partially purified chalone collected in step two. Cell counts will be performed in order to obtain growth curves to determine how different strains behave in the presence of the purified chalone. This experiment will determine if the missing component in the each different knockout strains is necessary for the chalone signal transduction pathway to function by evaluating any deviations from the normal behavior of the axenic cell proliferation patterns.

Step One: Dictyostelium discoideum cell culture

Cells used came from the *D. discodium* Ax2 line, which were obtained from the *dictyBase* stock center [15]. The cells were maintained in shaking culture flasks and grown at approximately 25°C (room temperature) in HL5 media supplemented with tetracycline and streptomycin. Old media was replaced by new media every 48 hours. A working stock culture had to be below 10 million cells per milliliter (10×10^6 cells/ml), and ideally would never get close to that high of a concentration. Cell counts for each shaking flask culture were preformed everyday in order to determine if the media had to be changed. Media exchange was performed routinely in order to

maintain cell character as outlined in the protocol found on *dictyBase* [15]. Each cell line was kept in shaking culture no longer than one and a half months to prevent compromise to the integrity of the cell lines and to reduce risk of mutation.

Step Two: Chalone isolation and purification

To understand the chalone mechanism, the growth kinetics of the amoebae *D. discoideum* will be studied. These axenic cells will be allowed to grow in synthetic defined minimal media (SIH) supplemented with tetracyline and streptomycin. Synthetic defined minimal media (SIH) is a synthetic media in which we know all the components, which is useful for purpose of purification [26]. The cells were cultivated in shaking culture in a 100ml volume flask with the synthetic defined minimal media (SIH). The media used to grow the cells was then collected and used to generate the conditioned media [26]. Cell counts were recorded daily until stationary phase was reached. Cell counts were preformed manually by taking aliquots of the sample and counting them using a hemocytometer under a microscope. Cells are always secreting the chalone. It is when the chalone is at its highest concentration that stationary phase is triggered. Thus, waiting for cells to reach stationary phase before collecting the media allows that chalone concentration to be at its highest when isolated, allowing the purification of the conditioned media to have a more efficient yield. Once the cells reach stationary phase, it is suspected that the cells will secrete the chalone. Thus, the media was collected at the stage of growth where the chalone is supposed to be at a high enough concentration to trigger the cells to enter stationary phase. The media containing the chalone at its highest concentration was extracted and purified by a series of steps. Firstly, the intact cells were removed by centrifuging the media at 3000 RPM. Secondly, the chalone will be partially isolated from the media via gel filtration

chromatography. Thirdly, the media containing the chalone will be filtered through a 2kDa filter via ultra-centrifugation. In preliminary research, it was observed that the chalone's enzymatic activity can still be observed after the media was purified via a 2kDa filter suggesting that the chalone is smaller than 2kDa; hence, this is the reason why this method was chosen to purify the chalone.

Step Three: Screening knockout strains at different chalone concentrations

Knockout cell lines were screened with different concentrations of the chalone at uniform time increments to identify if any of the missing components are necessary for the signal transduction pathway mechanism, which triggers a halt in cell proliferation, thus, initiating a stationary phase. Twenty different knockout strains were screened in 72-hour increments. Each knockout strain lacked a different pre-known component such as a gene encoding a specific protein. Each knockout strain was exposed to three different conditions comprised of different concentrations of conditioned media suspended in normal HL5 media supplemented with tetracycline and streptomycin. The three conditions tested will be 0%, 25%, and 50% conditioned media. Each flask initially contained 5ml of the designated combination of conditioned media and normal HL5 media supplemented with the tetracycline and streptomycin. Differences in proliferation rate, the maximum population density achieved, and total cell survival over a time period of 72 hours will be observed. Cell cultures will be seeded initially at 0.5×10^6 cells/ml on the first day. Cells will be counted in aliquots of the cultures using a hemocytometer in regular increments over the ten-day growth period. Then the growth curves of each sample will be analyzed. Each knockout cell line will undergo at least three trials. The data was analyzed using Prism Software Version 2.6. A standard growth curve of cell density vs. time (days) was determined. From this

information, we were able to determine which knockout cell lines had a missing component that potentially was involved in the chalone's signaling transduction pathway responsible for halting cell proliferation.

Step Four: Screens were repeated for a ten day time period for selected knockout strains

Screens for selected knockout cell lines that had shown results differing from the normal growth pattern were subjected to repeated screens (the same as before) except for an extended time increments of 10 days instead of 72 hours.

Is polyphosphate present in the conditioned media?

The team found polyphosphate to be present in the media collected to generate the conditioned media, which contains the chalone. Polyphosphate is smaller than 2kDa and was not filtered out, and therefore, was not filtrated out of the conditioned media.

Would a mutant lacking polyphosphate have chalone function?

The purpose of this experiment was to generate a a mutant lacking extracellular polyphosphate production and determine if it would exhibit chalone function. This could directly determine if polyphosphate could in fact be the chalone. Graduate student, Patrick Suess, generated random mutants via Restriction Enzyme Mediated Insertion (REMI) to attempt to find a mutant with a desired phenotype (exhibiting a lack of inhibition and no stationary phase) with the purpose to identify the missing component. Then, I used Inverse Polymerase Chain Reaction (IPCR) to identify the REMI generated mutations. Once the mutated gene had been located via molecular markers, then I attempted to generate a knockout of this gene via homologous recombination.

Then gel electrophoresis was preformed and the product was sent to Star Labs in Houston, Texas, for DNA sequencing to try to determine the mutation in the REMI mutant. However, this experiment was not successful and the gene responsible for the lack of extracellular polyphosphate is still unknown. However, we were able to prove that it was not the *ppk* gene.

What is observed via growing vegetative cells grown in shaking cultures?

Vegetative cells were grown in shaking culture and screened with different concentrations of inorganic polyphosphate to observe polyphosphate's effect cell development and cell proliferation behavior on a unicellular level. Standard growth curves were recorded and compared to the control growth curve screened with different concentrations of conditioned media containing the chalone to determine if there is a correlation.

How does polyphosphate affect cells on a multicellular level?

By observing cells in a multicelllular state, non-vegetative cells were grown on SM/5 plates and screened with different concentrations of inorganic polyphosphate to evaluate polyphosphate's effect on growth development and cell proliferation behavior on a multicellular level. If cells exposed to the polyphosphate race faster across the plate than the control plate without polyphosphate, this could support polyphosphate identity as also a potential chemoattractant. Using the edge plate technique, *D. discodium* wild-type Ax2 cells were plated with a lawn of KA bacteria on SM/5 agar plates comprised of three different concentrations of inorganic polyphosphate. The purpose of this assay is to observe difference in development and proliferation patterns due to the different concentration of inorganic polyphosphate in the SM/5 plates. Specifically, the three conditions are as follows: a control SM/5 agar plate, a 0.5 mg/1.0

ml inorganic polyphosphate SM/5 plate, and a 1.0 mg/1.0 ml inorganic polyphosphate SM/5 plate. This is a newly developed technique. Initially the SM/5 agar plates were made per the normal accepted protocol on *dictyBase* [25]. The only deviation form this protocol was to add different concentrations of inorganic polyphosphate to two of the three conditions. Inorganic polyphosphate was added at its appropriate concentration respectively and allowed to fully dissolve in solution after the SM/5 agar media had been autoclaved and allowed to cool to room temperature on a magnetic stir pad. KA was used to make bacteria lawn for the *D. discoideum* to consume. Then AX2 cells were plated to make an edge plate. By adding the polyphosphate to the agar, it is hypothesized that the *D. discoideum* exposure to inorganic polyphosphate in the media will cause the organism to develop at a faster rate than the control containing no polyphosphate.

CHAPTER III

RESULTS

The overall purpose of this project is to characterize the chalone of a chalone signal transduction pathway in *D. discoideum* and the potential genetic components vital to the chalone mechanism function. This project was divided into six different approaches to characterizing the chalone. The purpose of each approach was to characterize the size of the chalone, give insight into the chalone signal transduction pathway by determining necessary genetic components, to determine if polyphosphate fits the criteria to potentially be the chalone, to attempt to determine the genetic component responsible for polyphosphate regulation, to test inorganic polyphosphate as a possible component of the chalone and to observe its effect on vegetative cells, and to observe inorganic polyphosphate's effect on cell development on a multicellular level.

Chalone function exhibited after 2kDa filtration

Preliminary experiments done by Patrick Suess showed that the necessary components that make up the chalone mechanism must be all less than 2kDa in size since proliferation patterns did not change when the cells were exposed to conditioned media containing the chalone filtered with a 2kDa filter [Figure 6]. The assay described in *Figure 6* was performed to determine if the chalone was greater than 2kDa in size by filtering the chalone in a 2kDa filter. The conditioned media containing the chalone was filtrated with a 2kDa and a 10kDa filter. The 2kDa filtrate still showed enzymatic activity inhibiting cell proliferation showing that the chalone still was present and functional.

Figure 6 compares proliferation patterns for *D. discoideum* axenic cells exposed to conditioned media comprising the chalone at a 50% concentration. The chalone was collected from media from axenic cell culture that was allowed to reach stationary phase indicating chalone potency. The axenic cells were exposed to conditioned media filtered by different size filters to determine the size of the chalone by analyzing cell proliferation regulation function. The following are the five different conditions the axenic cells were exposed: normal conditioned media with no filter, 2kDa filtrated conditioned media, 2kDa non-filtrated conditioned media, 10 kDa filtrated conditioned media, and 10 kDa non-filtrated conditioned media.

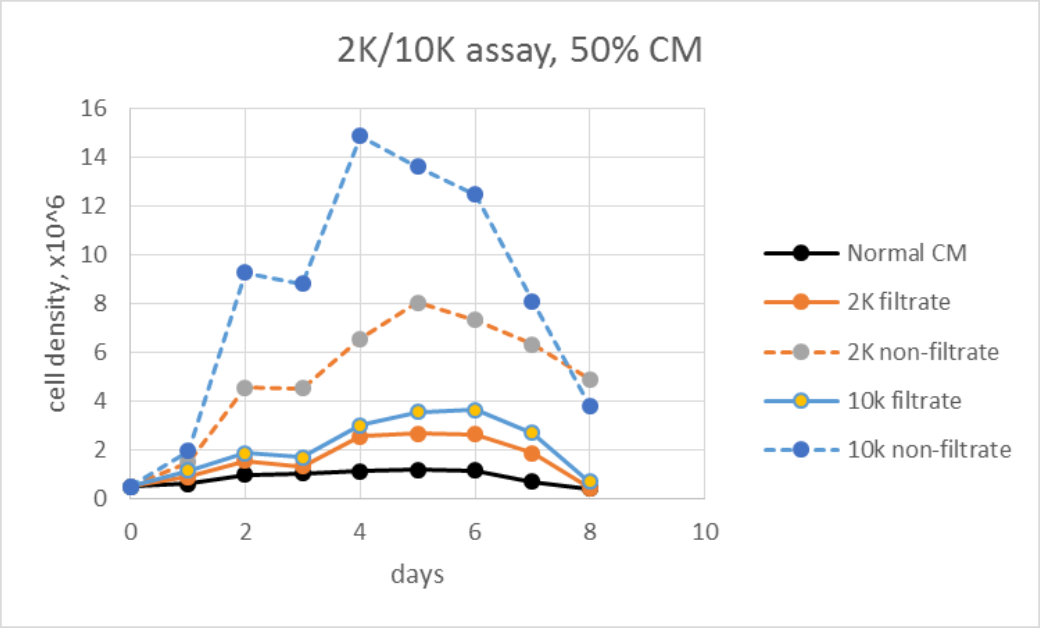

Figure 6: The chalone size is smaller than 2kDa. Standard Plot Showing Cell Density Proliferation Patterns in 2kDa Filtrated 50% Conditioned Media Verses 10kDa Filtrated 50% Conditioned Media.

Knockout cell lines growth curve analysis

72-hour knockout screenings in different conditioned media concentration

The purpose of this approach is to use "wreck and check" assays to determine which genetic components (genes, proteins, etc.) are necessary for the chalone signal transduction pathway. "Wreck and check" assays exposes cell lines that are missing a single genetic component to different conditions and then compares its growth curves to the growth curve of the control (wild-type AX2 cell line that is not missing any genetic components) exposed to the same conditions. The genetic components tested are specified in *Table 1*. Deviation from the normal growth curve pattern indicates that the missing genetic component is vital to the chalone function. The results from this approach displayed in *Figure 7* will allows us to better understand plausible components involved in cells experiencing unregulated proliferation as exemplified in cancer cells.

Knockout cell lines were screened with different concentrations of the chalone in shaking culture in SIH media that were counted at uniform time increments to identify if any of the missing components affected the signal transduction pathways ability to regulate cell proliferation. We screened in 72-hour increments knockout cell lines each lacking a pre-known component specified in *Figure 7* with three different concentrations of the chalone (0%, 25%, and 50% conditioned media). See *Table 1* to reference the missing genetic component and the resulting absent gene products specified for each knockout tested: *Ax2, pten-, tpp1-, Akt1-/2-, rasC-/rasG-, gcA/sgcA-, pldβ-, pkc-, pakD-, Sml A-, crlA-, gβ-, gdt4, and r2F-*. Observations were made based on differences in proliferation rate, the maximum population density achieved, and total cell survival over the 72 hours time period.

As seen in *Figure 7* and *Figure 8, pten-, Akt1-/2-, pldβ-, pakD-***,** and *gβ-* all showed deviation from the normal proliferation pattern. To prove a genetic component has a direct and vital interaction with the chalone mechanism, we would expect to see a situation where in the growth curves, the increase in chalone concentration does not trigger a significant amount of increase in inhibition in the cell knockout cell lines as normally expected in the growth curve of the cell line not missing any genetic components (Ax2 cells - Control). However, signal transduction pathways involve many components and alternate routes in pathways to compensate when a genetic component is absent and so results may not always yield an "all or nothing" response. Therefore, any deviations from the normal cell growth curve pattern classified the knockout cell line as candidates to be explored. These are the genetic components that potentially have some kind of direct or indirect interaction with normal chalone functions and should be researched further to validate a more solid conclusion. However, what we can conclude is that the knockout cell lines that do not show significant deviation from the normal growth curve pattern (inhibition increases as the chalone concentration increases) exhibited by normal Ax2 cells (Control) are not missing components that are vital to chalone function. Cell lines that demonstrated sensitivity to the chalone and exhibited the normal growth curve pattern, and therefore, are not missing vital component to chalone function are the following: *crlA-, gcA/sgcA-, gdt4-, pkc-, r2F-, rasC-/rasG-, SmlA-,* and *tpp1-. (*Results for cell lines for the following cell lines were confirmed by extended growth curve results in *Figure 8: crlA-, r2F-, rasC-/rasG-,* and *SmlA-.)*

Table 1: Name Descriptions, Gene Products, Gene ID, and Genetic Location of Null Genes in Knockouts.

Summary of *Dictyostelium discoideum* Knockout Strain Characteristics			
Abbrev.	Name Description	Gene Product	*Gene ID*/ Location
Ax2	Axenic strain (wildtype)	N/A	N/A
Akt1-/2-	*Also known as "pkbA".* pkb = **P**rotein **K**inase **B**	AKT/PKB protein kinase PkbA	*DDB_G0268620*; Chromosome **1** coordinates **1977388** to **1978925**, **Watson** strand
crlA-	**C**yclic AMP **R**eceptor-**L**ike	cAMP receptor-like protein G-protein-coupled receptor (GPCR) family protein	*DDB_G0280983;* Chromosome **3** coordinates **4253816** to **4255131**, **Crick** strand
gβ-	*glb1*	beta-galactosidase 1	*DDB_G0290217*; Chromosome **5** coordinates **3809656** to **3811671**, **Crick** strand
gcA/sgcA-	gc= **G**uanylyl **C**yclase	guanylyl cyclase	*DDB_G0275009*; Chromosome **2** coordinates **5230828** to **5235376**, **Watson** strand
	scg = **S**oluble **G**uanylyl **C**yclase		*DDB_G0276269;* Chromosome **2** coordinates **6582943** to **6592973**, **Crick** strand
gdt4-	**G**rowth-**D**ifferentiation **T**ransition	MLK family protein kinase Gdt4	*DDB_G0270550*; Chromosome **1** coordinates **2926042** to **2931347**, **Watson** strand
pakD-	**P**21-**A**ctivated protein **K**inase	serine/threonine protein kinase PakD	*DDB_G0269696*; Chromosome **1** coordinates **3345400** to **3350536**, **Watson** strand
pldβ-	**P**hospo**L**ipase **D**	glycosylphosphatidylinositol phospholipase D	*DDB_G0276919;* Chromosome **2** coordinates **7179136** to **7182209**, **Watson** strand
pkc-	*DDB_G0288147*	PKC domain-containing protein, PE/DAG binding protein kinase, TKL group tyrosine kinase-like protein	*DDB_G0288147;* Chromosome **5** coordinates **1147360** to **1151873**, **Watson** strand
pten-	**P**hosphatase and **TEN**sin homolog	protein tyrosine phosphatase 3-phosphatidylinositol 3-phosphatase	*DDB_G0286557*; Chromosome **4** coordinates **4733890** to **4735609**, **Crick** strand
r2F-	prf = **P**eptide chain **R**elease **F**actor RF2 = **R**elease **F**actor **2**	class I peptide chain release factor; peptide chain release factor 2	DDB_G0288835; Chromosome **5** coordinates **2047432** to **2048948**, **Watson** strand
rasC-/G-	*ddRASG or* **RA**t **S**arcoma viral oncogene homolog	Ras GTPase RasG	*DDB_G0293434*; Chromosome **6** coordinates **2877682** to **2878372**, **Crick** strand
	rasC or **RA**t **S**arcoma viral oncogene homolog	Ras GTPase	*DDB_G0281385;* Chromosome **3** coordinates **4519070** to **4520084**, **Watson** strand
Sml A-	**SM**a**L**l aggregates	Unknown	*DDB_G0287587*; Chromosome **5** coordinates **461888** to **462739**, **Watson** strand
tpp1-	tpp = **T**ri**P**eptidyl **P**eptidase I cln2 = similar to **C**eroid-**L**ipofuscinosis, **N**euronal **2**	tripeptidyl-peptidase 1	*DDB_G0269914*; Chromosome **1** coordinates **3852049** to **3853851**, **Crick** strand

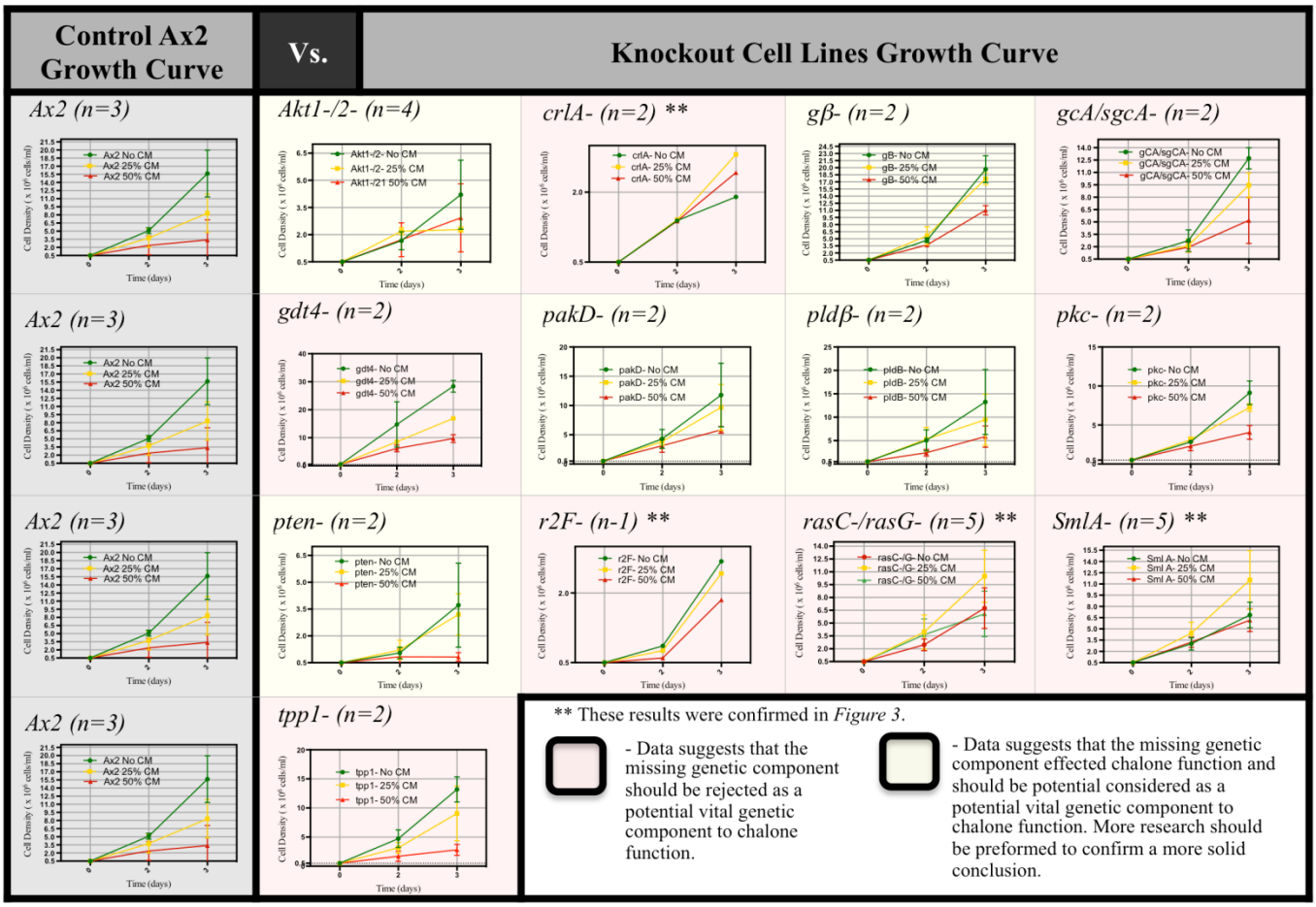

Figure 7: Standard Plots of Three Day Growth Curve Showing Ax2 Expected Cell Proliferation Patterns When Exposed to the Following Three Different Concentrations of the Chalone: 0%, 25%, 50% Conditioned Media.

240-hour knockout screenings in different conditioned media concentration

Figure 8 displays the results of the 240-hour knockout screenings in different conditioned media concentrations using the "wreck and check" assay. Some of the knockout cell lines that had shown significant differences from the normal growth pattern were subjected to repeated screens (the same as before) except for an extended time increment of 10 days (240 hours) for n=1 trial. The knockouts selected for extended 10-day screens were the following cell lines: *Ax2 (control), rasC/rasG-, Sml A-*, crlA-, and *r2F-*.

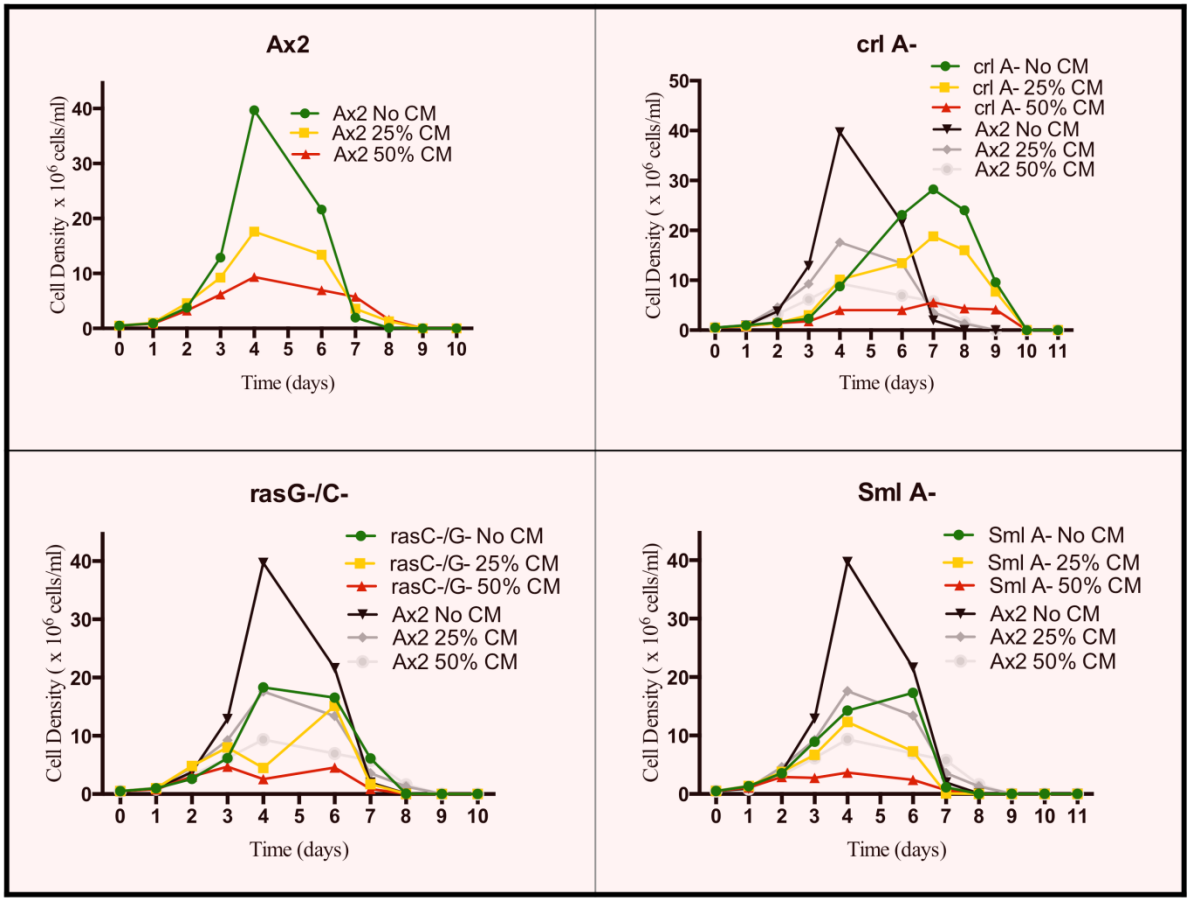

Figure 8: 10 Day Screenings with 0%, 25%, and 50% Conditioned Media Comparing Wild-type Ax2 Cell Line Versus the Following Knockout Cell Lines: *rasC-/G-*, *Sml A-*, *crl A-*, *r2F-*.

Polyphosphate is present in the conditioned media

Polyphosphate was found to be present in the conditioned media containing the chalone. Polyphosphate is also smaller that 2kDa and is assumed to still be present in the 2kDa filtrated conditioned media that still exhibited enzymatic activity even after being filtrated. Polyphosphate fits the criteria to be the potential chalone so far.

Attempts to produce true *ppk* gene knockout was unsuccessful

A knockout showing lack of extracellular polyphosphate was proven to not be missing *ppk* gene. This means there must be another unknown gene responsible for extracellular polyphosphate

regulation. This experiment was lead by graduate student, Patrick Suess, and only supplemented by my aid for a couple steps in the procedure as described in the methods section. The team generated a random mutant suspected of missing the polyphosphate kinase (*ppk*) gene that exhibited a lack of extracellular polyphosphate. However the gene affected was found to not be the *ppk* gene, even though the mutant produced less extracellular polyphosphate. This means that there is another unknown gene responsible for polyphosphate regulation. Identifying this gene could potentially help identify a homolog in the human genome responsible for polyphosphate regulation. Consequently, the conclusion was not definitive, but these findings were important in leading to our hypothesis and the exploration of an unknown genetic component responsible for regulating extracellular polyphosphate that could reveal a homolog in the human genome.

Polyphosphate inhibited cell proliferation in vegetative cells

Effects of Different Concentrations of Polyphosphate on Ax2 Cell Proliferation Pattern

The purpose of this assay was to test the hypothesis of inorganic polyphosphate as a being a possible component of the chalone. To explore this hypothesis, inorganic polyphosphate was added at different concentration to vegetative cells in shaking culture in liquid SIH media to determine the role it had on cell proliferation observed on a unicellular level. Axenic cells (Ax2) were exposed to the following four different concentrations of polyphosphate: 0.28 mM, 0.14 mM, 0.07mM, and no polyphosphate control. This resulted in an obvious deviations form the normal expected proliferation pattern [Figure 9]. The results in *Figure 9* show that polyphosphate at concentrations higher than 0.14 mM inhibits cell proliferation.When cells were treated with a higher concentration of 0.28 mM, cell proliferation was completely inhibited [Figure 9].

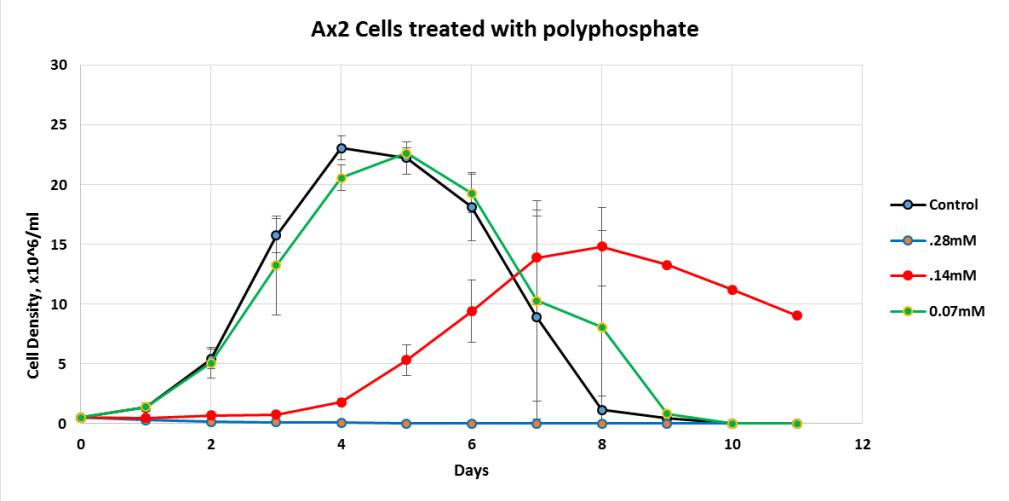

Figure 9: Standard Plot Showing the Effects of Different Concentrations of Inorganic Polyphosphate on Cell Density Proliferation Patterns.

Polyphosphate had effects of multicellular development

The purpose of this assay was observe *Dictyostelium discoideum* developmental patterns and proliferation rate across when exposed to SM/5 edge plates laced with different concentrations of inorganic polyphosphate (0.0mg/ml, 0.5mg/ml, and 0.1mg/ml). If results were to support the hypothesis that polyphosphate is the chalone, we would expect to see a deceleration in cell proliferation of the Ax2 *D. discoideum* plated on edge plates with SM/5 agar infused with inorganic polyphosphate across the plate as polyphosphate concentration is increased. *Trial A* [Figure 10] and *Trial B* [Figure 11] were preformed with the purpose to test this hypothesis.

Trial A: Effects of microwaved/polymerized polyphosphate infused media

However, during *Trial A*, we discovered two characteristics about inorganic polyphosphate interactions that we were not looking for at the time that are quite interesting and didn't contradict our hypothesis, but rather supplements the complexity of polyphosphate's potential role in cell multicellular growth and development. Firstly, we found that omitting the method of

microwaving the SM/5 media containing the polyphosphate in the procedure in *Trial B* yielded extremely different results from *Trial A* when the technique of microwaving was included. It is possible that the inorganic phosphate was polymerized into long chains by the microwaving method. A *Klebsiella aerogenes* (KA) bacteria is a genus of non-motile, Gram-negative, oxidase-negative, rod-shaped bacteria used to grow a bacteria lawn on agar plates to serve as a food source for *D. discoideum*. KA consumes polyphosphate. Polymerized inorganic polyphosphate yields a heighted energy food source for KA that in return increases the amount and quality of bacteria lawn yielded. *D. discoideum* eats the larger bacteria lawn, thus providing more energy for growth and development. Therefore, we propose that the reason there is an increase in *D. discoideum* growth in *Trial A* is because the inorganic phosphate had been polymerized by microwaving.

Figure 10 shows pictures taken on day 5 of assay growing AX2 on edge plates laced with different concentrations of polymerized polyphosphate via microwaving. (Our intention was to heat the agar for pouring plates. Polymerization of polyphosphate was a discovery that we unintentionally discovered.) Observations showed that increasing polymerized polyphosphate (0.0 mg/ml. 0.5 mg/ml, 1.0 mg/ml polymerized polyphosphate) concentration in SM/5 agar plates increased cell proliferation rate and development across the plate and generated larger stalks. Surprisingly, polymerized polyphosphate increased the rate of growth and development on a multicellular level, an increased cell proliferation rate, more rapid collection of unicellular amoebae into a multicellular slug, a larger fruiting body, and taller and more developed stalks. Secondly, we found that polymerized polyphosphate showed signs of potentially being a chemoattractant for *D. discoideum*. The cells exposed to the polymerized inorganic

polyphosphate were observed to cross and consume the lawn of bacteria grown on the SM/5 plates supplemented with polymerized polyphosphate approximately 75% faster than the cells not exposed to polymerized inorganic polyphosphate. The cells exposed to the polymerized inorganic polyphosphate also showed more uniform cell proliferation across the plate. The organism seemed to have directionality in its movement indicating polymerized inorganic polyphosphate possibly to play a role as a chemotaxis attractant. We aim to explore this idea further in future experiments.

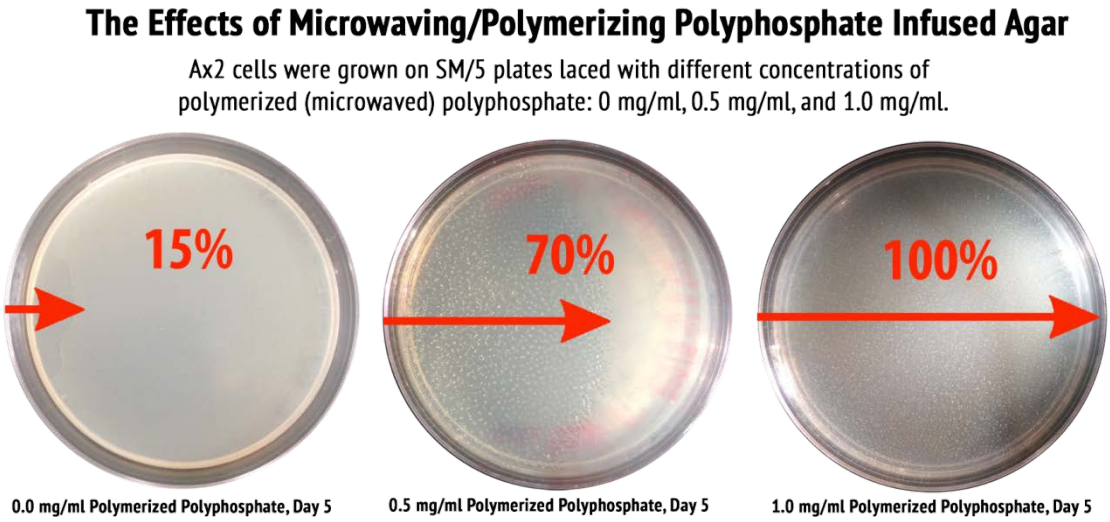

Figure 10: *Trial A* - Photograph of *Dictyostelium discoideum* Ax2 Edge Plate on Microwaved SM/5 Plate Laced with Different Concentrations of the Polymerized Inorganic Polyphosphate (0.0mg/ml, 0.5mg/1ml, and 1.0mg/ml) Displaying Cell Proliferation and Development Differences (n=2, day 5).

Trial B: Effects of inorganic polyphosphate infused SM/5 edge plates on Dictyostelium discoideum on multicellular development

Trial B [Figure 11] followed an identical procedure to *Trial A* except for omitting microwaving as a heating method. *Figure 11* displays the results of the distance of the bacteria lawn consumed by Ax2 *D. discoideum* cells across the plate per time on edge plates laced with different concentrations of polyphosphate (not microwaved). Observations showed that increasing

polyphosphate (0.0 mg/ml. 0.5 mg/ml, 1.0 mg/ml, and 2.0mg/ml inorganic polyphosphate) concentration in SM/5 agar plates decreased cell proliferation rate and development across the plate and generated smaller stalks and smaller fruiting bodies. These results support of the hypothesis of polyphosphate potentially being the chalone.

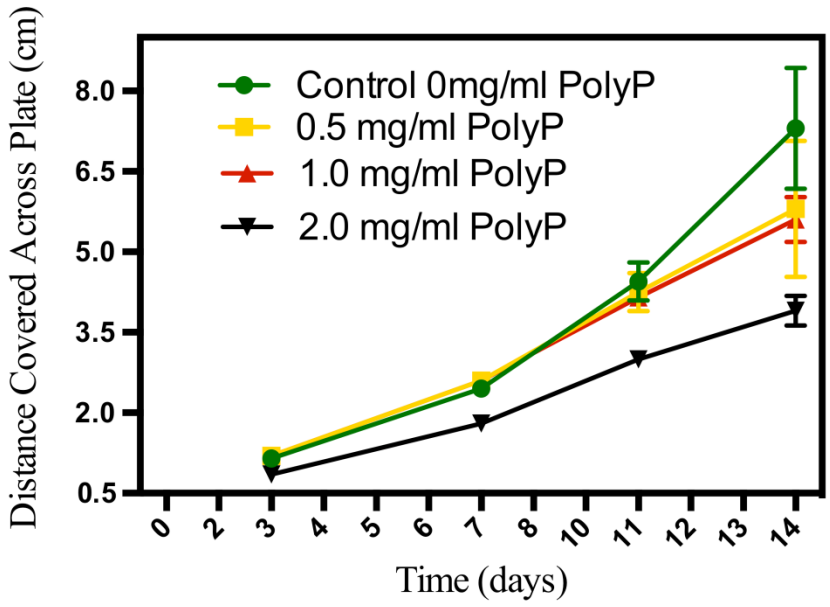

Figure 11: *Trial B* - Graph of distance covered across SM/5 D*ictyostelium discoideum* Ax2 plates supplemented with increasing concentrations (0.0mg/ml, 0.5mg/1ml, 1.0mg/ml, and 2.0mg/ml) of inorganic polyphosphate showed an increasing proliferation inhibition on multicellular level (n=2, day 14)

CHAPTER IV

CONCLUSION

The chalone mechanism is supported as a key component of cell proliferation regulation in the signal transduction pathway that inhibits cell proliferation and possibly plays a role in tissue-size regulation. The primary goal of this project was to identify the chalone and to characterize necessary genetic components of the chalone mechanism. We report that cells treated with polyphosphate shows similar behavior to cells treated with the unidentified chalone suggesting polyphosphate could potentially be the chalone. As chalone concentration is increased when exposed to vegetative wild-type cells, inhibition is increased [Figure 2]. As polyphosphate concentration is increased, inhibition is increased on a unicellular level [Figure 9] and a multicellular level [Figure 11].

However, shockingly, despite finding that inorganic polyphosphate (that was not polymerized by microwaving) inhibited cell proliferation correlating with the behavior of the chalone, we found the quite a different effect to be induced when cells are exposed to polymerized polyphosphate. It was discovered that polymerized polyphosphate (via microwaving) actually increased cell growth and development in non-vegetative cells grown on agar plates and almost appeared to have a chemoattractant effect when observed on a multicellular level [Figure 10]. This finding demonstrates the complexity of the polyphosphate's role in cell proliferation and multicellular development.

In addition to studying polyphosphate as the potential chalone, potential vital genetic components to chalone function was analyzed via “wreck and check” growth curves. Results showed that *crlA-, gcA/sgcA, gdt4, pkc-, rasC/rasG-, r2F-, SmlA-, and tpp1-* are not necessary genetic components in the chalone mechanism. However, the *pten-, Akt1-/2-, pldβ-, pakD-***,** and *gβ-* knockout cell lines all showed some degree of insensitivity to the chalone and are candidates to be further explored as possible vital components to chalone function [Table 1, Figure 7, Figure 8]. Further evidence is needed to support this conclusion, but this data could pave the way to a new approach to charactering the signal transduction pathway responsible for cell proliferation regulation ultimately yielding valuable information to our understanding of tissue size regulation and aid in uncovering genetic targets for cancer therapeutics development.

In summary, the following conclusions listed below can be deduced:

1. The chalone is smaller that 2kDa [Figure 6].
2. Genetic components vital to chalone function do not include the missing genetic components of the following knockout cell lines: *crlA, gcA/sgcA, gdt4, pkc, rasC/rasG, r2F, SmlA, and tpp1*. Plausible candidates of being potential necessary components have been narrowed down to include the following genetic components *pten, Akt1/2, pldβ, pakD,* and *gβ* [Table 1, Figure 7, Figure 8].
3. Polyphosphate is present in the conditioned media containing the chalone.
4. Attempts to produce *ppk* gene knockout was unsuccessful. However, a different randomly generated REMI mutant proven to not be a *ppk* gene knockout that also showed a lack of extracellular polyphosphate was discovered. The gene of the this mutant

responsible for the extracellular polyphosphate could potentially lead us to identify a homolog gene in the human genome responsible for polyphosphate regulation in humans.

5. Polyphosphate inhibits cell proliferation in vegetative cells in shaking culture [Figure 9]. As observed, increasing the concentration of either inorganic polyphosphate or the chalone shows an inverse correlation to cell density; and, therefore, both appear to have a similar inhibiting effect on *D. discoideum* cell proliferation [Figure 2, Figure 9, Figure 11].
6. In *Trial A*, it was discovered that using microwaving as a heating method polymerized polyphosphate in the SM/5 edge plates. Surprisingly, polymerized polyphosphate increased the rate of cell proliferation and multicellular development when cells were in a non-vegetative state. Polymerized polyphosphate also showed evidence of being a potential chemo-attractant. In *Trial B*, the microwaving step was omitted from the procedure. Results showed that increasing polyphosphate in SM/5 edge plates increased multicellular cell proliferation inhibition which matches the behavior of the chalone.

Overall, the hypothesis of polyphosphate being the potential chalone has been supported by results. Although, not enough evidence has been collected to fully accept this hypothesis, observation look encouraging of the hypothesis and more experiments should be conducted to further explore polyphosphate's role in the chalone mechanism.

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