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Molecular and Genetic Analysis of a Conserved Transcription Factor with a Role in Promoting the Completion of Cytokinesis in *Schizosaccharomyces pombe*

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

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**Molecular and Genetic Analysis of a Conserved Transcription Factor with a
Role in Promoting the Completion of Cytokinesis in
*Schizosaccharomyces pombe***

(Thesis format: Monograph)

by

Bidhan Chakraborty

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
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ABSTRACT

The fission yeast *Schizosaccharomyces pombe* activates regulatory networks that promote the faithful execution of cytokinesis in response to drugs that perturb the cytokinesis machinery. In order to identify novel components of these networks, a screen for mutants hyper-sensitive to the actin depolymerizing drug LatrunculinA (LatA) was previously performed. This screen identified a transcription factor, Pap1p, which is orthologous to the mammalian stress activated transcription factor, AP-1. Through molecular and genetic analysis, I showed that the deletion mutant of *pap1* is sensitive to LatA and that it cannot maintain the integrity of the actomyosin ring upon LatA treatment leading to cytokinesis failure. I also demonstrated that Pap1p is imported to the nucleus upon LatA challenge. Finally, I showed that nuclear translocation of Pap1p is important for its role in the cytokinesis monitoring system. Translocation was also important to the colony forming abilities of *S. pombe* cells and for their viability. In summary, I concluded that nuclear import of Pap1p is crucial for the proper function and regulation of the cytokinesis monitoring system.

Keywords: cytokinesis, *Schizosaccharomyces pombe*, cell cycle, checkpoint, transcription factor, Pap1p, Imp1p

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LIST OF ABBREVIATIONS

Ade: adenine

APC: anaphase promoting complex

BRAC2: breast cancer 2

Byr: bypass of *ras*

Cdc: cell division cycle

Cdk: cyclin-dependent kinase

Cdr: changed division response

Clp1: Cdc14p-like protein

DAPI: 4',6,-diamidino-2-phenylindole

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

EDTA: ethylenediaminetetraacetic acid

EMM: Edinburgh minimal medium

G2: gap 2 phase

GFP: green fluorescent protein

LatA: Latrunculin A

LATS1: large tumor suppressor

Leu: leucine

LOH: loss of heterozygosity

M: mitosis

MgcRacGAP: male germ cell RacGAP

Mid1: division in the middle 1

ORF: open reading frame

PBS: Phosphate buffered saline

PCR: polymerase chain reaction

Plk4: polo-like kinase 4

Pom1 : polarity misplaced 1

PRC1: protein regulator of cytokinesis 1

Rad: radiation-sensitive

Rng2: ring 2

Ser: serine

Sid: septation initiation-deficient

SIN: septation initiation network

SPAS: sporulation agar

Spg: septum-promoting GTPase

Ura: uracil

YES: yeast extract and supplements

CHAPTER 1: INTRODUCTION

1.1 Cytokinesis in eukaryotes

Cytokinesis is the last step of the cell cycle in which a cell physically divides to generate two independent daughter cells. It is a highly conserved process with few major differences between distantly related eukaryotes. Unfortunately, the complicated mechanism of cytokinesis makes it almost impossible to study by *in vitro* methods (Guertin et al. 2002). As a result, cytokinesis has been studied in a number of model systems including *Drosophila*, *Caenorhabditis elegans*, invertebrate marine animals, mammalian cell culture, budding yeast, and fission yeast. Although a fundamental biological process, the molecular details underlying cytokinesis and its control are not fully defined. However, recent advancements in molecular genetics methods and the availability of genetically tractable organisms have made the task of unveiling the molecules involved in cytokinesis, and to explain their mechanisms of action, easier (Balasubramanian et al. 2004; Eggert et al. 2006; Glotzer 2005; Pollard 2010; Pollard and Wu 2010). Although there are minor differences between organisms, the major events involved in cytokinesis are universal and shown in Figure 1-1.

In animal cells, negative regulation of the mitotic cyclin dependent kinase Cdk1 initiates cytokinesis. Cdk1 blocks the function of protein complexes essential for cytokinesis by means of phosphorylation during early mitosis (Barr and Gruneberg 2007). The targeted proteolysis of cyclin B by the anaphase promoting complex (APC) triggers the inactivation of Cdk1 (Eggert et al. 2006; Peters 2002). This mechanism ensures that cytokinesis coincides with the onset of anaphase (Niiya et al. 2005). Coordinating cytokinesis initiation with anaphase onset ensures cell division occurs after chromosome segregation.

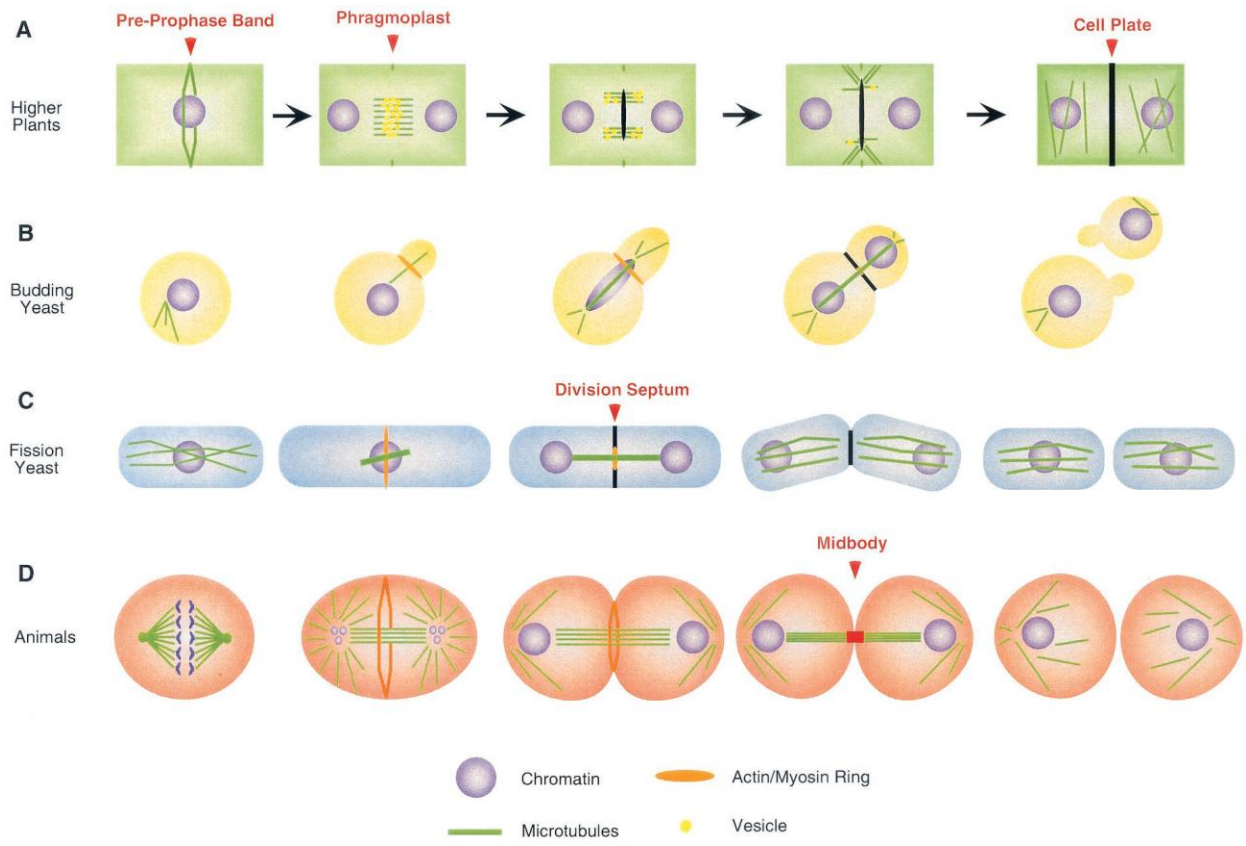
Once anaphase is completed in metazoans, the spindle midzone (the region of overlapping microtubules in the center of the spindle) is assembled by the microtubule associated protein, PRC1 (Barr and Gruneberg 2007). The spindle midzone offers an important spatial cue for the formation of the cleavage furrow, which is the site where the plasma membrane squeezes the cytoplasm. The cleavage furrow is also the site for the assembly of the actomyosin ring required for cytokinesis (Glotzer 2004; Guertin et al. 2002). The actomyosin ring is conserved in most

eukaryotes and is composed of actin filaments, the motor protein myosin II, and several other proteins (Balasubramanian et al. 2004). The actomyosin contractile ring begins to depolymerize and contract upon furrow ingression. Finally, when furrow ingression has advanced to the spindle midzone, cytokinesis is completed. An intercellular bridge also known as midbody physically attaches the two cells through the microtubules of the spindle midzone. At the very end, abscission continues with membrane targeted to the site of spindle midzone disassembly (Figure 1-2) (Barr and Gruneberg 2007).

1.2 *Schizosaccharomyces pombe*: a model system to study cytokinesis in eukaryotes

The fission yeast *Schizosaccharomyces pombe* is a unicellular, rod shaped fungus belonging to the phylum Ascomycota. In recent years, it has become a popular system to study the cell cycle and cytokinesis (Egel 2004). The organism provides a variety of advantages to the researcher. First, it has a simple haploid genome of 3 chromosomes that facilitates easy genetic analysis and manipulation. The genome of the organism is completely sequenced, which has led to the availability of a wide array of genomic tools (Wood et al. 2002). In fact, both classical genetics and molecular genetics approaches can be easily applied to this organism. Most importantly, many *S. pombe* genes are conserved in higher eukaryotes including humans. For example, 172 genes out of the ~ 5000 genes in *S. pombe* share remarkable sequence similarity to human genes involved in disease. Mis-regulation of these genes in humans leads to different types of diseases, including cancer development (Wood et al. 2002). Apart from genetic manipulation, *S. pombe* has a short generation time of 2.5-3 hours when grown in rich media, offering rapidness in laboratory based research. Lastly, *S. pombe* is particularly useful in the study of the cell cycle and cytokinesis because it maintains a conventional eukaryotic cell cycle and mitotic nuclear divisions.

Figure 1-1: General mechanisms of cytokinesis in different eukaryotes. Although the ultimate outcome of cytokinesis is the physical separations of a mother cell into two daughter cells, the mechanisms of cell division differ slightly between model organisms as illustrated below. (A) Higher plants, after separating the nuclei, use microtubules to deliver Golgi-derived vesicles to the equatorial region. Vesicles fuse to form the phragmoplast, which, through continued vesicle fusion, grows outward to the cell cortex, ultimately building a physical barrier between daughter cells called the cell plate. (B and C) Yeast and animal cells, unlike plant cells, divide through use of an actomyosin-based contractile ring. (B) In budding yeast cells, the ring is positioned at the interface between the mother cell and daughter bud, termed the bud neck. (C) In fission yeast and animal cells, the contractile ring is centrally placed, as both cell types divide by medial fission. Both budding and fission yeasts synthesize a division septum behind the leading edge of the constricting ring, which is eventually degraded, resulting in physical cell separation. (D) In animals, the ingressing furrow constricts the spindle midzone components into a dense structure called the midbody (adapted from Guertin et al. 2002).



All the above mentioned advantages have made *S. pombe* an excellent model system to study a diverse range of biological processes including cell cycle control, RNA interference, cytoskeletal dynamics and DNA damage/repair (Chang and Martin 2002; Dang et al. 2011; Kovar et al. 2011; Kuntz and O'Connell 2009; McInerny 2011; Wood et al. 2002; Yanagida 2002). In fact, the fundamental mechanisms of cell cycle control were elucidated by Noble Laureate Paul Nurse using this organism (Nurse and Thuriaux 1980; Lee and Nurse 1987; Nurse et al. 1976).

1.3 Cytokinesis in *S. pombe*

Cytokinesis in fission yeast can be separated into three distinct phases: positioning the site of cell division, actomyosin contractile ring assembly, and contractile ring constriction (Figure 1-3). At first, the anilin-like Mid1p protein determines the site of cell division by localizing to the center of the cell in late G2 phase (Bahler and Pringle 1998; Bahler et al. 1998; Daga and Chang 2005; Huang et al. 2007). Just before the onset of mitosis, Mid1p exits the nucleus and localizes to a series of ~65 membrane-bound cortical nodes in the middle of the cell (Bahler et al. 1998; Paoletti and Chang 2000; Wu et al. 2006). Along with Mid1p, other cell cycle regulatory kinases Cdr1p, Cdr2p and Wee1p co-localize to the nodes (Almonacid et al. 2009; Martin and Berthelot-Grosjean 2009; Moseley et al. 2009). In addition, another protein kinase called Pom1p forms a polar gradient extending from the cell ends to restrict the interphase nodes to the middle of the cell (Celton-Morizur et al. 2006; Moseley et al. 2009). It is crucial that cortical nodes are positioned at the middle of cell since nodes are the precursors to the actomyosin ring (Pollard and Wu 2010). Eventually, when the cell continues growing, the local concentration of Pom1p decreases at the middle of the cell which in turn lessens its inhibitory activity on Cdr1p and Cdr2p. Reduced inhibition of Cdr1p and Cdr2p results in the phosphorylation and inhibition of Wee1p (Martin and Berthelot-Grosjean 2009; Moseley et al. 2009). Next, inactivation of Wee1p leads to the reduction of inhibitory phosphorylation of Cdc2p, the key regulator of mitotic entry (Morgan 1997). Consequently, Pom1 helps in positioning the division plane and coordinating cell growth with the cell cycle.

Figure 1.2 Generalized schematic of cytokinesis in eukaryotes. Image adapted from (Pollard and Wu 2010). .

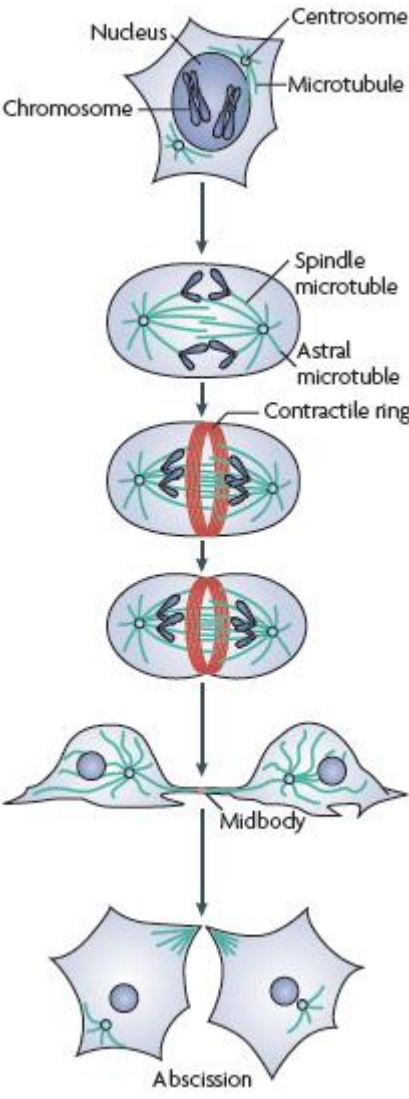
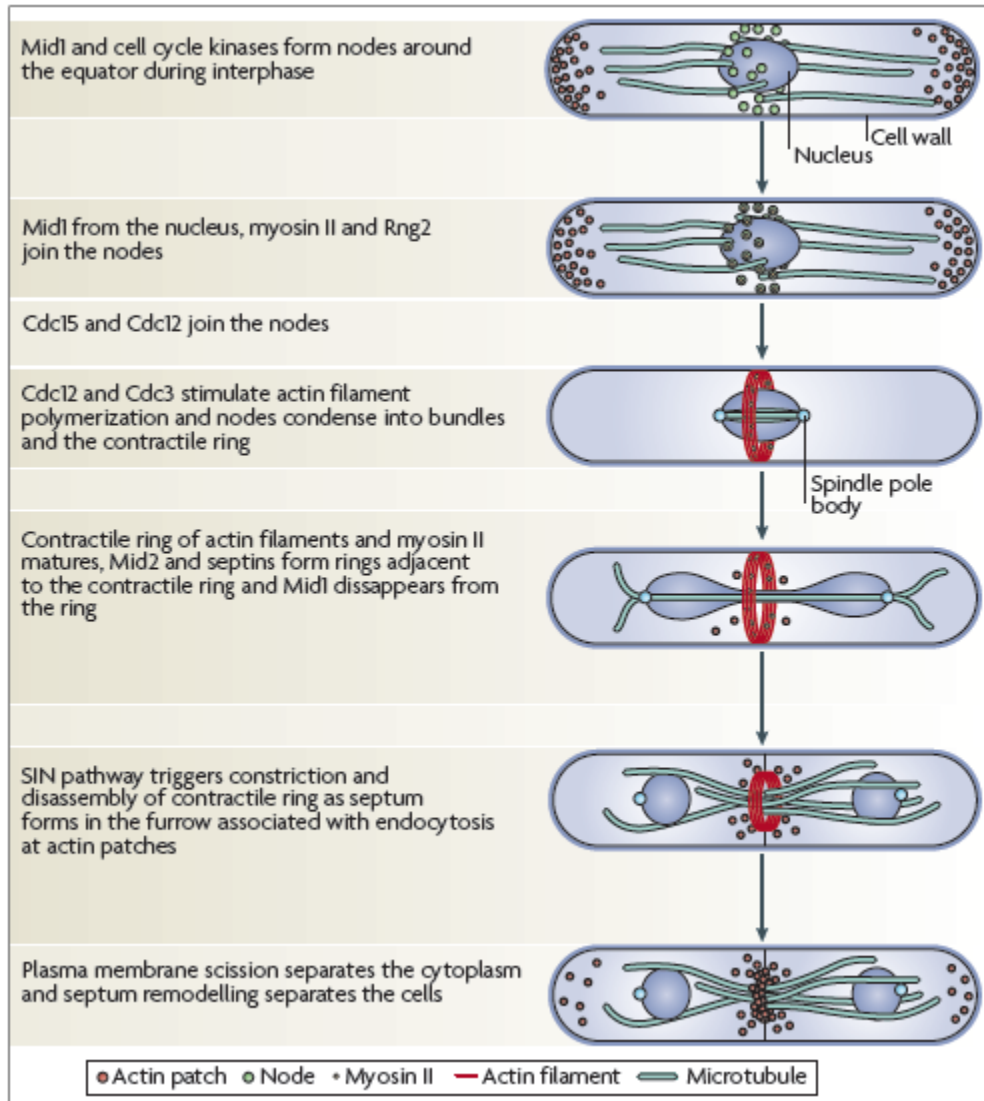


Figure 1-3: Mechanism of cytokinesis in fission yeast. Mid1p forms nodes at the cell equator, which recruit contractile ring assembly proteins. Cdc12p nucleates actin filament polymerization, and nodes condense to form bundles with aid of myosin II motor activity. SIN triggers ring constriction and division septum formation between daughter cells. Membrane scission and septum cleavage completes cell division. The depicted spindle pole bodies are analogous to animal centrosomes. Image adapted from (Pollard and Wu 2010).



Following the placement of the division plane, assembly of the contractile ring takes place. At the beginning of contractile ring assembly, Myosin II, the ring assembly protein Rng2p, the F-domain-containing protein Cdc15p, and the formin Cdc12p localize to Mid1p nodes to form mature cortical nodes (Wu et al. 2006). Cdc12p mediates nucleation and elongation of actin filaments from the mature nodes (Kovar et al. 2003; Wu and Pollard 2005; Wu et al. 2006). Eventually, the pulling forces of myosin II on actin filaments moves nodes together on the cylindrical surface of the cell creating the ring structure (Coffman et al. 2009; Vavylonis et al. 2008).

Once ring formation is achieved, constriction of the mature actomyosin ring coincides with reduced Cdk1 activity at the end of anaphase (Wolfe and Gould 2005). Essentially, a regulatory module known as the septation initiation network (SIN) governs the temporal regulation of ring constriction and formation of the septum (Krapp and Simanis 2008). The SIN is a GTPase-regulated signaling cascade consisting of three kinases (Cdc7, Sid1p, and Sid2p) and one GTPase, Spg1p (Fankhauser and Simanis 1994; Guertin et al. 2000; Hou et al. 2000). This protein kinase network associates with the spindle pole body (SPB) – an analogue of the centrosome in *S. pombe* – during mitosis through the scaffolding proteins Sid4p and Cdc11p (Krapp et al. 2001; Rosenberg et al. 2006). The upstream regulator of SIN is Spg1p, which is regulated through its nucleotide status by the bipartite GTPase activating protein, Cdc16p-Byr4p (Furge et al. 1998). Cdc16p-Byr4p regulates SIN signaling by negatively promoting GTP-hydrolysis by Spg1p. The final player of the cascade is Sid2p, which translocates from the SPB to the division site to initiate ring constriction upon the commencement of cytokinesis (Hou et al. 2000; Sparks et al. 1999).

1.4 Cytokinesis in *S. pombe* vs cytokinesis in metazoans

Since cytokinesis has some species-specific features, the suitability of *S. pombe* as a reliable model that can be used to produce significant predictions in metazoans, depends on the similarities and differences between *S. pombe* and metazoan cytokinesis. Fortunately, *S. pombe* uses an actomyosin ring as do the metazoans, and the basic components of the ring are very comparable in both systems. These features make *S. pombe* an excellent model with which to

study cytokinesis. Conversely, the order of the ring assembly constituents is quite different, which likely reflects the need for the actomyosin ring to coordinate with species-specific features (Balasubramanian et al. 2004).

One of the major differences between fission yeast and metazoan cells is the growth pattern. *S. pombe* grows by length extension alone, whereas animal cells do not change cell shape, but generally increase in volume. This means that animal cells face more challenges regarding membrane re-organization and deposition during cytokinesis. Another structural dissimilarity between these two species is that *S. pombe* has a cell wall, but animal cells do not. As a result, *S. pombe* cells have to deposit a septum at the division site in addition to membrane material, and it must be coupled with actomyosin ring constriction. In addition, the nuclear envelope stays intact in yeast whereas the animal cells lose it during mitosis (Balasubramanian et al. 2004).

Apart from structural differences, there are also temporal differences between yeast and animal cell cytokinesis. As an example, the division site is determined in anaphase in the case of animal cells. On the other hand, fission yeast determines the site in G2. One similarity both species share is their coordination of cytokinesis with the nuclear cycle. In both systems, ring constriction cannot take place until chromosome segregation is completed. However, the timing of ring assembly differs; in *S. pombe*, ring assembly is dependent on mitotic entry and in animal cells, ring assembly generally does not begin until anaphase onset (Balasubramanian et al. 2004). In addition, both these species have similarities at the molecular level of regulation. There are elements of the fission yeast SIN which are conserved in animal cells, and preliminary studies indicate that these animal counterparts are also needed for cytokinesis. Considering all these facts, it can be concluded that while exhibiting some distinctions, *S. pombe* is still a great model system for modeling metazoan cytokinesis (Balasubramanian et al. 2004).

1.5 Cytokinesis checkpoints in *S. pombe*

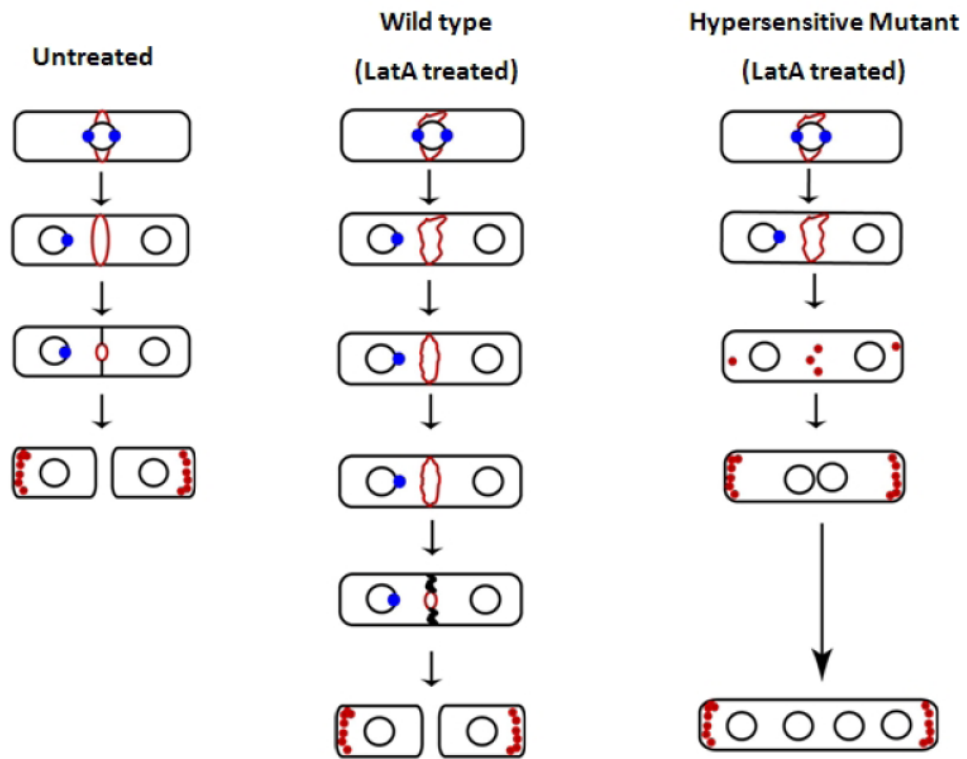
The progression from one step to the next in the cell cycle is actively controlled by cell cycle checkpoints. Checkpoints monitor cell cycle transitions and maintain fidelity by ensuring that the

previous phase is completed before moving to the next one. They are of utmost importance because the abolition of checkpoints may result in cell death, abnormal chromosome distribution and so on. They are not an integral part of the process they control; rather they are external mechanisms monitoring a key process (Hartwell and Weinert 1989).

While extensive research has been conducted on the assembly and constriction of the actomyosin ring, less is known about the mechanisms (if any) monitoring the completion of cytokinesis. Given the complexity of cytokinesis and its importance to cellular proliferation, it is not surprising that recent research has provided evidence that a cytokinesis monitoring system exists in *S. pombe*. Once cytokinesis is perturbed in this organism, the monitoring system has the ability to delay the cell cycle and stabilize the contractile actomyosin ring (Liu et al. 2000; Le Goff et al. 1999; Mishra et al. 2004; Trautmann et al. 2001; Mishra et al. 2005; Chen et al. 2003). The SIN, the Cdc14 family phosphatase, Clp1p, the 14-3-3 protein, Rad24p, and the kinase, Lsk1p are some of the already characterized members of this monitoring system. Together they ensure that cytokinesis occurs once every cell cycle and before a new mitotic cycle commences (Mishra et al. 2004; Trautmann et al. 2001; Mishra et al. 2005; Karagiannis and Balasubramanian 2007; Karagiannis et al. 2005; Saberianfar et al. 2011).

Loss of function mutations in these genes makes fission yeast cells hyper-sensitive to what is referred to as cytokinetic stress. The most common of these is induced by treating cells with Latrunculin A (LatA), a compound that causes the depolymerization of actin (Liu et al. 2000; Mishra et al. 2004). When used at low concentration (0.2-0.5 μ M), LatA has been shown to activate the cytokinesis monitoring system because it slightly perturbs the actomyosin ring that forms during cytokinesis (Ayscough et al. 1997; Mishra et al. 2004). Cells deleted for the checkpoint proteins Clp1p, Rad24p, or Lsk1p show no obvious phenotypic manifestation in normal growth conditions. However, if treated with low doses of LatA, these deletion mutants become inviable, multi-nucleated cells (Figure 1-4) (Karagiannis et al. 2005; Mishra et al. 2005; Mishra et al. 2004). Thus, using LatA to screen gene deletion mutant libraries has proven useful in identifying novel regulators of the cytokinesis monitoring system (Karagiannis et al. 2005; Mishra et al. 2005).

Figure 1-4: Treatment with low doses of Latrunculin A can be used as a tool to screen for mutants defective in the cytokinesis checkpoint response. Upon exposure to Lat A, the majority of wild-type cells are able to successfully complete cytokinesis due to Clp1p mediated cell-cycle delay, and SIN hyperactivation (Mishra et al. 2005; Mishra et al. 2004). Mutants lacking Clp1p or components of the SIN are unable to maintain a functional contractile ring leading to cytokinesis failure (Karagiannis et al. 2005; Mishra et al. 2005; Mishra et al. 2004). Nuclear divisions continue in the absence of cell division resulting in lethality. The nuclei are depicted as black circles, actin patches and rings are shown in red. The blue dot represents the Cdc7p protein (spindle pole body localized Cdc7p is a cytological marker of active SIN signaling). Figure adapted from (Karagiannis et al. 2005).



1.6 Cytokinesis failure, aneuploidy and cancer development

The faithful and reliable execution of cytokinesis is of utmost importance for the maintenance of genomic integrity. One hundred years ago the German cell biologist, Theodor Boveri hypothesized that cytokinesis failure might lead to the generation of tetraploid cells with twice the normal number of centrosomes (Harris 2008). He also proposed that this abnormal condition would then result in chaotic, multipolar mitoses resulting in aberrant chromosome segregation and aneuploidy due to the presence of a spindle apparatus with four (instead of the normal two) spindle poles (Figure 1-5). Due to advancements in the science of genetics and cell biology, there is now adequate evidence to support a connection between cytokinesis failure, aneuploidy, and tumour formation *in vivo* (Fujiwara et al. 2005; Ganem et al. 2009; Ganem et al. 2007; Holland and Cleveland 2009; Sagona and Stenmark 2010).

By transplanting tetraploid p53^{-/-} mouse mammary epithelial cells derived from cytokinesis failure into an immunodeficient mouse model followed by the observation of tumour formation, Fujiwara confirmed Boveri's hypothesis experimentally in 2005 (Fujiwara et al. 2005). Large-scale numerical and structural chromosome aberrations were observed in tetraploid-derived tumour cells. This indicated that tetraploid intermediates have a greater likelihood of developing aneuploid genetic complements, and thus are more likely to develop into tumours (Ganem et al. 2009; Ganem et al. 2007; Holland and Cleveland 2009). In recent years, it was shown that loss of heterozygosity (LOH) of the polo family kinase Plk4 results in cytokinesis failure and to a high incidence of multinucleation, supernumerary centrosomes, and a near-tetraploid karyotype (Rosario et al. 2010). Additionally, injection of these cells into mice leads to *in vivo* tumour formation. Lastly, LOH in Plk4 is associated with 50% of human hepatocellular carcinomas (Ko et al. 2005; Rosario et al. 2010). Overall, these studies strongly support a role for cytokinesis failure leading to tetraploid intermediates that can initiate tumorigenesis. Interestingly, tetraploidy has also been shown precede aneuploidy and genomic instability in various pre-malignant tissues (Galipeau et al. 1996; Olaharski et al. 2006). Furthermore, several well established oncogenes and tumour suppressors have been implicated in the normal function of cytokinesis. These include the Aurora A kinase, BRCA2, and LATS1 (Daniels et al. 2004; Meraldi et al. 2002; Yang et al. 2004). These examples support a model in which the faithful and reliable execution of cytokinesis is important in the maintenance of

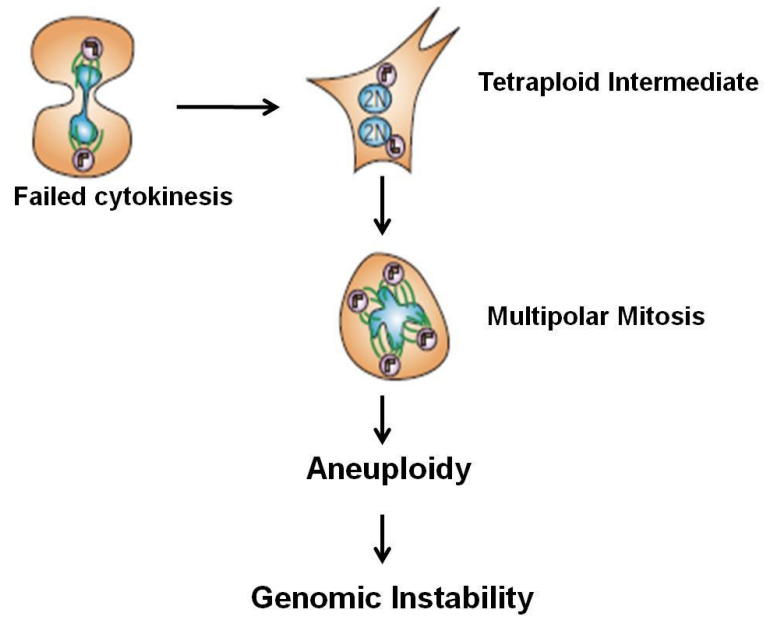
genomic integrity.

1.7 Pap1p, the stress activated transcription factor and its importer, Imp1p

A recent genome-wide genetic screen of *S. pombe* gene deletion mutants has led to the identification of a set of genes with roles in responding to LatA treatment (Mishra, McCollum, and Karagiannis, unpublished). All these LatA sensitive mutants represent putative regulators of the cytokinesis monitoring system. Among the LatA sensitive mutants is *pap1Δ*, which was initially characterized as a stress activated transcription factor involved in MAP kinase stress responsive pathway (Shiozaki and Russell 1995; Toone et al. 1998). *pap1* confers resistance to multiple drugs, such as brefeldin A, staurosporine or caffeine, when constitutively activated or over-expressed (Toda et al. 1991, Turi et al. 1994, Benko et al. 2004). Besides, Pap1p activates the expression of antioxidant genes when cells are treated with H₂O₂ (Toone et al. 1998; Quinn et al. 2002).

Pap1p is a bZip containing transcription factor that has homology to and similar DNA binding specificity as the mammalian c-Jun transcription factor – AP1 (Toone et al. 1998). AP1 is one of the two targets of mammalian stress activated kinases (Toda et al. 1991; (Hibi et al. 1993; Derijard et al. 1994; Kyriakis et al. 1994). Pap1p, like its budding yeast (*Saccharomyces cerevisiae*) counterpart Yap1p, is regulated at the level of nuclear localization. This means, although predominantly cytoplasmic, Pap1p is imported to the nucleus upon exposure to stresses (Kuge et al. 1997; Toone et al. 1998). Pap1p contains a complex nuclear localization signal (NLS) consensus sequence which consists of two overlapping bipartite-type NLSs (Umeda et al. 2005). Having these NLSs, the import of Pap1p from the cytoplasm to the nucleus is in turn dependent on an import factor importin- α , which is coded for by *imp1* in fission yeast. Eukaryotes have multiple importin- α proteins that have been categorized on the basis of amino-acid sequence comparisons (Malik et al. 1997; Mason et al. 2002). So far, three subfamilies including members of different expression patterns, different functions, and/or different cargo-binding specificities, have been identified in plants and animals (Kohler et al. 1999; Talcott and Moore 2000; Geles and Adam 2001; Geles et al. 2002; Mason et al. 2002). Interestingly, the importin- α deletion mutant *imp1Δ* was also LatA sensitive in the genome wide deletion mutant screening study (Mishra, McCollum, and Karagiannis, unpublished).

Figure 1-5: Progression from cytokinesis failure to aneuploidy. Cytokinesis failure leads to the formation of tetraploid intermediates containing double the normal number of centrosomes. Upon progression into mitosis, chaotic multipolar chromosome segregation and aneuploidy result. In this model the inherent genomic instability of aneuploid cells leads to tumourigenesis. Image adapted from (Storchova and Pellman 2004).



1.8 Rationale of the study

In eukaryotic cells, the molecular response to exposure to adverse environmental conditions involves modification at the level of gene transcription. This change in gene expression solely results from the change in the activity of a particular transcription factor (Treisman 1996). Certain MAP kinase signaling pathways mediate this link between adverse external stimuli and transcriptional regulation (Waskiewicz and Cooper 1995; Kyriakis and Avruch 1996). Interestingly, these kinase pathways share a high degree of conservation across different species including *Drosophila*, budding yeast and fission yeast (Riesgo-Escovar et al. 1997; Sluss et al. 1996; Brewster et al. 1993; Millar et al. 1995; Shiozaki and Russell 1995; Kato et al. 1996). Other than kinases, significant structural and functional similarities have also been found in transcription factors that serve as the substrates for the stress activated kinases in mammalian cells, *Drosophila*, and fission yeast. Thus, it is not surprising that these MAP kinase pathways share a significant degree of similarity in their structural and mechanistic features among different species. Pap1p, which is one of the target transcription factors of the MAP kinase pathway, is sensitive to LatA treatment. Moreover, Imp1p, the importer of Pap1p is also LatA sensitive. Taking together the high extent of similarities in the MAP kinase pathway across species and *pap1Δ* and *imp1Δ* being LatA sensitive, I propose the following two hypotheses:

1. Pap1p is responsible for the transcriptional activation of genes required to respond to LatA induced perturbation of the cell division machinery and
2. Imp1p is required for the nuclear import of Pap1p in response to LatA induced cytokinetic stress.

What follows are the results of experiments aimed at testing the model in which Pap1p and Imp1p work together.

CHAPTER 2: MATERIALS AND METHODS

2.1 Strains, growth media, and culture conditions

Escherichia coli strain XL1-Blue was used for all molecular cloning experiments. *E. coli* cells were made electrocompetent via cold shock by washing the cells with ice-cold dH₂O and 10% glycerol at 4 °C and then electro-transformed with the desired plasmids. Following transformation, cells were grown overnight on Luria Bertani (LB) agar media containing 100 µg/ml ampicillin at 37 °C. The transformed colonies were then isolated and grown overnight in LB broth media containing 100 µg /ml ampicillin with constant shaking at 200 rpm. Plasmid DNA was extracted from 1.5ml of culture using the High-Speed Plasmid Mini Kit (GeneAid).

S. pombe wild-type strain JK 484 (Table 1) was used for constructing all the mutant and fusion protein tagged strains in this study. *S. pombe* cells were cultured in Yeast Extract (YES) media at 30 °C with constant shaking at 150 rpm (Alfa et al. 1993). The cells were grown to mid-log phase (OD₆₀₀ of 0.2-0.4) before lithium acetate transformation (Forsburg and Rhind 2006), treatment with low doses of the anti-cytokinetic drug Latrunculin A, or used for microscopy. For mutant selection, strains were grown on Edinburgh Minimal Media (Forsburg and Rhind 2006) (US Biologicals) with the appropriate supplementary amino acids (adenine, uracil, leucine or histidine). For constructing the double mutants, SPAS mating media (Forsburg and Rhind 2006) was used when mating was needed. Synchronous cultures of *cdc25-22* cells were prepared by transient temperature shifts to 36 °C for 4 hours followed by growing the cultures at 27 °C to release the cell cycle block.

2.2 Molecular techniques

Creation of the *pap1Δ* gene deletion mutant

To construct the *pap1Δ* deletion mutant, a PCR based cloning method was used followed by homologous recombination in wild-type *S. pombe* (Figure 2-1). A 606 bp and 285 bp sequence located just upstream and just downstream, respectively, of the *pap1* open reading frame

SPAC1783.07c were PCR amplified. The primers used in this PCR are listed in Table 2. These primers incorporated specific restriction sites to the upstream and downstream regions of the target gene. For instance, the primers used for upstream region incorporated the restriction sites *NheI* and *SspBI* in the 5' and 3' end of the amplicon, respectively. On the other hand, the primers used for the downstream region incorporated the restriction sites for *NheI* and *BamHI* in the 5' and 3' end of the amplicon, respectively. Then both the PCR products were digested with *NheI* and subsequently ligated using the first ligation kit (Thermo Scientific). Following that, the ligated PCR product was double digested with *SspBI* and *BamHI* and cloned in the cloning vector pCloneHYG1 carrying a gene conferring resistance to hygromycin. Next, the cloned vector was transformed into electro-competent *E. coli*. Once isolated, the cloned vector was treated with *NheI* to make it linear. Once linearized, it was transformed into *S. pombe* cells where it integrated into the *S. pombe* genome by homologous recombination. The hygromycin resistant transformants were isolated and subjected to colony PCR using primers JK 283, JK 284 and vector specific universal primers upch-uni and dwch-uni to identify clones in which the linear dsDNA fragment had replaced the endogenous *pap1* gene via homologous recombination. All of the restriction enzymes used in this study were purchased from Thermo Scientific and used according to the manufacturer's recommended buffers and protocols (Double Digest™: <http://www.fermentas.com/doubledigest/index.html>).

Verification of Bioneer gene deletion mutant

The *imp1Δ* deletion mutant was commercially bought from Bioneer Corporation (<http://pombe.bioneer.co.kr/>). This mutant had the *imp1* gene replaced by a KanMX selectable marker conferring resistance to antibiotic G418 (Kim et al. 2010). The strain was verified by colony PCR method using gene specific primers JK 304 and JK 305 and primers CPC1, CPC3, CPN1, CPN10 provided by the supplier (Figure 2-2).

Table 2-1: *S. pombe* and *E. coli* strains used in this study

Strain	Relevant Genotype	Source
BC1	<i>pap1:: hphMX4 ura4-D18 leu-32 ade6-216 his3-D1</i>	This Study
BC2	<i>imp1::KanMX ura4-D18 leu-32 ade6-216</i>	This Study
BC3	<i>pap1:: hphMX4 imp1::KanMX ura4-D18 leu-32 ade6-216 his3-D1</i>	This Study
BC4	<i>pap1:: hphMX4 clp1::ura4⁺ ura4-D18 leu-32 ade6-216 his3-D1</i>	This Study
BC5	<i>pap1:: hphMX4 rlc1GFP::ura4⁺ ura4-D18 leu-32 ade6-216 his3-D1</i>	This Study
BC6	<i>pap1-GFP::ura4⁺ ura4-D18</i>	This Study
BC7	<i>pap1-SV40NLS-GFP::ura4⁺ ura4-D18</i>	This Study
BC8	<i>pap1-NES-GFP::ura4⁺ ura4-D18</i>	This Study
BC9	<i>pap1:: hphMX4 cdc25-22 ura4-D18 leu-32 ade6-216 his3-D1</i>	This Study
JK9	<i>clp1::ura4⁺ ura4-D18</i>	JK collection
JK402	<i>E. coli XLI-Blue strain</i>	JK collection
JK484	<i>ura4-D18 leu-32 ade6-216 his3-D1</i>	JK collection
MBY175	<i>cdc25-22, ura4-D18, h-</i>	JK collection
MBY624	<i>rlc1::GFP ura4⁺ leu1-32 h-</i>	JK collection

Table 2.2 Primers used in this study

Oligos	Sequence	Description
JK 279	5-AAAAGCTAGCCGAAACGAAACGCGTTTTTCAGG-3	Forward, upstream sequence amplification for <i>pap1Δ</i> mutant construction
JK 280	5-AAAATGTACATTCACAAA GCAATGCAA GATGAA GG-3	Reverse, upstream sequence amplification for <i>pap1Δ</i> mutant construction
JK 281	5-AAAAGCTAGCGCCTTTGGTCGACGAATATTAGCATC-3	Forward, downstream sequence amplification for <i>pap1Δ</i> mutant construction
JK 282	5-AAAAGGATCCCCTTTTTGTTTTCTTCGCCTCGTG-3	Reverse, downstream sequence amplification for <i>pap1Δ</i> mutant construction
JK 283	5-TGGAGGCTAGACTCAGCGCA-3	Forward, verification of integration of the deletion construct in of <i>pap1Δ</i>
JK 284	5-GCCAA GCGTTTTTCGAGTTC-3	Reverse, verification of integration of the deletion construct in of <i>pap1Δ</i>
JK 304	5-CCCGTTTTATGGTGCGAAGG-3	Forward, verification of <i>implΔ</i> strain
JK 305	5-TGACCACGAAAACAACCCAAA-3	Reverse, verification of <i>implΔ</i> strain
JK 291	5-GGCGGGAATTCTCTAACGAAAATGGA-3	Forward, cloning of fusion protein tagging in <i>pap1</i> and verification of fusion protein tagging
JK 292	5-GGCGGCCCGGGATTAAATTGATTTAA-3	Reverse, cloning of fusion protein tagging in <i>pap1</i>
JK 293	5- GGCGGCCCGGGTACCTTTCTCTTCTTTTTTGGTACCTT TCTCTT CTTTTTGGATTAAATTGATTTAA-3	Reverse, cloning of fusion protein tagging (NLS) in <i>pap1</i>
JK 294	5-GGCGGCCCGGGTCTAGGGTGA GACGTTCTAG CGGGGGGAGCTGTAGATTAAATTGATTTAA-3	Reverse, cloning of fusion protein tagging (NES) in <i>pap1</i>
ME 10	TGGACAACCTCCAGTGAAAA	Reverse, verification of <i>GFP</i> construct integration
CPC 1	5-TGATTTTGTAGACGAGCGTAAT-3	Forward, verification of downstream junction of Bioneer <i>impl</i> gene deletion
CPC 3	5-GGCTGGCCTGTTGAACAA GTCTGGA-3	Forward, verification of downstream junction of Bioneer <i>impl</i> gene deletion
CPN 1	5-CGTCTGTGAGGGGAGCGTTT-3	Reverse, verification of upstream junction of Bioneer <i>impl</i> gene deletion
CPN 10	5-GATGTGAGAACTGTATCCTAGCAAG-3	Reverse, verification of upstream junction of Bioneer <i>impl</i> gene deletion

Figure 2-1: Schematic presentation of the construction of the *pap1*Δ deletion mutant. (A) PCR amplification, digestion and cloning of the up and downstream sequences of *pap1*. (B) Homologous recombination of the cloned *pap1* gene into *S. pombe* genomic DNA.

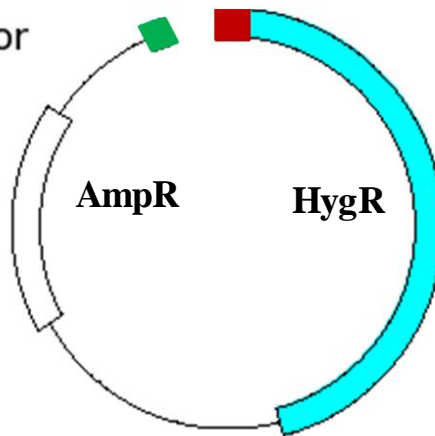


Digestion with *NheI*

Ligation by fast ligation kit

Double digestion of PCR product with
SspBI and *BamHI*

Plasmid
vector



Double digestion of vector
by *SspBI* and *BamHI*

B)

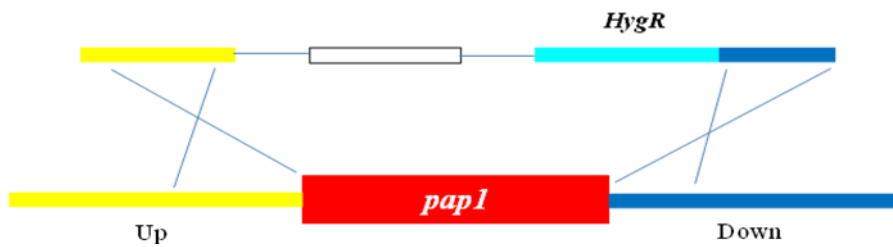
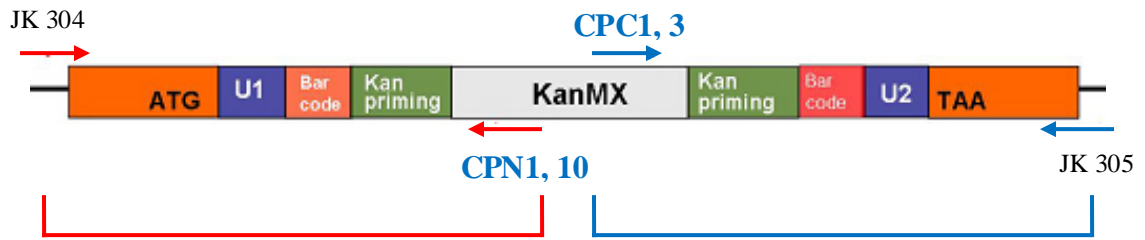


Figure 2-2: Verification of the Bioneer *imp1*Δ deletion mutant. The Bioneer gene deletion mutant has the *imp1* ORF replaced with a KanMX selectable marker conferring resistance to the antibiotic G418 (Kim et al. 2010). Primers JK 304 and JK 305 are specific to the region upstream and downstream of the *imp1* ORF, respectively. Primers CPN1, CPN10, CPC1 and CPC3 are specific to the KanMX module. Four PCR reactions are performed. The red and blue arrows indicate primer pairs used in individual PCR reactions to generate an amplicon (indicated below the schematic in red or blue). Generation of all four amplicons provides evidence for deletion of the *imp1* ORF with the KanMX deletion cassette. Figure 2-2 was adapted from Bioneer website (http://pombe.bioneer.co.kr/technic_infomation/Verification.jsp).



2.3 Creation of *pap1*-GFP integrant strain

The *pap1*-GFP integrant strain was created using a PCR based cloning method. First, a carboxy terminal fragment of the *pap1* gene was PCR amplified from genomic DNA using primers JK 291 and JK 292 (Table 2) using a high fidelity PCR system (Figure 2-3). Following PCR amplification, the amplicon was cloned in frame to the upstream of the GFP gene located in the pJK210-GFP cloning vector. The construct was confirmed by sequencing. Finally, the plasmid was transformed into *S. pombe* wild-type strain JK 484 using the lithium acetate method (Forsburg and Rhind 2006). The transformed cells were then selected by growing them on EMM solid media lacking uracil. The Ura⁺ transformants were further verified for locus specific homologous recombination by colony PCR reaction.

2.4 Genetic techniques

Creation of double mutants

To create the double mutant strains of the desired genotype, the individual single mutants of the opposite mating type were mated and subsequently analyzed for meiotic products. In brief, both the single mutants were mixed with each other for genetic crossing in a 20 µl drop of sterile dH₂O on SPAS mating medium (a medium lacking nitrogen specifically formulated to induce sporulation in diploids) (Forsburg and Rhind 2006). The media were incubated at room temperature for 48 hours. Upon sporulation, the cells of the opposite mating types underwent meiosis to produce four haploid spores contained within an ascus, also known as tetrads. To analyze the products of meiosis, tetrad dissection or free spore analysis was employed afterwards.

Tetrad dissection

After mating, the mix was spread out on a YES plate and individual tetrads isolated using a micromanipulator attached to a Zeiss Axioskop 40 microscope. The tetrads were then separated on the YES plate arranged in rows and allowed to germinate by growing the media at 30 °C.

Germination resulted in the breakdown of the asci cell wall and the individual spores were sorted out in rows and the YES plate was incubated at 30 °C to allow the spores to grow and produce colonies. Once colonies appeared on to the YES plate, these isolated colonies were then replica plated onto selective media. When needed, temperature sensitive mutants were selected by growing the culture at 36 °C. Double mutants could be isolated by comparing the growth of the colonies on both the selectable plates or growth at high temperature.

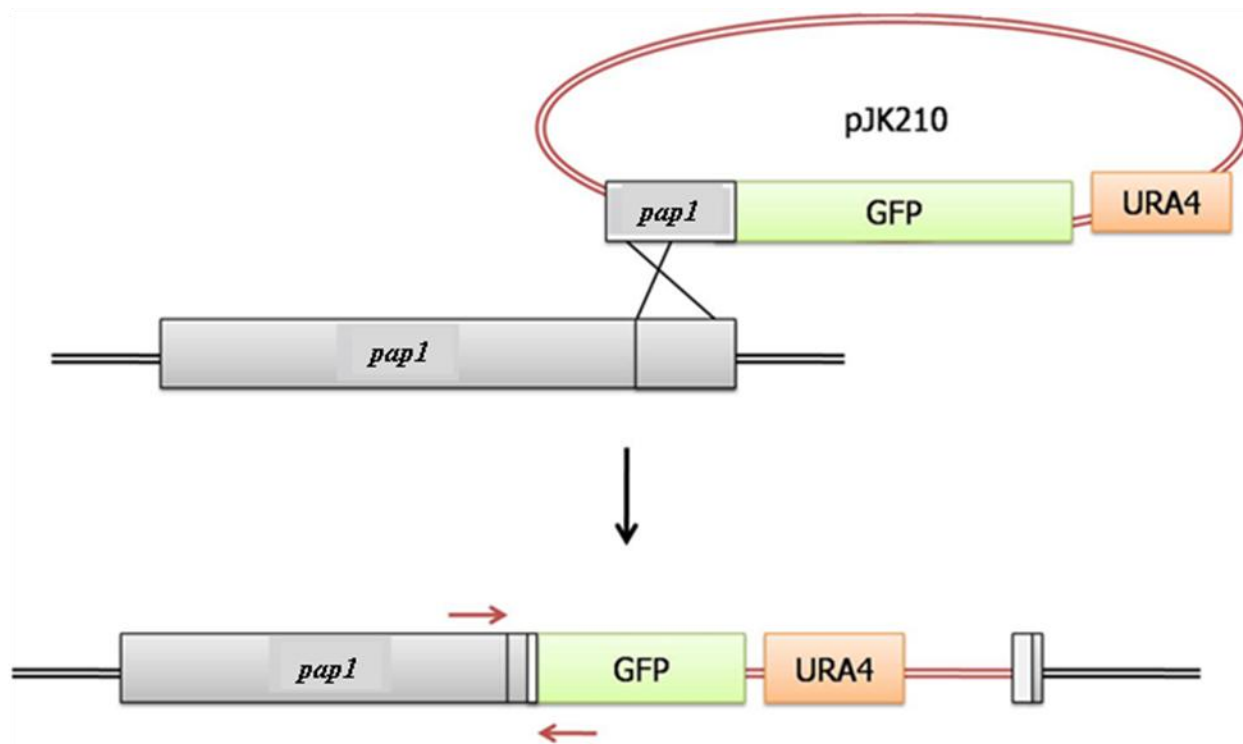
Free spore analysis:

Asci were treated with 0.5µL of glusulase, a cell wall degrading enzyme. Upon treatment of glusulase, it is expected that only spores will remain and that all the vegetative cells will die. The spores were then washed with water and diluted using a 10-fold dilution. Finally, the diluted spores were plated onto YES media and incubated at 30 °C to promote colony formation. Colonies usually appeared within 3-5 days of incubation. Once colonies appeared, the double mutants were selected as described above.

2.5 Staining and fluorescence microscopy

S. pombe cells were grown to mid-log phase and were fixed with ethanol (Forsburg and Rhind 2006). The cells were eventually stored in 15% glycerol and PBS pH 7.4 before staining. The nucleus and cell wall/septa were then stained with a mixture of 0.02 µg/µl 4',6,-diamidino-2-phenylindole (DAPI) and 1 µg/µl aniline blue, respectively. Images were taken under fluorescent light using the Zeiss Axioskop 2 microscope along with ImageJ 1.41 software (National Institute of Health) and a Scion CFW Monochrome CCD Firewire Camera (Scion Corporation, Frederick Maryland). For cells that were GFP tagged, they were grown to mid-log phase and images were taken using a Leica DMI 6000B microscope with a BD CARV II Confocal Imager fitted with a GFP filter (BD Biosciences, San Jose, CA) and a Quantem: 512SC camera (Photometrics, Tucson, AZ). For the ring dynamics experiment, live cells were filmed by growing them on a glass slide with YES media containing 2% Agarose and by immobilizing the cells with a cover slip.

Figure 2-3: Schematic presentation of the construction of the *pap1*-GFP strain. The carboxy terminal region of *pap1* was cloned upstream of GFP in the cloning vector pJK210-GFP and transformed into wild-type strain JK 484. Positive transformants were selected using the *ura4* gene as the selectable marker. Finally, colony PCR using one primer specific to a region upstream of the carboxy terminal fragment used in cloning, and another GFP specific primer were used to confirm integration at the *pap1* locus (data not shown).



2.6 Latrunculin A treatment

S. pombe cells were grown to mid-log phase (OD₆₀₀ of 0.2-0.8) and eventually treated with low doses (0.2-0.5 μM) of Latrunculin A dissolved in DMSO (Karagiannis and Balasubramanian 2007; Karagiannis et al. 2005). The treated cells were grown at 30 °C with shaking at 150-200 rpm for variable hours depending on the objective (0.5-4 hrs). Cells were separately treated with DMSO as a negative control in experiments involving LatA. Latrunculin A was purchased from Enzo Life Sciences International Inc. (Plymouth Meeting, Pennsylvania).

CHAPTER 3: RESULTS

3.1. *pap1Δ* & *impl1Δ* deletion mutants exhibit similar cytokinetic defects upon LatA treatment which is consistent of their involvement in the same genetic pathway

If Pap1p and Imp1p play a pivotal role in the cytokinesis monitoring system, then *pap1* and *impl1* deletion mutants would be expected to undergo cytokinetic failure when exposed to low doses (0.2-0.5 μ M) of Latrunculin A (LatA). The *pap1Δ* deletion mutant was constructed using a PCR based cloning method and verified by colony PCR (See Methods and Materials). The *impl1Δ* strain, on the other hand, was purchased from Bioneer Corporation (Kim et al. 2010) and verified by colony PCR.

Once both the deletion mutants were verified, they were tested for their sensitivity to LatA in order to determine their involvement in the cytokinesis monitoring system. First, both mutants were grown overnight in liquid YES media at 30 °C to mid-log phase. When the desired OD was reached, LatA was added to a final concentration of 0.2 μ M (or treated with the solvent negative control DMSO). Consequently, the cells were fixed and then stained with DAPI and aniline blue to visualize the nucleus and cell wall/septum, respectively (Balasubramanian et al. 1998). Upon LatA treatment, most of the wild-type cells showed resistance to LatA. The cells were either uninuclear or binuclear with functional septa (cell plate, that cleaves the cell at its midpoint) that often appeared slightly misshapen. On the other hand, both the *pap1Δ* and *impl1Δ* were LatA sensitive. Although the cells were binuclear, their septa were completely fragmented (Figure 3-1) indicating that the actomyosin ring had been unable to properly constrict.

To determine the extent of LatA sensitivity, the cellular phenotypes were quantified. Cell were categorized into four groups: i) uninucleate; ii) binucleate with a functional septum; iii) binucleate with a fragmented septum and iv) tetranucleate. Upon DMSO treatment, all strains irrespective of genotypes, showed similar behaviour, most being uninuclear and binuclear with a functional septum. When the strains were treated with LatA, wild-type cells showed uninuclear or binuclear cells with functional septa. In contrast, the *pap1Δ* and *impl1Δ* mutants showed a significantly higher number of binuclear cells with fragmented septa (ranging from 85% to 95%) (Figure 3-2).

Since Imp1p is responsible for Pap1p import to the nucleus (Umeda et al. 2005), when compared to the respective single mutant, the *pap1Δimp1Δ* double mutant would be expected to show a similar response upon LatA treatment. To test this verification, both the *pap1Δ* and *imp1Δ* single mutants were crossed to make the *pap1Δimp1Δ* double mutant and treated with 0.2 μM LatA. Microscopic examination revealed that the *pap1Δimp1Δ* double mutant behaved similarly to the *pap1Δ* and *imp1Δ* single mutants (binuclear with fragmented septum) (Figure 3-1). This result was further supported by the cell quantitation studies; the *pap1Δimp1Δ* double mutant showed comparable percentage of binuclear cells (76%) with fragmented septa as the *pap1Δ* and *imp1Δ* single mutants (95% and 85% respectively) (Figure 3-2).

3.2 The nuclear cycle delay seen in *pap1Δ* cells is dependent on Clp1p, the cytokinesis checkpoint master regulator

My data (Figure 3-1 and 3-2) showed that *pap1Δ* mutant cells were unable to maintain their ring integrity for extended periods. However, the cells were binucleate which indicates that they were able to delay cell cycle progression. Thus, *pap1Δ* can be categorized as a group I cytokinetic mutant (a cytokinesis mutant with the capacity to delay cell cycle progress) (Mishra et al. 2004). It has been shown that the nuclear cycle delay exhibited by group I mutants is dependent on group II mutants (cytokinesis mutants unable to delay cell cycle progress) (Liu et al. 2000; Le Goff et al. 1999; Trautmann et al. 2001; Cueille et al. 2001). Clp1p, a previously well characterized cytokinetic regulator plays a central role in this delaying process. When treated with low doses of LatA, *clp1Δ* cells show a higher number of tetranucleate cells indicating that they cannot delay the nuclear cycle upon cytokinesis failure. To determine if the nuclear cycle delay showed by *pap1Δ* cells is Clp1p dependent, the *pap1Δclp1Δ* double mutant was made and treated with 0.2 μM LatA. Interestingly, while a high number of binucleate cells with fragmented septum were observed for *pap1Δ*, the *pap1Δclp1Δ* double mutant showed a dramatic increase in the number of tetranucleate cells (Figure 3-3 and 3-4).

Figure 3-1: *pap1Δ* and *imp1Δ* are unable to complete cytokinesis upon LatA treatment. Wild-type and mutant strains were grown to mid-log phase in YES at 30°C and treated with 0.2 μM LatA or DMSO (solvent control) for 4 hrs. Cells were fixed and stained with DAPI (DNA) and aniline blue (cell wall/septum) and observed using a fluorescent microscope.

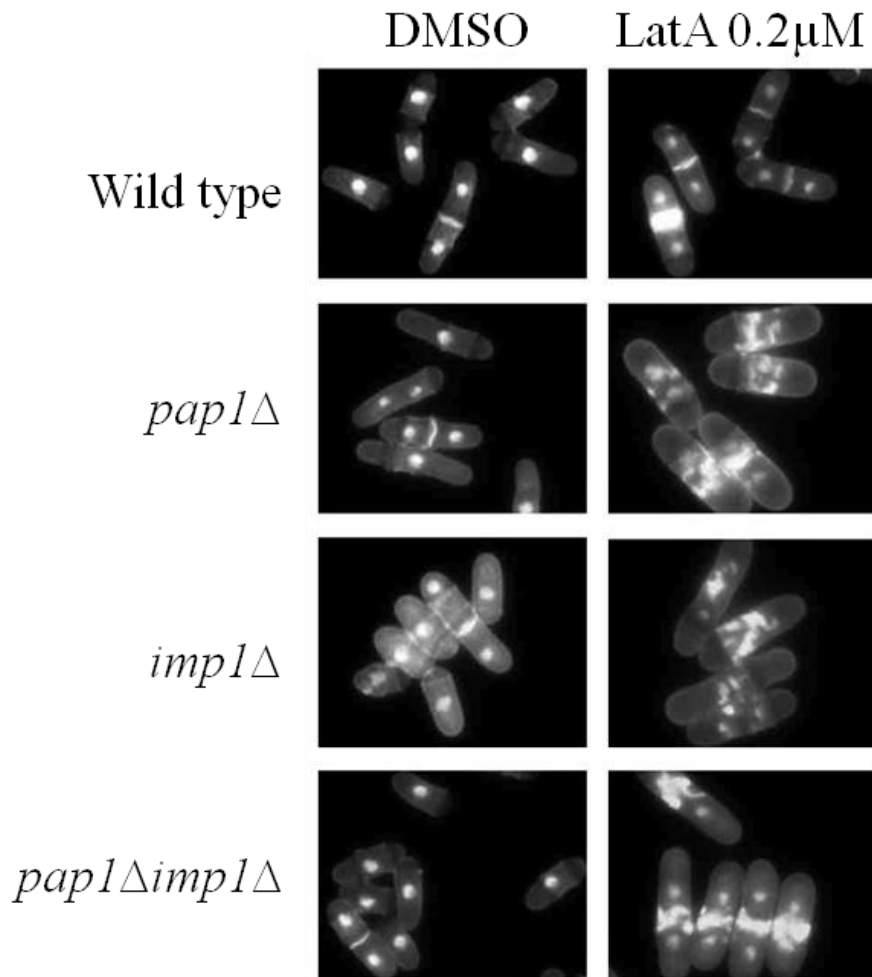


Figure 3-2: Quantitation of phenotypes of cells treated with DMSO and/or LatA.
Quantitation was done in triplicate by counting 200 cells each time for each genotypic class.

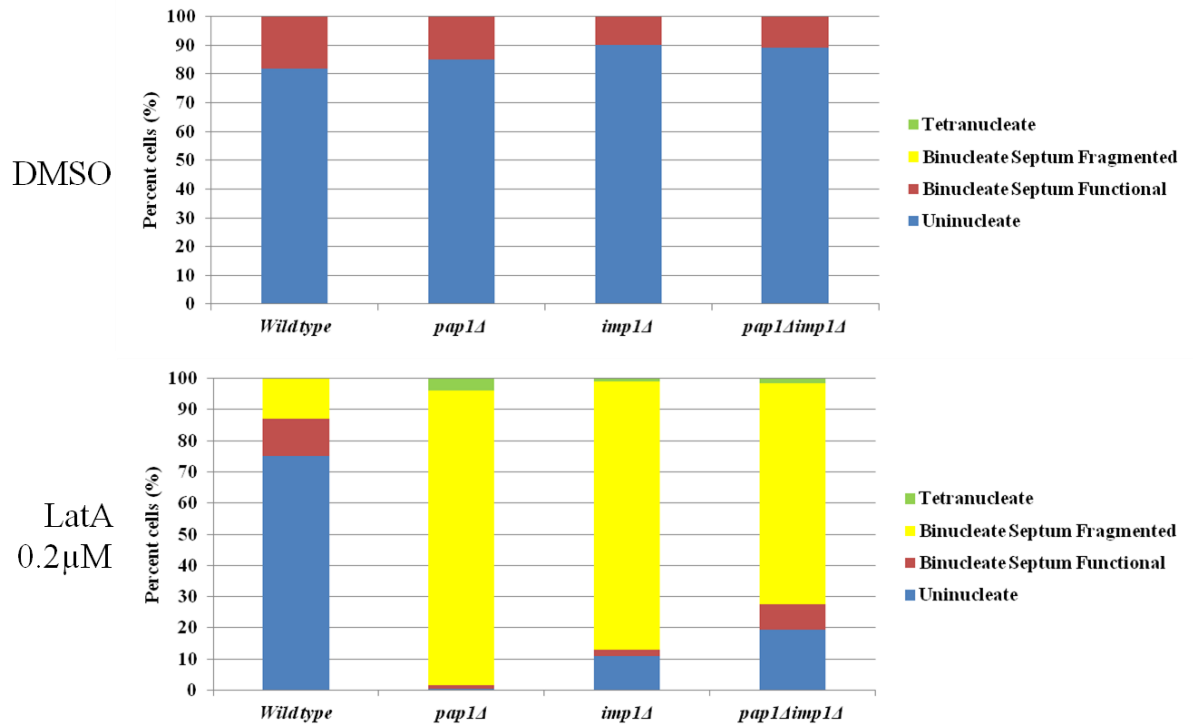


Figure 3-3: The nuclear cycle delay seen in *pap1Δ* cells is Clp1p dependent. The microscopic examination showed tetranucleate cells for *pap1Δclp1Δ* double mutant whereas the single *pap1Δ* mutant showed binuclear cells with broken septa upon LatA treatment for 4 hrs.

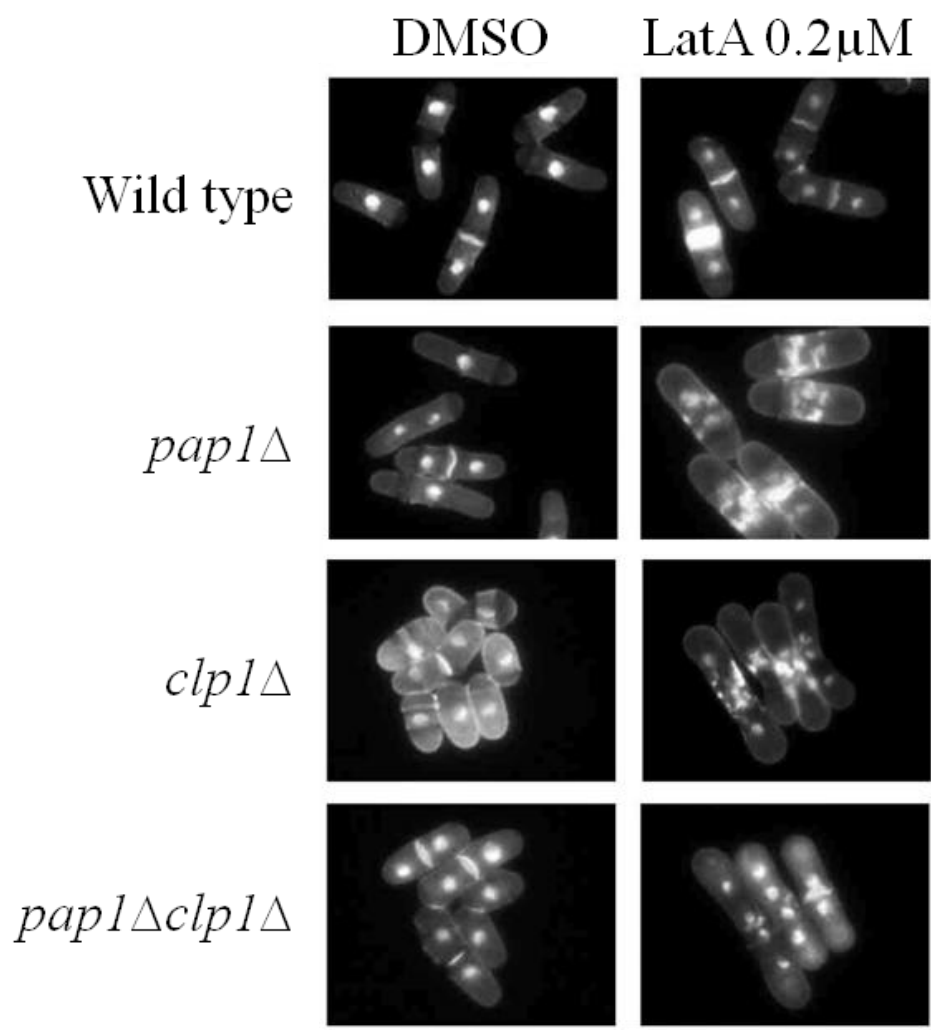
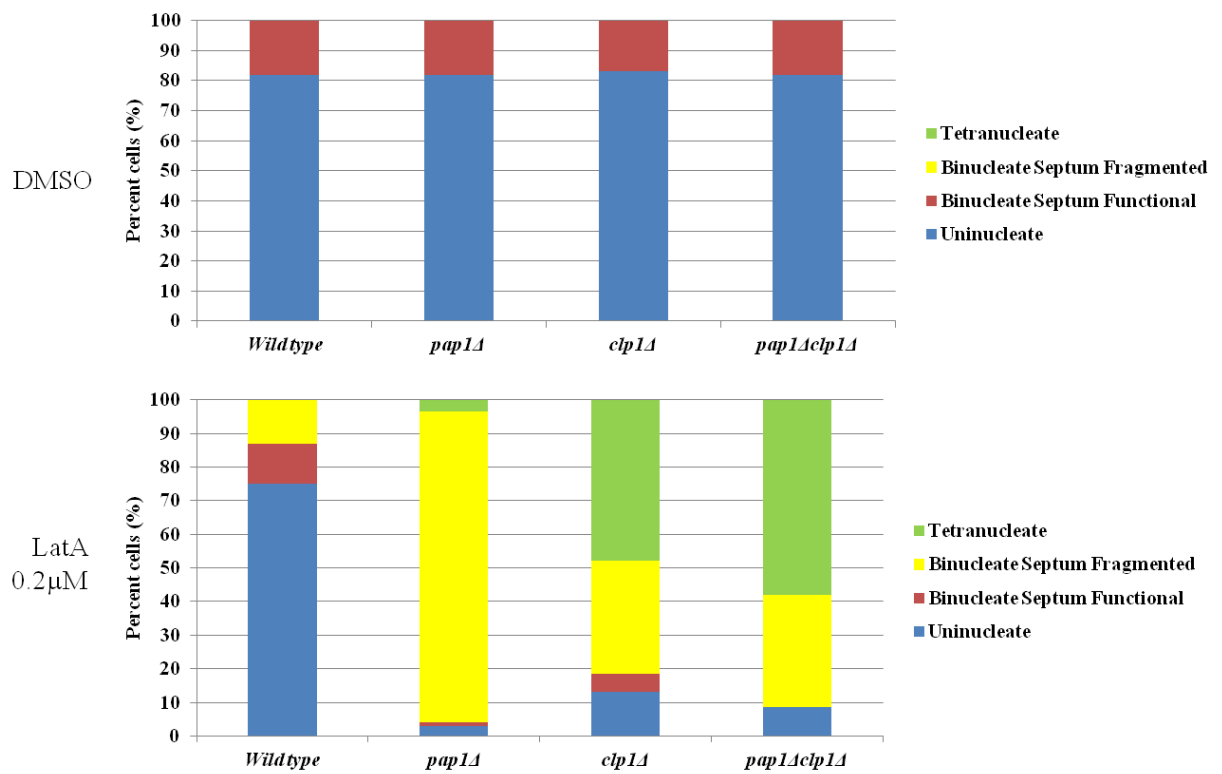


Figure 3-4: Quantitation of phenotypes of cells treated with DMSO and/or LatA. Quantitation was done in triplicate by counting 200 cells each time for each genotypic class. Cell quantitation also showed significantly higher number of tetranuclear cells for the *pap1Δclp1Δ* double mutant. The *pap1Δ* single mutant was predominantly binuclear with fragmented septa.



3.3 The *pap1Δ* mutant can form the actomyosin ring, but is unable to maintain ring integrity upon LatA treatment

Since the *pap1Δ* showed sensitivity to LatA and failed to execute cytokinesis, a *pap1Δ* mutant strain expressing a marker of the actomyosin ring, Rlc1-GFP (Le Goff et al. 2000) was constructed in order to more closely examine the effects of LatA on cytokinesis. Both the *rlc1*-GFP and mutant *pap1Δrlc1*-GFP strains were grown to mid-log phase and then live cells were observed using time lapse microscopy after treating them with DMSO/LatA. Upon DMSO treatment, both the *rlc1*-GFP and *pap1Δrlc1*-GFP strains showed comparable kinetics of ring constriction (ranging from 30 to 38 minutes) (Figure 3-5, top two rows). However, when grown in LatA media, *pap1Δ* cells displayed significant differences in phenotype compared to wild-type. The wild-type cells were still able to constrict their ring upon LatA treatment (as the ring constricts towards the middle of the cell, which can be visible from the decreasing diameter of the ring), although over a longer time frame (~46 minutes). In contrast, the *pap1Δ* mutants started their ring formation upon LatA treatment, but expectedly could not maintain ring integrity for extended periods of time. At around 12.5 minutes, their ring started fragmenting and finally around 17.5 minutes, their rings were completely lost (Figure 3-5, bottom two rows).

3.4 Pap1p localizes to the nucleus upon LatA treatment

If Pap1p is necessary for the functioning of the cytokinesis checkpoint, and if Pap1p is a stress activated transcription factor, then one would expect it to localize to the nucleus upon LatA treatment. To test this prediction, a strain was created that expressed a C-terminal GFP-tagged allele of Pap1p driven by its native promoter. The *pap1*-GFP strain was grown over night to mid-log phase and treated with DMSO and 0.2 μM LatA. In this experiment, the strain was also treated with 0.003% H₂O₂ as a positive control because it was previously shown that Pap1p migrates from the cytoplasm to the nucleus upon H₂O₂ treatment (Toone et al. 1998). It should be mentioned here that the dark region of the cells under the fluorescent images is the nucleus. Fluorescence microscopy (over a period of 0 to 120 minutes) revealed no nuclear accumulation of Pap1p upon DMSO treatment as expected. On the other hand, when treated with H₂O₂, all the cells showed nuclear localization of Pap1p irrespective of time. Interestingly, when treated with

0.2 μ M LatA, approximately 68% of the cells showed nuclear accumulation; the rest did not (data not shown) over a time period of 60 to 90 minutes (Figure 3-6).

3.5 Pap1p is a cell cycle dependent regulator of cytokinesis monitoring system

My earlier data showed that Pap1p is a nuclear protein, but that not all the cells showed nuclear accumulation upon LatA treatment because the cells were not synchronized at the same phase of the cell cycle (Figure 3-4). This raised the question of whether Pap1p is a cell cycle dependent protein, that is, it is imported to the nucleus upon LatA treatment only at certain times during the cell cycle. To test this prediction, a *pap1-GFPcdc25-22* strain was constructed. *cdc25-22* is a temperature sensitive mutant that allowed synchronization of the cells at the G2/M phase transition. After releasing the block by lowering the temperature to permissive temperature of 27°C, the cells were stained with DAPI to view their nuclear status. If the synchrony was successful, all the cells would be expected to be synchronized at late G2/M phase with uninuclear status. Microscopic observation revealed that almost all the cells were uninuclear, which was further confirmed by the cell quantitation study (Figure 3-7). As illustrated in Figure 3-7A, the cells were examined for their nuclear status after releasing the block. Right after releasing the block, at 0 min, all the cells were uninuclear. Then eventually the cells started being binuclear; from 60 to 90 minutes, almost 90% cells showed binuclear status. In due course, the cells started executing cytokinesis and as a consequence became uninuclear at around 150 minutes (~93%).

Having shown that the method used to synchronize the cells was successful; cells were then treated separately with DMSO, 0.003% H₂O₂ and 0.2 μ M LatA and then subjected to fluorescence microscopy over a time period of 0 min to 180 minutes. For the control, the cells did not show any nuclear accumulation of Pap1 at any given time. On the other hand, when the cells were treated with 0.003% H₂O₂, the cells showed nuclear localization of Pap1 from 30 to 120 minutes. However, upon LatA treatment, none of the cells showed nuclear localization of Pap1p until 60 minutes. Interestingly, around 90 minutes, when all the cells were binuclear, the cells started importing Pap1p in the nucleus from the cytoplasm. Pap1p was retained in the nucleus until 120 minutes when the cells were still binucleate. Eventually at 150 minutes, when

the cells became uninuclear, which confirms the successful completion of cytokinesis, Pap1p was exported back to the cytoplasm. All these findings confirm that Pap1p is a cell cycle dependent protein, which is imported to the nucleus at late mitotic phase right before cytokinesis upon LatA treatment (Figure 3-8).

3.6 Nuclear localization of Pap1p is important for the cytokinesis regulatory function and cell viability of *S. pombe*

Once it was confirmed that Pap1p localizes to the nucleus upon LatA treatment, it was important to know if this nuclear localization is important for its function. To test this, additional *pap1* constructs were generated by cloning a SV40 nuclear localization signal (NLS) and a nuclear export signal (NES) downstream of *pap1* followed by GFP. The prediction was that constitutive nuclear accumulation of Pap1p would show resistance to LatA like the wild-type cells. On the contrary, when Pap1p was constitutively restricted at the cytoplasm, the cells would show sensitivity to LatA like the *pap1Δ* mutant. To test this hypothesis, both strains were grown to mid-log phase and subsequently treated with 0.2 μM LatA. After the treatment, the cells were stained with DAPI/aniline blue and visualized for their nuclear status and septa, respectively by fluorescence microscopy. Similar to *pap1Δ* mutant strain, the *pap1*-NES cells showed hypersensitivity to LatA, which was characterized by binuclear cells with fragmented septa. On the other hand, the *pap1*-NLS strain exhibited resistance to LatA, which was characteristic of the wild-type strain (Figure 3-10A). The cells had functional septa and were able to carry out cytokinesis, which is reflected by an increased number of uninuclear cells. According to the cell quantitation study, *pap1*-NLS cells like the wild-type cells, showed higher number of uninuclear cells whereas the *pap1*-NES cells, like the *pap1Δ* cells, showed significantly higher number of binuclear cells with broken septa (Figure 3-10B).

Figure 3-5: *pap1Δ* cells are unable to maintain the integrity of the actomyosin ring upon LatA treatment. Wild-type and *pap1Δ* cells expressing the Rlc1-GFP fusion protein (marker of the actomyosin ring) were imaged upon treatment with 0.2 μM LatA or DMSO using time lapse microscopy. Arrow indicates fragmented ring.

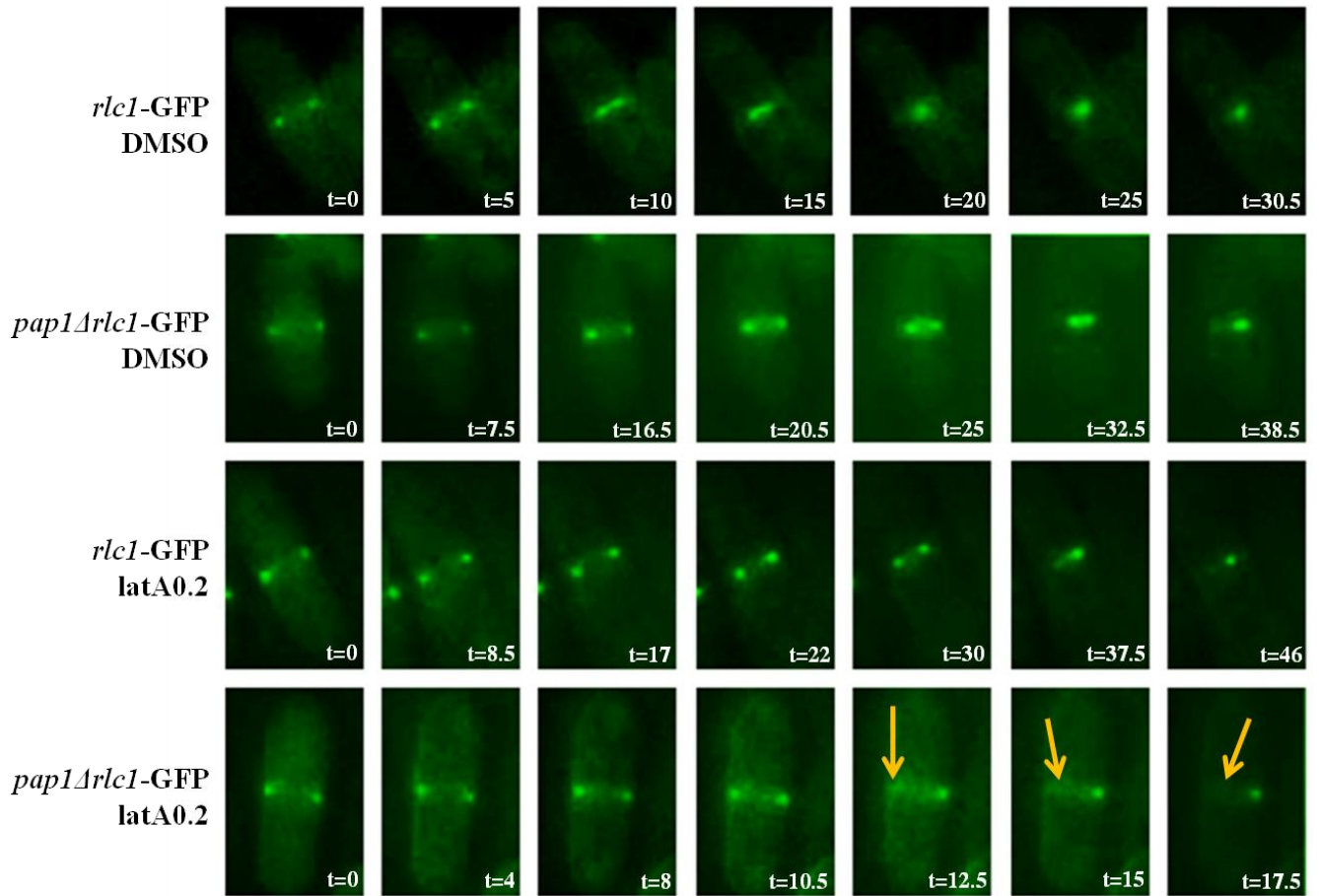


Figure 3-6: Pap1p localization to the nucleus upon LatA treatment. The *pap1p*-GFP strain was grown to mid-log phase in liquid YES, and then treated with either 0.2 μ M LatA or DMSO (solvent control) and also with 0.003% H₂O₂ as a positive control. Cells were visualized using fluorescence microscopy and a GFP filter as described in Materials and Methods. Arrow indicates nuclear accumulation of Pap1p.

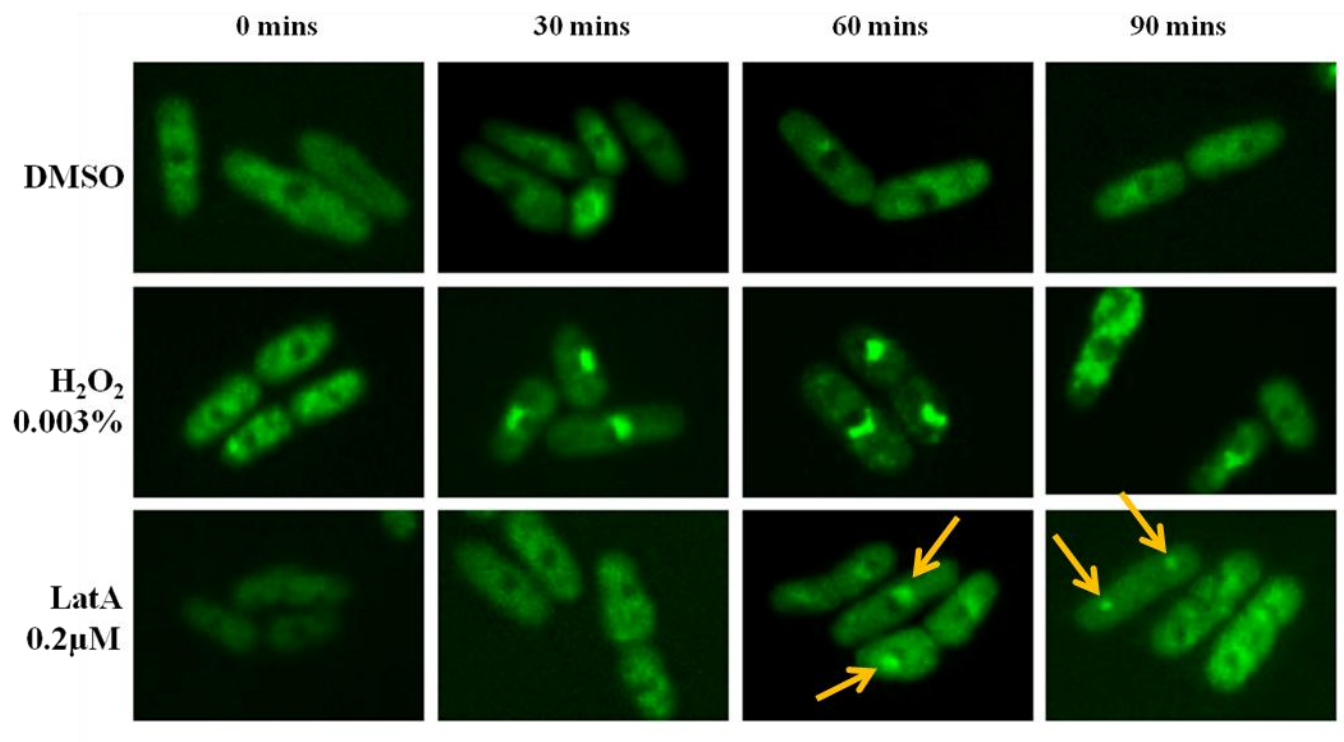
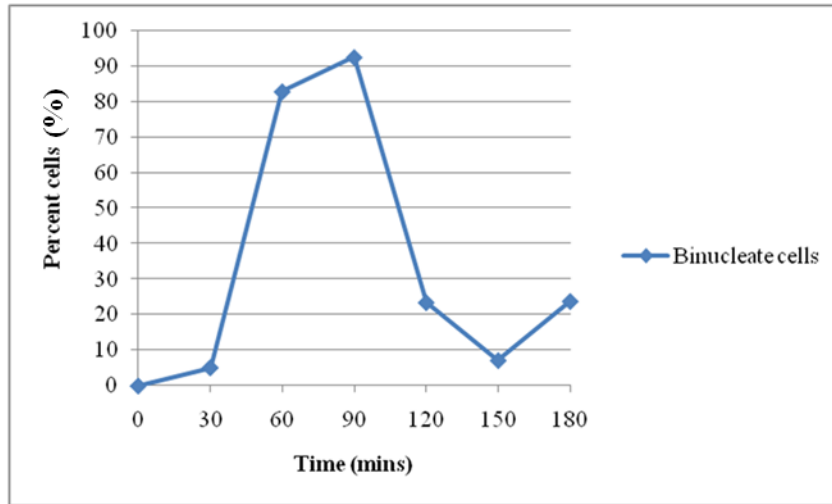


Figure 3-7: Verification of the synchrony of the *pap1*-GFP cells after mating with *cdc25-22*.

The cells were grown to mid-log phase at 27°C followed by incubation at 36°C for 4 hrs to block cell cycle progression at late G2/M phase. After releasing the block by reducing the temperature back to 27°C, the binucleate cells were counted at every 30 minutes interval up to 180 minutes. The experiment was done in three consecutive replicates. The nuclear status of the cells at different time periods is shown which relates to the different mitotic cell division events (A). Microscopic examination of the cells also confirmed the results of the cell quantitation study (B).

A)



B)

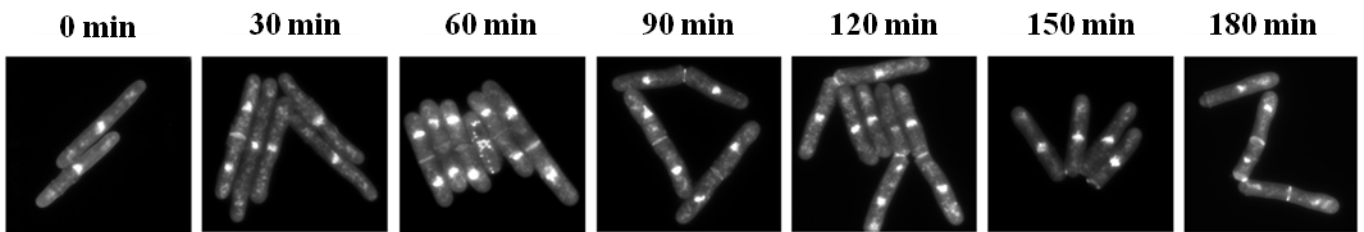
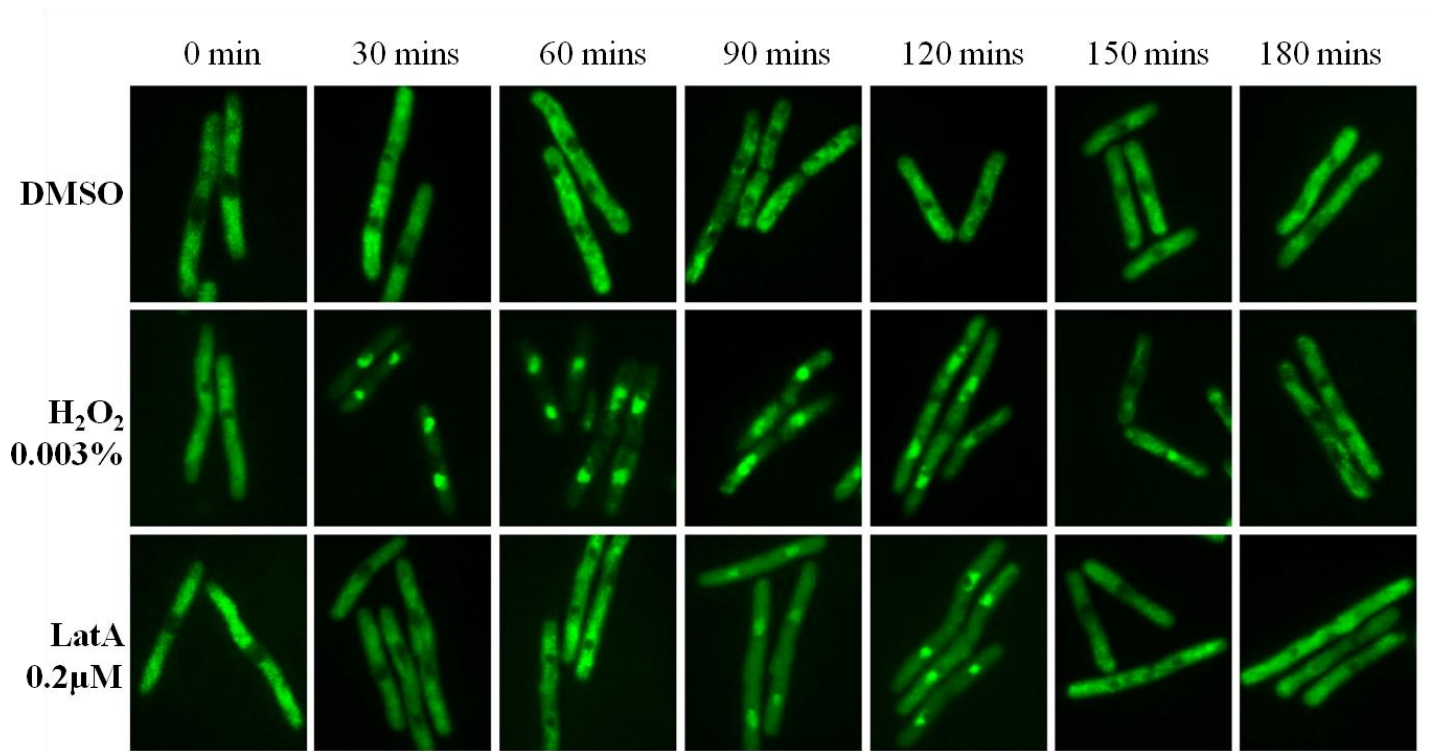
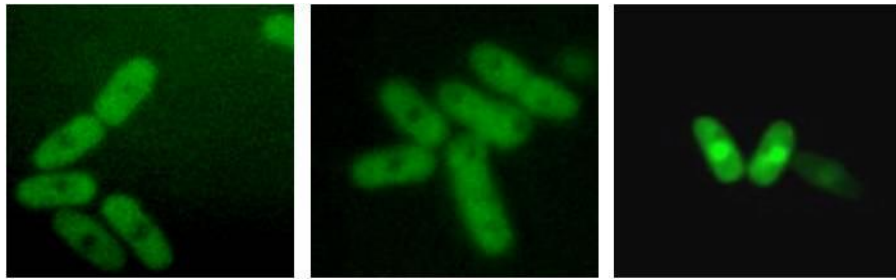


Figure 3-8: Confirmation of Pap1p as a cell cycle dependent protein. The cells were treated with DMSO, 0.003% H₂O₂ and 0.2 μM LatA after synchronizing them at late G2/M phase. Finally, the cells were observed by fluorescence microscopy to determine the nuclear localization of Pap1p at different time period.



So far, it was confirmed that Pap1p nuclear localization was important for the functioning of the cytokinesis monitoring system upon LatA treatment. Next, I examined if this nuclear localization of Pap1p was also important for the cells to form colonies and for their viability since colony formation is a direct outcome of cytokinesis and Pap1p is important for cytokinesis. To this end, the cells were streaked on YES agar media containing LatA or DMSO and were incubated overnight at 30°C. Following incubation, they were observed under bright-field microscopy. As illustrated in Figure 3-11, all the strains were able to form colonies when exposed to DMSO. However, when treated with 0.2 μ M LatA, the wild-type cells and the *pap1*-NLS cells were able to form colonies. On the other hand, the *pap1* Δ and the *pap1*-NES strains unlike the wild-type cells could not form colonies. Finally, a spot test was employed to confirm the importance of nuclear localization of Pap1p for cell viability. The cells were grown to mid-log phase and 10 fold serial dilutions of wild-type, *pap1*-NLS, *pap1* Δ and *pap1*-NES were spotted onto YES agar media containing 0.2 μ M LatA or DMSO and incubated for 3 days at 30 °C. As expected, the wild-type cells and the *pap1*-NLS cells formed colonies even after treated with LatA whereas the other two strains, *pap1* Δ and *pap1*-NES, did not (Figure 3-12). All these findings confirm that it is important for the *S. pombe* cells to import Pap1p from the cytoplasm to the nucleus upon LatA treatment to deal with the anti-cytokinetic stress and to sustain viability.

Figure 3-9: Constitutive localization of Pap1p upon NLS and NES tagging. In the absence of LatA, Pap1p remained in the cytoplasm for both the *pap1*-GFP and *pap1*-NES strains, On the other hand, even without LatA treatment, *pap1*-NLS strain showed nuclear accumulation of Pap1p because of the additional nuclear localization signal cloned downstream of *pap1*.



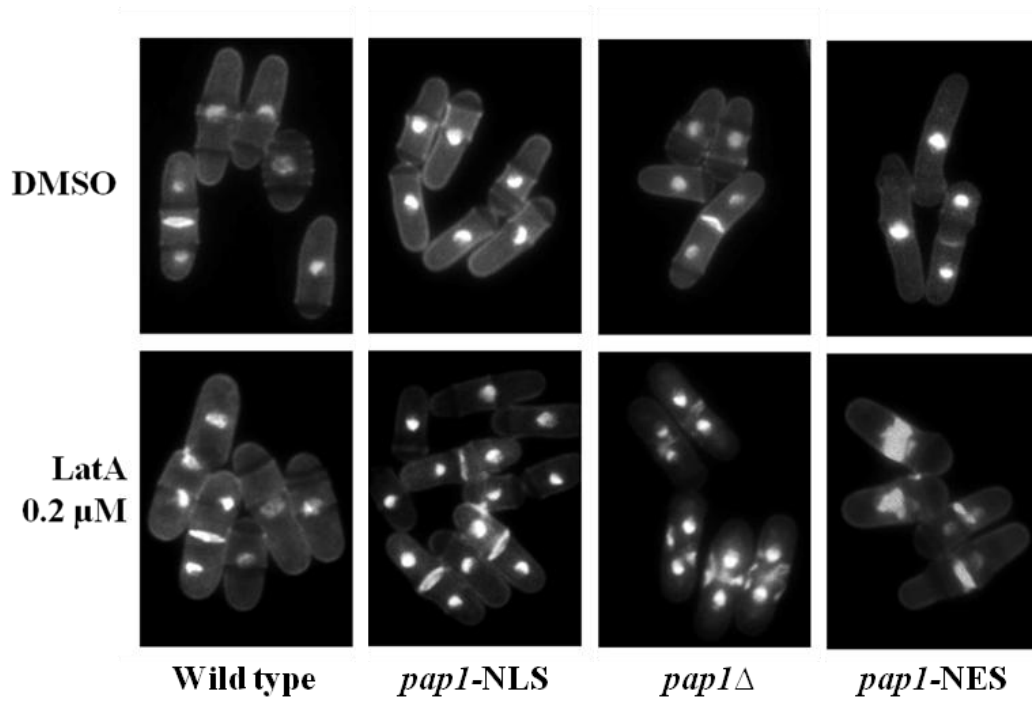
pap1-GFP

pap1-NES

pap1-NLS

Figure 3-10: Nuclear localization of Pap1p is important to carry out its function. Cells of the indicated genotypes were grown to mid-log phase followed by DAPI/aniline blue staining and examined by fluorescence microscopy (A). Cell quantitation study (done in triplicate) validated the outcomes of the fluorescence microscopy results (B). Fluorescence microscopy of the cells confirmed the importance of Pap1p localization.

A)



B)

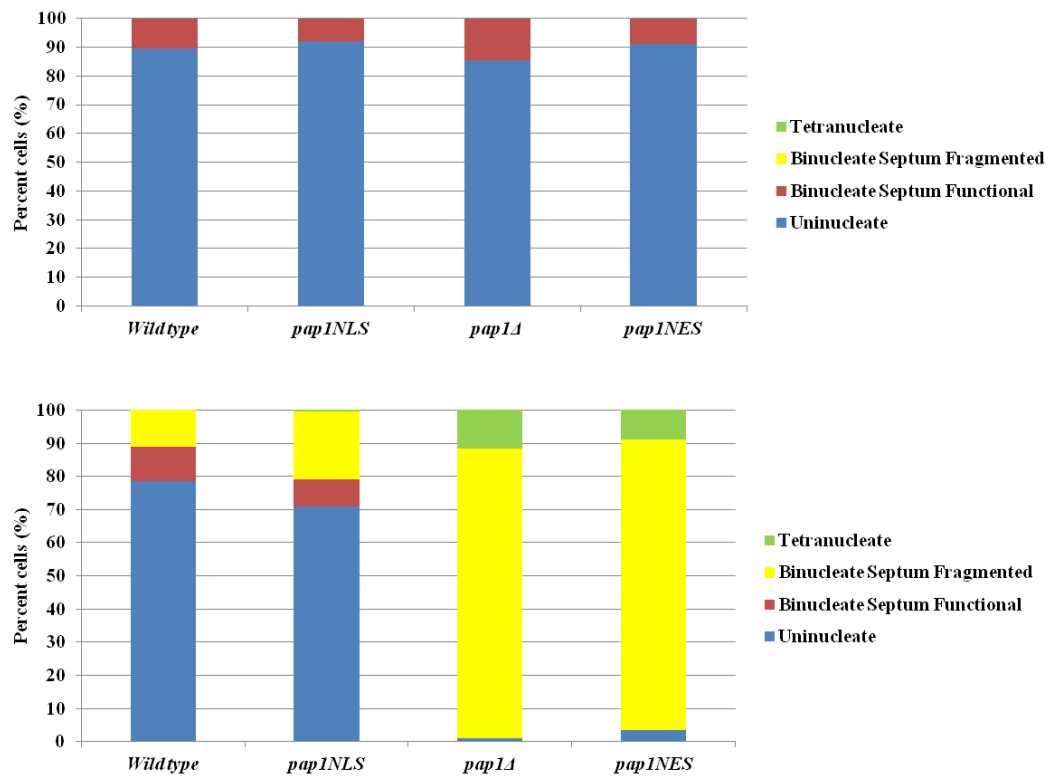


Figure 3-11: Nuclear localization of Pap1p is important for the colony forming abilities of the cells. Cells were streaked out on YES agar media containing DMSO and/or LatA and observed under bright-field microscopy.

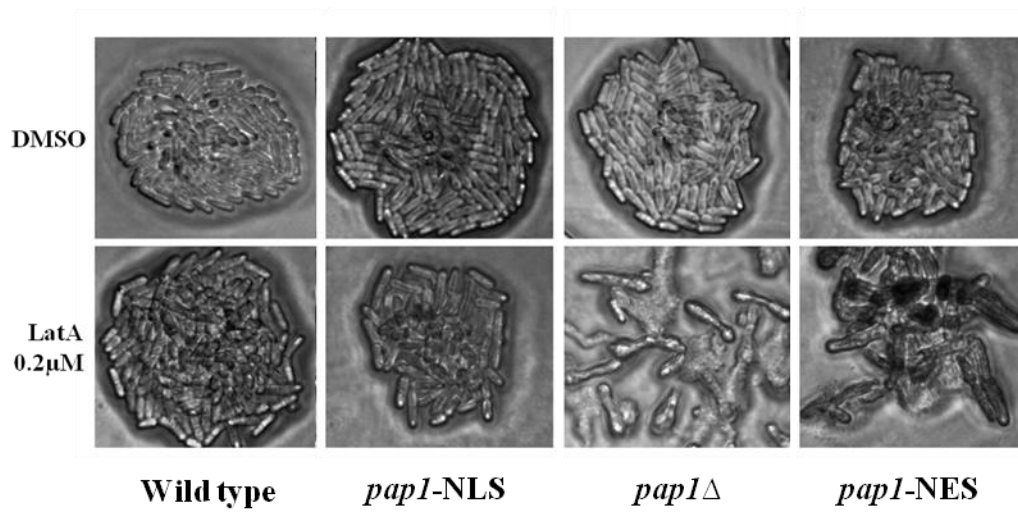
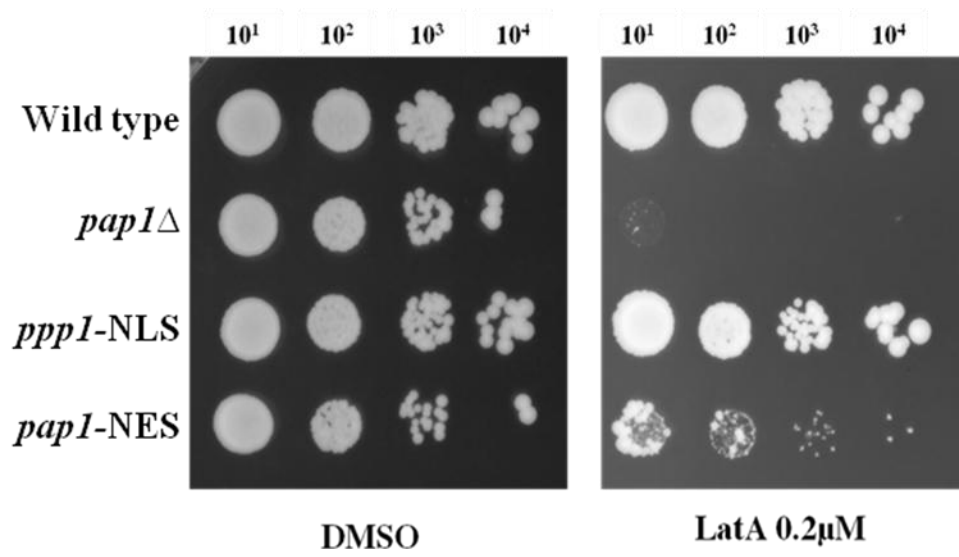


Figure 3-12: Nuclear localization of Pap1p is important for cell viability. Cells were grown to mid-log phase and serially diluted by 10 fold. Spot test was done on solid YES media containing DMSO and/or LatA and observed under bright-field microscopy.



CHAPTER 4: DISCUSSION

The process of cytokinesis is an ideal example of eukaryotic cellular complexity. It was Theodor Boveri who proposed an association between cytokinesis failure and the destruction of genomic integrity that could eventually lead to tumorigenesis (Harris 2008). A variety of researchers have provided adequate evidence to support his prediction in recent times (Fujiwara et al. 2005; Ganem et al. 2009; Ganem et al. 2007; Holland and Cleveland 2009; Sagona and Stenmark 2010; Rosario et al. 2010). Hence, the study of cytokinesis demands both widespread scientific research and attention.

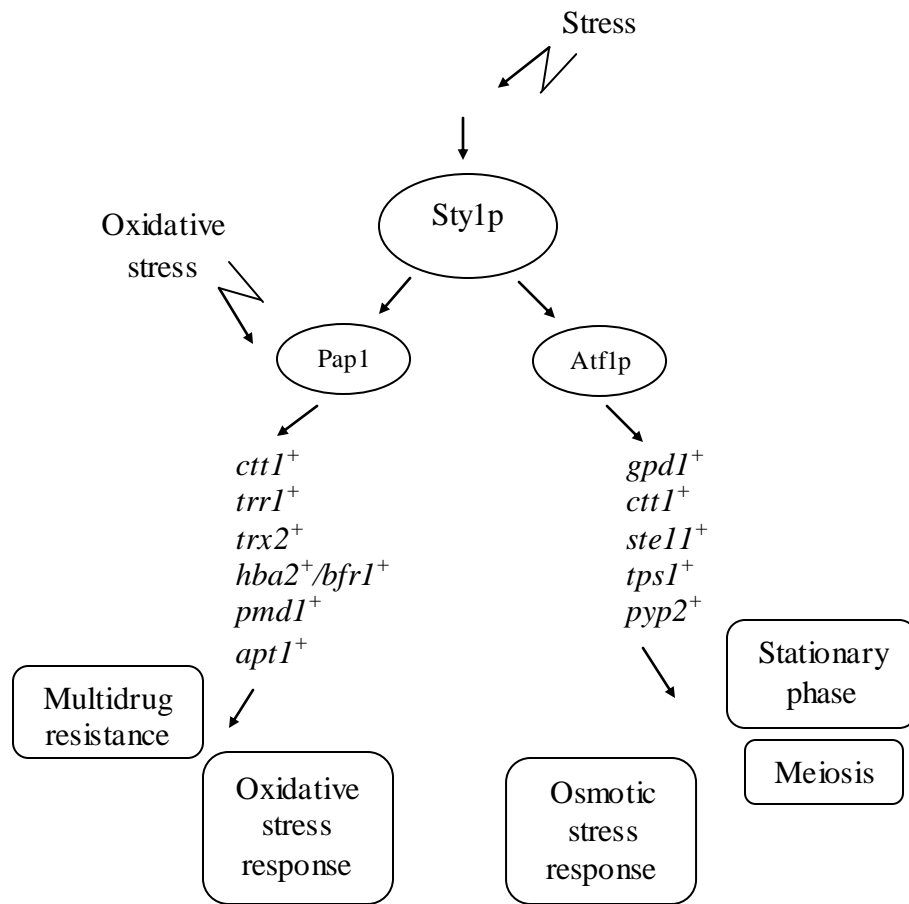
Extensive research in the field of cytokinesis has identified many of the molecular components of the division machinery. However, when it comes to how cytokinesis is regulated, little is known. Investigations over the last decade have established *S. pombe* as a valuable tool to study cytokinesis in higher eukaryotes (Balasubramanian et al. 1998; 2004; Karagiannis et al. 2005; Karagiannis and Balasubramanian 2007; Mishra et al. 2004; 2005). Only recently was it established that a cytokinesis monitoring system exists in *S. pombe*. This monitoring system delays the cell cycle and stabilizes the contractile actomyosin ring when the division machinery is perturbed (Liu et al. 2000; Le Goff et al. 1999; Mishra et al. 2004; Trautmann et al. 2001; Mishra et al. 2005; Chen et al. 2003).

Latrunculin A (LatA) is an actin depolymerizing drug produced by certain sponges. LatA disrupts the F-actin cytoskeleton by binding and damaging a region of the actin monomer. By doing so, it sequesters the monomers in an assembly-incompetent complex (Ayscough et al. 1997). With this ability to depolymerize actin filaments, LatA has been employed (at low doses) by researchers to study cytokinesis in *S. pombe*. Treating fission yeast cells with LatA induces the activation of the cytokinesis monitoring system. Upon exposure to LatA, wild-type cells with a functional checkpoint system will halt the cell cycle while simultaneously stabilizing the ring. On the other hand, cytokinetic checkpoint mutants will experience cytokinetic failure due to disintegration of the ring, along with repeated rounds of nuclear division, leading to inviable, multinucleate cells (Figure 1-4). Thus, LatA treatment can be used as a tool to screen genome wide deletion mutant libraries for genes with putative roles in cytokinesis (Mishra, McCollum and Karagiannis, unpublished).

Pap1p, which was identified in the aforementioned genome wide screen, encodes a stress activated transcription factor that is involved in the MAP kinase stress responsive pathway (Shiozaki and Russell 1995; Toone et al. 1998). Pap1p, although predominantly cytoplasmic, shuttles between the nucleus and cytoplasm. Upon exposure to cellular stresses, it remains in the nucleus until the cell deals with the particular stress. For example, when cells are treated with H₂O₂, Pap1p goes to the nucleus within 15 minutes and triggers >2-fold the transcription of 50 genes (Toone et al. 1998; Chen et al. 2003).

In mammalian cells, two transcription factors, referred to as AP-1 and ATF2, are the major targets of the stress activated MAP kinases (Toda et al. 1991; Hibi et al. 1993; Derijard et al. 1994; Kyriakis et al. 1994). Moreover, these kinase pathways display considerable conservation across species (Riesgo-Escovar et al. 1996; Sluss et al. 1996; Brewster et al. 1993; Millar et al. 1995; Shiozaki and Russell 1995; Kato et al. 1996). Interestingly, the fission yeast Pap1p is a bZip containing transcription factor with significant sequence homology and similar DNA binding specificity to the mammalian c-Jun transcription factor AP1, (hence its name *pombe-AP1*) or Pap1p (Toone et al. 1998). *S. pombe* also contains another transcription factor called Atf1p, which is homologous to mammalian ATF2 (Shiozaki and Russell 1996; Wilkinson et al. 1996). Pap1p was shown to be the downstream target of the stress responsive kinase Sty1p/Spc1p in *S. pombe* when treated with H₂O₂ and a number of other drugs (Toone et al. 1998). In fact, Sty1p regulates the activity of these two *S. pombe* transcription factors - Pa1p and Atf1p depending on the type of the cellular stress (Figure 4-1) (Toone et al. 1998). In addition, Sty1p has a role in the regulation of cell cycle progression. The *sty1Δ* deletion mutant was found to take an extended time for completing cell division and was shown to be synthetically lethal with *cdc25* mutants (*cdc25* encodes a known regulator of entry into mitosis; Warbrick and Fantes 1991; Millar et al. 1992, 1995; Shiozaki and Russell 1995). These data suggest the existence of a conserved pathway comprised of Pap1p that is involved in the cytokinesis monitoring system. This ultimately formed the basis of this proposed study to elucidate the putative role of Pap1p in cytokinesis monitoring system.

Figure 4-1: Model demonstrating the role of the Sty1 pathway in the fission yeast stress response. The Sty1 signaling pathway is involved in the response of cells to a variety of different stress conditions. The role of Sty1 is to regulate the activity of the two transcription factors Atf1 (Shiozaki and Russell 1996; Wilkinson et al. 1996) and Pap1 (Toone et al. 1998), which in turn regulate the expression of a number of genes encoding products that mediate different stress responses. The physiological events controlled by each factor are indicated. Adapted from Toone et al. 1998.



Before getting into the detailed characterization of Pap1p, it was necessary to verify the involvement of Pap1p in the cytokinesis regulatory system. The *pap1Δ* deletion mutant was created using a PCR based method (see Methods and Materials) and compared with wild-type strain upon LatA challenge. As illustrated in Figure 3-1, unlike wild-type cells, *pap1Δ* mutant was LatA sensitive (0.2μM), which was characterized by binuclear cells with completely fragmented septa. The cell quantitation study supported the observation (Figure 3-2). When LatA treated cells were quantified in triplicates, *pap1Δ* mutants showed significantly higher number of binuclear cells with broken septa (93%). On the other hand, the wild-type cells were able to carry out cytokinesis properly with either uninuclear cells (76%) or binuclear cells with functional septa (10%).

It was previously established that upon cellular stress, Pap1p is imported from the cytoplasm to the nucleus and that this import is dependent on the carrier protein importin-α encoded by *imp1* in *S. pombe* (Umeda et al. 2005). In order to conclude that Imp1p is indeed responsible for transporting Pap1p from the cytoplasm to the nucleus upon LatA treatment and they work in the same biological pathway, the *imp1Δ* deletion mutant was treated with 0.2μM LatA. As expected, the *imp1Δ* cells displayed a phenotype as did *pap1Δ*. In addition, I found no synthetic lethality when I treated the *pap1Δimp1Δ* double mutant with 0.2μM LatA (figure 3-1). Moreover, in the cell quantitation study, the *pap1Δ*, *imp1Δ* and *pap1Δimp1Δ* cells showed significantly comparable outcomes in terms of percentage of binuclear cells with broken septa (93%, 85% and 76%, respectively). These data are consistent with Imp1p and Pap1p working in a linear pathway, that is, they are involved in the same pathway.

Another interesting outcome of this physiological study is the dependency of Pap1p on the key cytokinetic regulator Clp1p to maintain the binuclear status upon LatA challenge. Clp1p offers a survival advantage upon LatA treatment through both delaying cell cycle progression and stabilizing the actomyosin ring (Mishra et al. 2004). The *pap1Δ* mutant, although sensitive to LatA, managed to delay the nuclear cycle progression (Figure 3-1). It was confirmed in this study that this nuclear delay exhibited by *pap1Δ* is Clp1p dependent. When the *pap1Δclp1Δ* double mutant was treated with LatA, they continued the second round of mitosis and had significantly higher number of tetranucleate cells (58%) (Figure 3-3 and 3-4). On the other hand, the *pap1Δ* single mutant showed few tetranucleate cells (3%). This suggests that Pap1p affects

only the ring integrity component of the cytokinesis monitoring system and plays no role in the cell cycle delay aspect of the system.

Determining Pap1p was LatA sensitive, I was interested to know the effect of LatA on actomyosin ring dynamics. To this end, *pap1Δ* was mated with a biomarker for ring constriction, Rlc1p-GFP to more closely examine the dynamics of cytokinesis in *pap1Δ* mutants. Both the *rlc1*-GFP and the *pap1Δrlc1*-GFP strains were treated with LatA and subjected to time lapse microscopy. Upon the control treatment of DMSO, both the strains showed comparable ring dynamics in terms of constriction (30.5 and 38.5 minutes, respectively). Moreover, upon LatA treatment, the wild-type cells were able to constrict the ring, albeit over a longer period of time (46 minutes). However, the *pap1Δrlc1*-GFP strain initiated ring assembly, but could not maintain the integrity of the ring for longer when treated with LatA. At around 12.5 minutes, the ring started disintegrating and was finally lost by 17.5 minutes (Figure 3-5). I thus concluded that Pap1p has a role in maintaining the physical structure of the ring upon LatA treatment. The nature of this role remains unknown at this time.

Since the *imp1Δ* mutant showed similar phenotypic features as *pap1Δ* when exposed to LatA, and the activity of Pap1p is regulated at the level of nuclear localization (Toone et al. 1998), I next wanted to confirm if Pap1p was imported to the nucleus upon LatA treatment. To this end, the *pap1* gene was tagged with GFP at the C-terminal end and treated with DMSO and/or 0.2μM of LatA. In this experiment, H₂O₂ was used as a positive control since Pap1p was shown to localize to the nucleus upon H₂O₂ treatment (Toone et al. 1998). When treated with DMSO, Pap1p remained in its non-stressed regular cellular location, the cytoplasm. On the contrary, treatment of the cells with H₂O₂ led to the import of Pap1p to the nucleus for almost all the cells from 30 minutes to 60 minutes. However, there was no nuclear accumulation of Pap1p at 30 minutes when the cells were treated with LatA. Eventually, it started accumulating in the nucleus beginning at 60 minutes (Figure 3-6). Interestingly, not all the cells (which are unsynchronized) showed this accumulation at the same time. This suggests that Pap1p import might be cell cycle dependent.

In order to verify this hypothesis, the *pap1*-GFP cells were synchronized by blocking them at late G2/M phase using the temperature sensitive *cdc25-22* mutant. Cdc25p is a mitotic

inducer that regulates the progression of cell cycle from late G2 to M phase (Berry and Gould 1996). Once the synchronization was confirmed by transient temperature shift to 36°C (figure 3-7), the *pap1-GFPcdc25* strain was treated with DMSO, H₂O₂ and 0.2 μM LatA and examined by fluorescence microscopy. From 30 to 90 minutes, the cells showed nuclear localization of Pap1p when treated with H₂O₂. However, after treating with LatA, nuclear accumulation of Pap1p started at 90 minutes and it was retained until 120 minutes. It should be mentioned here that at 90 and 120 minutes, the cells were binucleate, that is, chromosomal segregation was completed. The cells started exporting Pap1p back to cytoplasm at 150 minutes when they became uninuclear (Figure 3-8). This clearly confirmed that Pap1p goes to the nucleus once chromosomal segregation is completed and remains there until cytokinesis concludes. One could speculate that after nuclear segregation to the completion of cytokinesis, Pap1p triggers the expression of the genes required to deal with LatA stress. Once cytokinesis is finished, Pap1p leaves the nucleus and returns to the cytoplasm and does not start accumulating in the nucleus until the next round of cytokinesis. The confirmation of Pap1p being a cell cycle dependent protein is one of the major results of this study.

The data clearly showed that Pap1p enters the nucleus after LatA treatment. I thus wanted to assess if this nuclear localization is required for the cytokinetic regulatory function of Pap1p. To this end, either a SV40 nuclear localization signal or a nuclear export signal were cloned downstream of full length *pap1* sequence followed by GFP tagging. By doing so, I was able to constitutively regulate the cellular localization of Pap1p even at the absence of any forms of stress (figure 3-9). Pap1p remained in the cytoplasm for the *pap1*-NES strain and was imported to the nucleus for the *pap1*-NLS strain without any treatment. Finally, the wild-type, *pap1*-NLS and *pap1*-NES strains were treated the same with DMSO and LatA. Fluorescence microscopy showed that both the wild-type and the *pap1*-NLS strains had Pap1p in the nucleus when treated with LatA. Further, these strains exhibited similar phenotypes in that they were resistant to LatA treatment. On the other hand, the *pap1Δ* deletion mutant and the *pap1*-NES strains were sensitive to LatA and exhibited binuclear cells with fragmented septa (Figure 3-10). Wild-type and *pap1*-NLS strains had very low percentage of binucleate cells with fragmented septa (10% and 7%, respectively). Conversely, the *pap1Δ* and *pap1*-NES strains had significantly higher number of binuclear cells with broken septa (88% and 87%, respectively) (Figure 3-10).

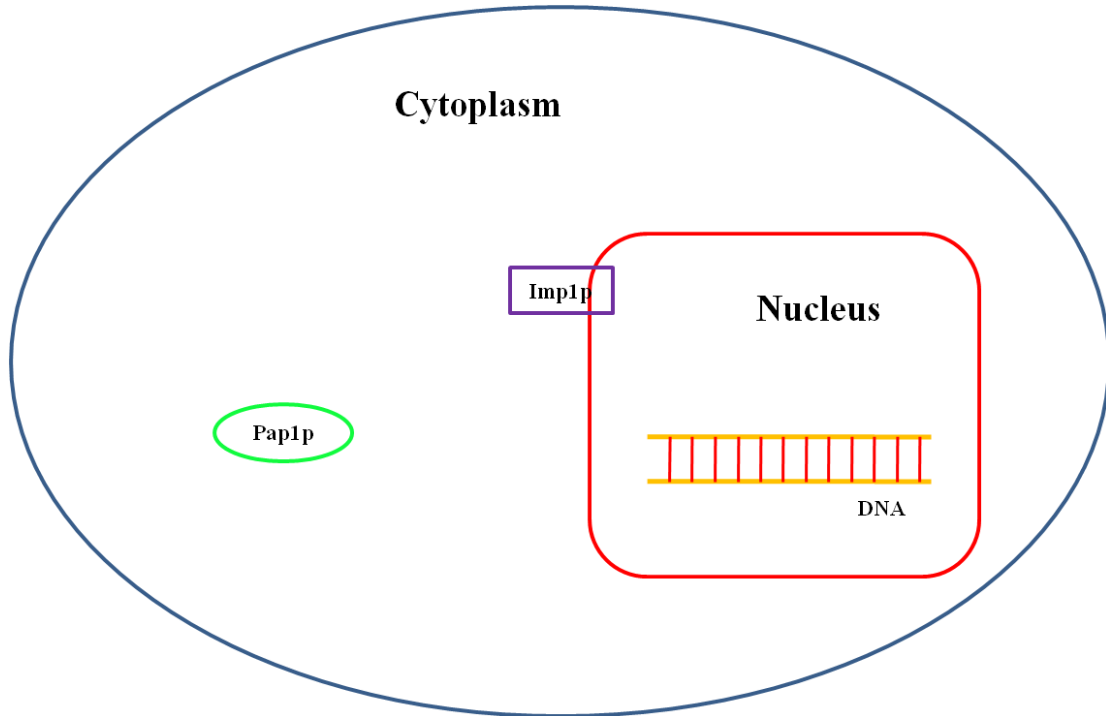
In addition, all the strains were investigated for their colony forming ability by growing them on solid YES media containing DMSO and/or 0.2 μ M LatA. Similar to earlier observations, both the wild-type and the *pap1*-NLS strains were able to form colonies when grown on media containing LatA. However, the *pap1* Δ and the *pap1*-NES strains could not form colonies (Figure 3-11). The strains were also tested for their ability to grow upon LatA treatment. 10 fold serial dilutions of all the strains were spotted on solid YES media with DMSO and/or LatA. As expected, the wild-type and the *pap1*-NLS cells were viable, but the *pap1* Δ and *pap1*-NES strains were not upon exposure to LatA (Figure 3-12). In summary, it can be stated that if Pap1p is not transported to the nucleus, the cells shows hypersensitivity to LatA and lose their viability, which reinforces the importance of nuclear localization of Pap1p upon cytokinetic stress.

All the outcomes of this study established reasonably the role of Pap1p in a cytokinesis monitoring system. The results discussed so far can be summarized in a Pap1p-dependent model of cytokinesis regulation as illustrated below in Figure 4-2. In typical growth conditions, Pap1p remains in the cytoplasm; once the cells are exposed to LatA treatment, some sort of modifications in Pap1p restrict it in the nucleus where it regulates the expression of certain sets of genes required for cytokinesis maintenance.

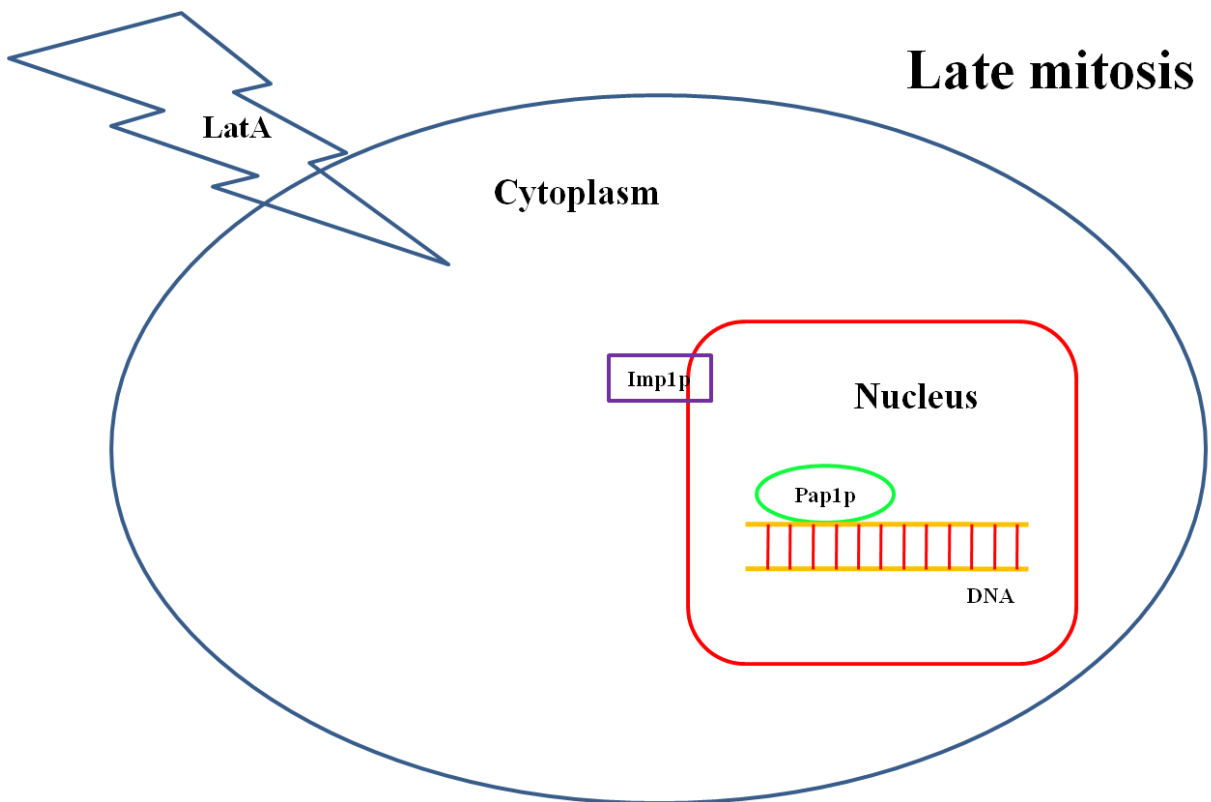
At this end, the Pap1p-dependent genes are still unknown, which demands further investigation. In addition, further analyses are important to understand the modification, which leads to nuclear retention of Pap1p upon LatA treatment. It is phosphorylation which enables AP-1, the mammalian counterpart of Pap1p to stay in the nucleus upon cellular stress (Toda et al. 1991; Hibi et al. 1993; Derijard et al. 1994; Kyriakis et al. 1994). It would be interesting to examine if phosphorylation leads to nuclear retention of Pap1p upon LatA treatment in *S. pombe*. In addition, although based on background information and the results of this study suggest a conserved pathway consisting of Sty1 and Pap1p, it necessitates further study to validate this association of Sty1p and Pap1p in the cytokinesis monitoring system.

Figure 4-2: Mechanism of action of Pap1p upon LatA treatment. Under normal growth conditions, Pap1p remains in the cytoplasm (A). When treated with LatA, Pap1p is imported into the nucleus during late mitosis in a Imp1p dependent manner where it transcribes the group of genes required for maintaining a cytokinesis competent state. The identity of these genes remains unknown (B).

A)



B)



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