May 2014

The APL5 Subunit of the AP3 Adaptor Protein Complex is Required for Cytokinesis Checkpoint Function in Schizosaccharomyces Pombe

Joy Wang  
*The University of Western Ontario*

Supervisor  
Dr. Jim Karagiannis  
*The University of Western Ontario*

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

© Joy Wang 2014

Follow this and additional works at: [https://ir.lib.uwo.ca/etd](https://ir.lib.uwo.ca/etd)

Part of the *Biology Commons*

**Recommended Citation**

[https://ir.lib.uwo.ca/etd/2071](https://ir.lib.uwo.ca/etd/2071)

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca.
THE APL5p SUBUNIT OF THE AP3 ADAPTOR PROTEIN COMPLEX IS REQUIRED FOR CYTOKINESIS CHECKPOINT FUNCTION IN *SCHIZOSACCHAROMYCES POMBE*

(Thesis format: Monograph)

By

Joy Menghan Wang

Graduate Program in Biology

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

© Joy M. Wang 2014
ABSTRACT

This study investigates the role of Apl5p in the complex regulatory network of Schizosaccharomyces pombe, which ensures the faithful and reliable completion of cytokinesis. This network, referred to as the cytokinesis checkpoint, ensures successful cell division upon perturbances to the cytokinetic machinery (e.g. disruption of the actin cytoskeleton). Apl5p has been identified as a putative regulator of the cytokinesis checkpoint based on the hyper-sensitivity of apl5Δ mutants to the actin depolymerizing drug, Latrunculin A. Apl5p is an essential subunit of the conserved AP3 adaptor complex, which is suspected to be involved in vesicular trafficking. Thus, I hypothesized that Apl5p mediates the transport of materials which are necessary for cytokinetic regulation during stress. In this report, I show that apl5Δ mutants are inviable in the presence of LatA due to their inability to complete cytokinesis. Using live-cell imaging, I show that the mutant’s failure to complete cytokinesis results from an underlying inability to maintain the physical integrity of the actomyosin ring upon the initiation of constriction. Over-expression of Apl5p resulted in a dominant negative effect and impeded cell viability upon treatment of LatA (rather than conferring LatA resistance). Lastly, I determined the intracellular localization of Apl5p by monitoring Apl5-YFP fusion proteins. Given its role in vesicular trafficking, Apl5p was expected to localize to cytoplasmic vesicles at, or near, the site of cell division. Surprisingly, Apl5p-YFP fusion proteins were instead found to localize to small punctate structures within, or on, the cell nucleus. This contradicts both my hypothesis and our current understanding of the functions of the AP3 complex. Therefore, further research is necessary to determine the role of Apl5p in cytokinetic regulation, especially concerning its localization.

KEY WORDS

Schizosaccharomyces pombe, fission yeast, cytokinesis, cell cycle, checkpoint mechanism, AP3 adaptor protein complex, vesicular transport
ACKNOWLEDGEMENTS

I would first like to acknowledge and thank my academic supervisor, Dr. Jim Karagiannis, for his immeasurable contributions to my project. I am deeply grateful for his support, knowledge, and guidance throughout my studies.

I would like to thank my advisory committee members, Dr. Kathleen Hill, Dr. Rob Dean, and Dr. Brenda Murphy for their unique perspectives on my work and causing me to reflect on my project more than before. I would also like to Dr. Ben Rubin who was so kind and helpful even after the third time I returned to him with a question about the same statistics problem.

I would like to express my gratitude towards my lab mates (past and present) without whom I could not have completed this thesis. To Bidhan, Vanessa, and Simrat, thank you for lending a helping hand or ear, for taking time out of your own research to help me with mine, and for making the lab such a fun place to work in.

I would like to thank my family and friends for their continuous support over the last two years. To my parents, thank you for being always willing to offer wise advice on my research and deliver food when I was too busy to cook. To my friends, thank you for your encouragement during my times of stress. Lastly, thank you to my wonderful fiancé, Jordan, who helped me during every aspect of my studies; from helping me do background research at the beginning, to helping me fill pipette boxes, to collecting my autoclave trays, to editing this thesis, and finally, to acting as a willing audience while I practiced my defense talk.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................................................. ii

ACKNOWLEDGEMENTS ............................................................................................................................ iii

TABLE OF CONTENTS ................................................................................................................................. iv

LIST OF TABLES .......................................................................................................................................... vi

LIST OF FIGURES ...................................................................................................................................... vii

LIST OF ABBREVIATIONS ........................................................................................................................... viii

CHAPTER ONE: INTRODUCTION ................................................................................................................ 1

1.1 The Cytokinetic Monitoring System is Necessary to Ensure the Faithful and Reliable Execution of Cell Division .......................................................................................................................... 1

1.2 Cytokinesis in Eukaryotes ....................................................................................................................... 2

1.2 Using Schizosaccharomyces pombe as a Model Organism for the Study of Cytokinesis in Eukaryotes .................................................................................................................................................. 5

1.3 Cytokinesis in Schizosaccharomyces pombe .......................................................................................... 6

1.4 Differences in Cytokinesis Between Schizosaccharomyces pombe and Animal Cells................. 9

1.5 The Schizosaccharomyces pombe Cytokinesis Checkpoint ................................................................. 10

1.6 Use of Latrunculin A as a Tool to Screen for Regulators of the Cytokinesis Checkpoint in Schizosaccharomyces pombe ................................................................................................................. 11

1.8 Apl5p, an Essential Subunit of the Conserved AP3 Adaptor Protein Complex ......................... 13

1.9 Rationale of the Study ............................................................................................................................ 15

1.10 Aims of the Study ................................................................................................................................ 16

CHAPTER TWO: MATERIALS AND METHODS .......................................................................................... 17

2.1 Strains, Media, and Growth Conditions ............................................................................................... 17

2.2 Verification of the Bioneer apl5Δ Gene Deletion Mutant ................................................................... 17

2.3 Verification of Riken pDUAL-YFH1c Vectors ..................................................................................... 20

2.4 Plasmid Integrations .............................................................................................................................. 21

2.5 DNA Sequencing .................................................................................................................................. 21

2.6 Genetic Techniques ............................................................................................................................... 23

2.7 Fluorescence Microscopy ..................................................................................................................... 23

2.8 Monitoring the Constriction of the Actomyosin Ring ......................................................................... 24

2.8 Latrunculin A Treatment ...................................................................................................................... 24
LIST OF TABLES

Table 2.1: *S. pombe* and *E. coli* strains used in this study .......................................................... 18

Table 2.2: Primers used in this study .................................................................................................. 19

Table 3.1: Quantification of Apl5p, Apl6p, Aps3p, Apm3p overexpression after treatment with LatA. ......................................................................................................................... 43
LIST OF FIGURES

Figure 1.1: A comparison of cytokinesis in plants, animals, and the fission yeast, Schizosaccharomyces pombe. ................................................................. 4

Figure 1.2: Cytokinesis in S. pombe. ............................................................... 7

Figure 1.3: Morphological defects associated with LatA treatment. .................. 12

Figure 1.4: Schematic representation of a heterotetrameric AP complex. .......... 14

Figure 2.1: Schematic representation of the Bioneer deletion cassette. ............. 19

Figure 2.2: pDUAL-YFH1c plasmid map. ....................................................... 20

Figure 2.3: Strategy for chromosomal integration of pDUAL-YFH1c vectors. ....... 22

Figure 3.1: Verification of the Bioneer apl5Δ deletion mutant by colony PCR. ........ 27

Figure 3.2: Structure of the apl5 locus in the Bioneer apl5Δ strain ................... 28

Figure 3.3: The apl5Δ mutant is exquisitely sensitive to LatA............................. 29

Figure 3.4: The colony forming ability of the apl5Δ mutant is abolished upon LatA treatment... 30

Figure 3.5: apl5Δ mutants are unable to complete cytokinesis upon LatA treatment ........ 31

Figure 3.6: Quantification and statistical analysis of phenotypic effects of DMSO and LatA treatment. .................................................................................................................. 33

Figure 3.7: apl5Δ mutants are unable to maintain the physical integrity of the actomyosin ring upon LatA treatment. ................................................................. 35

Figure 3.8: Intracellular localization of Apl5p, Apl6p, Apm3p, Aps3p ................. 37

Figure 3.9: Over-expression of Apl5p, Apl6p, Apm3p, or Aps3p impedes cell growth in the presence of LatA ................................................................. 39

Figure 3.10: DAPI/aniline blue staining of cells over-expression Apl5p, Apl6p, Aps3p, and Apm3p ................................................................. 41

Figure 3.10 (continued): DAPI/aniline blue staining of cells over-expression Apl5p, Apl6p, Aps3p, and Apm3p ................................................................. 42

Figure 3.11: Schizosaccharomyces pombe kin1Δ mutants are sterile .................. 45
LIST OF ABBREVIATIONS

Ade: Adenine
AP: Adaptor Protein
BRCA2: Breast Cancer 2
CDK: Cyclin-Dependent Kinase
Cdr: Changed Division Response
DAPI: 4',6-diamidino-2-phenylindole
DMSO: Dimethyl Sulfoxide
DNA: Deoxyribonucleic Acid
EMM: Edinburgh Minimal Medium
G2: Gap 2 Phase
GFP: Green Fluorescence Protein
His: Histidine
LatA: Latrunculin A
Leu: Leucine
M: Mitosis
PBS: Phosphate Buffered Saline
PCR: Polymerase Chain Reaction
Ser: Serine
Sid: Septation Initiation-Deficient
SIN: Septation Initiation Network
SPAS: Sporulation Agar
SPB: Spindle Pole Body
Spg: Septum-Promoting GTPase
Thia: Thiamine
TGN: Trans-Golgi Network
Ura: Uracil
YES: Yeast Extract with Supplements
YFP: Yellow Fluorescent Protein
CHAPTER ONE: INTRODUCTION

1.1 The Cytokinetic Monitoring System is Necessary to Ensure the Faithful and Reliable Execution of Cell Division

Cytokinesis is the essential process by which a mother cell physically separates into two independent daughter cells. This process involves dramatic remodelling of the cytoskeleton and must be carefully monitored, both spatially and temporally, to ensure equal division of genetic material between the two daughter cells (Prekeris & Gould, 2008).

The interest in developing a better understanding of cytokinetic regulation is underscored by the impact of cytokinesis on genomic integrity. In 1914, Theodor Boveri hypothesized that cytokinetic failure might result in tetraploid intermediate cells containing extra centrosomes (the main microtubule organizing center) (Harris, 2008). He reasoned that the extra centrosomes might lead to chaotic multipolar mitoses, in which sister chromatids are not equally segregated between daughter cells. Consequently, the daughter cells would be more susceptible to structural breaks and rearrangements, leading to increased rates of aneuploidy and tumorigenic growth (Harris, 2008).

Over the last century, research in a variety of model organisms has uncovered considerable evidence supporting the idea that failure in cell division leads to multipolar mitosis, chromosomal rearrangements, aneuploidy, and tumorigenic growth (Harris, 2008; Ganem et. al., 2007; Fujuwara et. al., 2005; Olaharski et. al., 2006; Holland & Cleveland, 2009). In experiments using mouse mammary epithelial cells, tetraploid intermediates, created through drug induced cytokinesis failure, were shown to form tumours at greater rates than diploid controls (when transplanted into nude mice (Ganem et. al., 2007; Fujuwara et. al., 2005).
Other evidence to support this theory includes the discovery of tetraploid intermediates in early stage cancers and in the premalignant condition, Barrett's oesophagus (Olaharski et. al., 2006; Galipeau et. al., 1996). Furthermore, known tumor suppressor proteins such as BRCA2, P53, and Aurora A kinase are necessary for the faithful completion of cytokinesis (Daniels et. al., 2004; Yang et. al., 2004; Vitale et. al., 2011; Bae et. al., 2005; Sherr & McCormick, 2002). Much is now known about the components of the division machinery. However, the complex process of cytokinesis, particularly the mechanisms that regulate and control the division process, is still not fully understood. Further research into cytokinetic regulation will be relevant not only to our understanding of basic eukaryotic cell biology, but also to our understanding of how eukaryotes maintain the integrity of their genomes.

1.2 Cytokinesis in Eukaryotes

Unlike other cell cycle processes, cytokinesis has been particularly difficult to study in vitro (Guertin et. al., 2002). Therefore, the use of model organisms has proven immensely beneficial in revealing the essential protein components of cytokinesis. These model organisms include Drosophila melanogaster (fruit fly), Caenorhabditis elegans (roundworm), Saccharomyces cerevisiae (budding yeast), and Schizosaccharomyces pombe (fission yeast).

Although differences in cytokinetic processes exist between organisms, the major events are highly conserved; this is especially true when comparing cytokinesis in animal cells and in the fission yeast, Schizosaccharomyces pombe (Figure 1.1). In animal cells, cytokinesis is dependent on an actomyosin contractile ring, as well as remodelling of the plasma membrane through vesicular trafficking. Preparation for cell division begins in early anaphase when negative regulation of cyclin dependent kinase Cdk1 lifts the inhibition of cytokinetic proteins (Eggert et. al., 2006; Peters, 2002; Niiya et. al., 2005; Barr & Gruneberg, 2007). Upon completion of anaphase, anti-parallel microtubules are organized into a spindle midzone (located at the center of the cell) that serves as a spatial guide for the site of the cleavage furrow. The cleavage furrow
triggers the formation of a contractile actomyosin ring using actin filaments, myosin II and other essential factors (Glotzer, 2005; Guertin et. al., 2002; Balasubramanian et. al., 2004). The actomyosin ring constricts, driving the ingress of the plasma membrane to create a physical barrier within the dividing cell. The ingressing furrow compresses the spindle midzone microtubules into an intracellular bridge, called the spindle midbody, that recruits proteins for the final abscission of the plasma membrane. The addition of newly synthesized membrane material through vesicular trafficking is coordinated with the constriction of the actomyosin ring (Wang et. al., 2002). It is noteworthy that many proteins involved in membrane trafficking are also components of the midbody (Skop et. al., 2004; Albertson et. al., 2005; Barr & Gruneberg, 2007; Doxsey et. al., 2005).

Cytokinesis in S. pombe is considered to be functionally comparable to animal cells, since they also divide via the formation and subsequent constriction of an actomyosin ring at the medial region of the cell (Bezanilla et. al., 1997; Kitayama et. al., 1997; Marks & Hyams, 1985; May et. al., 1997; Motegi et. al., 1997; Simanis, 1995). However, unlike animal cells, all fungi (including S. pombe) cells possess a cell wall. Therefore, a multilayered polysaccharide septum must be deposited behind the actomyosin ring as it constricts, guiding new cell wall material. The inner layer of the septum is eventually degraded, disconnecting the daughter cells (Karagiannis, 2012, Bowman & Free, 2006).
Figure 1.1: A comparison of cytokinesis in plants, animals, and the fission yeast, *Schizosaccharomyces pombe*.

In plants (A), the site of cell division is selected by Golgi-derived vesicles which are delivered to the central region of the cell. In contrast to plant cells, both *S. pombe* and animals cells (B and C) divide by medial fission and initiate cytokinesis through a contractile actomyosin ring at the medial region of the cell. Reproduced from Pollard and Wu (2010) under the fair use provision of the *Canadian Copyright Modernization Act* (2012).

While animal and *S. pombe* cells require an actomyosin ring to complete cytokinesis, plants do not; instead, cell division is dependent on the assembly of a new cell wall. Plant cell division involves vesicular trafficking from the Golgi to the medial region of the cell to assemble a unique organelle, called the phragmoplast, which is surrounded by a pre-prophase band of microtubules. This band marks the site of the future mitotic
spindle (which organizes the separation of daughter chromosomes) as well as the site of fusion between new cell plate material and the existing cell wall. Continued vesicular trafficking of new cell wall and plasma membrane material, guided by microtubules extending from the phragmoplast, results in cell division (Jürgens, 2005; Dhonukshe et. al., 2007; Staehelin & Moore, 1995).

In most animal and fungi cells, cytokinesis is initiated and maintained by regulatory systems, which help position and form the actomyosin ring as well as facilitate its constriction. It is critical to the integrity of the cell cycle that the actomyosin ring constricts only after all genetic material in the cell has been replicated and chromosomal segregation is complete. Many key proteins essential to cytokinesis have been identified. However, our current understanding of the mechanisms governing the coordination of cytokinetic processes is lacking (Karagiannis, 2012). Therefore, further research is required in order to develop a deeper understanding of this critical biological mechanism.

1.2 Using *Schizosaccharomyces pombe* as a Model Organism for the Study of Cytokinesis in Eukaryotes

The unicellular fission yeast, *Schizosaccharomyces pombe*, has become a commonly used model organism in the study of a diverse range of biological processes, especially for studies concerning the cell cycle (Yanagida, 2002). Indeed, the fundamental mechanisms of eukaryotic cell cycle control were elucidated using *S. pombe* by Nobel Laureate, Paul Nurse (Nurse & Thuriaux, 1980; Lee & Nurse, 1987; Nurse et. al., 1976). Approximately 3% of *S. pombe* genes are homologous with human genes that play a role in a variety of diseases, including cancer (Wood et. al., 2002). Similarities between *S. pombe* and human cells are especially evident when considering the mechanisms and regulation of cell division. For example, similar to human cells, *S. pombe* cells execute cell division by way of medial fission. In both *S. pombe* and animal cells, regulatory mechanisms control the progression of the cell cycle and ensure that
cytokinesis only begins after the completion of the previous cycle of mitosis (Balasubramanian et. al., 2004; Hartwell & Weinert, 1989).

Aside from the conservation of gene function, the simplicity of the haploid *S. pombe* genome and its ease of manipulation offer numerous advantages in a research setting. The *S. pombe* genome was fully sequenced in 2002 and a large majority of the genome has been functionally and structurally annotated (Wood et. al., 2002). This has led to the development of an array of molecular, genetic and functional genomic techniques that allow for the detailed characterization of identified mutants (Verde et. al., 1995). Additionally, *S. pombe* contains the smallest number of protein coding genes for a eukaryote (4824 genes) and has a short generation time of 2.5-3 hours, allowing for expedited molecular and genetic studies (Wood et. al., 2002). Due to these advantages, the *S. pombe* cell cycle has emerged as an excellent model with which to study the complex regulatory networks required for cell division (Gould & Simanis, 1997; Le Goff et. al., 1999).

### 1.3 Cytokinesis in *Schizosaccharomyces pombe*

Cytokinesis in *S. pombe*, just as in more complex eukaryotes, is dependent on the formation and constriction of an actomyosin ring together with the deposition of a polysaccharide septum (Figure 1.2). The position of the actomyosin ring in *S. pombe* is determined before onset of mitosis through Plp1p kinase mediated nuclear export of anillin-like Mid1p and subsequent relocation of Mid1p to the center of the cell as part of interphase nodes (Bähler & Pringle, 1998; Daga & Chang, 2005; Huang et. al., 2007). Pom1p (a DYRK-type protein kinase) forms a polar gradient from both cell ends that restricts Mid1p and the interphase nodes to the medial region of the cell (Celton-Morizur et. al., 2006; Moseley et. al., 2009). The interphase nodes act as a marker for the site of the actomyosin ring and the future site of cell division (Pollard & Wu, 2010). Additional proteins, Wee1p, Cdr1p, and Cdr2p, also relocate to the interphase nodes as mitosis begins (Bathe & Chang, 2010; Almonacid & Paoletti, 2010; Wu & Pollard, 2005).
Figure 1.2: Cytokinesis in S. pombe.
Mid1p re-localization triggers the assembly of interphase nodes at the median of the cell. The nodes serve as a spatial marker for the formation of the actomyosin contractile ring. Additional proteins are then recruited to the interphase nodes. Cdc12p stimulates polymerization of actin filaments, which trigger matured cytokinesis nodes to condense into the bundled actomyosin ring. With the assistance of Myosin II, the contractile ring matures. Ring constriction is initiated by the SIN after the completion of mitosis. Constriction of the contractile ring also guides the deposition of a division septum. Adapted from Pollard and Wu (2010) under the fair use provision of the Canadian Copyright Modernization Act (2012).

As S. pombe cells grow lengthwise, Pom1p moves away from the medial region, relieving its inhibition on Cdr1p and Cdr2p. Reduced inhibition of Cdr1p and Cdr2p leads to the phosphorylation and inhibition of Wee1p (Martin & Berthelot-Grosjean, 2009; Moseley et al., 2009). Inhibition of Wee1p results in increased activity of Cdc2p.
and Cdc13p (also known as Cdk1-CyclinB), which promotes entry into mitosis (Morgan, 1997). Upon entry into mitosis, the interphase nodes mature into cytokinesis nodes through the recruitment of the following components of the actomyosin ring: Myosin II, Rng proteins (ring assembly protein), Cdc15p (PCH family protein), and Cdc12p (formin) (Wu et. al., 2006). Cdc12p and Cdc3p (profilin) promote polymerization of actin filaments, which condense into a bundled actomyosin ring with the aid of tropomyosin, fimbrin, and a-actinin (Kovar et. al., 2003; Wu & Pollard, 2005; Wu et. al., 2006).

Constriction of the mature actomyosin ring is coupled with the completion of the preceding mitosis and the inactivation of Cdc2p-Cdc13p activity at the end of anaphase (Wolfe & Gould, 2005). This coordination, as well as the deposition of a multilayered polysaccharide septum, is maintained by a regulatory module known as the septation initiation network (SIN) (Krapp & Simanis, 2008). The SIN consists of protein kinases including Cdc7p, Sid1p, and Sid2p, which are assembled on the spindle pole body (SPB), the functional equivalent to the human centrosome (Fankhauser & Simanis, 1994; Guertin et. al., 2000; Hou et. al., 2000). The SIN is regulated through a GTPase signalling cascade starting with the GTPase activating protein (GAP) Cdc16p-Byr4p at the SPB. Cdc16p-Byr4p represses the Spg1p GTPase during interphase. However, Spg1p is activated during metaphase (through decreasing levels of Cdc16p) and recruits Cdc7p to the SPBs, leading to the recruitment of the Sid4p-Cdc11 complex. Components of the SIN are bound to the SPB with the help of scaffolding proteins Sid4p and Cdc11p (Schmidt et. al., 1997; Tomlin et. al., 2002; Mishra et. al., 2005).

Recruitment of Sid4p—Cdc11p to the SPB triggers Sid2p-Moblp to re-localize from the SPB to the site of cell division. Sid2p-Moblp is required to promote actomyosin ring maintenance, ring constriction and the assembly of the division septum. Sid2p is also part of a positive feedback loop (through mediating the phosphorylation of scaffolding protein, Cdc11p) that supports SIN signalling during anaphase (Balasubramanian et. al., 2004). Activation of the SIN pathway also promotes the activity of Cps1p, a synthase important for the assembly of the division septum. After the assembly of the primary
and secondary septum, the primary septum is degraded, liberating the daughter cells from each other (Wang et. al., 2002).

1.4 Differences in Cytokinesis Between *Schizosaccharomyces pombe* and Animal Cells

The suitability of *S. pombe* as a model organism for animal cells is dependent on the similarity between the cytokinetic processes of both organisms. Both animal and *S. pombe* cells undergo cell division through the use of a contractile actomyosin ring. Furthermore, cell division in both of these eukaryotes requires the targeted deposition of new cellular membrane material. While highly similar overall, there are differences in both the regulatory and the structural organization of the cellular machinery driving cytokinesis (Balasubramanian et. al., 2004).

In both *S. pombe* and animal cells, membrane remodelling is inherent to the process of cell division because the single plasma membrane of one cell must separate into two. In addition, new membrane proteins must be generated to accommodate the growing surface area as one cell splits into two. Generally, new membrane materials are supplied by the Golgi or Golgi-like vesicles (Albertson et. al., 2005; Wang et. al., 2001). *S. pombe* cells also possess cell walls, necessitating the construction of a polysaccharide septum after the constriction of the actomyosin ring. In *S. pombe*, the majority of added surface area is generated throughout interphase, when the cell is undergoing polarized growth; thus, only the small amount of plasma membrane and cell wall material that form the partition between the daughter cells must be added during cytokinesis (Albertson et. al., 2005; Pollard & Wu, 2010; Marks et. al., 1986). Animal cells lack cell walls and grow spherically during metaphase, posing different requirements for membrane reorganization during cytokinesis (Balasubramanian et. al., 2004).

Apart from structural differences, there are also temporal differences between *S. pombe* and animal cell cytokinesis. For example, the division site in *S. pombe* is determined in the G2 phase of the cell cycle and is dependent on the position of the nucleus during interphase. In animal cells, on the other hand, the site of cell division is determined
during anaphase and reflects the position of the mitotic spindle (Rapoport, 1996; Glotzer, 2001). Furthermore, the timing of actomyosin ring assembly differs. Actomyosin rings in *S. pombe* are not assembled until cells enter into mitosis, although the components for the ring are medially localized in the G2 phase. Conversely, animal cells do not initiate ring assembly until the beginning of anaphase (Balasubramanian et al., 2004).

For both *S. pombe* and animal cells, there are regulatory mechanisms in place to ensure the coordination of cytokinesis with the nuclear cycle. Ring constriction cannot take place until after the complete and equal segregation of daughter chromosomes and other cytoplasmic material. Moreover, similarities are seen between *S. pombe* and animal cells at the molecular level through conserved elements of the SIN, which are important for cell division in both organisms (Balasubramanian et al., 2004). Hence, despite structural and temporal differences, *S. pombe* remains as an excellent model organism for the study of cytokinesis in animal cells.

### 1.5 The *Schizosaccharomyces pombe* Cytokinesis Checkpoint

In *S. pombe*, it has been demonstrated that progression through the cell cycle is controlled by cell cycle checkpoints. These checkpoints are essential for monitoring cell cycle transitions and ensure that previous steps are completed before subsequent steps are initiated (Hartwell & Weinert, 1989). Checkpoint failure results in abnormal cell cycle progression and can ultimately lead to cell death. An example of such a regulatory mechanism is the SIN-dependent process that ensures cytokinesis only begins once the preceding round of mitosis is complete (Krapp & Simanis, 2008; Karagiannis, 2012).

More recently discovered is the existence of a *S. pombe* cytokinetic monitoring checkpoint that coordinates dependency of subsequent rounds of mitosis upon the completion of the preceding round of cytokinesis. This cytokinetic regulatory mechanism is not an integral part of the cell cycle process, but is essential only if the cell division machinery is stressed. Upon such perturbances, the cytokinesis checkpoint
activates two pathways, one responsible for promoting the reassembly and stabilization of the actomyosin ring and a second for enforcing a G2 delay that persists until cytokinesis is completed successfully (Liu et. al., 2000; Le Goff et. al., 1999; Mishra et. al., 2004; Trautmann et. al., 2001; Mishra et. al., 2005). Critical members of this monitoring system include the SIN, the Cdc14 family phosphatase Clp1p, the 14-3-3 protein Rad24p, and the Lsk1p kinase (Mishra et. al., 2004; Trautmann et. al., 2001; Mishra et. al., 2005; Karagiannis & Balasubramanian, 2007; Karagiannis et. al., 2005; Saberianfar et. al., 2011).

1.6 Use of Latrunculin A as a Tool to Screen for Regulators of the Cytokinesis Checkpoint in Schizosaccharomyces pombe

Loss-of-function mutations in genes critical to the cytokinesis checkpoint render S. pombe cells particularly sensitive to cytokinetic stresses. An example of such a stress is treatment with a Red Sea sponge toxin, Latrunculin A (LatA) (Liu et. al., 2000; Mishra et. al., 2004). LatA sequesters actin monomers, thereby causing actin filament depolymerisation and inhibition of the contractile actomyosin ring. At low concentrations (0.2 - 0.5 µM), LatA mildly disrupts the stability of the actomyosin ring and activates the cytokinesis checkpoint system (Ayscough et. al., 1997; Mishra et. al., 2004; Mishra et. al., 2005).

Wild-type cells suffer only minor effects upon LatA treatment since the cytokinesis checkpoint system delays cell cycle progression, thereby providing cells with the time needed to stabilize the actomyosin ring before cytokinesis is initiated. Cells bearing mutations in cytokinetic checkpoint regulators show normal phenotype under normal growth environments. However, upon LatA treatment, these deletion mutants are inviable and form elongated, multinucleate cells with fragmented septa (Figure 1.3) (Karagiannis et. al., 2005; Mishra et. al., 2005; Mishra et. al., 2004). Hence, treatment with LatA can be used as a simple tool to isolate novel mutants affecting cytokinesis checkpoint function (Mishra et. al., 2004).
To identify novel components of the *S. pombe* cytokinesis monitoring system, a genome-wide *S. pombe* deletion library was screened for hypersensitivity to LatA (Mishra, McCollum, & Karagiannis, unpublished). The *apl5* gene deletion mutant was identified in this screen.

![Diagram showing morphological defects associated with LatA treatment.](image)

**Figure 1.3: Morphological defects associated with LatA treatment.**

Wild-type cells are generally able to complete cytokinesis despite division machinery perturbation. However, mutants lacking a functional cytokinetic checkpoint system are unable to stabilize the contractile ring, resulting in degeneration of the actomyosin ring, cytokinetic failure and multinucleate cells. Adapted from Karagiannis et. al. (2005) under the fair use provision of the *Canadian Copyright Modernization Act* (2012).
1.8 Apl5p, an Essential Subunit of the Conserved AP3 Adaptor Protein Complex

Apl5p is an essential subunit of the heterotetrameric AP3 adaptor protein complex, which is highly conserved (Pollard et. al., 2001; Boehm & Bonifacino, 2001). All AP (adaptor protein) complexes are heterotetrameric collections of subunits called adaptins (Boehm & Bonifacino, 2002). The AP1, AP2 and AP3 complexes exist in all eukaryotic organisms examined to date (Pollard et. al., 2001). Like all AP complexes, the AP3 complex is composed of two large subunits (Apl5p, Apl6p), 1 medium subunit (Apm3p), and 1 small subunit (Aps3p) (Figure 1.4).

Generally, the AP complexes play a role in the trafficking of vesicles as part of the secretory and endocytic networks (Pollard et. al., 2001; Nakatsu & Ohno, 2003). Vesicles are responsible for the intracellular exchange of materials between the trans-Golgi network (the sorting station on the trans face of the Golgi which prepares proteins for their final destinations), endosomes (an organelle of the endocytic transport pathway), lysosomes (or the related vacuoles in *S. pombe* and plants) and the plasma membrane (Boehm & Bonifacino, 2002). AP complexes form part of the protein coat that surrounds the vesicles and assist in both vesicle formation and the selection of specific cargo protein.

The exact role of the AP3 complex and its four subunits in *S. pombe* has not been determined. However, the AP3 complex is proposed to have two possible functions in mammalian cells: 1) a role in the formation of vesicles that participate in intracellular material transport and 2) involvement at an early sorting endosome to prepare cargo proteins for recycling (Boehm & Bonifacino, 2001; Boehm & Bonifacino, 2002; Dell’Angelica et. al., 1998). In *S. cerevisiae*, the AP3 complex has been shown to mediate an alternate pathway for selected cargo from the TGN to the vacuole or lysosome endosome (Odorizzi et. al., 1998; Stepp et. al., 1997).
Figure 1.4: Schematic representation of a heterotetrameric AP complex.

All AP complexes are composed of two large subunits (named \(\gamma/\alpha/\delta/\varepsilon/Apl5p\) and \(\beta1-4/Apl6p\), respectively), 1 medium subunit (\(\mu1-4/Apm3p\)), and 1 small subunit (\(\alpha1-4/Aps3p\)). Reproduced from Boehm and Bonifacino (2002) under the fair use provision of the Canadian Modernization Act (2012).

Based on its role in other organisms, the AP3 complex is also expected to be involved in vesicular transport or protein sorting in *S. pombe*. The recent identification of *apl5* in the *S. pombe* LatA screen suggests an additional role for the gene, as well as the whole AP3 complex, in the regulation of cytokinesis. The functions and localizations of the four AP3 complex subunits have not been studied in *S. pombe*, nor have the cargo proteins mediated by the complex been elucidated. This lack of understanding illustrates a gap in our knowledge concerning the basic functions of Apl5p, and the other subunits, which must be addressed before a role for Apl5p can be definitively assigned to cytokinesis regulation. The goal of this study was to characterize the functions of Apl5p and give insight on the how Apl5p, and ultimately the AP3 complex, is involved in the cytokinesis monitoring checkpoint.
In *D. melanogaster*, mutants in the individual subunits of the AP3 complex led to defects in pigmentation, which is in agreement with the established similarity between pigment granules and lysosomes (Mullins et. al., 2000). In humans and mice, AP3 complex mutants exhibit defects in lysosome and lysosome-related organelle function and the missorting of membrane proteins (Peden et. al., 2004; Dell’Angelica et. al., 2000; Boehm & Bonifacino, 2002). Mice bearing mutations in the δ subunit (homologous to *S. pombe apl5*) display a condition called mocha, which results in hypopigmentation of the coat, lysosomal abnormalities, and neurological defects (Kantheti et. al., 1998).

**1.9 Rationale of the Study**

In *S. pombe*, the constriction of the actomyosin ring during cell division requires the deposition of new membrane and cell wall material. Recycling vesicles, which transport cargo from the trans-Golgi network to the plasma membrane, are an important source of such materials (Wang et. al., 2002; Ai & Skop, 2009; Bluemink & De Last, 1973). It thus stands to reason that membrane trafficking components contribute to the proper execution of cytokinesis in *S. pombe*. While it has been suggested to participate in vesicular transport and cargo selection based on work conducted with other organisms, the exact function(s) of Apl5p in *S. pombe* has yet to be elucidated (Cowles et. al., 1997; Stepp et. al., 1997; Peden et. al., 2004; Dell’Angelica et. al., 1998). If Apl5p does indeed play a role in vesicular transport, its contribution towards the function of the cytokinesis monitoring system may be related to the transport of material required for cytokinesis to the site of cell division. Through molecular and genetic analysis, I aim to characterize the role of Apl5p with respect to the *S. pombe* cytokinesis checkpoint. I hypothesize that Apl5p-mediated vesicular transport to the site of cell division is required for successful cytokinesis in *S. pombe*. 
1.10 Aims of the Study

To assess the role of *apl5* in cytokinetic regulation, I aimed to confirm the sensitivity of the *apl5* deletion mutant to the drug, LatA. Fluorescence microscopy was used to visualize the phenotype of the *apl5* deletion mutant. The constriction of the actomyosin ring was assessed in real-time using a GFP-tagged marker for ring constriction, in an *apl5* mutant background. This demonstrated the impact of Apl5p on maintaining the integrity of the actomyosin ring during cytokinetic stress. I also determined the localization of Apl5p and observed the movement of the protein throughout the cell cycle. Lastly, I performed over-expression studies to determine if the function of Apl5p is dosage dependent. Ultimately, I hoped to provide further insight on the contribution of Apl5p and vesicular transport to the cytokinesis checkpoint in *S. pombe*.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Strains, Media, and Growth Conditions

The *S. pombe* strains used in this study (Table 2.1) were obtained from commercial sources (Bioneer Corporation, Alameda, CA) or derived from the Karagiannis laboratory collection. Unless otherwise noted, *S. pombe* strains were cultured in liquid yeast extract medium with supplements (YES) (Forsburg & Rhind, 2006). Cultures were grown at 30°C, with shaking at 150 rpm to mid log phase (OD_{600} 0.2-0.4). A selection of strains carrying auxotrophic markers was carried out by growth in Edinburgh Minimal Media (EMM) supplemented with the appropriate nutrient supplements (adenine, histidine, leucine, and/or uracil) (Forsburg & Rhind, 2006). Strains were induced to mate by culturing in sporulation medium with supplements (SPAS) (Forsburg & Rhind, 2006).

The *Escherichia coli* strain, XL1-Blue, was used for all molecular cloning experiments. These cells were made electro-competent through cold shock. Briefly, cells were washed with ice-cold dH2O and then 10% glycerol at 4°C. After electro-transformation, cells were plated and grown overnight on solid Luria Bertani (LB) agar media with 100 µg/ml ampicillin, at 37°C.

2.2 Verification of the Bioneer *apl5Δ* Gene Deletion Mutant

The *apl5Δ* deletion mutant was obtained from Bioneer Corporation (Alameda, CA). Bioneer constructed the *apl5Δ* strain by replacing the *apl5* open reading frame (ORF) with a deletion cassette carrying the *kanMX* selectable marker (conferring resistance to the drug G418) (Kim et. al., 2010). Gene disruption was verified by colony PCR using primers JK 407 and JK 408 together with primers CPC 1, CPC 3, CPN 1, and CPN 10 (provided by the supplier) (Table 2.2). Primers JK 407 and JK 408 were designed to be specific to sequences 119 bp upstream and 274 bp downstream of the gene of interest,
respectively. Primers CPC 1, CPC 3, CPN 1, and CPN 10 on the other hand, were specific to sequences inside the kanMX deletion cassette. Fermentas Taq DNA polymerase and Taq buffer were used for all PCR reactions. Gel electrophoresis of the amplicons was performed for 1 hour through a 1% agarose gel containing Gel Red to visualize the PCR products under UV light (365nm). Analysis of the size of the PCR products demonstrated the apl5Δ strain to be a gene disruption (Figure 2.1).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JK 9</td>
<td>clp1::ura4⁺ ura4-D18 h⁺</td>
<td>JK collection</td>
</tr>
<tr>
<td>JK 402</td>
<td>E. coli XL1-Blue</td>
<td>JK collection</td>
</tr>
<tr>
<td>JK 484</td>
<td>ura4-D18 leu-32 ade6-216 his3-D1 h⁻</td>
<td>JK collection</td>
</tr>
<tr>
<td>JK 485</td>
<td>ura4-D18 leu1-32 ade6-210 his7-366 h⁺</td>
<td>JK collection</td>
</tr>
<tr>
<td>JK 824</td>
<td>apl5::kanMX ura4-D18 leu-32 ade6-216 h⁺</td>
<td>Bioneer</td>
</tr>
<tr>
<td>JW 1</td>
<td>apl5-YFP::leu1⁺ leu1-32 ade6-210 his7-366⁺</td>
<td>This study</td>
</tr>
<tr>
<td>JW 2</td>
<td>apl6-YFP::leu1⁺ leu1-32 ade6-210 his7-366⁺</td>
<td>This study</td>
</tr>
<tr>
<td>JW 3</td>
<td>apm3-YFP::leu1⁺ leu1-32 ade6-210 his7-366⁺</td>
<td>This study</td>
</tr>
<tr>
<td>JW 4</td>
<td>apm3-YFP::leu1⁺ leu1-32 ade6-210 his7-366⁺</td>
<td>This study</td>
</tr>
<tr>
<td>JW 14</td>
<td>apl5-YFP::ura4⁺ ura4-D18 leu1-32 h⁻</td>
<td>This study</td>
</tr>
<tr>
<td>JW 18</td>
<td>apl5::kanMX rlc1-GFP::ura4⁺ leu1-32</td>
<td>This study</td>
</tr>
<tr>
<td>MBY 624</td>
<td>rlc1-GFP::ura4⁺ leu1-32 h⁻</td>
<td>JK collection</td>
</tr>
</tbody>
</table>
### Table 2.2: Primers used in this study

<table>
<thead>
<tr>
<th>Oligos</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>JK 407</td>
<td>5'-CTG CTT CAG CTC GAT AAT TCC T-3'</td>
<td>Forward, verification of Bioneer <em>apl5Δ</em></td>
</tr>
<tr>
<td>JK 408</td>
<td>5'-ACT TCG CAA AGT CCC ATA CAG T-3'</td>
<td>Reverse, verification of Bioneer <em>apl5Δ</em></td>
</tr>
<tr>
<td>CPC 1</td>
<td>5'-TGA TTT TGA TGA CGA GCG TAA T-3'</td>
<td>Forward, verification of downstream junction of Bioneer <em>apl5Δ</em></td>
</tr>
<tr>
<td>CPC 3</td>
<td>5'-GGC TGG CCT GTT GAA CAA GTC TGG A-3'</td>
<td>Forward, verification of downstream junction of Bioneer <em>apl5Δ</em></td>
</tr>
<tr>
<td>CPN 1</td>
<td>5'-CGT CTG TGA GGG GAG CGT TT-3'</td>
<td>Reverse, verification of upstream junction of Bioneer <em>apl5Δ</em></td>
</tr>
<tr>
<td>CPN 10</td>
<td>5'-GAT GTG AGA ACT GTA TCC TAG CAA G-3'</td>
<td>Reverse, verification of upstream junction of Bioneer <em>apl5Δ</em></td>
</tr>
<tr>
<td>pUC/M13 Forward</td>
<td>5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'</td>
<td>Forward, sequencing and verification of ORF-YFP constructs</td>
</tr>
<tr>
<td>pUC/M13 Reverse</td>
<td>5'-TCA CAC AGG AAA CAG CTA TGA C-3'</td>
<td>Forward, sequencing and verification of ORF-YFP constructs</td>
</tr>
</tbody>
</table>

![Primer pairs](image)

**Figure 2.1: Schematic representation of the Bioneer deletion cassette.**

The annealing sites of primers JK 407 and JK 408 (specific to the regions downstream and upstream of the *apl5 ORF*) and the annealing sites of primers, CPN 1, CPN 10, CPC 1, and CPC 3 (specific to the *kanMX module*) are shown. Primer pairs are represented by color-matched arrows. Adapted from the Bioneer website, [http://pombe.bioneer.co.kr/technic_infomation/Verification.jsp](http://pombe.bioneer.co.kr/technic_infomation/Verification.jsp).
2.3 Verification of Riken pDUAL-YFH1c Vectors

Plasmids expressing C-terminal YFP fusions to apl5, apl6, apm3, and aps3 were purchased from Riken Bioresource Centre (Saitama, Japan). Each ORF was fused to sequences encoding YFP at the 3’ end, and the tagged ORF was cloned into a derivative of the S. pombe pDUAL expression vector, pDUAL-YFH1c (Figure 2.2). The genes were expressed under the control of the thiamine repressible promoter, nmt1, which allows controlled expression of the cloned ORFs. The pDUAL series of vectors carries a leu1 selectable marker separated by an intervening fragment consisting of the autonomous replication element, ars1 and ura4+. Additionally, the vector carries two NotI restriction sites on either side of the ars1-ura4+ fragment. Confirmation of vector identity was performed through DNA sequencing using the pUC/M13 forward and reverse primers (Table 2.2).

![Figure 2.2: pDUAL-YFH1c plasmid map.](image)

MCS (multiple cloning site), nmt1 (thiamine repressible nmt1 promoter), ORF (open reading frame), YFP (yellow fluorescent protein), ars1 (autonomously replicating sequence). Arrows indicate NotI restriction sites. Adapted from Matsuyama et. al. (2004) under the fair use provision of the Canadian Copyright Modernization Act (2012).
2.4 Plasmid Integrations

Chromosomal integration for localization study. *S. pombe* strain, JK 485 (Table 2.1), was used for chromosomal integration of the pDUAL-YH1c vectors obtained from Riken Bioresource Centre (Saitama, Japan). The identity of the plasmid was verified by DNA sequencing. Once verified, the plasmid was intra-chromosomally integrated into a wild-type *S. pombe* strain, JK 495. The *leu1*-32 allele present in JK 485 cells carries a single base substitution within the *leu1*+ ORF that renders it inviable on media lacking leucine (Figure 2.3). Endonuclease digestion of pDUAL-YFH1c plasmids with the NotI restriction enzyme released the *ars1-ura4*+ fragment, generating a linearized plasmid carrying a *leu1* targeting fragment. The linearized plasmids were then homologously integrated into the *leu1*-32 locus of JK 485 cells using the lithium acetate method (Forsburg & Rhind, 2006). Correct chromosomal integration of the vectors in JK 485 conferred leucine prototrophy. Transformed cells were selected through plating on EMM solid media lacking leucine. The strain was then grown in EMM liquid media to mid-log phase and treated with DMSO (solvent control) and 0.2 µM LatA.

Extra-chromosomal expression of pDUAL-YH1c plasmids. The pDUAL-YH1c plasmids are dual purpose, in that they can be utilized as extra-chromosomal multicopy vectors as well as integrative vectors. This is possible due to the presence of an autonomous replication sequence *ars1* and the selectable marker *ura4*+ (refer to Figure 2.2). For multicopy expression, each plasmid was transformed into the *S. pombe* strain, JK 484, using the lithium acetate method (Forsburg & Rhind, 2006). Transformed cells were selected by plating on EMM media lacking uracil.

2.5 DNA Sequencing

All DNA samples to be sequenced were prepared according to Robarts Sequencing Facility guidelines (http://www.robarts.ca/gateway.php?id=99). After sequencing at the London Regional Genomics Center, results were analyzed using GENtle software (University of Cologne, Version 19.4)
Figure 2.3: Strategy for chromosomal integration of pDUAL-YFH1c vectors.
A) Each pDUAL-YFH1c vector was digested with NotI as indicated with arrows. B) Release of the \textit{ars1-ura4}$^+$ fragment generates the \textit{leu1} targeting fragment which can be integrated into the chromosomal \textit{leu1-32} locus of \textit{S. pombe} strain, JK 485, through homologous recombination. The asterisk represents the single base substitution in the \textit{leu1} gene conferring leucine auxotrophy. C) Proper integration of the fragment is indicated by generation of leucine prototrophy in transformants. Reproduced from Matsuyama et. al. (2004) under the fair use provision of the \textit{Canadian Copyright Modernization Act} (2012).
2.6 Genetic Techniques

Creating double mutants. To create double mutant strains, single mutants of opposite mating types were mixed on SPAS medium (a medium lacking nitrogen that induces sporulation in diploids) in a 20 µl drop of sterile dH₂O (Forsburg & Rhind, 2006). The SPAS plates were then incubated at room temperature for 48 hours. Asci were then analysed by free spore analysis (Forsburg & Rhind, 2006).

Free spore analysis. Asci were treated with glusulase (0.5 units/mL) and left overnight at 30°C (Forsburg & Rhind, 2006). Glusulase treatment lysed vegetative cells, but does not affect the viability of spores. Spores were then washed with water and serially diluted. The dilutions were plated onto solid YES media and incubated at 30°C. Once colonies appeared after 3-5 days of incubation, double mutants were selected by plating spores on selectable YES or on EMM plates containing the appropriate nutrients/drugs.

2.7 Fluorescence Microscopy

For LatA/DMSO assays, S. pombe strains were grown overnight to mid-log phase and fixed with 2 volumes of ice-cold ethanol (Forsburg & Rhind, 2006). The cells were stored in PBS pH 7.4 (with 15% glycerol) at 4°C. The cells were stained with 0.02 µg/µl DAPI and 1 µg/µl aniline blue to view the nucleus and cell wall/ septa, respectively (Balasubramanian, 1998). Images were taken using the Zeiss Axioskop 2 microscope along with ImageJ 1.41 software (National Institute of Health) under fluorescent light, using the DAPF filter, and a Scion CFW Monochrome CCD Firewire Camera (Scion Corporation, Frederick, Maryland). By visualizing the emitted signal via fluorescence imaging, it thus becomes possible to obtain detailed information with respect to cell cycle position of the assayed cells.

Cells expressing YFP or GFP fusions were grown to mid log phase and imaged using a Leica DMI 600B microscope with a BD CARV II Confocal Imager fitted with a YFP or GFP Filter (BD BioSciences, San Jose, CA) and a Quantum: 512SC camera (Photometrics, Tucson, AZ).
2.8 Monitoring the Constriction of the Actomyosin Ring

To monitor the effect of non-functional *apl5* on actomyosin ring constriction, *apl5Δ* was mated with strains expressing Rlc1p-GFP fusion proteins to produce *apl5Δ-rlc1-GFP*. I then monitored actomyosin ring constriction by tracking the localization of the Rlc1p protein and the dynamics of ring constriction were then observed in a +/- LatA background. Both wild-type and *apl5Δ* mutants expressing Rlc1p-GFP fusion proteins were grown to mid-log phase in YES media. Live cells were then filmed using time-lapse microscopy after the addition of DMSO or 0.2 µM LatA.

2.8 Latrunculin A Treatment

Latrunculin A was purchased from ENZO Life Sciences International Inc. (Plymouth Meeting, Pennsylvania). To perform LatA treatments, *S. pombe* strains were first grown overnight in liquid media with shaking (150-200 rpm) to mid-log phase (OD$_{600}$ 0.2-0.4). YFP tagged strains were grown in liquid EMM with selectable supplements at 30°C while all other strains were grown in liquid YES at 30°C. Strains were then treated with Latrunculin A (0.2 - 0.5 µM) dissolved in DMSO (Karagiannis & Balasubramanian, 2007; Karagiannis et. al., 2005). The strains were also treated with DMSO alone (solvent control). The treatment and control cultures were grown for 5 hours with shaking. Treated cells were then fixed with ethanol, and subsequently stained with DAPI/aniline blue and viewed by fluorescence microscopy (Section 2.7). The *clp1Δ* strain is a known checkpoint mutant and was thus used as a positive control in these experiments (Mishra et. al., 2004).

2.9 Statistical Analysis

All phenotypic assays were performed in triplicate. After each trial, the cellular phenotypes resulting from the treatments (DMSO or LatA) were quantified by scoring 500 cells for each genotype. Cells were categorized into four groups based on
phenotype: i) uninucleate, ii) binucleate with a functional septum, iii) binucleate with a non-functional septum (i.e., does not fully bisect the cell) and iv) tetranucleate.

Statistical analysis was performed using the R language and environment to determine whether a difference in phenotypes was present between various genotypic pairings after treatment with LatA or DMSO (R Development Core Team, 2013). Two-way ANOVA was performed for all comparisons ($\alpha = 0.05$). Pairwise differences on all figures are marked with an asterisk (*).
CHAPTER THREE: RESULTS

3.1 Genotypic Verification of the Bioneer \textit{apl5}\textDelta Mutant

Based on a previously conducted genome wide screen using a \textit{S. pombe} deletion library, the \textit{apl5}\textDelta deletion mutant was identified to be hypersensitive to LatA, a drug used to identify mutants affecting the cytokinesis checkpoint (McCollum, Mishra, & Karagiannis, unpublished). To further investigate the role of Apl5p in the cytokinesis checkpoint, the \textit{apl5}\textDelta deletion mutant was purchased from Bioneer Corporation (Alameda, CA). The mutant strain was expected to be carrying the \textit{kanMX} deletion cassette in place of the wild-type \textit{apl5} gene. To verify this replacement, colony PCR was performed using primer pairs amplifying the upstream and downstream regions of \textit{apl5} (Table 2.2).

Based upon information provided by Bioneer, the expected amplicon sizes for each primer pair were as follows: JK 407-CPN 1 (414 bp), JK 407-CPN 10 (533 bp), JK 408-CPC 1 (860 bp), JK 408-CPC 3 (838 bp). However, the resulting amplicons obtained from colony PCR were the following sizes: JK 407-CPN 1 (896 bp), JK 407-CPN 10 (1051 bp), JK 408-CPC 1 (940 bp), JK 408-CPC 3 (918 bp) (Figure 3.1). To determine the cause of this discrepancy, I sequenced the PCR amplicons. Analysis of the sequences revealed that the \textit{apl5}\textDelta mutant still carries 482 bp of the 5’ region of the \textit{apl5} open reading frame, and 80 bp of the 3’ region of the \textit{apl5} open reading frame. This demonstrated that the \textit{apl5}\textDelta gene deletion strain was not, as advertised by Bioneer, a true gene deletion but instead a gene disruption (Figure 3.2). Since the disruption removes \textasciitilde 86\% of the \textit{apl5} open reading frame, I continued with the analysis of the strain since the allele almost certainly represents a loss of function.
Figure 3.1: Verification of the Bioneer apl5Δ deletion mutant by colony PCR.

Colony PCR was performed as described in the Materials and Methods. The PCR amplicons were visualized by running the samples on a 1% agarose gel for 1 hour. Lanes 1 and 10 represent the DNA ladder (fragment sizes in bps are shown to the left of lane 1). Lanes 2-5 represent colony PCR reactions using apl5Δ genomic DNA as template. Lanes 6-9 represent colony PCR reactions using wild-type genomic DNA as template. In lanes 2 and 6, the primer pair JK 407-CPN 1 was used; in lanes 3 and 7, JK 407-CPN 10 was used; in lanes 4 and 8, JK 408-CPC 1 was used; in lanes 5 and 9, JK 408-CPC 3 was used.
3.2 Apl5p is Essential for Cell Proliferation Upon Latrunculin A Treatment

After verifying that the Bioneer apl5Δ deletion mutant did indeed represent an apl5 loss-of-function allele, I turned my attention to confirming the original results of the genome-wide gene deletion screen performed by McCollum, Mishra, and Karagiannis (unpublished). To this end, apl5Δ, clp1Δ, and wild-type cells were grown to mid log phase and streaked out onto YES agar plates containing varying concentrations of LatA.

As shown in Figure 3.3, all strains were fully viable in the presence of DMSO. In the presence of LatA, however, wild-type cells maintained viability at 0.1 µM and 0.2 µM LatA, but were strongly impaired at 0.35 µM LatA. clp1Δ cells, on the other hand, were capable of growth at 0.1 µM, displayed poor growth at 0.2 µM, and were inviable at 0.35 µM LatA. In contrast, apl5Δ cells were inviable at all LatA concentrations tested. These experiments thus demonstrated that apl5Δ cells display an exquisite sensitivity to LatA, being unable to grow at LatA concentrations where other known checkpoint mutants remain viable. Upon inspection of the current literature, it is apparent that the apl5Δ mutant exhibits the highest degree of LatA hypersensitivity observed to date.
Figure 3.3: The *apl5Δ* mutant is exquisitely sensitive to LatA. Strains of the indicated genotype were grown overnight in liquid YES media, and then streaked onto YES agar containing DMSO or the indicated concentration of LatA. Photographs were taken after incubation at 30°C for 72 hours.

To examine the cells more closely, wild-type, *clp1Δ*, and *alp5Δ* cells were viewed under bright field microscopy at 2000X magnification. Cells of all the tested genotypes were capable of forming colonies after DMSO treatment (Figure 3.4). While colony size decreased with increasing LatA concentration, wild-type cells could form small micro-colonies even at 0.35 µM LatA. *clp1Δ* cells could form small colonies at 0.1 µM LatA, but were strongly inhibited at 0.2 µM LatA, and could not form colonies at all at
0.35 µM LatA. The colony forming ability of apl5Δ cells, on the other hand, was completely abolished even at 0.1 µM LatA. These results support the drastic decrease in proliferative ability observed for apl5Δ mutants upon LatA treatment in the streak assay described above (Figure 3.3).

![Figure 3.4: The colony forming ability of the apl5Δ mutant is abolished upon LatA treatment.](image)

Strains of the indicated genotype were grown overnight in liquid YES media then streaked onto YES agar containing DMSO or the indicated concentration of LatA. Micrographs (2000X) were taken after incubation at 30°C for 24 hours.

### 3.3 apl5Δ Mutants are Unable to Complete Cytokinesis Upon Perturbation of the Cell Division Machinery

At this point, it was clear that the apl5Δ mutant was indeed hypersensitive to LatA. However, the underlying etiology of this phenotype was still unknown. To determine if the observed LatA hypersensitivity was due to an underlying defect in cytokinesis, I
employed the technique of DAPI/aniiline blue staining together with fluorescence microscopy.

Microscopic observation showed that DMSO treated wild-type, apl5Δ, and clp1Δ cells behaved similarly, with most cells being uninucleate and the remainder being bi-nucleate. As expected, bi-nucleate cells in the DMSO treated samples possessed a functional septum indicating that these cells were in the cytokinetic phase of the cell cycle (Figure 3.5). There was no statistically significant phenotypic difference between wild-type and clp1Δ strains after DMSO treatment (F(4,20)=2.271, p=0.0976; two-way ANOVA) or between wild-type and apl5Δ strains (F(4,20)=1.245, p=0.324; two-way ANOVA) (Figure 3.6).

Figure 3.5: apl5Δ mutants are unable to complete cytokinesis upon LatA treatment.

apl5Δ, wild-type, and clp1Δ, were grown to mid-log phase in liquid YES media 30ºC and treated with 0.2 µM LatA or DMSO (solvent control) for 5 hrs. Cells were fixed with ethanol and stained with DAPI (nucleus) and aniline blue (cell wall/septum) and observed using fluorescence microscopy with a DAPI filter.
Upon LatA treatment, wild-type cells appeared resistant to the cytokinetic perturbation relative to clp1Δ and apl5Δ mutants. Approximately 75% of the wild-type cells were uni-nucleate, while ~12% were bi-nucleate (with a functional septum). The remaining 13% were bi-nucleate and possessed a fragmented septum indicating failed cytokinesis (Figure 3.6).

In contrast to wild-type, both the apl5Δ and clp1Δ strains were found to be highly sensitive to LatA. Both apl5Δ and clp1Δ mutants showed a noticeably higher number of cells with an abnormal phenotype. Approximately 88% of apl5Δ cells were binucleate (with a non-functional septum) while ~10% were tetranucleate (indicating that cytokinesis failed two times in succession). For clp1Δ mutants, ~43% were binucleate with a damaged septum and ~47% were tetranucleate (Figures 3.5 and 3.6). A statistically significant phenotypic difference was seen between wild-type and apl5Δ mutants (F(4,20) = 35.624, P < 0.001; two-way ANOVA), and between wild-type and clp1Δ (F(4,20) = 45.65, P < 0.001; two-way ANOVA). There was also a statistically significant difference between the phenotypes exhibited by clp1Δ and apl5Δ cells (F(4,20)=3.375, p=0.0289; two-way ANOVA).
Figure 3.6: Quantification and statistical analysis of phenotypic effects of DMSO and LatA treatment.

Cell counts were performed in triplicate by counting and categorizing 500 cells for each genotypic class: wild-type, apl5Δ or clp1Δ. The colors in each bar correspond to the number of cells of each phenotype: uninucleate, binucleate with a functional septum, binucleate with a fragmented septum, and tetranucleate. Statistical analysis was performed using two-way ANOVA (α = 0.05). Statistically significant differences are indicated by asterisks (*).
3.4 The Structural Integrity of the Actomyosin Ring is Compromised in \textit{apl5\Delta} Mutants in the Presence of Latrunculin A

Upon DMSO treatment, wild-type cells were typically able to constrict the ring within 25-40 minutes. \textit{apl5\Delta} mutants showed similar kinetics and were able to complete ring constriction over a similar time frame (Figure 3.7).

Upon LatA treatment, wild-type cells were still able to completely constrict the actomyosin ring, albeit over a longer time period; typically, ring constriction was completed between 60-90 minutes. In contrast, \textit{apl5\Delta} mutant were unable to maintain the integrity of the ring after the addition of LatA. Typically, a few minutes after \textit{apl5\Delta} cells initiated constriction, the rings became slanted, then eventually fragmented and were lost (Figure 3.7).
Figure 3.7: *apl5Δ* mutants are unable to maintain the physical integrity of the actomyosin ring upon LatA treatment.

Wild-type and *apl5Δ* cells expression Rlc1p-GFP fusion proteins were grown to mid-log phase in YES media and imaged in real time using fluorescence time-lapse microscopy with GFP filter. The time (in minutes) is indicated in white type in the bottom right of each image. In "*apl5Δ* LatA (1)”, the arrow at 5 minutes indicates the slant of the ring and the arrow at 30 minutes highlights the break in the actomyosin ring. In "*apl5Δ* LatA (2)”, a similar slant and break is seen at 10 minutes and 25 minutes, respectively.
3.5 Intracellular Localization of Apl5p, Apl6p, Apm3p, Aps3p

To continue the initial characterization of Apl5p, I next decided to determine the subcellular localization of the protein. Given the role of the AP3 complex in vesicular transport, I expected Apl5p to localize to small vesicles at, or near, the site of cell division. However, Apl5p was found to localize, not to the site of cell division, but to small punctate structures within the cell nucleus (Figure 3.8). This localization was observed irrespective of treatment with DMSO or LatA, and was also independent of cell cycle phase (data not shown).

To determine if this localization was specific to Apl5p, or was true of all other members of the AP3 complex, I purchased plasmids from Riken Bioresource Centre (Saitama, Japan) expressing Apl6p-YFP, Apm3p-YFP, and Aps3p-YFP. These plasmids were also confirmed by DNA sequencing and integrated into wild-type S. pombe cells. Apl6p-YFP showed similar staining to Apl5p-YFP in the form of punctate nuclear dots. Aps3p and Apm3p were also localized to the nucleus, but staining was more uniform (i.e. no punctate dots) (Figure 3.8). Similarly to Apl5p, the localization of the other three subunits was not affected by LatA, and was independent of cell cycle phase (data not shown).
Figure 3.8: Intracellular localization of Apl5p, Apl6p, Apm3p, Aps3p.

apl5-YFP, apl6-YFP, apm3-YFP, and aps3-YFP strains were grown to mid-log phase in liquid EMM-leucine and visualized under fluorescence microscopy using a YFP filter. Intracellular localization for all subunits was consistent throughout the cell cycle and after the addition of LatA.
3.6 Over-expression of Apl5p, Apl6p, Apm3p, and Aps3p Result in a Dominant Negative Effect on Growth

To complete the initial characterization of the \( apl5 \) gene, I next embarked upon a series of over-expression studies. Over-expression was accomplished using the same vectors that were used to determine the intracellular localization of Apl5p-YFP. In this family of vectors, the thiamine-repressible nmt1 promoter controls the expression of the YFP fusion proteins. Furthermore, when not chromosomally integrated, these vectors replicate with a copy number of between 10-30. Thus, very strong over-expression can be achieved using cells that are grown in the absence of thiamine, in which the plasmids are propagated extra-chromosomally. Since plasmids expressing Apl6p-YFP, Apm3p-YFP, and Aps3p-YFP were also on hand, I included an analysis of their over-expression as part of this study.

To assess the effects of over-expression, strains carrying extra-chromosomally replicating Apl5p-YFP, Apl6p-YFP, Apm3p-YFP, or Aps3p-YFP vectors, were streaked onto EMM media containing varying concentrations of LatA or DMSO (solvent control). As a negative control, the same strains were also streaked onto solid EMM containing 10 \( \mu \text{M} \) thiamine (repressing conditions), as well as LatA or DMSO. Plates were viewed using bright-field microscopy (Figure 3.9).

All strains were viable upon DMSO and 0.2 \( \mu \text{M} \) LatA treatment, regardless of being repressed or de-repressed (Figure 3.9). Upon treatment with 0.35 \( \mu \text{M} \) LatA, however, strains over-expressing Aps3p, Apl5p, and Apl6p, while viable in the presence of thiamine, were inviable in the absence of thiamine. The same pattern was observed for Apm3p over-expression, except that these cells were viable up to 0.5 \( \mu \text{M} \) LatA in the presence of thiamine, but were inviable at the same LatA concentration in the absence of thiamine. These observations suggest that rather than being beneficial, over-expression of Apl5p, Apl6p, Apm3p, or Aps3p has a dominant negative effect and impedes cell viability.
Figure 3.9: Over-expression of *apl5*, *apl6*, *apm3*, or *aps3* impedes cell growth in the presence of LatA.

Over-expression strains were streaked onto EMM-URA media with the indicated concentrations of LatA. The top row of plates also contains 10 µM thiamine (to repress over expression). The bottom row of plates does not carry thiamine (de-repressed). Cell growth was observed with bright field microscopy after three day incubation at 30°C.

To more closely analyze the effects of LatA treatment, the over-expression strains were grown in liquid EMM-URA media to mid-log phase. Strains were then treated with DMSO (solvent control) or LatA (at 0.2 µM, 0.35 µM, and 0.5 µM). Parallel cultures supplemented with 10 µM thiamine were also used as repressed controls. After a 5-hour treatment, cells were fixed with ethanol, stained with DAPI/aniline blue, and then viewed with fluorescence microscopy (Figure 3.10).

As expected, all strains regardless of overexpression were uninucleate or binucleate after treatment with DMSO control. However, microscopic visualization revealed that both repressed and de-repressed strains were sensitive to LatA treatment and showed phenotypic abnormalities in the form of fragmented septa, tetranucleate cells, multiple septa and abnormal branching (Figure 3.10).
To quantify the results, 500 cells from each strain were counted and placed into one of four phenotypic categories: uninucleate, binucleate with functional septum, binucleate with fragmented septum, and tetranucleate. Statistical analysis was performed using a two-way ANOVA to assess the statistical significance in phenotypic differences seen between overexpressed Apl5p, Apl6p, Apm3p, Aps3p and their respective thiamine repressed controls at the same concentrations of LatA. As seen in Table 3.1, statistically significant differences (P < 0.05) were seen only between the overexpressed Apl5p strain and its thiamine-repressed control at 0.2 µM LatA and 0.5 µM LatA, as well as between overexpressed Apm3p strain and its thiamine repressed control at 0.35 µM LatA. In all of these cases, the thiamine-repressed control resulted in more cells with a normal phenotype (uninucleate or with a functional septum). In addition, it is important to note that (with all strains +/- thiamine) there is a clear downward trend in the number of phenotypically normal cells with increasing LatA concentration. This supports the cell viability streak assay that showed decreasing cell proliferation with increasing LatA concentration.
Figure 3.10: DAPI/aniline blue staining of cells over-expression *apl5, apl6, aps3, and apm3.*

Overexpression plasmids expressing multicopy Apl5p, Apl6p, Apm3p, and Aps3p were treated with the indicated concentrations of LatA or DMSO (solvent control) for 5 hours. Cultures supplemented with thiamine (+ Thia) acted as repressed controls. Cells were fixed with ethanol and stained with DAPI (nucleus) and aniline blue (cell wall/septum) and observed with fluorescence microscopy using a DAPI filter. Arrows point to multiple septa or bulging/branching septa.
Overexpression plasmids expressing multicopy Apl5p, Apl6p, Apm3p or Aps3p were treated with indicated concentrations of LatA or DMSO (solvent control) for 5 hrs. Cultures supplemented with thiamine (+ Thia) acted as repressed controls. Cells were fixed with ethanol and stained with DAPI (nucleus) and aniline blue (cell wall/ septum) and observed with fluorescence microscopy using a DAPI filter. Arrows point to multiple septa or bulging/branching septa.

<table>
<thead>
<tr>
<th></th>
<th>DMSO</th>
<th>0.2 μM LatA</th>
<th>0.35 μM LatA</th>
<th>0.5 μM LatA</th>
</tr>
</thead>
<tbody>
<tr>
<td>apo3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+Thia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-Thia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>am3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+Thia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>am3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-Thia)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3.10 (continued):** DAPI/aniline blue staining of cells over-expression apo5, apl6, apo3, and am3. Overexpression plasmids expressing multicopy Apl5p, Apl6p, Apm3p or Aps3p were treated with indicated concentrations of LatA or DMSO (solvent control) for 5 hrs. Cultures supplemented with thiamine (+ Thia) acted as repressed controls. Cells were fixed with ethanol and stained with DAPI (nucleus) and aniline blue (cell wall/septum) and observed with fluorescence microscopy using a DAPI filter. Arrows point to multiple septa or bulging/branching septa.
Table 3.1: Quantification and statistical analysis of the phenotypic effects of DMSO and LatA treatment on cells over-expressing *apl5*, *apl6*, *aps3*, or *apm3*.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>+</td>
<td>1209</td>
<td>291</td>
<td>0</td>
<td>0</td>
<td>0.998</td>
<td>0.033</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1215</td>
<td>285</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 uM LatA</td>
<td>+</td>
<td>1233</td>
<td>174</td>
<td>92</td>
<td>1</td>
<td>&lt;0.001</td>
<td>4.696</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>985</td>
<td>173</td>
<td>338</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35 uM LatA</td>
<td>+</td>
<td>368</td>
<td>101</td>
<td>993</td>
<td>38</td>
<td>0.0513</td>
<td>2.842</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>348</td>
<td>34</td>
<td>1088</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 uM LatA</td>
<td>+</td>
<td>273</td>
<td>38</td>
<td>1152</td>
<td>37</td>
<td>&lt;0.001</td>
<td>6.782</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>218</td>
<td>6</td>
<td>1254</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>+</td>
<td>1254</td>
<td>246</td>
<td>0</td>
<td>0</td>
<td>0.0885</td>
<td>2.356</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1197</td>
<td>303</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 uM LatA</td>
<td>+</td>
<td>1109</td>
<td>98</td>
<td>286</td>
<td>7</td>
<td>0.222</td>
<td>1.567</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>829</td>
<td>254</td>
<td>404</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35 uM LatA</td>
<td>+</td>
<td>342</td>
<td>104</td>
<td>1017</td>
<td>37</td>
<td>0.222</td>
<td>4.604</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>408</td>
<td>140</td>
<td>925</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 uM LatA</td>
<td>+</td>
<td>117</td>
<td>60</td>
<td>1189</td>
<td>134</td>
<td>0.333</td>
<td>1.229</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>215</td>
<td>37</td>
<td>1223</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>+</td>
<td>1226</td>
<td>274</td>
<td>0</td>
<td>0</td>
<td>0.442</td>
<td>0.977</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1173</td>
<td>327</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 uM LatA</td>
<td>+</td>
<td>942</td>
<td>300</td>
<td>256</td>
<td>2</td>
<td>0.116</td>
<td>1.994</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>976</td>
<td>227</td>
<td>285</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35 uM LatA</td>
<td>+</td>
<td>575</td>
<td>107</td>
<td>750</td>
<td>68</td>
<td>0.0835</td>
<td>2.407</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>409</td>
<td>155</td>
<td>919</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 uM LatA</td>
<td>+</td>
<td>306</td>
<td>33</td>
<td>1086</td>
<td>75</td>
<td>0.189</td>
<td>1.703</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>204</td>
<td>21</td>
<td>1239</td>
<td>36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>+</td>
<td>1164</td>
<td>336</td>
<td>0</td>
<td>0</td>
<td>0.998</td>
<td>0.033</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1175</td>
<td>325</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 uM LatA</td>
<td>+</td>
<td>1047</td>
<td>95</td>
<td>328</td>
<td>30</td>
<td>0.639</td>
<td>0.641</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>962</td>
<td>130</td>
<td>373</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35 uM LatA</td>
<td>+</td>
<td>480</td>
<td>79</td>
<td>777</td>
<td>164</td>
<td>&lt;0.001</td>
<td>4.77</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>257</td>
<td>50</td>
<td>1134</td>
<td>59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 uM LatA</td>
<td>+</td>
<td>278</td>
<td>57</td>
<td>999</td>
<td>166</td>
<td>0.0963</td>
<td>2.282</td>
<td>4,20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>209</td>
<td>23</td>
<td>1213</td>
<td>55</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cell counts were done in triplicate by counting and categorizing 500 cells for each genotypic class. Asterisks indicate significant differences. Statistical analysis was performed using a two-way ANOVA.
3.7 The Kin1p Kinase: Potential Apl5p Cargo Protein?

A critical question regarding the function of the Apl5p protein, and the AP3 complex in general, is the identity of the cargo carried in the vesicles with which the AP3 complex may be associated. Interestingly, recent research has suggested an interaction between \textit{apl5} and \textit{kin1}, which encodes an evolutionarily conserved protein kinase with functions in the maintenance of cell polarization (Ryan et. al., 2012). I was thus interested in determining if Kin1p was a potential cargo mediated by Apl5p and the AP3 complex. I initially set out to construct \textit{apl5Δkin1Δ} double mutants to assess the morphology of the double mutant after treatment with LatA.

Surprisingly, I discovered that \textit{kin1Δ} mutants were sterile during my attempts to mate the strain to both wild-type and \textit{apl5Δ} cells. As seen in Figure 3.11, matings between \textit{apl5Δ} and wild-type cells resulted in the formation of asci with four spores (indicating that the wild-type and \textit{apl5Δ} strains are capable of mating. However, no spores were produced when \textit{kin1Δ} cells were mated to either a wild-type strain or to an \textit{apl5Δ} strain.
Figure 3.11: *Schizosaccharomyces pombe kin1Δ* mutants are sterile. Single mutant controls (top row) and mixtures of cells of the indicated genotypes were grown on SPAS media for 3 days at 22°C. Microscopic inspection revealed that *apl5* and wild-type cells were able to mate and produce asci (bottom left). However, *kin1Δ* cells were unable to mate with either *apl5Δ* or wild-type cells.
4.1 Apl5p Has a Dual Role in Preventing Cytokinesis Failure

Existing research has revealed two roles for the cytokinesis checkpoint system when stressed with LatA. The first role is to repair the actomyosin ring and the second is to initiate a cell cycle delay until the ring is stabilized and cytokinesis can be completed. These dual roles have led to the classification of two broad categories of cytokinesis mutants. Group I mutants (rng mutants) display defects in actomyosin ring assembly and constriction when the cytokinetic machinery is threatened. Additionally, Group I mutants show a delay in nuclei accumulation, revealing their ability to delay cell cycle progression. Group II mutants are unable to delay the nuclear cycle despite cytokinetic stressors, resulting in tetranucleate cells. The delay in cell cycle seen in Group I mutants is lost in a Group II mutant background (Karagiannis, 2012). An example of a Group II mutant is the Cdc14p family phosphatase Clp1p, which has been shown to delay cell cycle progression upon cell division stress. clp1Δ mutants are known to be hypersensitive to LatA stress and result in cells with accumulated nuclei (Cueille et. al., 2001; Trautmann et. al., 2001). Therefore, clp1Δ is used as a positive control in these studies.

A previously conducted S. pombe genome wide screen, using low doses of LatA, identified mutants that are missing the function of this cytokinetic checkpoint (Mishra, McCollum, & Karagiannis, unpublished). This screen identified apl5 as a potential novel regulator for the cytokinesis checkpoint function.

Using the apl5Δ knockout mutant, it was confirmed that Apl5p function is necessary for maintaining cell viability and colony-forming abilities when the cell is under cytokinetic stress (Figure 3.4 and Figure 3.5). However, similar to clp1Δ, apl5Δ appears phenotypically normal under typical growth conditions (Figure 3.6). This confirms that functional Apl5p is not necessary for cell growth in sufficient growth conditions. Therefore, the cargo proteins dependent on Apl5p-mediated transport are not inherently needed for cell survival.
A statistically significant difference was seen between the phenotype of wild-type cells, \textit{apl5}\textsuperscript{Δ}, and \textit{clp1}\textsuperscript{Δ} mutants upon LatA treatment (Figure 3.7). Both mutant strains displayed severe phenotypic defects, resulting in tetraneuculate cells and binucleate cells with fragmented septa. This distribution is similar to those observed in other studies in which low doses of LatA was used to treat wild-type and checkpoint mutant strains (Mishra et. al., 2004; Karagiannis et. al., 2005). This suggests that Apl5p offers an advantage to \textit{S. pombe} survival during LatA stress, both through enhancing the integrity of the actomyosin ring and issuing the cell cycle delay. Therefore, along with \textit{clp1}\textsuperscript{Δ}, \textit{apl5}\textsuperscript{Δ} can be categorized as a Group II cytokinesis mutant.

### 4.2 Apl5p is Essential to Ring Alignment and Stability Upon Cytokinetic Stress

While my previous analysis clearly indicated that the \textit{apl5}\textsuperscript{Δ} mutant was defective in cytokinesis, it did not provide any clues as to why cell division failed. Upon cytokinesis stress, one of the reactions of the cytokinetic monitoring system is to stabilize the actomyosin ring. Thusly, I then wished to observe the exact effect of \textit{apl5}\textsuperscript{Δ} on actomyosin ring dynamics. To examine cytokinesis in real time, I utilized Rlc1p-GFP as a biomarker for ring constriction. The \textit{rlc1} gene encodes a myosin regulatory light chain that binds the Type II myosins, Myo3p and Myo2p, which are responsible for ring formation (Le Goff et. al., 2000). Thus, Rlc1p-GFP fusion proteins can be used as a useful tool for monitoring actomyosin ring constriction.

Upon DMSO treatment, the actomyosin ring in \textit{apl5}\textsuperscript{Δ} cells and wild-type cells both displayed similar ring constriction dynamics (taking 30 minutes on average, with a range of 10-50 minutes). Upon LatA treatment, wild-type cells were able to maintain the integrity of the actomyosin ring, albeit ring constriction occurred over a much longer period of time (around 90 minutes). In contrast, \textit{apl5}\textsuperscript{Δ} cells displayed abnormal ring kinetics; around 5 minutes after the initiation of ring constriction, the actomyosin ring begins to slant and fragment, and the ring becomes completely broken after 25-30
minutes. These results suggest that Apl5p-mediated cargo is not an integral part of ring constriction but is essential during times of cytokinetic stress. These observations clearly demonstrate that Apl5p is required to maintain the physical integrity and positioning of the actomyosin ring upon perturbation of the cell division machinery. The exact nature of this role is currently unclear.

4.3 AP3 Subunits Localize to the Nucleus Uniformly or in the Form of Nuclear Dots, Both in Normal Growth and Under LatA Stress

Studies using mammalian cells have identified the localization of the AP3 complex to be concentrated in the perinuclear region (cytoplasmic region surrounding the nucleus), partially co-localizing with TGN markers. As well, the AP3 complex was observed at the periphery of the cell, co-localizing with endocytic markers such as the transferrin receptor, TfR (Simpson et. al., 1996; Dell’Angelica et. al., 1997; Dell’Angelica et. al., 1999). Additionally, the AP3 complex was found to dually localize to the TGN as well as to endosome vacuoles in mouse cells (Simpson et. al., 1996; Dell’Angelica et. al., 1998). In S. cerevisiae, an antibody to the δ subunit of the AP3 complex (analogous to Apl5p in S. pombe) placed the AP3 complex at early endosome associated tubules, rather than endosomes vacuoles (Peden et. al., 2004). Species-specific features can likely explain this discrepancy in the exact localization of the AP3 complex.

Based on previous research of the AP3 complex localization in other organisms, we expected to see the complex localize to the cytoplasm of S. pombe. As well, since the AP3 complex is involved in vesicular transport or protein sorting in other organisms, we expected to witness a similar function in S. pombe. To assess localization, four plasmids each containing one of the four AP3 complex subunits were tagged with YFP and transformed into a wild-type S. pombe strain. Both Apl5p and Apl6p were found to preferentially localize to the nucleus and form 2–4 distinct dots, which moved around inside the nucleus but never left it. Apm3p-YFP and Aps3p-YFP both revealed uniform nuclear staining with no sign of movement throughout the cell cycle (Figure 3.8).
Subunit localizations that were observed in our study are supported by a previous *S. pombe* genome-wide ORF protein localization study (Matsuyama et. al., 2006). The results of the ORFeome localization study are consistent with our observations that there is a lack of AP3 complex subunit localization in the cytoplasm, to the Golgi apparatus, or to the endosomes/vacuoles. The findings of this ORFeome study are in support of the idea that, in *S. pombe*, the function of the AP3 complex does not occur in the cytoplasm. However, further research is needed to determine the nature of the nuclear dots formed by Apl5p-YFP and Apl6p-YFP.

Since there was no evidence of movement of AP3 complex subunits in the cytoplasm, it suggests that these proteins (in *S. pombe*) are not physically involved in a protein transport pathway from the Golgi to the vacuole, as it is in *S. cerevisiae* (reviewed in Odorizzi et. al., 1998). As well, the nuclear localization of all four subunits, rather than cytoplasmic localization, suggests that the AP3 complex in *S. pombe* does not participate in synaptic vesicle formation from the TGN or from endosomes, as it had been implicated in mice and rat studies (Odorizzi et. al., 1998; Faundez et. al., 1998; Boehm & Bonifacino, 2001; Boehm & Bonifacino, 2002). Although all subunits localized to the nucleus, two subunits appear uniformly over the nucleus while the other two appeared as distinct dots. This brings about the possibility that although the four subunits work in concert, individual subunits may have a separate role outside of the complex.

The idea that the AP3 complex does not physically participate in vesicular transport is further supported by the similarity in AP3 complex subunit localization, even after LatA treatment. We had hypothesized that Apl5p mediates vesicle transport to the site of cell division to assist with the cytokinesis monitoring checkpoint. Therefore, we expected to see a stress induced change in AP3 complex localization to the cytoplasm, or an accumulation of Apl5p to the site of cell division, upon activation of the cytokinesis monitoring system by LatA. The fact that neither re-localization to the cytoplasm nor accumulation was seen, acts as further evidence that the AP3 complex is not responsible for stress induced physical transportation of cargo proteins.
4.4 Overexpression of the Subunits of the AP3 Complex Results in Decreased Cell Viability Upon Cytokinetic Stress

To explore whether the AP3 complex subunits play a dosage dependent role in cytokinesis monitoring, we tested the effect of over-expressing the subunits of the AP3 complex. Since the loss of function apl5 mutant conferred hypersensitivity to LatA, I reasoned that the over-expression of the apl5 gene, and the other subunits, might confer resistance to the drug. Over-expression of Apl5p, Apl6p, Apm3p, or Aps3p was accomplished using a multicopy vector that can produce numerous copies of the inserted ORF. After transformation of the constructs into a wild-type S. pombe strain, cells were grown in DMSO or increasing concentrations of LatA, +/- thiamine. Due to the “leaky” effect of nmt1, the addition of thiamine does not fully inhibit plasmid expression; cells grown in the presence of thiamine will likely still have higher levels of expression of the respective AP3 complex subunit than wild-type cells (Bøe et. al., 2008; Maundrell, 1990).

In the presence of DMSO and 0.2 µM LatA, thiamine-repressed cells as well as de-repressed cells over-expressing Apl5p, Apl6p, Apm3p, or Aps3p displayed similar growth. Compared to the repressed controls, overexpression of any AP3 complex subunit caused a decrease in cell viability at lower LatA concentrations (Figure 3.9). This suggests that overexpression of various AP3 complex subunits has a dominant negative effect and is hazardous to the cell’s ability to repair cytokinesis stresses.

Upon DMSO treatment, all cells, regardless of thiamine presence, appeared phenotypically normal. However, over-expression of the AP3 complex subunits led to unusual morphology after LatA treatment (Figure 3.10). Cells carrying overexpression of Apl5p exhibited significantly more tetranucleate cells and binucleate cells with broken septa compared to cells carrying repressed Apl5p (Table 3.1). As well, more overexpression cells were seen with morphological differences outside of phenotypes assessed by the quantification assay; more cells displayed multiple septa and abnormal branching (Figure 3.10). While some cells with repressed Apl5p contained broken or slanted septa, this number was significantly less and there were no cells with multiple
septa or abnormal bulging. Therefore, overexpression of Apl5p results in excessive septa material deposition, unusual cellular branching, as well as increased injury to the cytokinetic checkpoint system. This further supports a model in which Apl5p plays a role in the regulation of cytokinesis and suggests that Apl5p may also have another role in regulating cell morphology.

Compared to cells grown in thiamine, strains overexpressing Apl6p or Aps3p did not result in a significantly different number of tetranucleate cells or cells with fragmented septa (Table 3.1). However, a statistically significant difference was seen in strains with an overexpression of Apm3p, albeit only at 0.35 μM LatA. Interestingly, abnormally branched cells and multiple septa were observed in cells allowing overexpression of any of these three subunits (Figure 3.10). Cells with repressed overexpression of Apl6p did not show these morphological abnormalities irrespective of increasing concentrations of LatA; cells with repressed Aps3p or Apm3p overexpression did, but only at the highest concentration of LatA tested (0.5 μM). Therefore, decreased cell proliferation seen in strains allowing overexpressed Apl6p, Aps3p, or Apm3p is not due to increased cytokinesis failure. Rather, overexpression of any of the three subunits modulates another important biological process vital to cell viability during cytokinesis stress but is not involved in cytokinesis monitoring. The nature of those biological processes is likely related to regulation of cell morphology or to other cell cycle checkpoints.

Together, these results imply that overexpression of any one of the four AP3 complex subunits results in a dominant negative effect that prevents normal cell function. Cells carrying overexpression plasmids showed decreased cell viability at a lower LatA concentration than their repressed controls. Additionally, overexpression of each subunit resulted in different phenotypic defects. These results suggest that, while all subunits of the AP3 complex have a functional role together, they may also have individual roles. This suggestion supports my previous localization study, which revealed differences in localization of the various subunits (Figure 3.8).
4.5 Novel Finding Concerning the Sterility of *kin1A*

A *S. pombe* interactome study identified 32 genes to have possible genetic interactions with *apl5* (Ryan et. al., 2012). Seeing as there has been no research of physical interactions with Apl5p, we decided to assess the protein products of those genes suggested to interact with *apl5*. Of the 32 genes, the protein product of *kin1*, Kin1p, stood out as a potential cargo protein for Apl5p-mediated transport. Kin1p is known to be essential for the maintenance of cell polarization and in correlating actomyosin ring formation during cytokinesis (Cadou et. al., 2009; Cadou et. al., 2010). Kin1p has also been shown to associate with Pomlp, Tea1p, and Tea4p, all of which regulate the interphase nodes that act as precursors to the contractile ring (Bähler & Pringle, 1998). The localization of Kin1p is dynamically regulated by the cell cycle. In early G2, Kin1p localizes at the new cell end, moving to both ends during late G2, and finally to the site of active wall growth during cytokinesis (Cadou et. al., 2009). Interestingly, *kin1A* mutants were also found to be hypersensitive to LatA treatment in the genome wide deletion library screening (Mishra, McCollum, & Karagiannis, unpublished).

We aimed to create an *apl5Δkin1Δ* double mutant and assess its morphology upon LatA treatment. If the double mutant cells resulted in a similar phenotype as either single mutant, it would suggest the two genes to work in the same pathway and for Kin1p to be a possible transport cargo mediated by Apl5p. In contrast, if they functioned in independent pathways, one would expect the double mutant to show a much stronger sensitivity to LatA than either single mutant. In *S. pombe*, double mutants are created through the mating of two single mutants. Generally, the process of sexual differentiation occurs upon nutritional stress, when cells are nitrogen deprived. Cells become arrested in the G1 phase and can enter in one of two stages; cells can either enter a G0 resting phase or cells of opposite mating types, *h−* and *h+*, will initiate sexual differentiation and conjugate into a diploid zygote (Kitamura et. al., 1990). Immediately, the zygote undergoes meiosis and sporulation into an ascus, which contains four haploid spores (Egel, 1989).
kin1Δ and apl5Δ single mutants of opposite mating types, both purchased from Bioneer Corporation (Alameda, CA), were mated together on mating media. Surprisingly, no asci were created from the cross (Figure 3.11). When both single mutants were mated with a wild-type S. pombe strain, the apl5Δ and wild-type cross resulted in asc formation but kin1Δ and wild-type did not. This suggests that the kin1Δ mutant was sterile. Thus, while I was unable to follow through with my initial line of reasoning, I was able to demonstrate the novel finding that kin1Δ mutants are sterile. This novel finding is not supported by literature so additional research would be needed to determine the mechanism by which kin1 is involved in sporulation or in meiosis.

4.6 Apl5p May Mediate Sorting, Rather Than Transport, of Various Proteins Necessary for the Cytokinesis Monitoring Checkpoint

The results of this study challenge the suggested functions of Apl5p in normal growth conditions, as well as our hypothesis concerning its role in the cytokinetic monitoring system. Since apl5Δ did not result in an abnormal phenotype under DMSO treatment, this leads to two possible explanations concerning its function in the normal cell cycle. The first possibility is that the cargo mediated by Apl5p is not needed for the cell growth or that the AP3 complex is simply not active. The second model addresses the possibility that Apl5p mediates the movement of cargo proteins that are necessary for normal cell growth. It is unlikely for Apl5p to be physically involved in the transport of said cargo due to its localization in the nucleus and its immobility throughout the cell cycle. However, if it is the case that Apl5p mediates these cargos, failure in the apl5-mediated pathway may be compensated for by an alternate pathway. This model is inspired by the knowledge that, in S. cerevisiae, mutations in any AP3 complex subunit will result in its cargo proteins (ALP and Vam3p) being rerouted through the alternate VPS pathway. Consequently, some ALP and Vam3p will still arrive at their usual destination of the vacuolar membrane and perform their normal functions (Stepp et. al., 1997). A similar system may exist in S. pombe, which would explain the normal phenotype seen in apl5Δ cells upon DMSO treatment.
From this study, it is shown that Apl5p function is necessary for cell viability under cytokinetic perturbation. Therefore, regardless of whether Apl5p is active during normal cell growth, its involvement as a part of the cytokinetic monitoring system is activated by a cytokinetic stress. Localization experiments showed no evidence of cytoplasmic localization of any of the AP3 complex subunits throughout the cell cycle when stressed with LatA. This rebuffs our hypothesis that the AP3 complex has a transport function as a response to cytokinetic stress. As well, none of the AP3 complex subunits accumulated at the site of cell division, suggesting that the AP3 complex is not physically involved in repairing the actomyosin ring. Instead, the AP3 complex may be involved in protein sorting, at the nuclear membrane, directing selected proteins to assist in the cytokinesis monitoring checkpoint. The rationale for this possibility is based on research showing that upon stress, certain genes exhibit cytoplasmic to nuclear movement, or vice versa, in order to facilitate repair mechanisms and participate in the cytokinesis monitoring system. Such genes include already known cytokinetic checkpoint regulators, Pap1p (movement from cytoplasm to nucleus), and Clp1p (relocation from the nucleus to the site of cell division, cytoplasm, and mitotic spindle) (Toone et. al., 1998; Cueille et. al., 2001; Trautmann et. al., 2001). Thus, we propose that Apl5p-mediates sorting, from it’s position at the nucleus, to facilitate the movement of other proteins essential for the cytokinetic monitoring checkpoint.

4.7 Future Research Concerning the Role of Apl5p in Protein Sorting and Determining the Localization of the AP3 Complex

Two important questions regarding the *S. pombe* Apl5p arise as a result of this study and can form the basis for future research. The first is concerning the exact nature of the nuclear dots shown by Apl5p-YFP and Apl6p-YFP staining; the second is to assess the sorting abilities of Apl5p and the AP3 complex.

Co-localization studies using fluorescence protein tagged markers for various cellular structures, in and around the nuclei, can reveal the association between Apl5p, Apl6p and intracellular compartments. If the AP3 complex is involved in mediating the
movement or sorting of proteins, then the nuclear dots formed by the Apl5p-YFP and Apl6p-YFP fusion proteins may be associated with the nuclear envelope. The nuclear envelope is a barrier between the nucleus and cytoplasm, which is impermeable to macromolecules (Yoshida & Sazer, 2004). Molecules can be transported through the nuclear envelope through channels formed by the nuclear pore complex (NPC), a large complex made of around 30 nucleoporins, located at the nuclear periphery (Yoshida & Sazer, 2004). Co-localization studies can be performed using Apl5-YFP, or Apl6-YFP, and GFP tagging any of the known nucleoporins (Asakawa et. al., 2014). In addition to assessing the localization of the AP3 subunits to the nuclear envelope, other nuclear compartments may also be tested. As seen in previous studies, Cdc31p, a member of the conserved CDC31 family, can be used as a marker for S. pombe spindle pole bodies (Matsuyama et. al., 2006; Paoletti et. al., 2003). Dnt1p can be used as a marker for the nucleolus, as it localizes in the nucleus as two or more punctate dots (Jin et. al., 2007; Wang et. al., 2012). Another possibility for the location of the nuclear dots is based on their resemblance to the localization of heterochromatin proteins (Kitano et. al., 2011). There are three heterochromatin-like domains in S. pombe, the centromeres, the silent mating-type loci, and telomeres, all three of which can be visualized using Swi6p-GFP fusion proteins (Sadaie et. al., 2004; Ekwall et. al., 1995). Determining the exact localization of Apl5p and Apl6p could give further clues to the function of those proteins.

To assess the sorting abilities of Apl5p and the AP3 complex, a similar methodology to those seen in previous studies, concerning the functions of other sorting proteins in S. pombe, could be employed. This would involve GFP tagging the proteins predicted to interact with Apl5p to determine if their localization changes in an apl5Δ mutant background and if it is affected by LatA treatment. The proteins of interest could be selected from genetic interaction studies because there is, as previously mentioned, a lack of research concerning the possible cargo proteins of an Apl5p mediated pathway. This method is similar to those used in the identification of the role of Crm1p in nuclear export, as well as the roles of protein sorting receptors in S. pombe (Fukuda et. al., 1997; Iwaki et. al., 2006).
The Ap3 complex, and its four subunits, is an evolutionarily conserved complex assumed to have similar roles in all eukaryotes. However, this study shows that the AP3 complex may have a different role in \textit{S. pombe}. Further research is necessary to identify the exact function of the AP3 complex in \textit{S. pombe} and to assess its relevance to higher eukaryotes.
Bibliography


Curriculum Vitae

Joy Wang, M.Sc Candidate
Department of Biology, Western University

Education:
M.Sc. Candidate, Cell and Molecular Biology 2012-2014
Department of Biology, Western University

B.Sc. (Honors Specialization), Genetics 2008-2012
Department of Biology, The University of Western Ontario

Honours and Awards:
Western Graduate Research Scholarship (WGRS) 2012-2014
Nominated for Graduate Student Teaching Award 2012

Related Work Experience:
Graduate Teaching Assistant, Western University 2012-2014
Summer Research Assistant, Ivey Eye Institute, London ON 2010-2011

Publications:

