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Understanding the Role of Polyamine N-Acetyltransferase in the Fission Yeast Response to Perturbation of the Cytokinetic Machinery

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Abstract

The mechanisms that ensure cytokinetic fidelity are important for cellular proliferation and the maintenance of genomic integrity. To better understand these mechanisms, the actin depolymerizing drug, LatA, was previously used to screen for fission yeast genes required to prevent cytokinesis failure. This screen identified the *pna1* polyamine-N-acetyltransferase. Here, I show that *pna1Δ* mutants display severe growth defects when exposed to even low doses of LatA. Furthermore, LatA-treated *pna1Δ* cells display increased rates of cytokinesis failure characterized by fragmentation of the cytokinetic actomyosin ring. Surprisingly, Pna1-GFP fusion proteins form cytoplasmic filaments that may represent cytoophidia (meaning "cellular snakes"), a class of structures implicated in metabolic regulation via compartmentalization. Interestingly, Pna1p filament length is dependent on growth phase, but is not affected by inhibition of the TOR pathway. I suggest that fission yeast respond to LatA-induced cytoskeletal perturbations through modulating polyamine interaction with actin via a mechanism involving Pna1p-mediated polyamine acetylation.

Keywords

Schizosaccharomyces pombe, Fission yeast, Cytokinesis, Actin, Polyamines, Cytoophidia

Summary for Lay Audience

Cells division is essential for the growth and development of living organisms. The process of cell division has many steps, the last being "cytokinesis", which involves the physical separation of a mother cell into two independent daughter cells. When cytokinesis fails, it leads to the formation of cells with an abnormal number of nuclei. This can result in genetic instability, which in turn contributes to tumorigenesis. Hence, understanding cytokinesis is of fundamental importance. One way to understand this process is to disrupt cytokinesis and observe how the cell reacts to the disturbance. For example, a drug called LatA can be used as a tool to perturb the cytokinetic machinery and to identify genes that are required to counter this stress. Using this strategy, a group of genes required for growth in the presence of LatA was identified in fission yeast (a simple, single-celled model organism that is often utilized by researchers to better understand basic biological processes). One gene from this group, *pna1*, encodes a protein that modifies an important class of cellular molecules called polyamines, which are known to interact with a variety of negatively charged molecules, including actin.

To better understand the role of the Pna1p protein in responding to LatA-induced cell-division stress, I assayed cytokinesis failure rates using fluorescence microscopy. Unlike normal cells, those lacking Pna1p display dramatically increased rates of failure. Furthermore, time-lapse imaging of live cells revealed that this failure was due to a severe fragmentation of the actomyosin ring (a contractile structure crucial for cytokinesis). Surprisingly, I also show that Pna1p forms unusual cytoplasmic filaments that may represent a novel example of a class of structures termed cytoophidia (meaning "cellular snakes"). These structures have been identified in organisms ranging from bacteria to humans and may play a role in metabolic regulation via the compartmentalization of proteins. Interestingly, the length of Pna1p-cytoophidia are dependent on growth phase, as I show that cells that are not actively growing display longer filaments than those that are actively growing. The continued characterization of this structure and its role in cytokinesis may provide further insight into the cellular mechanisms preventing cytokinesis failure.

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Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

ABP – Actin Binding Protein

Arp2/3 – Actin Related Protein 2/3

BPA – Acyclic Branched Polyamines

Caf5 – Caffeine resistant protein 5

CDC – Cell Division Cycle

CDK – Cyclin Dependent Kinases

Cdr – Changed Division Response

CIN – Chromosomal Instability

Clp1 – Cdc14 like protein

CPC – Chromosomal Passenger Complex

CTPS – Cytidine 5′- Triphosphate Synthetase

DAPI – 4′,6-diamidino-2-phenylindole

DMSO – Dimethyl Sulfoxide

DNA – Deoxyribonucleic Acid

EMM – Edinburgh Minimal Medium

F-actin – Filamentous actin

G-actin – Globular actin

GAP – GTP Activating Protein

GEF – Guanine Exchange Factor

GFP – Green Fluorescent Protein

Imp1 – alpha Importin

LatA – Latrunculin A

MBC – Methyl benzimidazole-2-yl-carbamate

MPA – Macrocylic polyamines

mRNA – messenger Ribonucleic Acid

OD – Optical Density

ORF – Open Reading Frame

Pap1 - pombe AP-1-like gene

PBS – Phosphate Buffer Saline

PCR – Polymerase Chain Reaction

Pna1 – Polyamine N-Acetyl-transferase

Rlc – Regulatory Light Chain

ROCK – Rho Kinase

SIN – Septation Initiation Network

SPA – Sporulation Agar

SPB – Spindle Pole Body

TOR – Target of Rapamycin

YES – Yeast extract plus supplements

Chapter 1

1 INTRODUCTION

The successful completion of cytokinesis requires the intricate interplay of gene products ranging from signaling proteins to elements of the actin cytoskeleton. Taken together these pathways ensure that cytokinesis occurs at the proper location within a cell and at the proper time during the cell cycle. Interestingly, past research in the model eukaryote *Schizosaccharomyces pombe* (commonly referred to as fission yeast) has revealed the existence of regulatory modules that act to promote the establishment of a "cytokinesis-competent" state (i.e. a state characterized by delayed progression into mitosis and the continuous repair/re-establishment of the actomyosin ring). These modules act to prevent cytokinesis failure when cells encounter stresses that perturb the cell division machinery. In the pages that follow I present a review of the scientific literature as it pertains to the actin cytoskeleton, cytokinesis, the consequences of cytokinesis failure, and the known cellular functions of polyamines and polyamine N-acetyl transferases. In the subsequent chapters I describe and discuss experiments aimed at better understanding the function of a fission yeast polyamine N-acetyl transferase (encoded by the *pna1* gene) that was identified as being required for cytokinesis when cells are challenged with low doses of the actin depolymerizing drug, Latrunculin A. While this research was conducted using the simple unicellular fission yeast model, it is hoped that it will nonetheless provide a theoretical framework for understanding the regulatory wiring required to ensure the successful execution of cytokinesis in more developmentally complex eukaryotes.

1.1 The actin cytoskeleton

Actin is among the most abundant proteins on Earth, making up between 1-5 percent of cellular protein in species ranging from amoebas to humans. All eukaryotes possess genes encoding actin, which is highly conserved and exhibits only a few amino acid differences between algae, amoeba, fungi and animals (Pollard, 2016). The actin cytoskeleton is involved in many cellular functions including cell motility,

differentiation, cancer metastasis, and vesicular trafficking (Lamouille et al., 2014; Lanzetti, 2007; Weng et al., 2010). Actin organization is tightly controlled by proteins that regulate its nucleation, assembly and disassembly, bundling, localization, and cross-linking (Paul & Pollard, 2009; Xu et al., 2004).

Actin exists in two states: globular monomeric (G-actin) and filamentous (F-actin) (Mishra et al., 2014). Filament nucleators like the Arp2/3 complex initiate actin polymerization (Campellone & Welch, 2010; Chesarone et al., 2009; Pollard, 2007), while positively charged polycationic proteins bundle F-actin through non-specific electrostatic interactions that counter the repulsion between negatively charged actin filaments (Muhlrad et al., 2011; Tang & Janmey, 1996). Importantly, the assembly and disassembly of actin filaments can be regulated by the cell, allowing it to remodel the cytoskeleton as needed (Pollard & Borisy, 2003).

The actin network in metazoans is more extensive, yet comparable to that observed in yeast. The yeast actin cytoskeleton assembles into three distinct structures: patches, cables, and the actomyosin ring (**Figure 1-1**) (Mishra et al., 2014). Actin filaments in these three structures are bound by partially overlapping sets of actin-binding proteins, which give them their unique properties (Mishra et al., 2014).

Actin patches are dense networks of actin filaments nucleated by the Arp2/3 complex and are involved in clathrin mediated endocytosis (CME) (Winter et al., 1997). Actin patches are made of short, branched actin filaments, which are highly dynamic and have a lifespan of 1-2 min (Mishra et al., 2014). They assemble at sites of endocytosis, accompanying polarized cell growth at the tips of interphase cells and at the centres of dividing cells (Kovar et al., 2011). Actin assembly causes the inward movement of actin patches from the cortex, and this can be prevented by inhibiting actin polymerization by the drug Latrunculin A (Kaksonen et al., 2003).

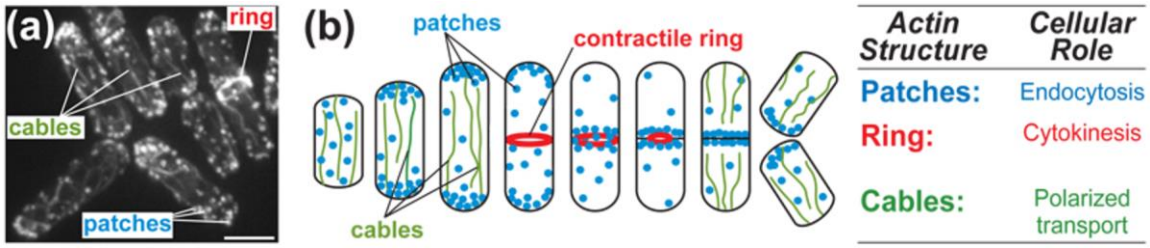

Figure 1-1: The fission yeast actin cytoskeleton.

(A) Actin staining of fission yeast cells highlighting patches, cables, and the actomyosin ring. Scale bar represents 5 µm. **(B)** Schematic illustrating the subcellular distribution of patches (blue), cables (green), and the contractile ring (red). Adapted from *Trends Cell Biol. 2011, 21(3): 177–187* under the "fair dealings" provision of the Canadian Copyright Act (2019).

Actin cables are polarized linear bundles of parallel actin filaments that provide polarized tracks for type V myosin-directed delivery of vesicles and organelles to the expanding cell tips (Kovar et al., 2011). Several cargos, like secretory vesicles, peroxisomes, the Golgi apparatus, mitochondria, vacuoles, and mRNAs use actin cables as tracks for movement. Actin cables extend along the long axis of the cell which make them suitable for polarized transport and cell growth. Actin cables are highly dynamic structures with a half-life of less than a minute (Mishra et al., 2014).

The third major type of actin structure is the actomyosin ring, which plays an indispensable role in cytokinesis (Balasubramanian et al., 2004). The actomyosin ring is a complex structure consisting of more than 100 proteins including F-actin and other modulators of actomyosin contractility (Mishra et al., 2014). The actomyosin ring signals cell wall assembly at the cell division site, and also provides the force needed for the cell to divide (Pollard & Wu 2010; Lee *et al.* 2012; Rincon and Paoletti 2012; Wloka and Bi 2012; Balasubramanian *et al.* 2012). The positioning of the actomyosin ring at the division site, its assembly, as well as its constriction are very tightly regulated by molecules such as cyclin dependent kinases, polo kinase, and the anaphase promoting complex, which ensures that events of cytokinesis are activated only at the appropriate point in the cell cycle (Mishra et al., 2014). All three types of actin structures – patches, cables, and the actomyosin ring – are bound by unique actin binding proteins (ABPs), thereby making them specialized for specific functions (Kovar et al., 2011).

1.2 Cytokinesis in eukaryotes

Cytokinesis is the last critical step in the process of cell division. It is initiated after chromosome segregation and proceeds until the mother cell separates into two independent daughter cells. The accurate partitioning of daughter nuclei is required for cell viability and the prevention of aneuploidy (Pollard & O'Shaughnessy, 2019). Cytokinesis is executed via constriction of a contractile ring, which is formed at the division site and is composed mainly of actin filaments (F-actin) and myosin-II (Kamasaki et al., 2007; Mabuchi, 1986; Narumiya & Mabuchi, 2002; Salmon, 1989).

While the process of cytokinesis is conserved in all dividing organisms – with considerable conservation of molecules between yeast and animal cells – there are some differences in the mechanisms involved across species (Guertin et al., 2002). Cytokinesis can be subdivided into four stages related to the function of the contractile actomyosin ring that mediates cell division: 1) the spatial positioning of the actomyosin ring within the cell, 2) actomyosin ring assembly, 3) actomyosin ring constriction, and 4) actomyosin ring disassembly (**Figure 1-2**).

The spatial positioning of the ring is the step that has diverged more than any other step over evolutionary time. Accurate placement of the cleavage plane is important since the aim of cytokinesis is to create two cells, each with its own nucleus (Pollard & Wu, 2010). Cyclin dependent kinases (CDKs) and Polo kinases control ring positioning in animal cells (Pollard & O'Shaughnessy, 2019). They do this by regulating the activity of two master complexes called the centralspindilin complex and chromosome passenger complex (CPC) (Pollard & O'Shaughnessy, 2019). CDKs and Polo kinases phosphorylate proteins and inhibit the cytokinetic machinery until anaphase (Canman et al., 2000; Niiya et al., 2005; Potapova et al., 2006). Transition to anaphase and furrow formation in animal cells depends on cyclin B degradation and termination of CDK kinase activity (Pollard, 2017). At anaphase, the two master complexes activate RhoGEF and Rho GTPases, which specify the location at which the actomyosin ring assembles. The two master complexes are in turn regulated by the mitotic spindle that signals the correct position for their accumulation, and hence the location of ring assembly (Basant & Glotzer, 2018).

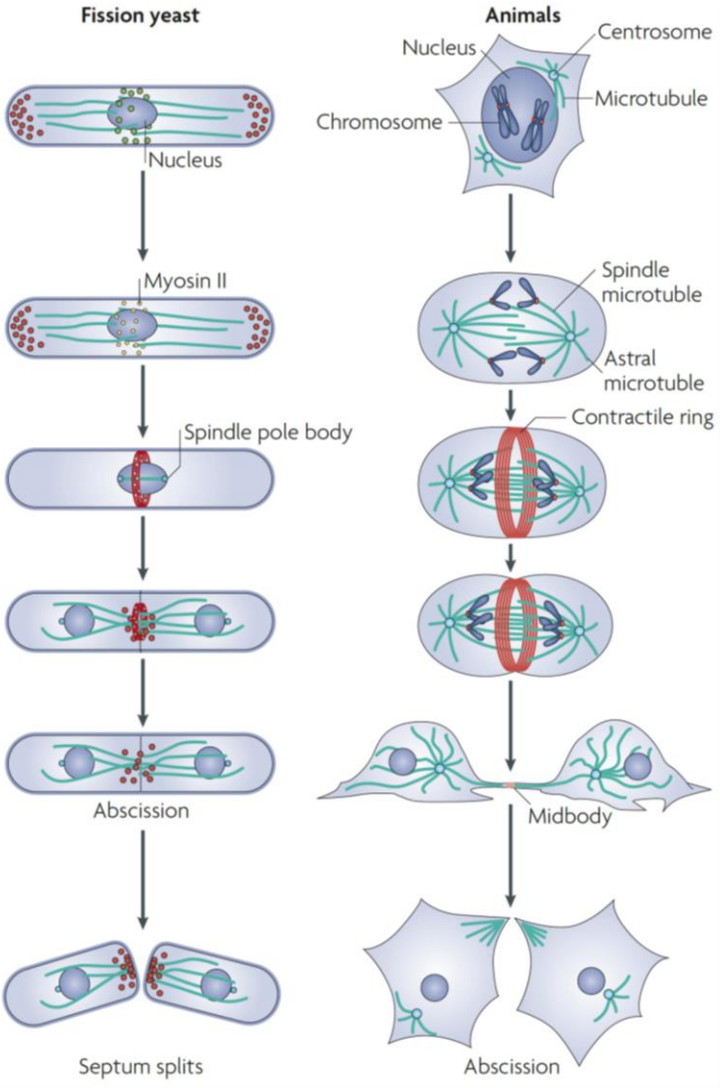

Figure 1-2: Cytokinesis in fission yeast and animal cells.

In *S. pombe*, the placement of actomyosin ring is determined by the position of the nucleus. In animal cells, the spindle and astral microtubules specify its position. Both *S. pombe* and animal cells assemble a ring comprised of both actin filaments and myosin II. After chromosome segregation, the ring constricts and the daughter cells separate by membrane fusion. Adapted from *Nat Rev Mol Cell Biol. 2010, 11(2):149–155* under the "fair dealings" provision of the Canadian Copyright Act (2019).

Once the location of ring assembly is determined, RhoGTP activates Rho-Kinase (ROCK) which phosphorylates regulatory light chains of myosin II, and also activates formins to assemble actin filaments (Pollard & O'Shaughnessy, 2019). It is speculated that pre-existing cortical actin, formed by the Arp2/3 complex, may also contribute to ring assembly (Chalut & Paluch, 2016). Myosin II and actin interact, producing a force that drives actin filaments and myosin clusters towards the equator (Reymann et al., 2016).

The next step of cytokinesis is the constriction of the formed actomyosin ring. Myosin II is one of the crucial elements required for ring constriction. The required tension for constriction of the ring is produced when myosin II exerts a force on the actin filaments that are membrane-anchored. These components together generate an inward mechanical force that drives furrow ingression and consequently cell division (Pollard & O'Shaughnessy, 2019).

Constriction of the ring and its disassembly occur simultaneously (Pollard, 2017). The polymerization and disassembly of actin in the ring happen together, where profilin and anillin help with disassembly and formin aids polymerization (Miller, 2011). Actin disassembly is needed for furrow ingression, and the ring is progressively disassembled with time (Miller, 2011). It is important to note that disassembly alone is not enough for the cell to divide; membrane insertion is also needed. After constriction, the ring starts disassembling, creating an intercellular bridge. The created bridge then tethers the two daughter cells until abscission (Miller, 2011). Abscission is defined as the process of physical separation of the two daughter cells by the splitting of their membranes (Pollard, 2017). Abscission requires the delivery of membrane vesicles to the intercellular bridge (Miller, 2011). In some rare cases, the final step of abscission can be suppressed, thereby generating two daughter cells that remain physically connected, but that function independently of each other (Glotzer, 2017).

1.3 Using *Schizosaccharomyces pombe* as a model organism

Schizosaccharomyces pombe, commonly known as fission yeast, is a single celled eukaryotic organism belonging to the kingdom, Fungi. *S. pombe* was first isolated from beer and is commonly used for fermentation. Each cell is cylindrical in shape having a diameter of approximately 3.5 µm. *S. pombe* cells are easy to culture, grow rapidly (generation time of 2 - 4 hours), and are genetically tractable, making them ideal for studying cell morphology and cell division in a laboratory environment (Hayles & Nurse, 2018). Following the complete genome sequencing of *S. pombe* in 2002, a range of new methodologies for genome-wide analysis were also developed, including a genome-wide set of gene deletion mutants (Hayles & Nurse, 2018; Wood et al., 2002). Since *S. pombe* is a unicellular eukaryote, it can be used to study processes that are conserved in more developmentally complex multicellular organisms including humans. *S. pombe* is commonly used as a model to study processes such as transcription, translation, DNA replication, cytoskeletal organisation, and the cell division cycle (Hoffman et al., 2016).

S. pombe gained popularity in cytokinetic studies following Nobel Laureate Sir Paul Nurse's work in understanding cell cycle control in fission yeast (Hoffman et al., 2016). An inventory of more than 130 cytokinesis genes as well as conditional mutants for many of these genes are available (Pollard & Wu, 2010). Sophisticated molecular tools and a high rate of homologous recombination in fission yeast allow for easy genetic manipulation and precise localization of tagged proteins expressed at endogenous levels (Kovar et al., 2011; Mishra et al., 2014). These features made it possible to chart a high-resolution timeline of cytokinetic events and to quantitate the intracellular distributions of key cytokinesis proteins (Pollard & Wu, 2010). All of these features make *S. pombe* an excellent organism with which to study the process of cell division (Pollard & Wu, 2010).

1.4 Cytokinesis in *S. pombe*

While the major steps of cytokinesis are similar between animal cells and *S. pombe*, there are some significant differences in its regulation and in the molecules participating in the

process (Glotzer, 2017). Unlike in animal cells, where all cytokinetic steps are inhibited until anaphase, some of the signals for the positioning of the ring start soon after the previous cell division in fission yeast (Glotzer, 2017).

Protein assemblies called nodes are precursors of the contractile ring (Pollard & Wu, 2010). The adaptor anillin-like protein (product of the fission yeast gene *mid1*) appears in interphase nodes along with Cdr1p, Cdr2p, and Wee1p, at the equator of the cell. These interphase nodes depend on the interaction between Cdr2p and Mid1p (Almonacid et al., 2009; Pollard & Wu, 2010). The precise location of these interphase nodes depends on another kinase, Pom1p, which concentrates at the two ends of the cell and inhibits Cdr1p and Cdr2p, thus restricting the nodes to the centre of the cell (Karagiannis, 2012; Pollard & Wu, 2010). This step is crucial since the location of the interphase nodes decides the location of the contractile ring. Before spindle pole body separation, myosin II, the ring assembly protein 2 (Rng2p), Cdc15p, and Cdc12p are added to interphase nodes, making them mature "cytokinetic" nodes (Pollard & Wu, 2010) (**Figure 1-3**).

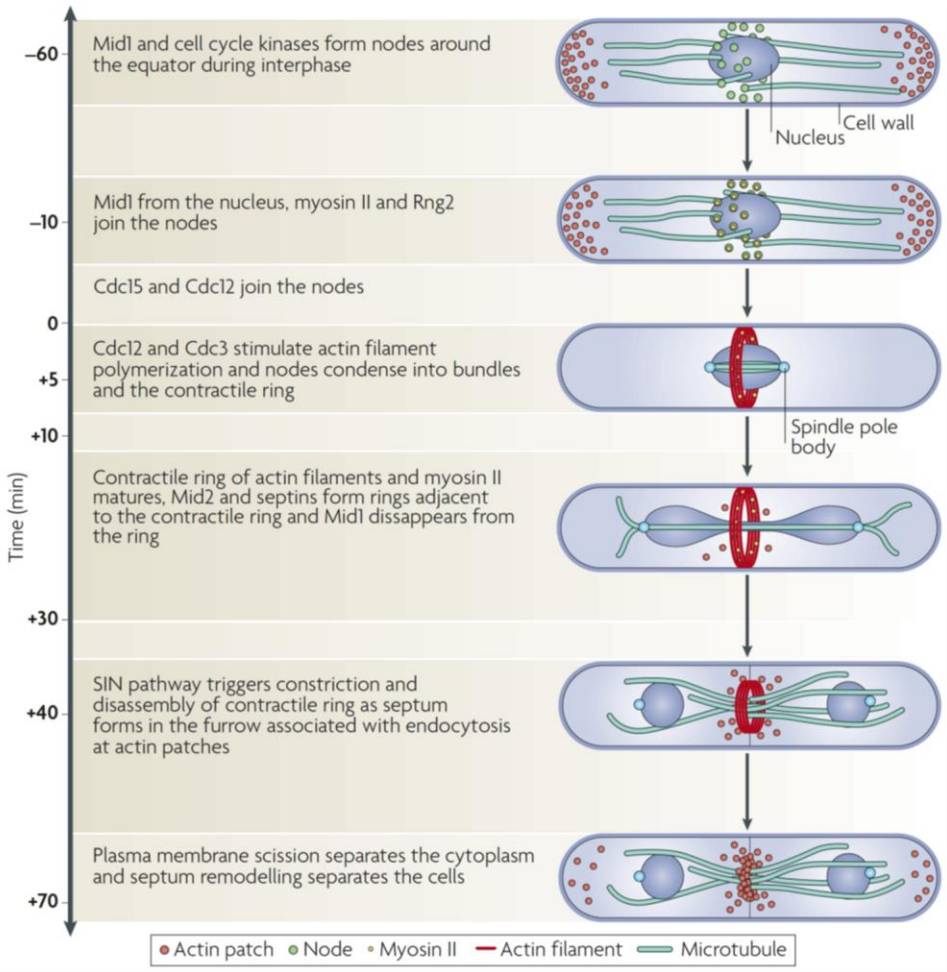

Figure 1-3: A high-resolution timeline of cytokinetic events in fission yeast.

Nodes containing Mid1p (anillin) form near the plasma membrane. These nodes mature by the addition of Myo2p (myosin II), Rng2p (ring assembly protein 2), Cdc15p (F-BAR domain protein), and Cdc12p (formin). Cdc12p and Cdc3p (profilin) stimulate the polymerization of actin filaments. During anaphase the nodes condense into a contractile ring. At the end of anaphase, a signalling pathway called the SIN (the septation initiation network) triggers ring constriction that guides the deposition of cell wall material to form the septum, which divides the cell into two independent compartments. Adapted from *Nat Rev Mol Cell Biol. 2010, 11(2):149–155* under the "fair dealings" provision of the Canadian Copyright Act (2019).

Meanwhile, actin filaments required for the formation of the contractile ring begin assembling. This is dependent on the formin Cdc12p and profilin (Chang et al., 1997; Kovar et al., 2003; Yonetani et al., 2008). Formin nucleates actin filaments from free actin monomers, and together with profilin, they elongate the filament (Pollard & Wu, 2010). Once the actin filaments appear around the equator, the cytokinetic nodes move continuously, eventually condensing along with actin filaments to form the contractile ring (Pollard & Wu, 2010). Once formed, the ring acquires proteins like myosin II, capping protein and Imp2p, and loses Mid1p (Pollard & Wu, 2010). A signalling pathway called the septation initiation network (SIN) regulates maturation of the contractile ring (Glotzer, 2017; Krapp & Simanis, 2008). In the absence of the SIN, the contractile ring is still able to form, but then disassembles prematurely in late anaphase (Karagiannis, 2012). The SIN consists of several proteins including the GTPase Spg1p, protein kinases Cdc7p, Sid1p, Sid2p, the scaffold proteins Cdc11p and Sid4p, and the phosphatase Cdc14p (Jiang & Hallberg, 2001; Karagiannis, 2012).

The Spg1p Ras-like GTPase triggers the SIN pathway (Jiang & Hallberg, 2001; Karagiannis, 2012). The GTP activating protein (GAP) consists of two proteins, Cdc16p and Byr4p, which negatively regulate the SIN pathway (Furge et al., 1998; Karagiannis, 2012). Cdc7p kinase is the immediate downstream target of GTP bound Spg1p. Cdc7p in turn recruits the Sid1p-Cdc14p protein kinase complex to the spindle pole body (Karagiannis, 2012). The final component in the SIN pathway is the Sid2p-Mob1p kinase complex, which localises to the cleavage site during ring constriction, and transmits a signal from the spindle pole body (SPB) to the actomyosin ring, thus triggering its constriction (Hou et al., 2000; Jiang & Hallberg, 2001; Karagiannis, 2012; Salimova et al., 2000; Sparks et al., 1999). Complete constriction of the ring followed by abscission and physical separation of the two new cells completes the process of cytokinesis.

1.5 A cytokinesis monitoring system in *S. pombe*

Cell-cycle progression is monitored and inhibited by checkpoint mechanisms that ensure the correct order of cell cycle events. For example, checkpoint mechanisms ensure that

DNA synthesis is complete before entry into mitosis. A spindle checkpoint also ensures that duplicated chromosomes do not segregate until each is attached to the mitotic spindle apparatus. Since cells lacking checkpoints display genomic instability, it is important for organisms to have these mechanisms operating at different stages of the cell cycle to ensure that the cell cycle does not proceed before the previous step has been successfully completed (Holland & Cleveland, 2009).

The existence of a cytokinesis monitoring system in fission yeast was first suggested by experiments in which the nuclear cycle kinetics of temperature sensitive cytokinesis mutants were carefully analyzed (Cueille et al., 2001; J. Liu et al., 2000; Trautmann et al., 2001). These experiments revealed two groups of cytokinetic mutant behaviour: Group I, comprised of mutants that delay their nuclear cycle upon cytokinetic perturbation, and Group II, comprised of mutants that proceeded with the nuclear cycle unaltered despite failure in cytokinesis (Karagiannis, 2012). The fission yeast Cdc14p family ortholog, Clp1p is a key regulator of the cytokinesis monitoring system since *clpΔ* cells are hypersensitive to a wide variety of mutations that disrupt the actomyosin ring (Karagiannis, 2012). Clp1p, which is usually a non-essential protein, becomes critical for maintaining cell viability when cytokinesis is perturbed (Mishra et al., 2004). In response to perturbation of the actomyosin ring, *S. pombe* cells enter a "cytokinetic competent state" which is characterized by the continuous repair and maintenance of the actomyosin ring (Mishra et al., 2005b). Clp1p and the SIN (Septation Initiation Network) form an integral part of this checkpoint system, which ensures that damaged cell division structures are repaired or reformed to allow cytokinesis (Mishra et al., 2004). In the absence of SIN signaling, the actomyosin ring forms upon entry into mitosis, but then disassembles prematurely in late anaphase (Karagiannis, 2012). Clp1p extends the duration of SIN signaling upon cytokinetic perturbations and helps maintain the integrity of the actomyosin ring, thereby promoting the successful completion of cytokinesis (Karagiannis, 2012).

It has also been shown that Clp1p, once phosphorylated, binds the 14-3-3 protein Rad24p and is thereby retained in the cytoplasm upon cytokinetic perturbations (Mishra et al., 2005a). In the absence of Rad24p, cells fail to maintain SIN signalling and are inviable even upon mild cytokinetic perturbations (e.g. treatment with low doses of Latrunculin A). Latrunculin A (LatA) is a toxin produced by the red sea sponge *Latrunculia magnifica*. LatA is the most extensively used reagent to depolymerize actin filaments in live cells (Fujiwara et al., 2018). LatA functions by sequestering actin monomers, thereby preventing the polymerization of actin. At high concentrations of 20 - 50 µM, LatA completely disrupts the actin cytoskeleton within 20 minutes, ultimately leading to cell death (Karagiannis, 2012). However, when used at lower concentrations (0.2 - 0.5 µM), it mildly perturbs the actin cytoskeleton and activates the cytokinesis monitoring system (Karagiannis, 2012) (**Figure 1-4**).

Interestingly, a recent genome-wide screen performed in *S. pombe* uncovered a set of gene deletion mutants hypersensitive to LatA, indicating that fission yeast cells possess mechanisms that prevent cytoskeletal collapse and cytokinesis failure when treated with the drug (Asadi et al., 2016). One of the genes identified in the screen was *pna1* (encoding a polyamine N-acetyltransferase). Cells deleted for *pna1* exhibit severe growth defects upon exposure to LatA (Asadi et al., 2016). It is also known that polyamines promote actin bundling, and that acetylation of polyamines inhibits their interaction with actin. Taken together, this suggests that polyamine regulation might play a role in responding to LatA induced cytoskeletal stress in fission yeast.

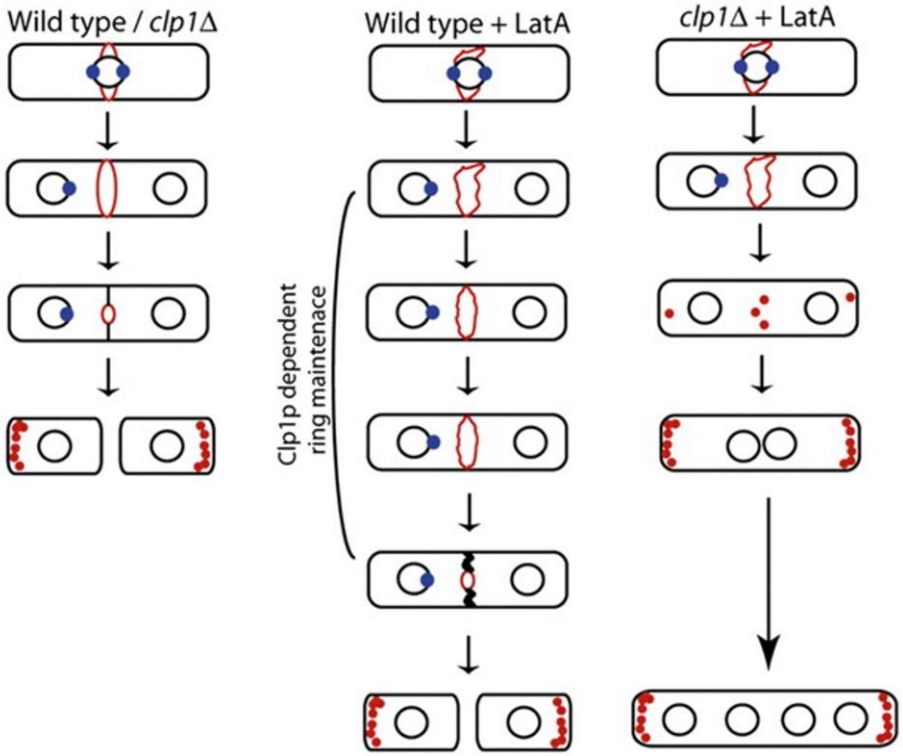

Figure 1-4: The effect of LatA on wild-type cells and *clp1Δ* mutants defective in the cytokinesis monitoring system.

Wild-type cells, as well as mutants defective in the cytokinesis monitoring system (e.g. *clp1Δ*), are viable under normal growth conditions (left). However, upon treatment with low doses of LatA, wild-type cells delay entry into mitosis and activate the Clp1p dependent extension of SIN signaling (middle). Mutants defective in this process are unable to delay mitosis and fail in ring constriction. As a result, cells become multinucleated and are inviable (right). The actomyosin ring is shown as a red circle. Actin patches are shown as red dots. The nucleus is shown as a black circle. The spindle pole body (active) is shown as a blue dot. Adapted from *J Cell Sci. 2004, 117:3897-3910* under the "fair dealings" provision of the Canadian Copyright Act (2019).

1.6 Polyamines and their regulation

Polyamines are low molecular weight cations present in all living cells. They interact with negatively charged molecules, including actin. Spermine, spermidine, and putrescine are among the most commonly occurring polyamines in bacteria and fungi (**Figure 1-5**) (Gevrekci, 2017). Biosynthesis of polyamines occurs through the coordinated action of two decarboxylases, ornithine decarboxylase (ODC), and S-adenosylmethionine decarboxylase (SAMDC), and two synthases, spermidine synthase, and spermine synthase (Firpo & Mounce, 2020; W. Sun et al., 2020). Polyamines play roles in a variety of cellular functions ranging from gene expression, cell growth, stress response, and proliferation (Michael, 2016; Miller-Fleming et al., 2015). The role of polyamines in cell growth is among the most important, and studies have shown that actively growing cells have higher levels of polyamines compared to cells that are not actively growing (Das & Kanungo, 1982; Yoshinaga et al., 1993).

Polyamines are conserved across organisms and altered levels of polyamines have been observed in several diseases in mammals including humans (Bewley et al., 2006). Because of the variety of functions that polyamines perform, it is important for cells to strictly regulate polyamine levels (Gevrekci, 2017). Polyamine transporters found in the plasma membrane help in maintaining an influx and efflux of polyamines, and this is one of the important ways in which the cell regulates intracellular polyamine levels (Gevrekci, 2017). Polyamine acetylation appears to be necessary for their breakdown and export from cells (Casero & Pegg, 1993). Acetylated polyamines are either exported from the cell or metabolized by polyamine oxidases (Casero & Pegg, 1993). Polyamine acetyltransferases are conserved across species. For example, the human gene aralkylamine N-acetyltransferase (AANAT), and the budding yeast gene polyamine acetyltransferase (*paa1*), among others, are orthologs of the fission yeast gene *pna1* that has been analysed for this study.

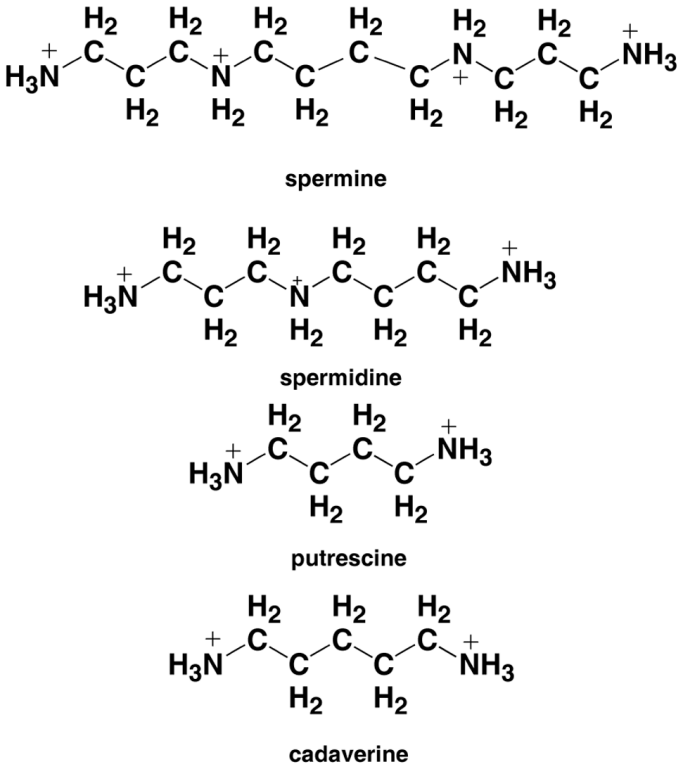

Figure 1-5 Chemical structure of the common natural polyamines (spermine, spermidine, putrescine, and cadaverine).

Adapted from *PLoS One. 2013, 8(7):e70510* under the "fair dealings" provision of the Canadian Copyright Act (2019).

Polyamines such as spermine and spermidine are also known to promote actin bundling in vitro (Grant et al., 1983; Oriol-Audit, 1978, 1982). Synthetic polyamines like macrocyclic polyamines (MPAs) and acyclic branched polyamines (BPAs) are known to promote actin bundling and subsequently promote rapid actin-rich lamellipodia (Nedeva et al., 2013). In *S. pombe*, it was observed that MPAs and BPAs slow down actomyosin ring constriction up to 50% (Riveline et al., 2014). Acetylation decreases the net positive charge of polyamines, thereby decreasing their ability to bind and bundle actin filaments. Thus, the acetylation of polyamines by Pna1p may inhibit their interaction with actin, subsequently preventing actin bundling.

1.7 Cytokinesis defects and genomic integrity

The complexity of the cytokinetic process makes it vulnerable to changes that result in cell division failure and the formation of abnormal binucleate cells (Potapova et al., 2013). If cytokinesis fails completely, the resulting tetraploid cell can be the starting point for the development of future aneuploidies (Lens & Medema, 2019). The consequences of such changes were first considered more than 100 years previously by Theodor Boveri who suggested a connection between failed cytokinesis and tumour development. Since then, evidence supporting this idea has accumulated leading to increased interest in the mechanisms mediating the failure of cytokinesis (Lens & Medema, 2019; Sagona & Stenmark, 2010).

Cytokinesis defects promote genetic diversification through a combination of chromosomal instability (CIN) and the buffering effects of tetraploidy, which supports the generation of aneuploid cells (Lens & Medema, 2019). Tetraploidy arises from three pathways: 1) endoreduplication – replication of the genome in the absence of an intervening mitosis, 2) cell fusion – the fusion of multiple uninuclear cells to form one multinuclear cell, 3) cytokinesis/mitotic failure (Lim & Ganem, 2014). Of these three pathways, cytokinesis or mitotic dysfunction is thought to be the most common cause of tetraploidy in human cells (Lim & Ganem, 2014). Cytokinetic failure can occur at any of the multiple steps involved in this complex process. First, it can fail when furrow

ingression is not initiated. This may happen when Plk1 (Polo kinase, activator of RHO GTPase), or GEF (guanine nucleotide exchange factor), or ROCK (Rho associated protein kinase) are inhibited at the onset of anaphase. Second, it can fail when ingression of the cleavage furrow is not completed; this happens when the chromosomal passenger complex (CPC) is disturbed. Lastly, it can fail at the last stage of cytokinesis, abscission when mediators of abscission like CEP55 or spastin are absent from the midbody (Lens & Medema, 2019).

Cytokinesis failure results in increased CIN because tetraploid cells often contain double the normal number of centrosomes, which results in the generation of multipolar spindles and chromosome segregation defects in the next mitosis (**Figure 1-6**). Proliferation of tetraploid cells is a major source of genomic instability since it leads to inaccurate mitosis and generates daughter cells with altered number of chromosomes (Ganem et al., 2009; Gisselsson et al., 2010; Godinho & Pellman, 2014; Nigg, 2006). CIN is a common feature of almost all tumours, and the level of instability is usually proportional to its level of aneuploidy (Duesberg et al., 1998; Gordon et al., 2012).

Aneuploidy is a condition in which cells gain or lose chromosomes. As described above, this condition arises as a consequence of CIN and is a common characteristic of many human cancers (Lv et al., 2012). There are many reports demonstrating that tumour cells have multiple centrosomes and centrosomal abnormalities (Sagona & Stenmark, 2010). Human breast tumours, for example, have increased number of chromosomes and microtubule nucleating capacity (Lingle et al., 2002).

Thus, tetraploidy (which may arise through cytokinesis failure) may have a significant and underappreciated role in tumour development. Computational analysis from around 4000 human cancers indicate that almost 40% of human tumours have undergone tetraploidization at some point during their progression (Zack et al., 2013). This makes the study of cytokinesis, its regulation, and the biological mechanisms that promote its reliable execution, fundamentally important.

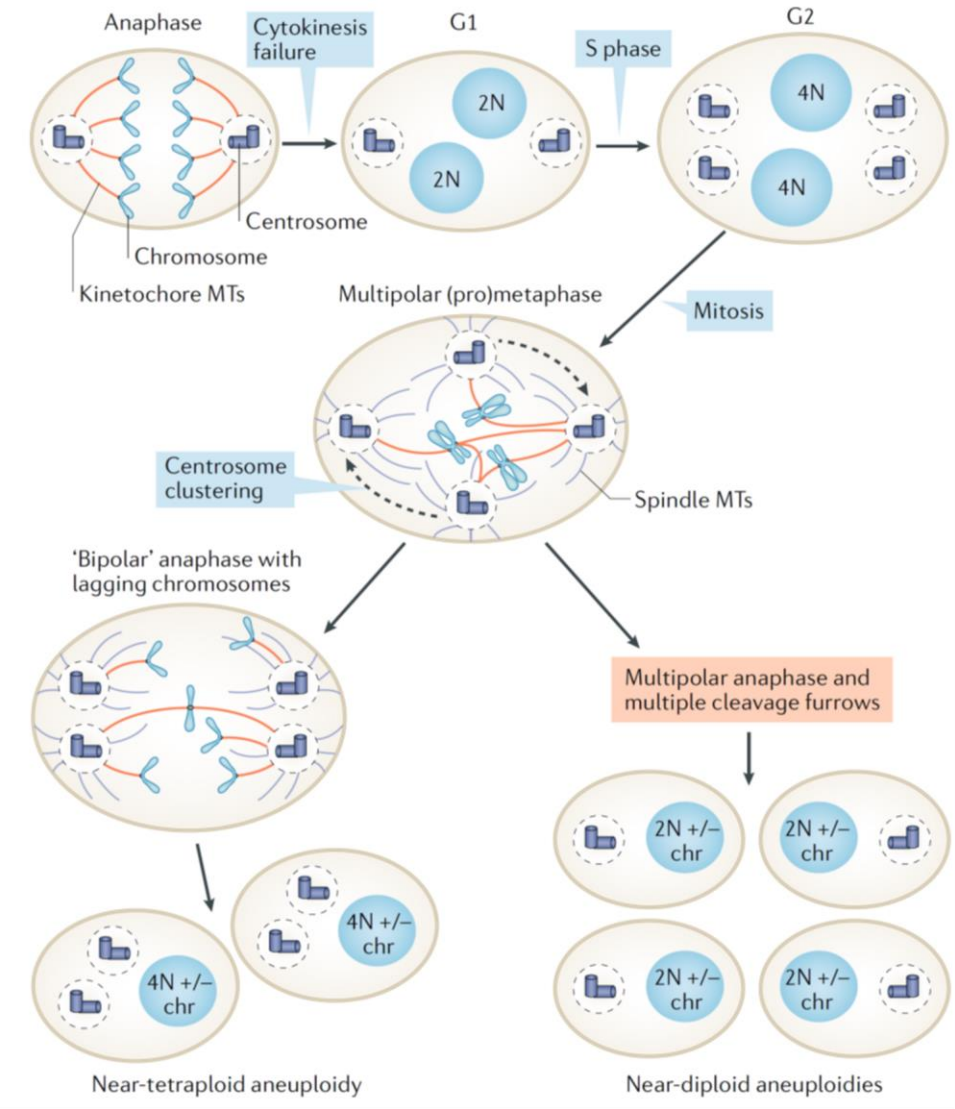

Figure 1-6: The consequences of cytokinesis failure.

Cytokinesis failure leads to the generation of a cell with twice the normal number of chromosomes and centrosomes. In the subsequent mitosis, the presence of the extra centrosomes can give rise to a multipolar spindle leading to multiple cleavage furrows and the production of aneuploid offspring. A "pseudo" bipolar spindle (due to centrosome clustering) leading to lagging chromosomes and aneuploidy is also possible. Adapted from *Nat Rev Cancer. 2019, 19(1):32–45* under the "fair dealings" provision of the Canadian Copyright Act (2019).

1.8 Rationale and objectives

Polyamines are essential for a variety of functions in the cell including gene expression, cell growth and proliferation, and stress response (Michael, 2016; Miller-Fleming et al., 2015). However, the role of the acetylation of polyamines has not been explored in the context of cytokinetic perturbation. Given that the polyamine-N-acetyltransferase gene deletion (*pna1Δ*) was hypersensitive to low doses of the actin depolymerizing drug LatA, displaying increased rates of cytokinesis failure (Asadi et al., 2016), I initiated a project aimed at characterizing the previously unstudied fission yeast *pna1* gene, and at better understanding the function of *pna1* with respect to its role in cell division. I hypothesised that loss of *pna1* confers sensitivity to LatA due to improper constriction of the actomyosin ring leading to cell division failure. In order to test this hypothesis, I set the following objectives initially:

1. To verify the genotype and phenotype of the Bioneer *pna1Δ* gene deletion strain via colony PCR and spot assays, respectively.
2. To provide a detailed and quantitative characterization of the effects of LatA on cytokinesis in the *pna1Δ* mutant by assessing cytokinesis failure rates via fluorescence microscopy.
3. To monitor the kinetics of actomyosin ring constriction in *pna1Δ* mutants in comparison to wild-type cells using strains expressing the actomyosin ring marker, Rlc1-GFP.
4. To characterize the subcellular localization of Pna1p by constructing a strain expressing a C-terminal Pna1-GFP fusion and visualising it using fluorescence microscopy.

While carrying out my fourth objective, we unexpectedly discovered that Pna1p formed unusual filamentous structures. Hence, I shifted the focus of the project towards experiments aimed at my fifth objective:

5. To better understand and characterize the observed Pna1p filaments.

By pursuing these objectives, I hoped to provide novel biological insight into the function of Pna1p with respect to its role in cell division.

Chapter 2

2 MATERIALS AND METHODS

2.1 Strains, growth media, and culture conditions

Chemically competent *E. coli* cells from New England Biolabs (High Efficiency NEB 5-alpha) were used for all bacterial transformations. Following transformation, cells were grown overnight on Luria-Bertani (LB) agar media containing 100 μg/mL ampicillin at 37ºC. 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 1.5 mL of culture using the High-Speed Plasmid MiniKit (GeneAid).

S. pombe strain JK484 (*ura4-D18 leu1-32 ade6-210 his3-D1 h*$^-$) 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 30ºC with constant shaking at 150 rpm (Alfa & Cold Spring Harbor Laboratory, 1993). The cells were grown to mid-log phase (OD_{600} of 0.2 - 0.4) for transformation by the lithium acetate method (Forsburg & Rhind, 2006). For selection, strains were grown on Edinburgh Minimal Media (US Biologicals) with the appropriate supplementary amino acids (adenine, uracil, leucine or histidine) (Forsburg & Rhind, 2006). For constructing the double mutants, SPAS mating media was used (Forsburg & Rhind, 2006).

2.2 Latrunculin A treatment

S. pombe cells were grown to mid-log phase (OD_{600} of 0.2 - 0.4) and treated with low doses (0.1 - 0.2 μM) or high doses (25 μM) of LatA dissolved in DMSO (Karagiannis et al., 2005; Karagiannis & Balasubramanian, 2007). The treated cells were grown at 30ºC with shaking at 150 rpm for the indicated durations before fixation with ethanol. Cells were separately treated with DMSO (solvent) as a negative control in experiments involving LatA.

2.3 Spot assays

S. pombe cells were grown to mid-log phase (OD_{600} of 0.2 - 0.4) and ten-fold serial dilutions of the respective cultures spotted onto YES-agar media containing DMSO (solvent control) (Karagiannis et al., 2005; Karagiannis & Balasubramanian, 2007) or varying concentrations of LatA. The plates were allowed to dry and then incubated in a 30ºC incubator for 3-4 days. The plates were then imaged using a Uvitec Firereader V10 gel documentation system.

2.4 Molecular techniques

2.4.1 Verification of the *pna1* gene deletion mutant

The *pna1* deletion mutant (*pna1::kanMX ura4-D18 leu1-32 ade6-210* h^+) was revived from the Bioneer gene deletion library. This mutant has the *pna1* open reading frame replaced with the *kanMX* selectable marker conferring resistance to the antibiotic G418 (Kim et al., 2010). The strain was verified by colony PCR using gene specific primers MR1 and MR2 and primers CPC1, CPC3, CPN1, CPN10 specific to the *kanMX* deletion cassette (**Table 2-1)**. Colony PCR reactions were performed using 2X FroggaBio Taq Mix according to the manufacturer's instructions. Amplicons were analyzed by gel electrophoresis (1% agarose).

2.4.2 Creation of Pna1-GFP integrant strain

The *pna1*-GFP integrant strain was created using a PCR based cloning method. A C-terminal fragment of the *pna1* gene was PCR amplified from wild-type *S. pombe* genomic DNA using primers MR5 and MR6 incorporating *EcoR*I and *Xma*I sites (**Table 2-1**). The amplicon was then digested with *EcoR*I and *Xma*I and cloned in-frame to the GFP gene in the pJK210-GFP vector. Successful ligation of the C-terminal fragment into the vector was confirmed by DNA sequencing. The confirmed pJK210-pna1-GFP plasmid was then linearized within the *pna1* sequence using *Bsp*EI (to promote integration at the *pna1* locus during the subsequent transformation). The linearized plasmid was then transformed into a *ura4-D18 S. pombe* strain and plated onto EMM

media lacking uracil to select for transformants that have integrated the vector (Forsburg & Rhind, 2006). To determine if the vector integrated at the *pna1* locus, a forward primer specific to *pna1* sequences upstream of the amplified fragment (MR7), and a reverse primer specific to sequences within the *gfp* gene (MR8) were used to perform colony PCR reactions on Ura^+ colonies derived from the transformation (**Table 2-1**). An untransformed wild-type yeast colony was used as a template for the negative control reaction. Colony PCR reactions were performed using 2X FroggaBio Taq Mix according to the manufacturer's instructions. Amplicons were analyzed by gel electrophoresis (1% agarose).

2.4.3 Creation of *pna1* overexpression constructs

The *pna1* overexpession construct was created using the pREP41 vector containing the thiamine repressible *nmt41* promoter. The full length *pna1* ORF was PCR amplified from wild-type *S. pombe* genomic DNA using primers (MR1a, MR1b) incorporating *Nde*I and *Xma*I restriction sites (**Table 2-1**). The fragment was then digested with *Nde*I and *Xma*I and cloned downstream of the *nmt41* promoter (Maundrell, 1993). Successful ligation of *pna1* into the vector was confirmed by DNA sequencing. The construct was then transformed into a *leu1-32 S. pombe* strain expressing Pna1-GFP and plated onto EMM media lacking leucine to select for transformants carrying the plasmid. To over-express *pna1*, strains carrying the vector were first grown in media containing thiamine, and then shifted to media lacking thiamine to allow induction of *pna1* expression. These cells, as well as controls maintained in the presence of thiamine were then fixed with 3.7% formaldehyde prior to visualisation.

Table 2-1: Primer List

Primer	Sequence (5'-3')	Use
MR1	TTTTGTTTTTGTTCATAAACCACCC	Forward – *pna1* genotyping, left junction
MR2	AGTTTTTACGGTTTATGTCTTGCAA	Reverse – *pna1* genotyping, right junction
CPC1	TGATTTTGATGACGAGCGTAAT	Forward – *pna1* genotyping, right junction
CPC3	GGCTGGCCTGTTGAACAAGTCTGGA	Forward – *pna1* genotyping right junction
CPN1	CGTCTGTGAGGGGAGCGTTT	Reverse – *pna1* genotyping, left junction
CPN10	GATGTGAGAACTGTATCCTAGCAAG	Reverse – *pna1* genotyping, left junction
MR5	GGCGGGAATTCTGAATTTGCTTTCCTGAAGCAGAG	Forward- *pna1* amplification, GFP fusion
MR6	GGCGGCCCGGGTATTTTGTGGGTCATGTCATACC	Reverse – *pna1* amplification, GFP fusion
MR7	CAGTTTAAGAGGTCGTTTTCTGAAC	Forward – *pna1-gfp* genotyping
MR8	TGGGACAACTCCAGTGAAAA	Reverse – *pna1-gfp genotyping*
MR1a	GGCGG CAT ATG GTCAACATTCGTAATATCGA	Forward – *pna1* amplification, overexpression
MR1b	GGCGGCCCGGGTTATATTTTGTGGGTCATGTCATACC	Reverse – *pna1* amplificaiton, overexpression

2.5 Genetic techniques

2.5.1 Co-segregation analysis

Wild-type (h^-) and *pna1*Δ cells (h^+) were mixed in 2 µL of sterile water on sporulation agar (SPA) plates (Forsburg & Rhind, 2006). The plates were then incubated at room temperature for 48 hours. Individual asci were isolated using a micromanipulator attached to a Zeiss Axioskop 40 microscope and transferred to YES-agar plates. The tetrads were arranged in rows and allowed to germinate by incubating at 30ºC. The individual spores were then separated using the micromanipulator and the plates incubated at 30ºC to form colonies. The colonies were then plated to media containing the antibiotic G418 (100 µg/mL) to identify strains bearing the *pna1* deletion, and to media containing LatA (0.2 µM LatA) to identify strains displaying LatA sensitivity.

2.5.2 Construction of *pna1Δ* cells expressing Rlc1-GFP

To create a *pna1*Δ strain expressing Rlc1-GFP, fission yeast cells expressing the *rlc1-gfp* gene (h^-) were allowed to mate with a *pna1*Δ strain (h^+) on SPA plates and progeny isolated as described in **Section 2.4.1**. Progeny were plated on EMM media lacking uracil to select for the *rlc1-gfp::ura4*$^+$ construct and on media containing the antibiotic G418 (100 µg/mL) to identify strains bearing the *pna1* deletion.

2.6 Fluorescence microscopy

2.6.1 DAPI/Aniline blue and actin staining

S. pombe cells were treated with the indicated concentrations of LatA. For DAPI/Aniline blue staining, cells were fixed by adding 500 µL of ice-cold ethanol to 1000 µL of culture (Forsburg & Rhind, 2006). The cells were washed once with PBS pH 7.4 containing TritonX-100, followed by three washes with PBS pH 7.4. The cells were then stained with a mixture containing 0.02 mg/mL 4′6,-diamidino-2-phenylindole (DAPI) and 1 mg/mL aniline blue, to stain the nucleus and cell wall material (septa) respectively. For staining with the actin stain Alexa Fluor™ 568 Phalloidin, cells were treated with

indicated concentrations of LatA and then fixed with 3.7% formaldehyde. Then, cells were stained with Alexa Fluor™ 568 Phalloidin at a final concentration of 1µg/µL (Hagan, 2016). Images were taken using a Leica DMI 6000B microscope with a BD CARV II Confocal Imager.

2.6.2 Visualising strains expressing Pna1-GFP fusion proteins

Cells expressing Pna1-GFP fusion proteins were grown in YES media and treated (+/- LatA, +/- MBC, +/- Torin-1, 0°C/37°/45°C, or variation in growth phase) as indicated in the Results section. The cells were then fixed with 3.7% formaldehyde and images taken using a Leica DMI 6000B microscope fitted with a GFP filter set. Where indicated, Z-series were obtained by imaging 21 slices, 0.4 µm apart. A maximum projection of all slices into one frame was created using Metapmorph software. For time-lapse experiments, cells were grown to mid-log phase and imaged after placing 2 µL of culture onto a 2% YES-agarose pad that had been laid on top of a microscope slide. Cells were then immobilized with a cover slip and imaged using a Leica DMI 6000B microscope fitted with a GFP filter cube.

2.6.3 Visualising strains expressing Rlc1-GFP fusion proteins

Cells expressing Rlc1-GFP fusion proteins were grown in YES media to mid-log phase and treated with LatA or DMSO as indicated in the Results section. The cells were imaged after placing 2 µL of culture onto a 2% YES-agarose pad that had been laid on top of a microscope slide. Cells were then immobilized with a cover slip and imaged using a Leica DMI 6000B microscope fitted with a GFP filter cube.

2.7 Statistical analysis

For cytokinetic assays and for monitoring ring constriction kinetics, one-way ANOVA tests followed by Tukey's post-hoc test were performed. For cytokinetic assays, three trials were performed with each trial having 200 cells per sample being counted. The ratio of cells with fragmented septa to cells with unfragmented (i.e complete and functional septa) for each of the samples and trials were analyzed by one-way ANOVA

test. For ring constriction kinetics, 15 cells were counted for each group and the average duration of ring constriction was analyzed by one-way ANOVA. For both experiments, null hypotheses were rejected if p-values ≤ 0.05.

Chapter 3

3 RESULTS

The model eukaryote *S. pombe* (commonly known as fission yeast) is known to possess cellular mechanisms that prevent cytokinesis failure upon perturbation of the actin cytoskeleton (e.g. treatment with low doses of the actin depolymerizing drug, LatA) (Asadi et al., 2016, 2017; Grewal et al., 2012; Karagiannis, 2012; Karagiannis et al., 2005; Karagiannis & Balasubramanian, 2007; Mishra et al., 2004, 2005b; Rentas et al., 2012; Saberianfar et al., 2011). To uncover the complete set of *S. pombe* genes with roles in adapting to LatA induced cytoskeletal perturbations, a genome-wide screen was previously conducted by members of the Karagiannis lab (Asadi et al., 2016). One of the genes identified in this screen, *pna1*, encodes a polyamine N-acetyltransferase. These results suggested that the regulation of polyamine metabolism might play a role in modulating actin cytoskeletal dynamics, thereby affecting cytokinesis. To explore this possibility further, I initiated a project aimed at further characterizing the role of *pna1* in preventing cytokinesis failure. The results of this study are described below.

3.1 Genotyping of the Bioneer *pna1Δ* gene deletion strain

Due to the possibility of 1) errors within the commercially available Bioneer gene deletion collection (Kim et al., 2010), or 2) strain cross-contamination during the course of the original genome-wide screen, the present study was initiated by first confirming the genotype of the Bioneer *pna1Δ* gene deletion strain. A yeast colony PCR was performed using cells of the *pna1Δ* mutant as template. Forward and reverse primers (MR1 and MR2) specific to sequences up- and down-stream of the deletion cassette, together with staggered primers specific to the *kanMX* gene deletion cassette (CPN1, CPN10, CPC1, CPC3) were used (**Figure 3-1A**). Bands of 750 bp (MR1 and CPN1), 850 bp (MR1 and CPN10), 1000 bp (MR2 and CPC1), and 980 bp (MR2 and CPC3) were predicted if the *kanMX* cassette was indeed present in the place of the *pna1* ORF. A wild-type strain was used as a control for non-specific amplification since this strain lacked the

kanMX cassette. As expected, no amplicons were observed in any of the PCR reactions that used wild-type cells as template, while amplicons of the predicted sizes were present in the respective reactions using *pna1Δ* cells as template (**Figure 3-1B**). These results confirmed that the putative *pna1Δ* strain from the Bioneer gene deletion collection was indeed deleted for *pna1*.

3.2 Phenotypic characterization of the *pna1Δ* mutant

The initial genome-wide screen involved the high-throughput screening of ~ 4000 gene deletion mutants for sensitivity to LatA (0.1 – 0.3 µM). As this phenotype formed the basis of the current study, I first confirmed the results of the screen with respect to the growth of *pna1Δ* mutants on LatA containing media. To this end, spot assays were conducted with both *pna1Δ* and wild-type (control) strains. The cells were grown overnight in YES media and harvested in mid-log phase. They were then serially diluted and spotted onto YES-agar media containing DMSO (solvent control) or varying concentrations of LatA. As shown in **Figure 3-2**, wild-type cells grow on the DMSO and LatA containing plates while the *pna1Δ* grows to the same extent as wild-type on the DMSO containing media, but struggled to grow beyond the first dilution upon exposure to LatA. This confirmed the originally observed hypersensitive growth phenotype of the *pna1Δ* mutant upon exposure to LatA.

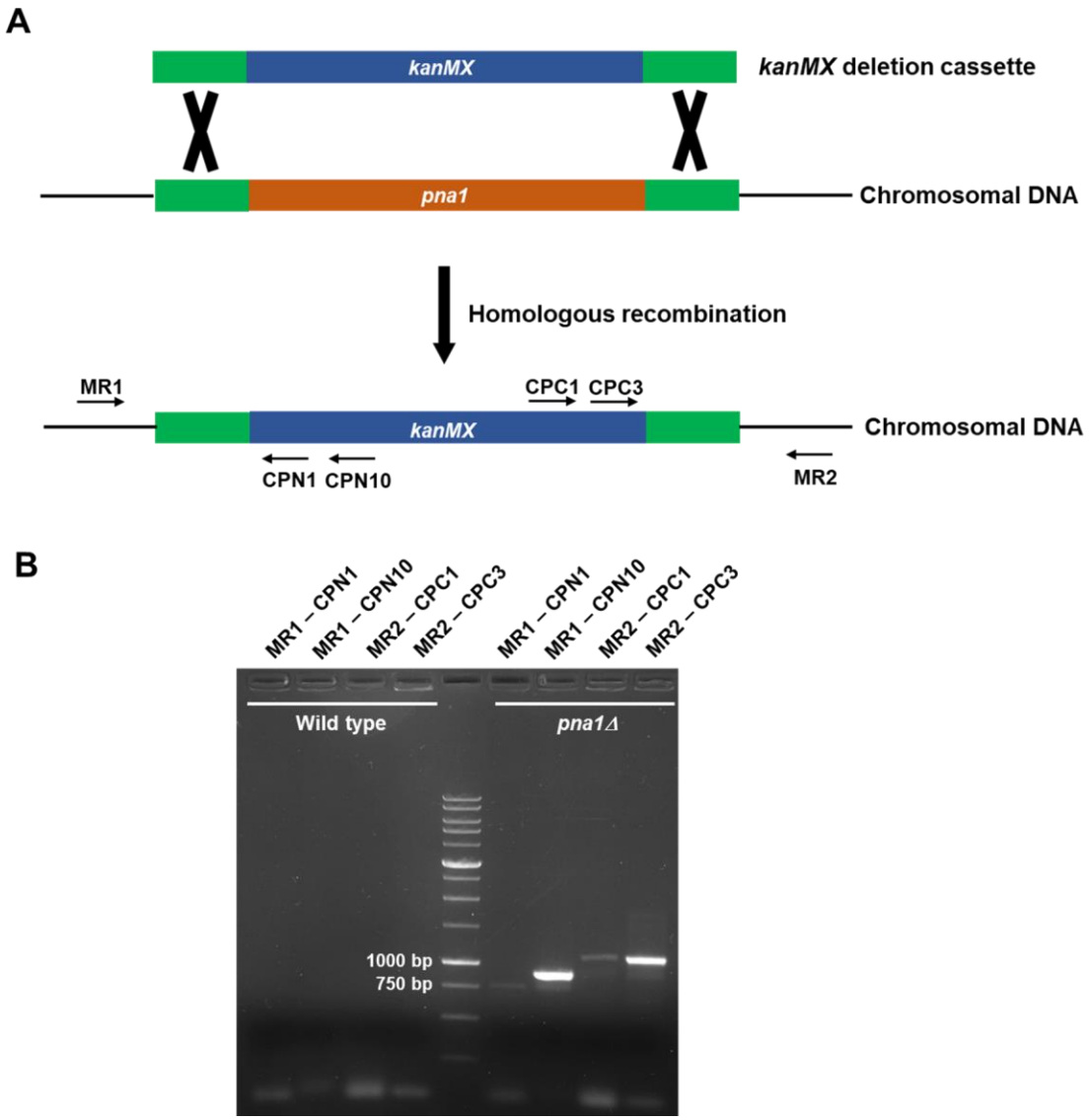

Figure 3-1: Genotyping of the Bioneer *pna1*Δ deletion strain.

(A) Schematic of the *pna1* locus before and after integration of the *kanMX* deletion cassette. The location and orientation of primers used for genotyping (MR1, MR2, CPN1, CPN10, CPC1, CPC3) are shown with black arrows. **(B)** Colony PCR reactions using *pna1Δ* and wild-type colonies as template were carried out with the indicated primer pairs. The resulting amplicons were run on a 1% agarose gel to confirm the presence/absence of the *kanMX* cassette at the *pna1* locus.

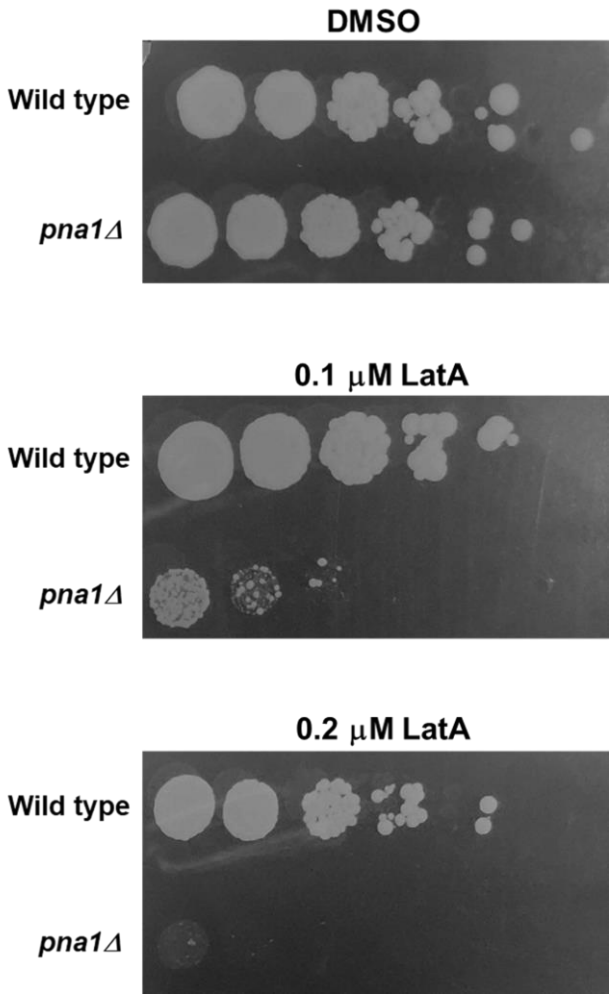

Figure 3-2: Fission yeast cells deleted for the *pna1* gene are inviable on media containing LatA.

Ten-fold serial dilutions of *pna1*Δ and wild-type (control) cells were plated on YES-agar media containing LatA or DMSO (solvent control) and incubated for three days at 30°C.

3.3 Co-segregation analysis

In order to confirm that the LatA-sensitive phenotype observed in *pna1Δ* mutants was indeed due to the deletion of the *pna1* ORF – i.e to rule out the possibility that a background mutation(s) might be the root cause of the growth defect – a cosegregation analysis was performed. Both wild-type and *pna1Δ* cells of the opposite mating type were allowed to mate on SPA media and the resulting asci dissected on YES-agar plates as described in **Section 2.4.1**. After dissection, the plates were incubated at 30°C for 5-7 days. Colonies derived from the individual spores of each tetrad were then picked and streaked to a YES-agar master plate and grown at 30°C overnight. The colonies were then streaked on LatA containing media (to observe the segregation of the LatA-dependent growth defect) and on media containing the antibiotic G418 (to observe the segregation of the $G418^R$ phenotype conferred by the *kanMX* deletion cassette). As shown in **Figure 3-3**, I observed a clear 2:2 segregation of growth:no growth on both the LatA, as well as the G418 containing plates. I also saw 100% co-segregation of LatA sensitivity to G418 resistance. This confirmed that the observed LatA sensitivity in *pna1Δ* was in fact monogenic and the deletion of *pna1* was responsible for the observed phenotype.

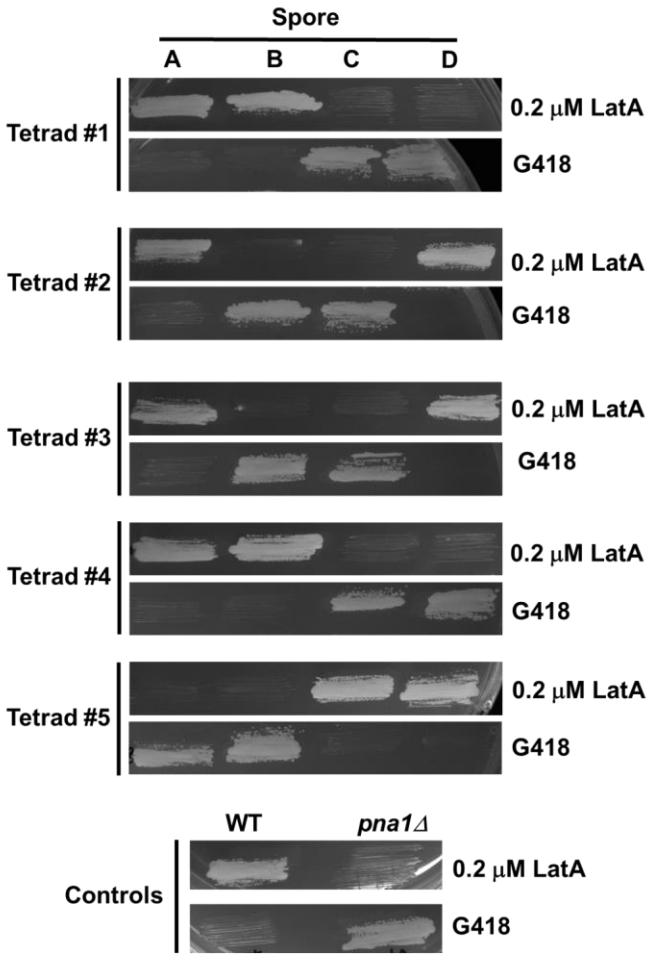

Figure 3-3: Co-segregation analysis.

Wild-type and *pna1Δ* cells of the opposite mating type were mated on SPA media and the resulting asci dissected on YES-agar plates. Colonies derived from the individual spores (labelled A – D) of each tetrad (labelled 1 – 5) were then picked and streaked to both LatA containing media and to media containing the antibiotic G418.

3.4 The *pna1* gene is required for the successful completion of cytokinesis upon exposure to LatA

Having confirmed the genotype and phenotype of the Bioneer *pna1* deletion strain, and having definitively demonstrated that deletion of the *pna1* ORF is indeed the root cause of the strain's LatA sensitivity, I turned my attention to characterizing the effects of LatA on cytokinesis in the *pna1Δ* mutant. LatA is commonly used as a tool to perturb the actin cytoskeleton. It prevents actin polymerization by sequestering actin monomers. Since the *pna1Δ* mutant struggled to grow in the presence of LatA, I hypothesized that this growth defect was due to the disruption of cytokinesis. In order to test this hypothesis, *pna1Δ* and wild-type cells were grown to mid-log phase in YES media. The cultures were then treated with DMSO (solvent control) or 0.2 µM LatA for 6 hours at 30ºC with constant shaking. The cells were then fixed with ethanol and stained with a mixture of DAPI (nuclear stain) and aniline blue (septum/cell wall stain) as described in **Section 2.5.1**. The cells were then examined using fluorescence microscopy under the DAPI filter to visualise both the nuclei and the septa of the cells. The cells were categorized into one of four groups: uninucleate cells, binucleate cells with a complete (i.e. functional) septum, binucleate cells with a fragmented (i.e. non-functional) septum, and tetranucleate cells (indicating two successive rounds of cytokinesis failure). The ratio of cells with fragmented septum to uninucleate cells or binucleate cells with a complete septum was calculated (**Table 3-1**).

Table 3-1: Mean percentage of cells (+/- SD) displaying the indicated phenotype after 6 hours treatment with 0.2 µM Latrunculin A (n=3).

Ratios not sharing a common superscript indicate a statistically significant difference.

					
Genotype	**Uninucleate**	**Binucleate (Complete Septum)**	**Binucleate (Fragmented Septum)**	**Tetranucleate (Fragmented Septum)**	**Ratio (Fragmented/Non-fragmented)**
Wild-type (DMSO)	86 +/- 7	13 +/- 6	1 +/- 1	0	0.01[a]
Wild-type (LatA)	65 +/- 8	18 +/- 6	16 +/- 13	1 +/- 1	0.17[a]
***pna1Δ* (DMSO)**	84 +/- 6	13 +/- 6	3 +/- 1	0	0.03[a]
***pna1Δ* (LatA)**	8 +/- 3	5 +/- 2	86 +/- 1	1 +/- 1	6.69[b]

As shown in **Figure 3-4**, *pna1Δ* and wild-type cells were predominantly uninucleate or binucleate with a fully formed septum when treated with DMSO (solvent control). However, unlike wild-type, the majority of *pna1Δ* cells were binucleate with fragmented septa or tetranucleate when treated with LatA. The ratio of cells with fragmented septum to uninucleate cells or binucleate cells with a complete septum was also much higher for the *pna1Δ* cells relative to wild-type cells treated with LatA. To determine if there is a significant difference between the different strains in their ability to complete cytokinesis, a one-way ANOVA test was carried out on the ratios of cells with fragmented/unfragmented septa. This was followed by a post-hoc Tukey test which indicated that there was a significant difference between *pna1Δ* treated with DMSO and *pna1Δ* treated with LatA, between wild-type cells treated with DMSO and *pna1Δ* treated with LatA, and between wild-type cells treated with LatA and *pna1Δ* treated with LatA (**Table 3-1**). These data indicate that the LatA sensitivity conferred by deletion of the *pna1* gene, results from the inability of these cells to successfully complete cytokinesis. Importantly, cytokinesis failure was not observed in *pna1Δ* or wild-type strains in the presence of the solvent control, DMSO. This indicates that *pna1Δ* mutants do not exhibit generalized defects in cytokinesis, but instead defects specific to conditions in which the cell division machinery is perturbed.

3.5 Monitoring the kinetics of actomyosin ring constriction

My previous work demonstrated that the deletion of *pna1* results in the failure of cytokinesis upon exposure to LatA. In order to more closely examine the cell division process, I constructed a *pna1Δ* mutant expressing Rlc1-GFP, a known marker of the actomyosin ring. A wild-type strain expressing Rlc1-GFP was used as a control. Wild-type and *pna1Δ* cells (both expressing Rlc1-GFP) were grown to mid-log phase and then treated with DMSO/LatA. Live cells were immobilized on a 2% agarose pad and observed through time lapse microscopy as described in **Section 2.5.3**.

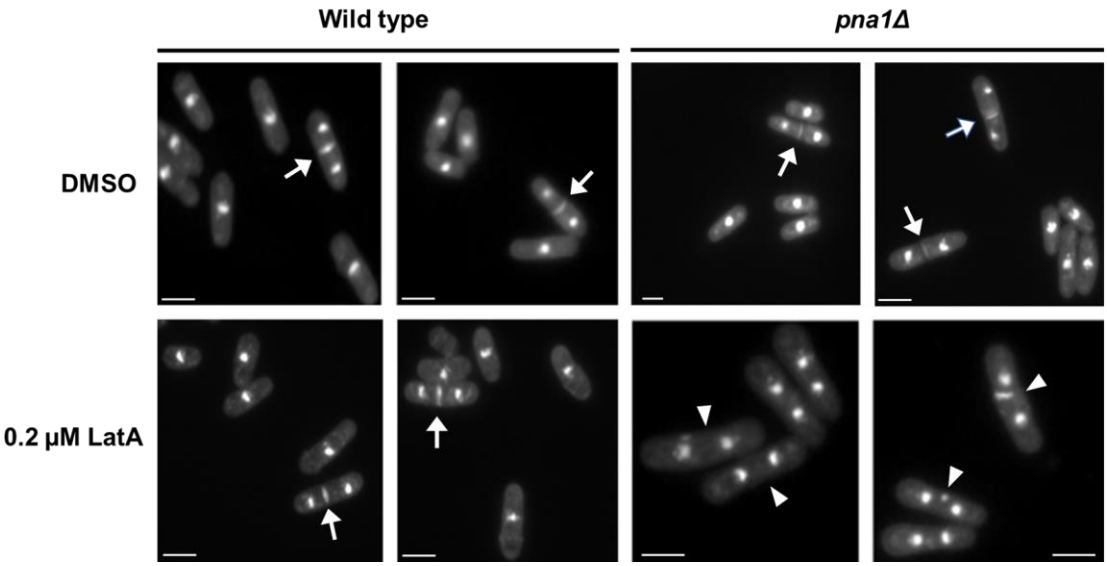

Figure 3-4: The *pna1* gene is needed for the successful completion of cytokinesis upon exposure to LatA.

Wild-type and *pna1*Δ strains were grown to mid-log phase in YES media at 30ºC and then treated with 0.2 µM LatA or DMSO (solvent control) for 6 hrs. Cells were fixed with ethanol and stained with a mixture of DAPI and aniline blue to visualise the nucleus and cell wall/septum respectively. Arrows indicate binucleate cells with functional septa. Arrowheads indicate binucleate cells with fragmented septa or binucleate cells with no observable septal material. Scale bars represent 5 µm.

Upon treatment with DMSO, the Rlc1-GFP strain took approximately 36 minutes to complete constriction of the ring, while the *pna1Δ* Rlc1-GFP strain took approximately 54 minutes. While the time taken for constriction was increased in the case of the *pna1Δ* mutant, the ring still constricted completely allowing for successful cell division. Upon treatment with LatA, the Rlc1-GFP strain took approximately 52 minutes for complete constriction of the ring. However, in the case of *pna1Δ* Rlc1-GFP, the ring began to constrict, but soon fragmented, and eventually disappeared after approximately 13 minutes. To determine the statistical difference between the average times taken for the ring to constrict in each case, a one-way ANOVA test was carried out, followed by a post-hoc Tukey test which indicated that there was a significant difference between Rlc1-GFP treated with DMSO and Rlc1-GFP treated with LatA, and also between Rlc1-GFP treated with DMSO and *pna1Δ* Rlc1-GFP treated with LatA. (**Figure 3-5**, **Table 3-2**, **Supplementary Movies 1-4**). Taken together, this indicates that, while the *pna1Δ* mutant is capable of forming the actomyosin ring, it is not able to maintain ring stability, thereby preventing constriction and leading to cytokinesis failure.

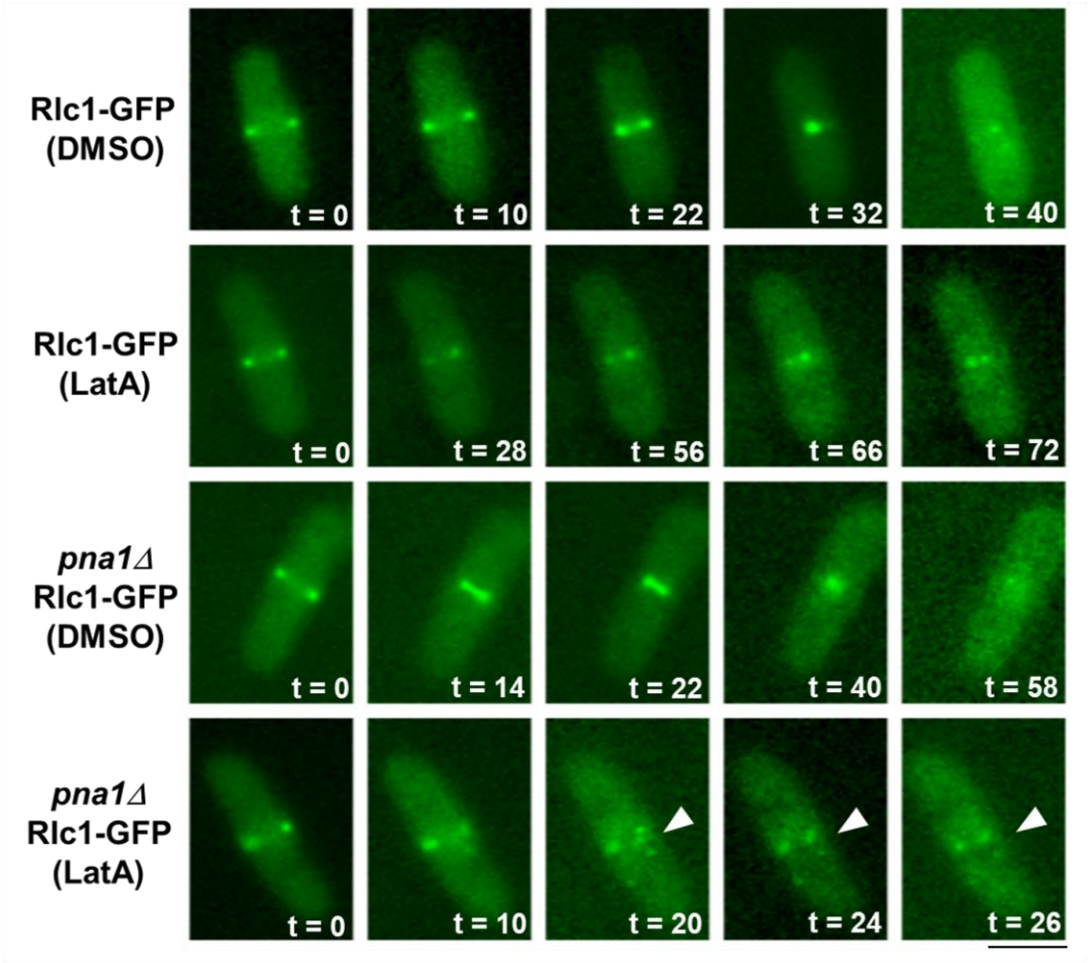

Figure 3-5: *pna1*Δ cells are able to form, but not properly constrict the actomyosin ring upon exposure to LatA.

Wild-type and *pna1Δ* cells expressing the Rlc1-GFP fusion protein (a marker of the actomyosin ring) were treated with 0.15 µM LatA or DMSO (solvent control) and imaged by time-lapse fluorescence microscopy to visualise the constricting ring. Time is indicated in minutes. Arrowheads indicate the fragmentation of the actomyosin ring. The full length movies from which these still images were taken are included as Supplementary Movies 1-4 and can be downloaded from the links in Appendix A. Scale bar represents 5 µm.

Table 3-2: Mean time needed (+/- SD) for cells of the indicated genotype to complete actomyosin ring constriction in the presence of 0.15 µM Latrunculin A or DMSO.

Fifteen cells were analyzed for each class. Means not sharing a common superscript indicate a statistically significant difference.

Genotype (Treatment)	Mean time (minutes) needed to complete actomyosin ring constriction
Rlc1-GFP (DMSO)	36 +/- 7[a]
Rlc1-GFP (LatA)	54 +/- 14[b]
***pna1Δ* Rlc1-GFP (DMSO)**	52 +/- 24[b]
***pna1Δ* Rlc1-GFP (LatA)**	N/A (rings fragment)

3.6 Construction of a Pna1-GFP integrant strain

Having observed that the deletion of *pna1* results in severe growth defects due to an inability to constrict the actomyosin ring upon exposure to LatA, I next wanted to complete a preliminary characterization of the gene by visualising its subcellular localization. In order to do this, I first created a strain expressing a C-terminal GFP fusion of *pna1* as shown in **Figure 3-6A** and described in **Section 2.3.3**. To confirm the integration of the construct at the *pna1* locus, a yeast colony PCR was performed with a forward primer upstream of the junction and a reverse primer specific to the *gfp* sequence (**Figure 3-6A**). Amplicons of the predicted size (~ 700 bp) were observed in putative *pna1-gfp* transformants, but were not observed in control reactions using the parent strain as template (**Figure 3-6B)**. This confirmed the integration of the construct at the *pna1* locus.

3.7 The GFP tag does not compromise the function of Pna1p in response to LatA induced cytokinetic defects

There has been evidence of fluorescent tags interfering with the functionality of proteins (Ausmees et al., 2003; Ma et al., 1996). In order to rule out this possibility in the *pna1-gfp* strain, a spot assay of *pna1-gfp*, wild-type, and *pna1Δ* cells on media containing DMSO/LatA was performed. As expected, wild-type grew successfully on DMSO as well as on LatA containing media, and *pna1Δ* cells struggled to grow upon exposure to LatA. Importantly, the *pna1-gfp* cells grew comparably to wild-type cells even upon exposure to LatA, indicating that the function of Pna1p is not compromised due to the addition of a C-terminal GFP tag (**Figure 3-7**).

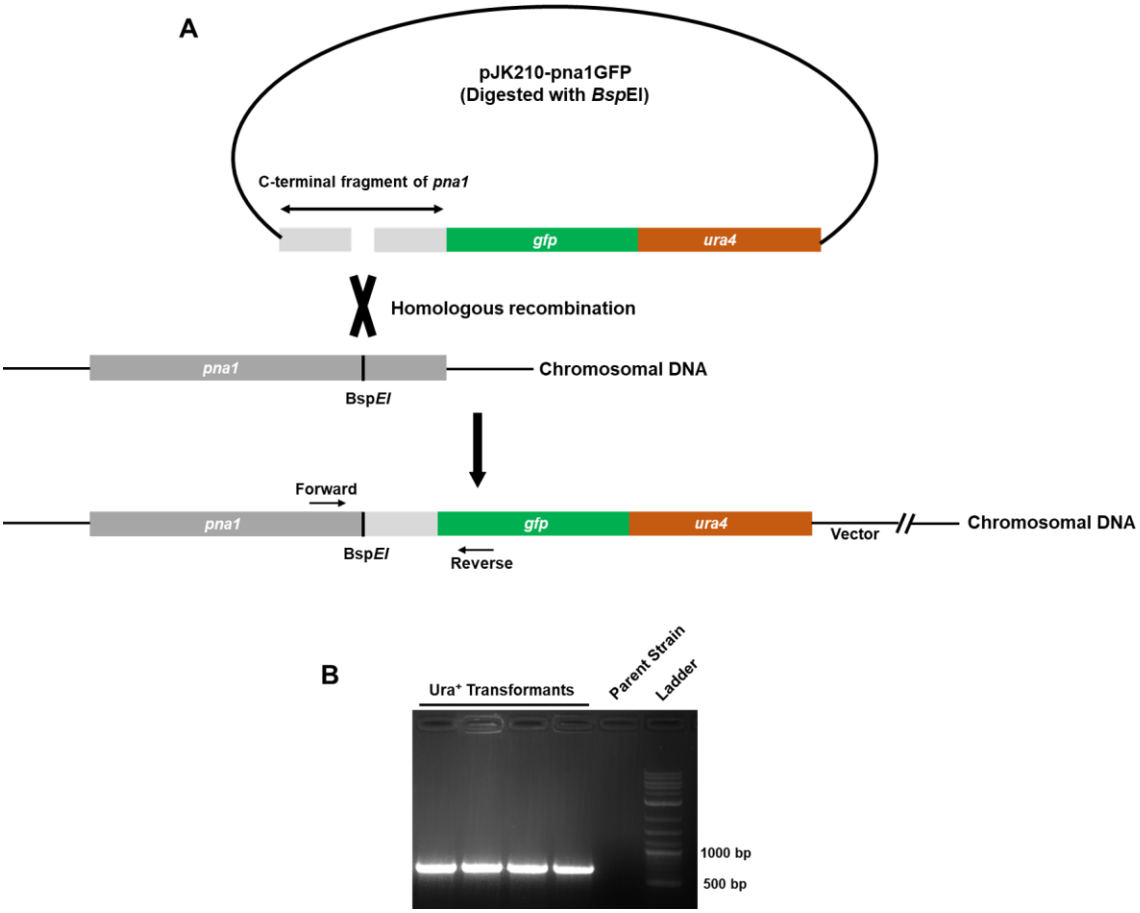

Figure 3-6: Construction of a fission yeast strain expressing a C-terminal Pna1-GFP fusion.

(A) A C-terminal fragment of the *pna1* gene was PCR amplified and ligated into the pJK210 vector in-frame with sequences encoding the *gfp* gene. The plasmid was digested within the *pna1* sequence using *Bsp*E1 (to stimulate subsequent homologous recombination at the *pna1* locus) and transformed into *a ura4-D18 S. pombe* strain. **(B)** Colony PCR was performed using Ura$^+$ transformants as template. The parental *ura4-D18* strain was used as a negative control. Primer locations are indicated in (A). The resulting amplicons were run on a 1% agarose gel to confirm integration at the *pna1* locus.

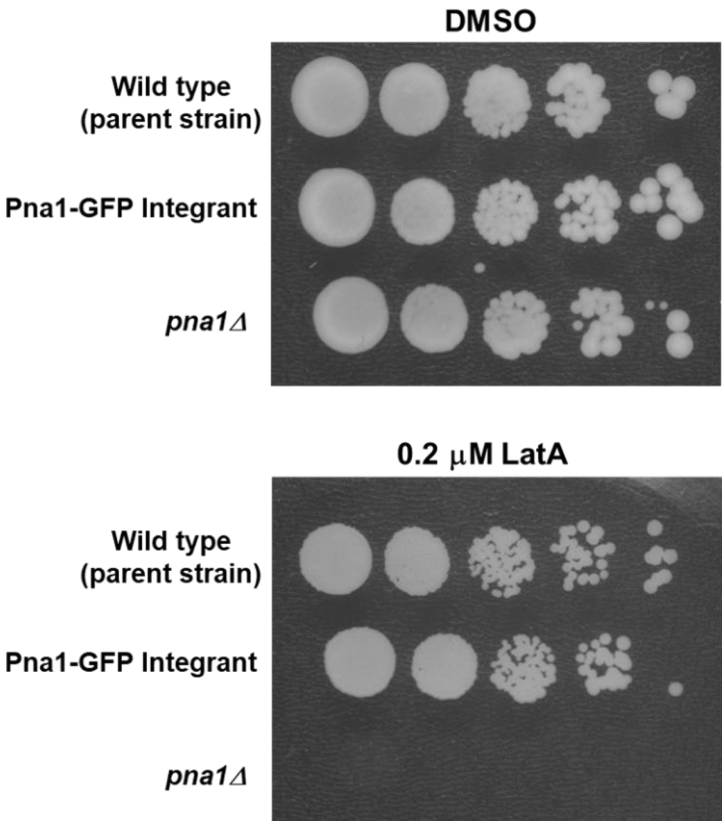

Figure 3-7: The presence of a C-terminal GFP tag does not compromise the function of Pna1p in response to LatA induced cytokinetic perturbation.

Ten-fold serial dilutions of a Pna1-GFP expressing strain, as well as *pna1*Δ and wild-type controls were plated on YES-agar plates containing DMSO or 0.2 μM LatA and incubated for three days at 30°C.

3.8 Pna1-GFP forms dynamic filaments in *S. pombe*

Once I confirmed the functionality of the Pna1-GFP fusion, I next visualised the protein within the cell. To this end, cells expressing Pna1-GFP from the native *pna1* promoter were grown to mid-log phase and then directly visualised using fluorescence microscopy. An untagged sample was used as a control to compare background signals. Since *pna1* codes for a polyamine N-acetyltransferase enzyme, I expected the protein to have a uniform cytoplasmic distribution within the cells. To my surprise, the protein formed unusual filamentous structures (**Figure 3-8**). I reviewed the literature to help us determine the identity of these structures and came across an enzyme filament called cytoophidia (meaning "cellular snakes" in Greek) which appeared similar to the observed Pna1p filaments.

To determine if the Pna1p filaments were static or dynamic, a *pna1-gfp* strain was grown to mid-log phase and then immobilized them on a 2% agarose pad. The cells were then visualised under the GFP filter cube using time lapse microscopy. I observed that the filaments were dynamic, with significant movement over a timescale of seconds to minutes (**Figure 3-9**; **Supplementary Movie 5**).

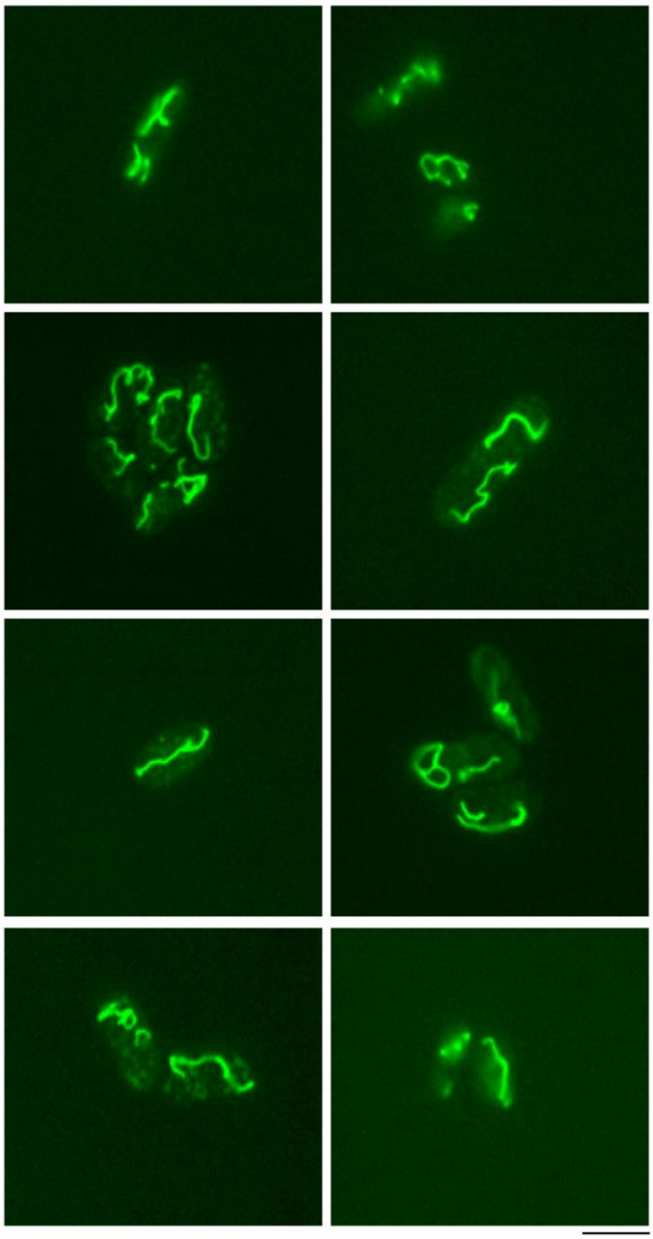

Figure 3-8: Pna1-GFP forms filamentous structures in *S. pombe.*

Pna1-GFP expressing cells were grown to mid-log phase and visualised by fluorescence microscopy using the GFP filter set. A collage of representative images illustrating the observed heterogeneity in the length, form, and sub-cellular location of the observed Pna1-GFP signal is shown. Scale bar represents 5 µm.

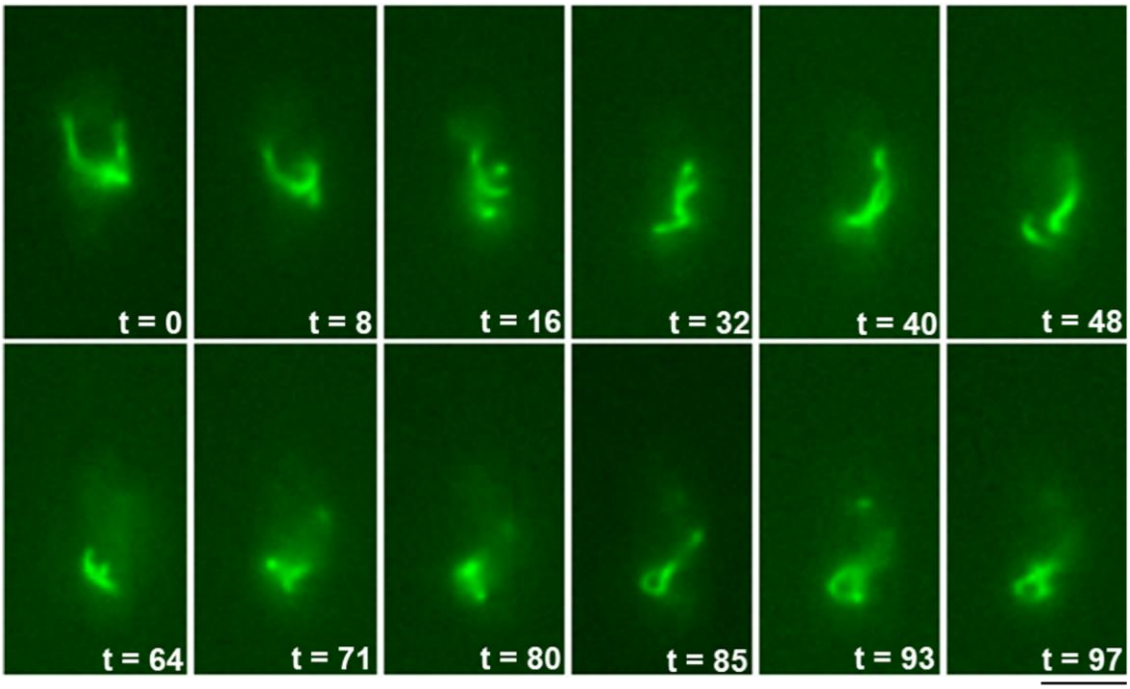

Figure 3-9: Pna1-GFP filaments are dynamic.

Cells expressing Pna1-GFP were grown to mid-log phase and then immobilised on a 2% agarose pad. Cells were then visualised by time-lapse fluorescence microscopy using the GFP filter cube over the course of two minutes. Representative images illustrating the rapid changes (time-scale of seconds) in the length, form and sub-cellular location of the Pna1-GFP signal are shown. The full length movie from which these still images were taken is included as Supplementary Movie #5 and can be downloaded from the link found in Appendix A. Scale bar represents 5 µm.

3.9 Pna1-GFP filaments are exclusively cytoplasmic

To understand if the observed Pna1p filaments localized to the nucleus, colocalization experiments were performed. Pna1-GFP expressing cells were grown to mid-log phase and then fixed with ice-cold ethanol. The cells were then stained with DAPI and visualised under the GFP filter cube (to visualise Pna1-GFP filaments) and under the DAPI filter cube (to visualise the nucleus). Z-series were obtained, and the flattened images overlayed to determine colocalization between the filaments and nucleus. As seen in the representative image in **Figure 3-10**, the filaments had the strong tendency to go around the nucleus, remaining exclusively cytoplasmic.

3.10 Pna1-GFP filaments are stable in the absence of cytoskeletal components

The cell uses its cytoskeletal components for the movement of several proteins within it. Actin and microtubules are known to aid this movement by forming cables that are tracks for this movement. Filamentous structures are known to use actin as their tracks. Since the deletion of *pna1* caused failure of cytokinesis upon exposure to LatA, and since the filaments were highly mobile, I wanted to test the effects of the absence of actin and microtubules on these filaments.

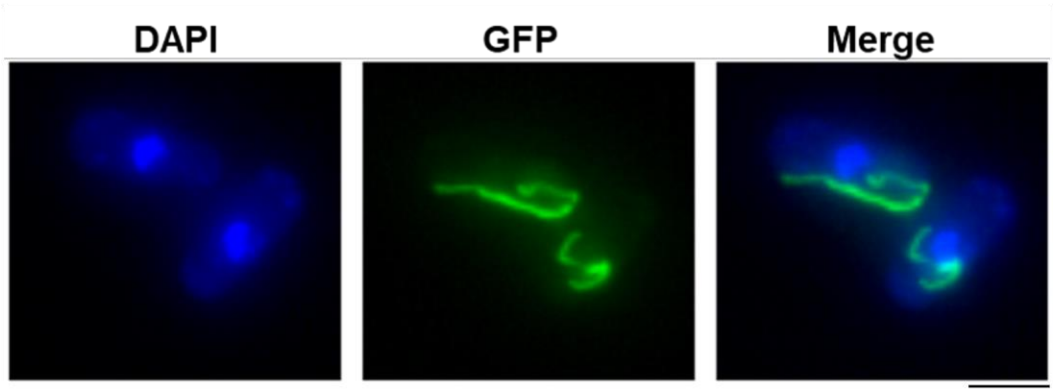

Figure 3-10: Pna1-GFP filaments are exclusively cytoplasmic.

Cells expressing Pna1-GFP were grown to mid-log phase, fixed with ice-cold ethanol and stained with DAPI. Cells were visualised by fluorescence microscopy using the GFP and DAPI filters to observe Pna1-GFP filaments and the nucleus, respectively. Z-stacks were collected, flattened, and then overlayed to determine colocalization between Pna1-GFP filaments and the nucleus. Scale bar represents 5 µm.

LatA treatment at low doses of around 0.2 µM is known to cause mild perturbation of the actin cytoskeleton. However, when used at higher doses of 25 µM and above, it is known to completely disrupt actin filaments. To disrupt actin, Pna1-GFP expressing cells were grown to mid-log phase and treated with 25 µM LatA/DMSO and then fixed with formaldehyde. The cells were then stained with the actin stain Alexa-568 Phalloidin and visualised. I observed that the actin cytoskeleton was completely disrupted upon treatment with 25 µM LatA, but remained intact in the DMSO containing media. The Pna1-GFP filaments looked comparable in the DMSO as well as LatA treated samples indicating that the filamentous structure of Pna1-GFP is independent of actin (**Figure 3-11**).

Methyl benzimidazole-2-yl-carbamate (MBC, also known as Carbendazim) is a known inhibitor of microtubules (Yenjerla et al., 2009). To disrupt microtubules, Pna1-GFP expressing cells were grown to mid-log phase and treated with 5, 10, 20, 25 µg/mL of MBC or DMSO and then fixed with formaldehyde. The cells were then visualised under the GFP filter to observe the Pna1-GFP filaments. The Pna1-GFP filaments looked comparable in the DMSO as well as MBC treated samples indicating that the filamentous structure of Pna1-GFP is independent of microtubules (**Figure 3-12**).

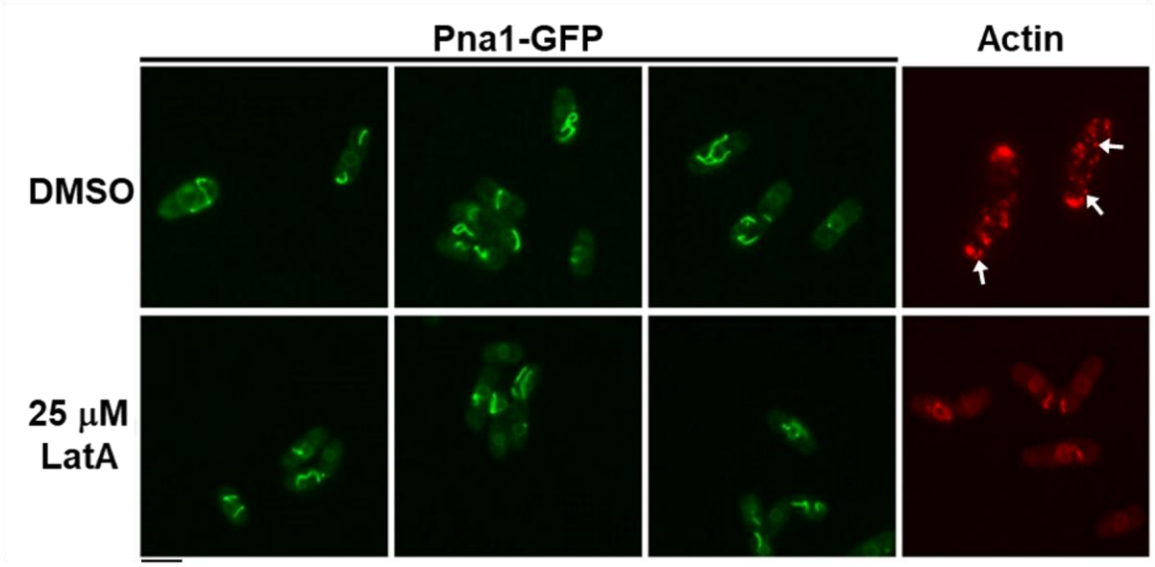

Figure 3-11: Pna1-GFP filaments are stable in the absence of actin.

Cells expressing Pna1-GFP were grown to mid-log phase and treated with 25 μM LatA or DMSO (solvent control). Cells were then fixed with formaldehyde and stained with Alexa-568 phalloidin and visualised by fluorescence microscopy to observe both Pna1-GFP filaments and actin. White arrows indicate actin patches. Scale bar represents 5 μm.

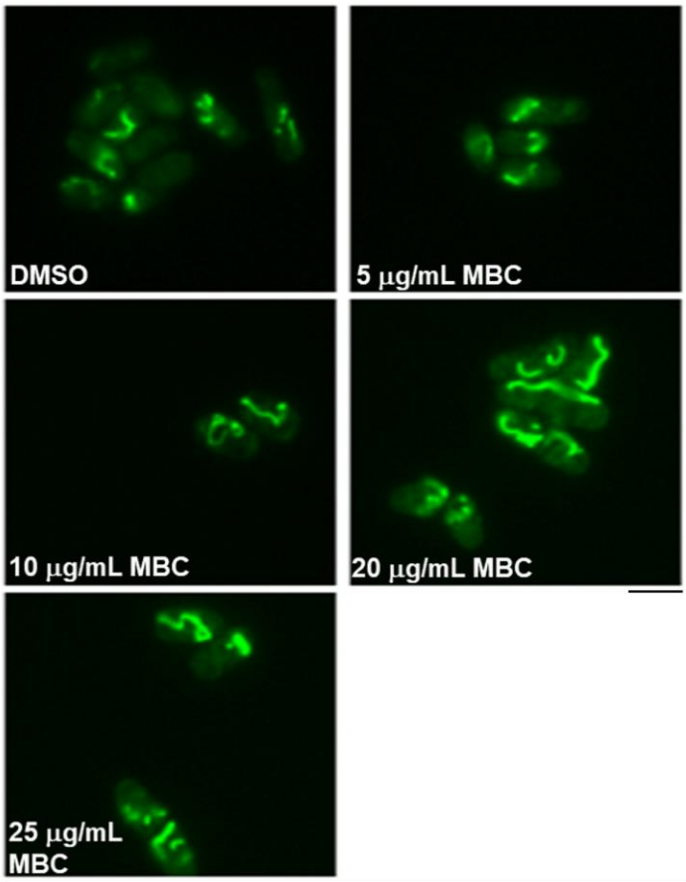

Figure 3-12: Pna1-GFP filaments are stable in the absence of microtubules.

Cells expressing Pna1-GFP were grown to mid-log phase and treated with 0, 5, 10, 20, or 25 μg/mL of Methyl benzimidazole-2-yl-carbamate. Cells were then fixed with formaldehyde and visualised by fluorescence microscopy to observe Pna1-GFP filaments. Scale bar represents 5 μm.

3.11 Pna1-GFP filament length changes as a function of growth phase

Since CTPS cytoophidia often display growth phase dependent properties (J.-L Liu, 2010; Noree et al., 2010; Shen et al., 2016; J. Zhang & Liu, 2019), I was interested in determining if the same was true of Pna1-GFP filaments. In order to test the effects of growth phase on Pna1-GFP filaments, I conducted an experiment to visualise the filaments as the cells went from the log-phase of growth to stationary phase. Briefly, *pna1-gfp* cells were inoculated into a liquid culture and harvested at different phases of growth according to their optical densities: early log phase (OD_{600} between 0.02 - 0.1), mid-log phase (OD_{600} between 0.2-0.8), and stationary phase (OD_{600} > 1.0). The cells were then fixed with formaldehyde and visualised under the microscope using the GFP filter to analyze the filaments. Interestingly, the GFP signal at early log phase appeared as very tiny dots, presumably the nascent form of the filaments. As the density increased, the filaments increased in length and appeared similar in length as originally observed. As the cells reached stationary phase, the filaments appeared much longer than originally observed, spanning the entire volume of the cell (**Figure 3-13**).

Since I observed that the filaments increased in length upon entering the stationary phase of growth, I wanted to determine if these filaments would change in length again when put into fresh media. To test this, I started a *pna1-gfp* culture and let it grow until it reached stationary phase. The cells were then transferred to fresh media and then harvested at different time points to see the effects of fresh media on the filaments. Interestingly, I saw that following 5-6 hours of shifting the cells to fresh media, the long filaments of stationary phase started to fragment and appeared as dots (similar to the dots observed in the early log phase). When allowed to grow further, the filaments started growing again in length, resembling the filaments of mid-log phase in about 11 hours (**Figure 3-14**). Taken together these data indicate that Pna1-GFP filament formation is dependent on growth phase.

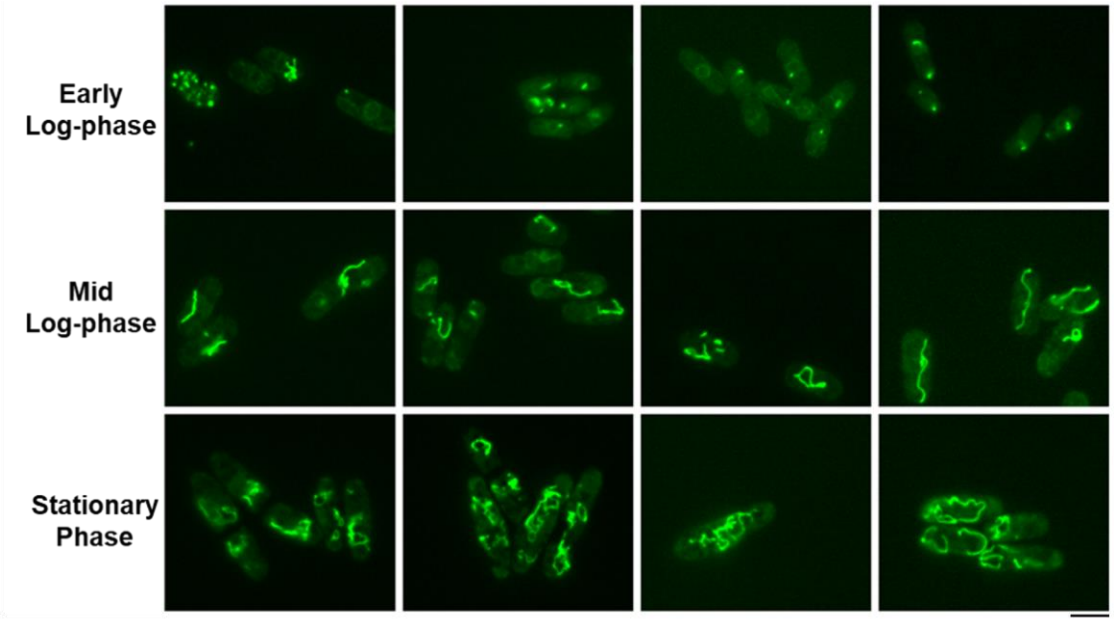

Figure 3-13: The length of Pna1-GFP filaments increases as cells grow from early-log to stationary phase.

A liquid culture of cells expressing Pna1-GFP cells was harvested at different growth phases based on their optical densities: early-log phase (OD_{600} between 0.02 - 0.1), mid-log phase (OD_{600} between 0.2 - 0.8), and stationary phase (OD_{600} > 1.0). Cells were then fixed with formaldehyde and visualised by fluorescence microscopy to observe Pna1-GFP filaments. Scale bar represents 5 µm.

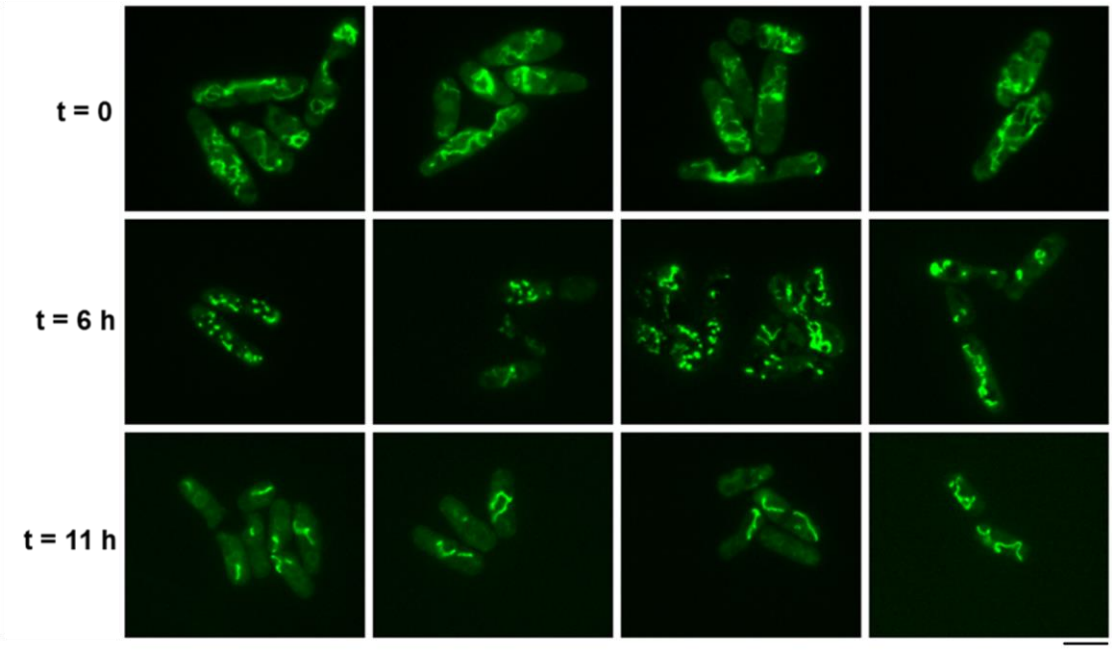

Figure 3-14: Pna1-GFP filaments in stationary-phase cells fragment and then re-grow when transferred to fresh media.

A liquid culture of cells expressing Pna1-GFP was first grown to stationary phase (OD_{600} > 1.0). Cells were then transferred to fresh YES media and harvested every hour. Cells were subsequently fixed with formaldehyde and visualised by fluorescence microscopy to observe Pna1-GFP filaments. Scale bar represents 5 µm.

3.12 Pna1-GFP filaments are independent of the TOR pathway

CTPS-cytoophidia in fission yeast are sensitive to the deletion of components of the TOR pathway (Andreadis et al., 2019). It is also known that the TOR pathway is most active during the active growth phase of yeast cells. With this background, and because of the observation that Pna1-GFP filaments change with change in growth phase, I wanted to check if the disruption of the TOR pathway had effects on the Pna1-GFP filaments. For this experiment, Pna1-GFP expressing cells were grown to mid-log phase and then treated with different concentrations of Torin-1 (a known inhibitor of the TOR pathway) or DMSO. The cells were then fixed with formaldehyde and visualised under the microscope using the GFP filter to analyse the filaments. The filaments looked comparable in both the DMSO or Torin-1 treated cells, indicating that they were independent of the TOR pathway (**Figure 3-15**).

3.13 Pna1-GFP filaments do not respond to heat shock or cold shock

Since previously characterized cytoophidia displayed stress sensitivity to alterations in temperature, I was interested to determine if this was also true of Pna1-GFP filaments. I thus assayed the response of Pna1-GFP filaments to both heat and cold shock. I grew Pna1-GFP expressing cells and harvested them at mid-log phase and subjected them to heat shock and cold shock at different temperatures and durations: heat shock at 37ºC and 45ºC for 20 minutes, as well as cold shock at 0ºC for 30 or 120 minutes. I harvested the cells after the respective temperature change and fixed them with formaldehyde. They were then visualised under the GFP filter of the microscope. A culture was kept at 30ºC as a control. I observed that the filaments looked comparable to the 30ºC control regardless of the temperature or the amount of time of heat/cold shock (**Figure 3-16**). This indicated that the Pna1-GFP filaments were independent of temperature stress.

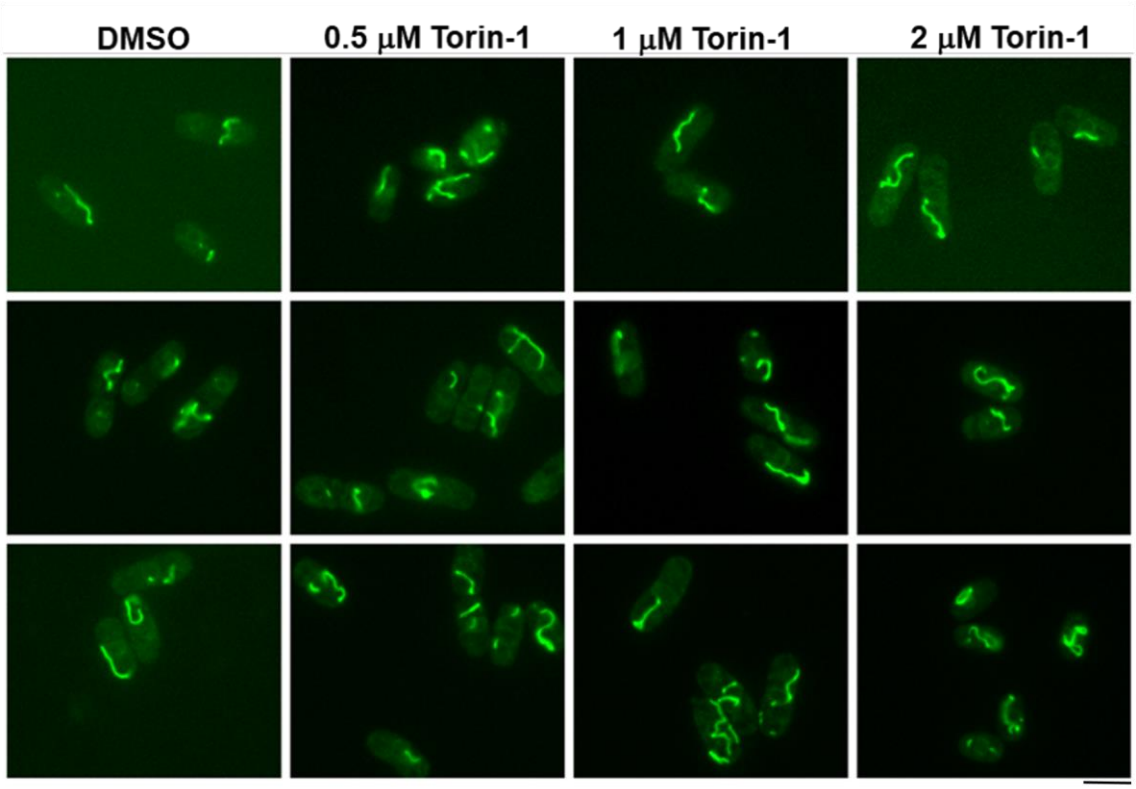

Figure 3-15: Pna1-GFP filaments are independent of TOR signalling.

Cells expressing Pna1-GFP were grown to mid-log phase and treated with 0, 0.5, 1.0, or 2.0 μM Torin-1 and then fixed with formaldehyde and visualised by fluorescence microscopy to observe Pna1-GFP filaments. Scale bar represents 5 μm.

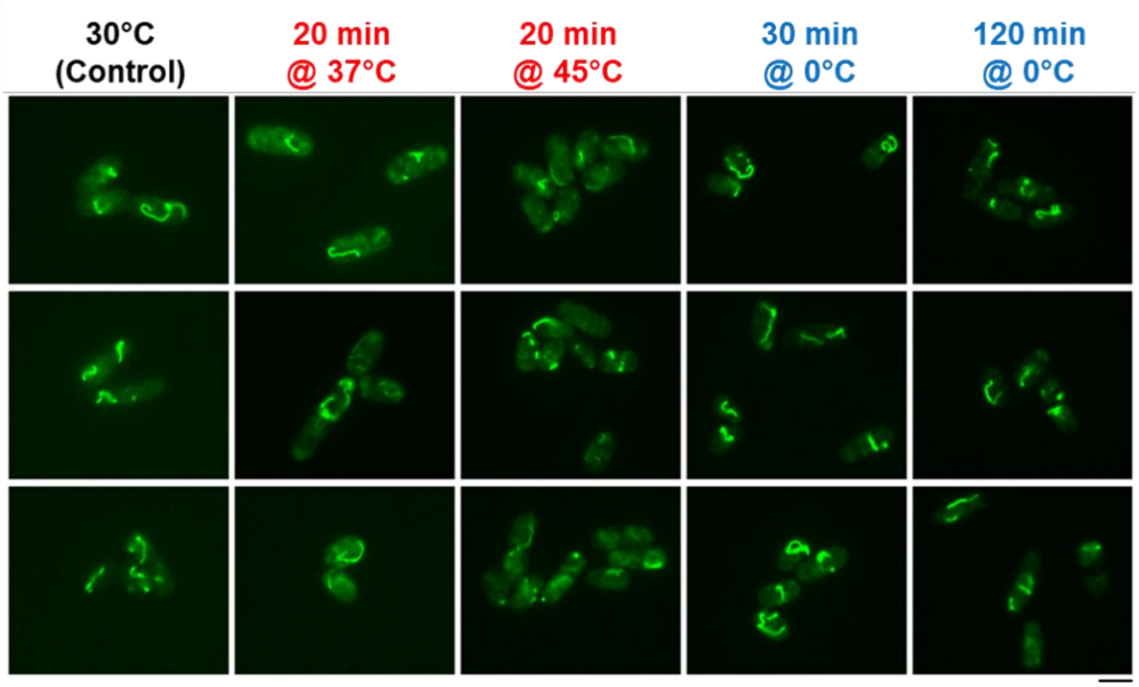

Figure 3-16: Pna1-GFP filaments are unaffected by heat/cold shock.

Cells expressing Pna1-GFP were grown to mid-log phase and then subjected to heat shock (37ºC/45ºC) or cold shock (0ºC) for the indicated duration. A control group of cells were incubated at 30ºC. Cell were then fixed with formaldehyde and visualised by fluorescence microscopy to observe Pna1-GFP filaments. Scale bar represents 5 µm.

3.14 Over-expression of *pna1* affects Pna1-GFP filament formation

Overexpression of *ctps* caused an increase in the intensity of CTPS cytoophidia in Drosophila (Azzam & Liu, 2013; Ingerson-Mahar et al., 2010). To test if the overexpression of *pna1* had a similar effect on the Pna1-GFP filaments in fission yeast, I transformed the *pna1* over-expression plasmid (constructed as described in **Section 2.3.2**) into the Pna1-GFP expressing strains. I then grew the cells to mid-log phase in EMM media containing thiamine to repress *pna1* expression. Then, the cells were transferred to EMM media lacking thiamine to induce overexpression of the gene. The cells were then fixed with formaldehyde and visualised using the GFP filter. Interestingly, after the gene was overexpressed, Pna1-GFP filaments were not formed, and the GFP signal was spread throughout the cell indicating that the protein was uniformly diffused in the cell and not concentrated in filaments (**Figure 3-17**).

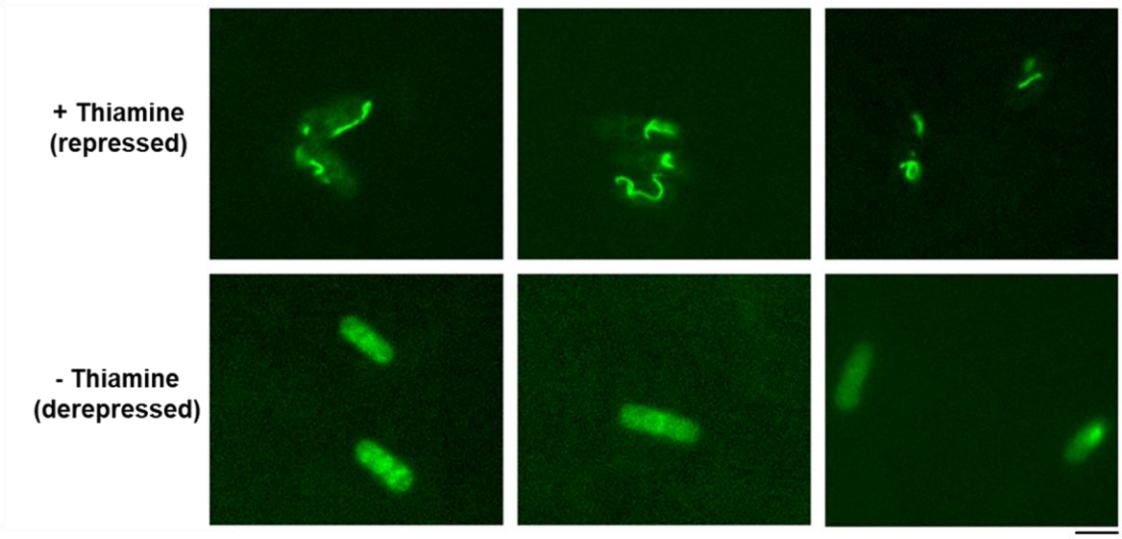

Figure 3-17: Pna1-GFP filament formation is inhibited upon *pna1* overexpression.

The *pna1* over-expression plasmid was transformed into fission yeast cells expressing Pna1-GFP. These cells were first grown to mid-log phase in EMM media containing thiamine to repress *pna1* expression. The cells were then transferred to EMM media lacking thiamine for 12 hours to induce expression of the *pna1* gene from the *nmt41* promoter. Cells were then fixed with formaldehyde and visualised by fluorescence microscopy to observe Pna1-GFP filaments. Scale bar represents 5 µm.

Chapter 4

4 DISCUSSION

Cytokinesis is one of the most crucial events in a cell's life since its successful completion is important for cell growth and the maintenance of genome integrity. Thus, a better understanding of the genetic factors important in ensuring its fidelity is of importance both to a fundamental understanding of biology, as well as an understanding of how cytokinesis failure relates to genetic instability and tumorigenesis. The current study has provided insight into the function of one genetic factor, the *pna1* gene (encoding a polyamine-N-acetyl-transferase), that is required for the successful completion of cytokinesis in the model eukaryote, *S. pombe*. In the current study, I clearly demonstrate that *pna1* is essential for cell division upon treatment with the actin depolymerising drug LatA (a commonly used tool that can be used to perturb the cell division machinery so that the cell's response to "cytokinetic stress" can be studied). Furthermore, I show that this failure is mediated by the inability of the cell to properly constrict the contractile actomyosin ring. However, questions remain as to the molecular mechanism(s) by which *pna1* acts to modulate cytokinesis (various possibilities are discussed below). Surprisingly, I also demonstrate that the *pna1* gene-product may form cytoophidia (intracellular filamentous structures containing metabolic enzymes). While not one of my initial objectives, I chose to further characterize these filamentous structures in detail in order to better understand this recently discovered class of proteins. The possible functional roles of cytoophidia, as well as how the formation of these filaments might modulate *pna1* regulation are discussed in detail below.

4.1 Polyamines and their role in modulating actin dynamics

Polyamines are aliphatic, organic polycations containing two or more amino groups, and are essential to all kingdoms of life. Even though polyamines are required for several functions in the cell, high concentrations can be toxic (Tome et al., 1997). Hence, they are tightly controlled at the levels of transcription, translation, protein turnover, and

transport (W. Sun et al., 2020). Budding and fission yeast mutants incapable of producing polyamines show several morphological abnormalities including enlargement of cells, increase in vesicle like bodies in the cytoplasm, and delocalized chitin and actin distribution (Balasundaram et al., 1991). Fission yeast mutants deprived of polyamines spermine and spermidine stop dividing after some generations, indicating that these polyamines are absolutely essential for their growth (Chattopadhyay et al., 2001, 2002).

F-actin is a negatively charged biopolymer which plays critical roles in cell division, motility, and structure. The ability of F-actin to overcome electrostatic repulsions and form bundles of parallel, closely-packed filaments is key to many of these functions (Sowa et al., 2006). There are several reports demonstrating that polyamines can induce actin bundling in mammalian cells and yeast (Pohjanpelto et al., 1981; Sowa et al., 2006; Sunkara et al., 1979; Tang & Janmey, 1996). A possible mechanism by which positively charged molecules – including poyamines – aid actin bundling, is the counterion-mediated attraction model, wherein cations condense along the negatively charged filaments, leading to electrostatic repulsions, which in turn cause an attraction force among closely packed filaments of actin (Sowa et al., 2006). Processes such as sperm activation and neuron repair in part rely on actin bundling. It has been observed that there is high concentration of spermine during these processes, suggesting that polyamine induced bundling of actin may play a physiologically significant role in cells (Muhlrad et al., 2011). Moreover, during cell division, the concentration of polyamines is elevated around the ring canal which pinches the cell into two. This process also depends on actin bundling, suggesting another physiological role of polyamine induced actin bundling (Sowa et al., 2006).

Studies have also shown that synthetic polyamines can induce actin bundling (Nedeva et al., 2013; Riveline et al., 2014). The macrocyclic polyamine C_7N_6 MPA, and acyclic branched polyamine C_8N_6 BPA promote lamellipodial protrusions and also have a profound effect on the actomyosin ring in fission yeast (Nedeva et al., 2013; Riveline et al., 2014). Ring closure was slowed down by 50% in the presence of C_7N_6 MPA and up

to 40% in the presence of C_8N_6 BPA, the reason for which is unknown (Riveline et al., 2014).

Acetylation of compounds makes them more negatively charged. Hence, acetylation of polyamines decreases their overall positive charge, thus inhibiting their interaction with negatively charged molecules. For example, the budding yeast *pna1* ortholog (*paa1*) (**Figure 4-1**) acetylates spermine, spermidine, and putrescine, inhibiting their interaction with the negatively charged chromatin, making the chromatin less condensed (B. Liu et al., 2005). It is thus interesting to speculate as to whether polyamine acetylation might affect polyamine interaction with actin, thereby modulating cytokinesis.

4.2 The possible role of the Caf5p mediated export pathway regulating the intracellular levels of polyamines

The original genome-wide fission yeast screen by Asadi et al. (2016) identified 38 genes (including *pna1Δ*) required to prevent cytokinesis failure upon LatA treatment. One pathway identified from this screen consisted of the transcription factor Pap1p, the alpha importin Imp1p, and the Caf5p efflux pump (Asadi et al., 2017). Interestingly, Caf5p exhibits homology with a family of polyamine transporters. The study showed that LatA mediated perturbation of the actin cytoskeleton translocates Pap1p from the cytoplasm into the nucleus via the importin Imp1p. Once in the nucleus, Pap1p induces the expression of *caf5* (**Figure 4-2**). The loss of any of these components: the Pap1p transcription factor, the Imp1p alpha-importin, or the Caf5p efflux pump, results in extreme sensitivity to LatA. Furthermore, these mutants also show striking cytokinesis defects and are unable to complete cell division in the presence of the drug (Asadi et al., 2017).

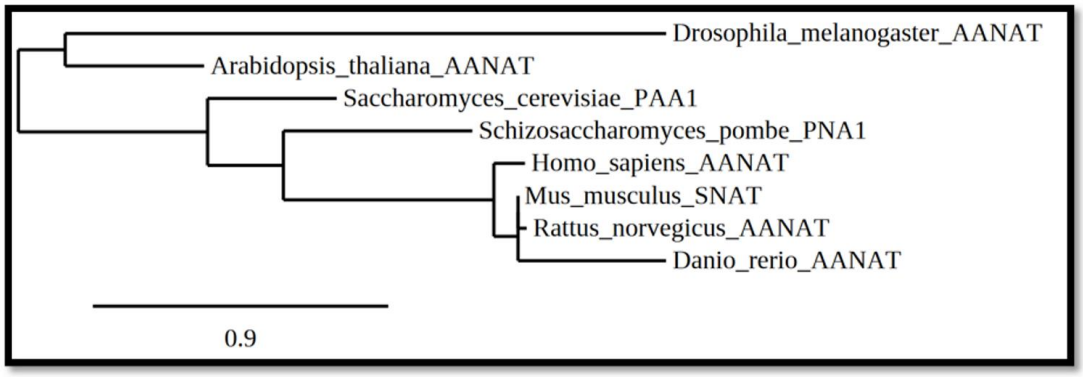

Figure 4-1 Phylogenetic analysis of Pna1p.

Protein sequences of Pna1p orthologs in different organisms were obtained from the NCBI database. MUSCLE, PHYML, and TreeDyn softwares were used for multiple alignment, phylogenetic analysis, and tree rendering respectively. Branch support value was kept at less than 50%.

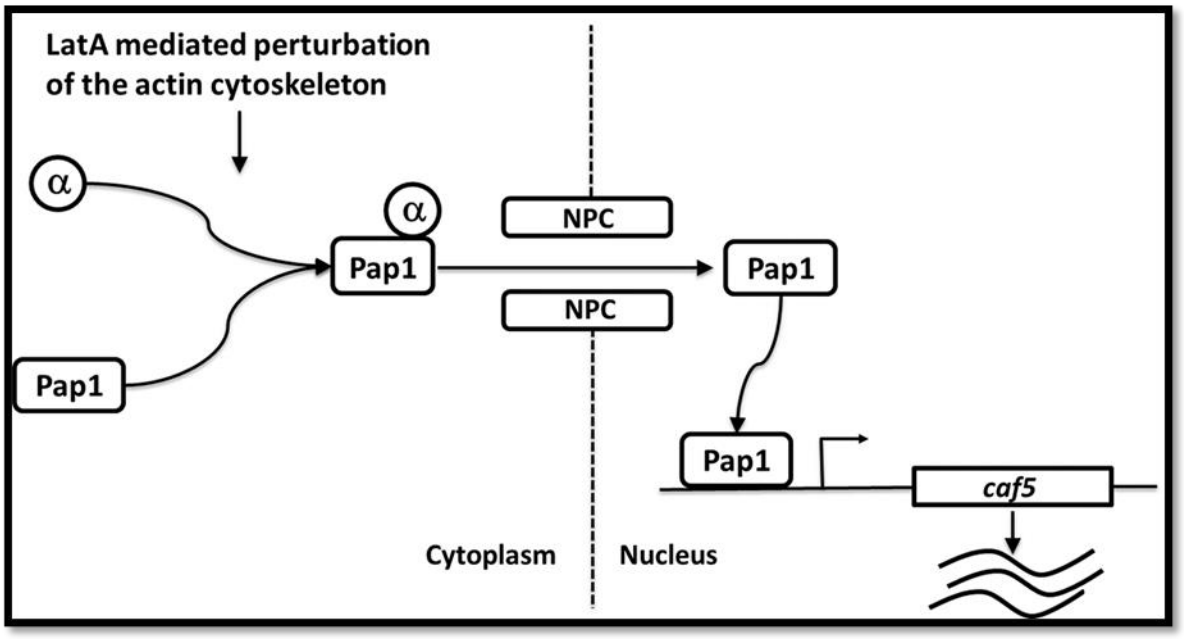

Figure 4-2 Pap1p-dependent induction of the Caf5p efflux pump

LatA mediated perturbation of the cytoskeleton translocates Pap1p from the cytoplasm to the nucleus via the α-importin Imp1p. In the nucleus, it induces expression of the gene *caf5* which belongs to a family of polyamine transporters. Acetylated polyamines are then transported out of the cell through Caf5p mediated efflux pump.

Given that Caf5p belongs to a family of polyamine transporters, and *pna1* codes for a polyamine N-acetyltransferase, one possible model is that Pna1p activity is stimulated upon perturbation of the actin cytoskeleton by LatA leading to the acetylation of polyamines which is known to promote export out of the cell (at least in human cell lines) (Wallace & Mackarel, 1998). Thus, one possibility is that *pna1* mediated acetylation of polyamines promotes their efflux from the cell via the Caf5p efflux pump, thus inhibiting their interaction with actin. This might further lead to active remodeling of the actin cytoskeleton to counter the effects of LatA and promote successful cell division.

Further experiments need to be conducted in order to test this hypothesis. For example, intracellular polyamine levels could be monitored using the dye polyamine RED in different gene deletion backgrounds in the presence/absence of LatA. There is evidence in fission yeast that synthetic polyamines such as BPAs (acyclic branched polyamines) and MPAs (macrocyclic polyamines) promote actin bundling and slow down actomyosin ring constriction (Nedeva et al., 2013; Riveline et al., 2014). The effects of these drugs can be tested in different gene deletion backgrounds to understand its effects on cell growth and cytokinesis. Further, using a targeted metabolomic approach, one could determine whether polyamine levels are indeed modified by acetylation in response to LatA treatment. If the Caf5p efflux pump indeed exported acetylated polyamines out of the cell, then one would expect to see an overall decrease in polyamine levels upon LatA treatment in wild-type cells. One would also predict to see that in *caf5Δ* and *pna1Δ* backgrounds, LatA dependent effects would be abrogated.

4.3 Filament forming proteins in fission yeast

Perhaps the most intriguing finding in this study was the observation of the filament forming capacity of Pna1-GFP fusion proteins. Importantly, I show that the Pna1-GFP fusion is fully functional with respect to its role in the cytokinesis monitoring system since strains expressing Pna1-GFP as the sole copy of *pna1* show similar levels of LatA resistance as wild-type cells. This leaves the question of the relevance of this filament forming ability to the function of Pna1p. Below I discuss the possible importance of

enzyme compartmentalization and how this might be relevant to the observations made during this study.

4.3.1 Compartmentalization of enzymes and its importance

Compartmentalization of biological processes within a cell is fundamental for their regulation (Gou et al., 2014; J.-L. Liu, 2011; Ovádi & Saks, 2004; Shen et al., 2016). Compartmentalization enables a cell to create specific microenvironments within itself to execute distinct functions efficiently without the interference of unrelated components. In the past, compartmentalization has been extensively studied through membrane-bound organelles such as Golgi bodies, endoplasmic reticulum, mitochondria. These subcellular compartments are dynamic since they constantly undergo reorganization in response to stimuli, both internal and external. They mediate novel biochemical functions that are predominantly involved in the fundamental metabolism of the cell (An et al., 2008; J.-L. Liu, 2010; J.-L. Liu & Gall, 2007; Sheth & Parker, 2006).

Each compartment is functionally specialized, containing distinct catalytic processes, specialized molecules, and specific microenvironments. High concentrations of macromolecules localize to these compartments, allowing the cell to perform various metabolic activities efficiently and simultaneously. Specific products are transported between compartments using a complex distribution system (J.-L. Liu, 2010). Macro- and micro-compartmentalization of metabolites and their channeling in organized cellular systems is the basis of cell life, including the cellular processes of energy conversion. The regulation of cellular energy metabolism is closely related to the structural organization of the cells (Ovádi & Saks, 2004). Compartmentalization, therefore, ensures increased structural and functional complexity within a cell’s organization (J.-L. Liu, 2010).

In recent years, compartmentalization has been discovered in novel non-membrane bound organelles such as cytoplasmic processing bodies (P-bodies), uridine-rich small nuclear ribonucleoprotein bodies (U-bodies), histone locus bodies (HLBs), and multi-enzyme complexes called purinosomes that are involved in the regulation of purine metabolism

(An et al., 2008; J.-L. Liu et al., 2006; J.-L. Liu & Gall, 2007; Sheth & Parker, 2006). It has been hypothesized that these compartments control the flux of different enzymes to a particular biochemical pathway (Aughey & Liu, 2016). This phenomenon has been demonstrated in the de novo biosynthesis of purine nucleotides. Key enzymes in this pathway reversibly co-localize to purinosomes, which are responsive to changing purine concentrations (An et al., 2008).

4.3.2 Compartmentalization via cytoophidia

In 2010, three research groups independently and simultaneously discovered a novel intracellular, filamentous structure containing the enzyme cytidine-triphosphate-synthase (CTPS). These structures were first identified in fruit flies, budding yeast and bacteria. Further research revealed the presence of filamentous CTPS structures even in human cell lines and archaea, indicating that they have been conserved across evolution. These structures were unlike anything previously described and were termed cytoophidia, meaning "cellular snakes" in Greek, because of their morphology (J.-L. Liu, 2010). These structures have been proposed to regulate enzyme function (at least in part) via compartmentalization.

CTPS cytoophidia have been observed in multiple cell types such as follicle cells, oocytes, and nurse cells, and in other fruit fly species such as *Drosophila virilis* and *Drosophila pseudoobscura*. These cytoophidia were present in two distinct sizes in the nurse cells and oocytes, and they were termed macro- and micro- cytoophidia. The macro cytoophidia are long and thick as opposed to the micro-cytoophidia which are short and thin (J.-L. Liu, 2010).

In bacteria, Ingerson-Mahar *et al.* (2010) identified filamentous structures formed by bacterial CTP synthase in *C. cresentus*, where they seemed to have a structural role in the uniquely shaped curved bacterial cell. Interestingly, this structural role was independent of the protein’s enzyme activity. In *S. pombe,* each cell contains a cytoplasmic and a nuclear CTPS cytoophidium (J. Zhang et al., 2014). These 2 separate pools of CTPS

cytoophidia independently get partitioned into one of the daughter cells during cell division. CTPS cytoophidia are highly dynamic in fission yeast (J. Zhang et al., 2014) and their movement happens along actin tracks (Li et al., 2018). Recently, CTPS cytoophidia in *S. pombe* were reported to be temperature sensitive (both to heat shock and cold shock) (J. Zhang & Liu, 2019). This study also revealed that CTPS cytoophidia require heat shock proteins for their assembly (J. Zhang & Liu, 2019). Subsequent research has revealed the presence of CTPS cytoophidia in humans, plants, and archaea suggesting that the filament forming property is conserved across evolution (Andreadis et al., 2019; Daumann et al., 2018; Huang et al., 2017; Z. Sun & Liu, 2019; B. Zhang et al., 2020; J. Zhang et al., 2014; Zhou et al., 2020).

Importantly, proteins other than CTPS can form cytoophidia. For example, a genetic screen performed in budding yeast identified nine proteins that form filaments and foci in vivo. The Ura7p and Ura8p proteins (which form the yeast CTP synthase) were part of this group (Noree et al., 2010). Later screens identified a total of 23 budding yeast proteins that formed filaments in vivo. These proteins were mostly metabolic enzymes involved in glucose and nitrogen metabolism as well as others involved in translation initiation (Shen et al., 2016). This has led to the suggestion that this observed protein filamentation may represent a novel mechanism for metabolic regulation through compartmentalization (Aughey & Liu, 2016).

4.3.3 Cytoophidia function

A structure must meet several criteria to be referred to as a cytoophidium. They must be filamentous as opposed to spherical bodies like P-bodies, U-bodies and Cajal bodies. They must contain a metabolic enzyme (unlike cytoskeletal components like actin, microtubules, and intermediate filaments), and they must also lack a membrane (unlike other organelles like the mitochondria and Golgi apparatus). Hence, cytoophidia can be defined as mesoscale, intracellular, filamentous structures containing metabolic enzymes (J.-L. Liu, 2016).

While the functions of cytoophidia are not fully understood, studies suggest that they may play a role in the metabolic regulation of the enzyme (Aughey et al., 2014). Several other functions, including metabolic channeling, intracellular transport, as well as possible structural roles have also been proposed, given their morphology (**Figure 4-3**).

4.3.4 Characterization of Pna1p filaments

The present work demonstrates that Pna1-GFP filaments form independently of cytoskeletal components and components of the TOR pathway. Further, they remain unaffected by temperature stress. Unlike, CTPS cytoophidia, which form two separate pools of cytoophidia in the nucleus and the cytoplasm (J. Zhang et al., 2014), Pna1-GFP filaments are exclusively cytoplasmic and go around the nucleus, thus avoiding it (**Figure 3-10**).

Several previous studies have shown that the formation and morphology of CTPS cytoophidia vary with the growth stage of cells in budding yeast, fission yeast, and Drosophila. A recent study by Zhang and Liu in 2019 in *S. pombe* showed that CTPS cytoophidia are abundant in log-phase cells and disappear in the stationary phase. However, Noree *et al*, in 2010, showed that Ura7p (CTPS ortholog in budding yeast) filaments greatly increased in saturated cultures. Importantly, it was also found that the protein levels remained unaltered, confirming that the increase in filament formation was not due to increased protein levels. A similar observation in budding yeast was made by Shen *et al* in 2016. In these cases, it was hypothesized that filament formation was dependent on the nutrient content of the growth media. Since the media in stationary phase is usually saturated with metabolites and is low in nutrition, the increased filaments in stationary phase was likely due to lack of nutrients in the media. There is also evidence of other structures such as processing bodies being induced in saturated cultures (Teixeira, 2005).

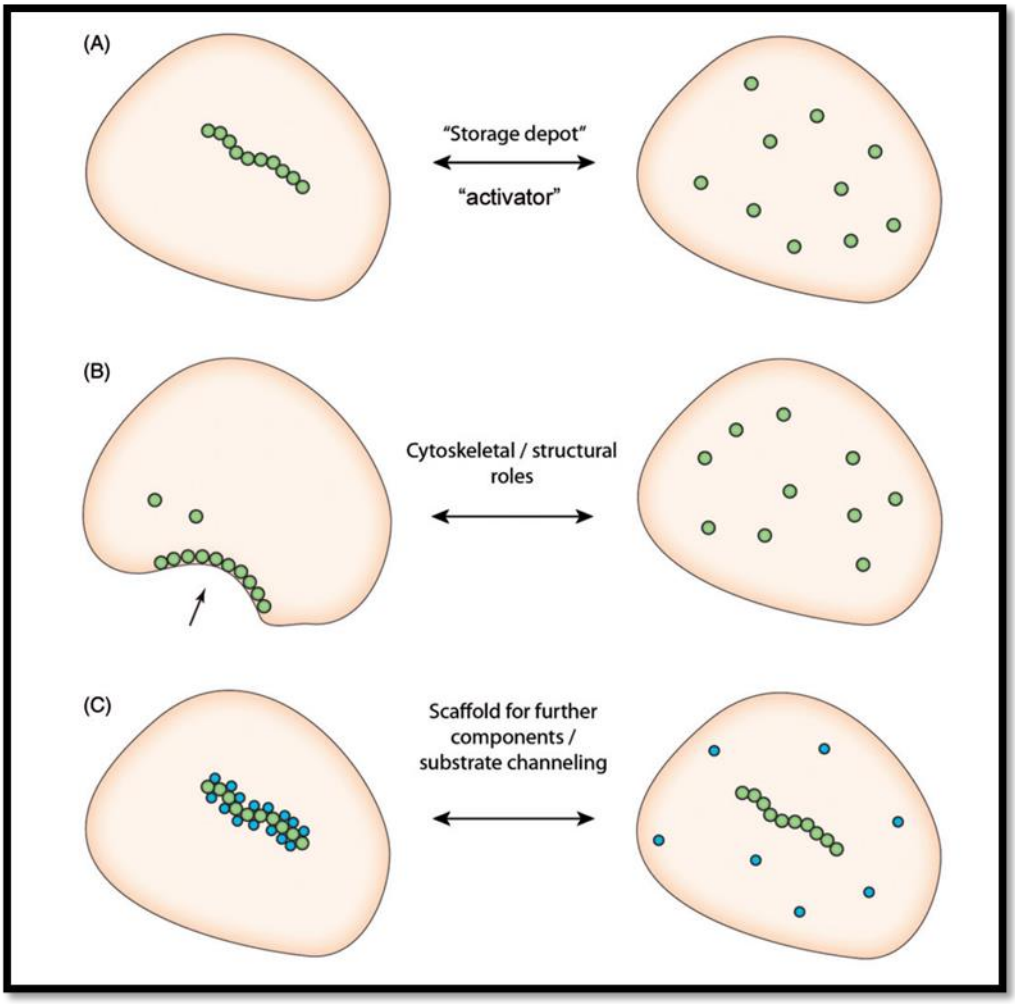

Figure 4-3: Possible roles of cytoophidia.

(A) They may be storage depots sequestering enzyme molecules and releasing them only when the need arises. **(B)** They may have structural roles such as maintaining stability of cell shape and curvature. **(C)** They may act as scaffolding structures that help in transport of regulation of other molecules within the cell. Adapted from *Nat Critical Reviews in Biochemistry and Molecular Biology. 2016, VOL. 51, NO.4 :282–293* under the "fair dealings" provision of the Canadian Copyright Act (2019).

In the current study I found that Pna1p filaments greatly increase in length when the culture transitions from early-log phase to mid-log to stationary phase (**Figure 3-13**). Furthermore, the long filaments in stationary phase are able to fragment and restart filament formation when introduced into fresh growth media (**Figure 3-14**). The reason for this maybe the nutrient content of the saturated cultures, however, further experiments need to be done to confirm this hypothesis. For example, the cells from stationary phase can be exposed to media with low glucose, which will tell us if variation in carbon source levels in the media is related to elongation of the filaments. Alternatively, cells can be exposed to growth media (plus/minus carbon and nitrogen sources) to determine the exact component responsible for the elongation of these structures in denser cell cultures.

In Drosophila and mammalian cells, it was shown that overexpression of *ctps* greatly increased the lengths and thicknesses of CTPS cytoophidia (Aughey et al., 2014; Azzam & Liu, 2013; Gou et al., 2014). In these studies, it was also observed that the overexpression of the *ctps* gene did not significantly affect the enzymatic function of the protein suggesting that cytoophidia formation is perhaps a way of sequestering the enzyme, thus regulating it. To see if there was a similar effect of overexpression on Pna1-GFP filaments, a *pna1* over-expression plasmid was transformed into a *pna1-gfp* and then induced in the absence of thiamine. In contrast to *ctps* overexpression, I saw that overexpressing *pna1* prevented the formation of Pna1-GFP filaments. This is to say, I observed the protein to be uniformly diffused throughout the cell. The reason for this phenotype is unknown, but does imply that a feedback mechanism may be involved.

4.4 Concluding Remarks

In this project, I found that *pna1* plays a crucial role in countering the effects of LatA mediated cytoskeletal perturbations. It is required for the successful completion of cell division and complete constriction of the ring. However, the exact molecular mechanism(s) by which this occurs are not yet fully elucidated.

Unexpectedly, I found that Pna1-GFP localises to filamentous structures which likely represent a novel intracellular structure called cytoophidia. I characterised this filament by subjecting it to different physiological stresses, as well as drugs inhibiting cytoskeletal components and the TOR pathway. I found that these filaments are quite stable when subjected to high doses of LatA (completely disrupts the actin cytoskeleton), MBC (inhibitor of microtubules), and Torin-1 (inhibitor of the TOR pathway). The filaments also did not respond to heat or cold shock. It is important to note, however, that all these experiments were done with the Pna1-GFP integrant strain. There has been evidence in literature that fluorescent tags can disrupt the function of the protein (Ausmees et al., 2003; Ma et al., 1996). However, the fact that the Pna1-GFP protein is fully functional with respect to LatA sensitivity (**Figure 3-7**) suggests that this is an unlikely possibility.

The most critical question arising from this work relates to the functional role of Pna1p filamentation. As described earlier, cytoophidia have been proposed to act as storage depots, sequestering enzyme molecules and releasing them only when the cell needs them. They may also have cytoskeletal or structural roles like the one described by Ingerson-Mahar et al. (2010). They may act as scaffolding structures that help sequestering other cytoplasmic components. They may even help in regulating traffic of the enzyme between different compartments of the cell. Polyamine levels are highly regulated within the cell. As discussed previously, acetylation of polyamines inhibits their interaction with actin, and I hypothesised that a Caf5p mediated efflux may be in place, helping regulation of polyamine levels in the cell. In such a scenario, Pna1p filaments likely sequester the enzyme, releasing Pna1p into the cytoplasm in order to allow acetylation of polyamines, promoting their efflux and thus modulating actin dynamics.

In this study, I found that Pna1-GFP forms filaments and I characterised the filaments by subjecting the cells to physiological stimuli and observing the change in morphology of the filaments. However, the functional significance of this filamentation is unclear. To study this, one approach would be to devise a genetic method to prevent formation of

Pna1p filaments and determine how this relates to the cytokinetic failure phenotype. For example, a PCR based random mutagenesis strategy could be used to generate a random set of *pna1* mutants. These mutants could then be screened for their ability to form filaments, and the mutants incapable of forming filaments sequenced to identify the specific portion of the protein affecting filament-formation. Such mutants could also be subjected to LatA mediated cytoskeletal stress to determine the phenotypic consequence. By further characterising Pna1p filaments, it may be possible to provide novel biological insight into both the regulation of cytokinesis, as well as the molecular functions of the mysterious and poorly understood intracellular structures called cytoophidia.

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Appendices

APPENDIX A: Links to folders containing downloadable Supplementary Movies.

NOTE: VLC media player is recommended for both Windows and MAC users.

For Windows users (.avi format):

https://uwoca-my.sharepoint.com/:f:/g/personal/jkaragia_uwo_ca/EjUuAchSChRCtOIhW2gOORkBK5hfLkLgK7pdF2c-yfqyPQ?e=blDjhj

For MAC users (.mov format):

https://uwoca-my.sharepoint.com/:f:/g/personal/jkaragia_uwo_ca/EiTyF3bmhJBKuwDRsunOiEoBTykhTptMC0Jx-MfAjaXexg?e=KAmzOt

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