MECHANISMS THAT CONTROL LOCALIZATION OF THE *SCHIZOSACCHAROMYCES POMBE* Clp1/Cdc14 PHOSPHATASE

BY

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LIST OF ABBREVIATIONS

1NM-PP1	4-Amino-1-*tert*-Butyl-3-(1'-Napthylmethyl) Pryazole(3,4-d) Pyrimidine
^{32}P	[$^{32}\gamma$]-phosphorous
A / ala	Alanine
ATP	Adenine triphosphatate
C	Carboxy / Celsius
Cdk	Cyclin dependent kinase
CISR	Cell Imaging Shared Resource
CR	Contractile Ring
D / asp	Aspartic acid
DiFMUP	6,8-difluoro-4-methylumbelliferyl phosphate
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose nucleic acid
EMM	Edinburgh minimal media
F	Phenylalanine
FEAR	Cdc14 early anaphase release
FRAP	Fluorescence recovery after photobleaching
G	Glycine
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
HA	influenza hemagglutinin epitope
H_2O	Water

H_2O_2	Hydrogen peroxide
Hr	Hour
HU	Hydroxyurea
Kan^R	Kanamycin resistant
KGY	Kathy Gould yeast
Kt	Kinetochore
LMB	Leptomycin B
M	Molar
MAPK	Mitogen-activated protein kinase
M_F	Mobile fraction
MBP	Maltose binding protein
MEN	Mitotic exit network
Min	Minute
mM	millimolar
mRFP	monomeric red fluorescent protein
MS	Mass spectrometry / mitotic spindle
MYC	MYC epitope
N	Number
ng	nanogram
NLS	Nuclear localization sequence
nmt	No message in thiamine
No	Nucleolus
NP-40	Nonidet® P-40

P / pro	Proline
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
R / arg	Arginine
S / ser	Serine / second
SDS	Sodium dodecyl sulfate
SIN	Septation initiation network
SPB	Spindle pole body
T / thr	Threonine / time
$T_{1/2}$	Half time of recovery
TAP	Tandem affinity purification
μg	microgram
μM	micromolar
μm	micrometer
X	any amino acid
YE	Yeast extract

CHAPTER I

INTRODUCTION

The eukaryotic cell cycle

A cell is considered the basic fundamental unit of life. All living organisms from simple bacteria and yeast, to complex metazoa are made up of cells. In order for new life to develop, cells must reproduce, and they do so by division from preexisting cells. For cells to undergo division they must first complete the cell cycle. Thus the cell cycle has been a major focus of scientific research to understand the fundamentals of life. Additionally, numerous diseases associated with developmental problems and/or cancer have been linked to errors within the cell cycle making this research important for our quality of life.

The typical eukaryotic somatic cell cycle is divided into four different phases: two gap phases, G1 and G2 that are important for cellular growth and monitoring the fidelity of the cell cycle, the synthesis phase (S phase) that is responsible for the replication of chromosomes, and M phase (mitosis), which controls even division of sister chromatids into both daughter cells (Figure 1) (Reviewed in Morgan, 2007). Once cells make the decision to enter the cell cycle, they are fully committed to undergo cellular division. An inability to complete the cell cycle once committed, results in cell death. Additionally, the order of each cell cycle phase is important and irreversible, ensuring that certain cellular events occur before others, and that certain events only occur once per cycle (e.g. chromosome must be duplicated only once before they can be equally

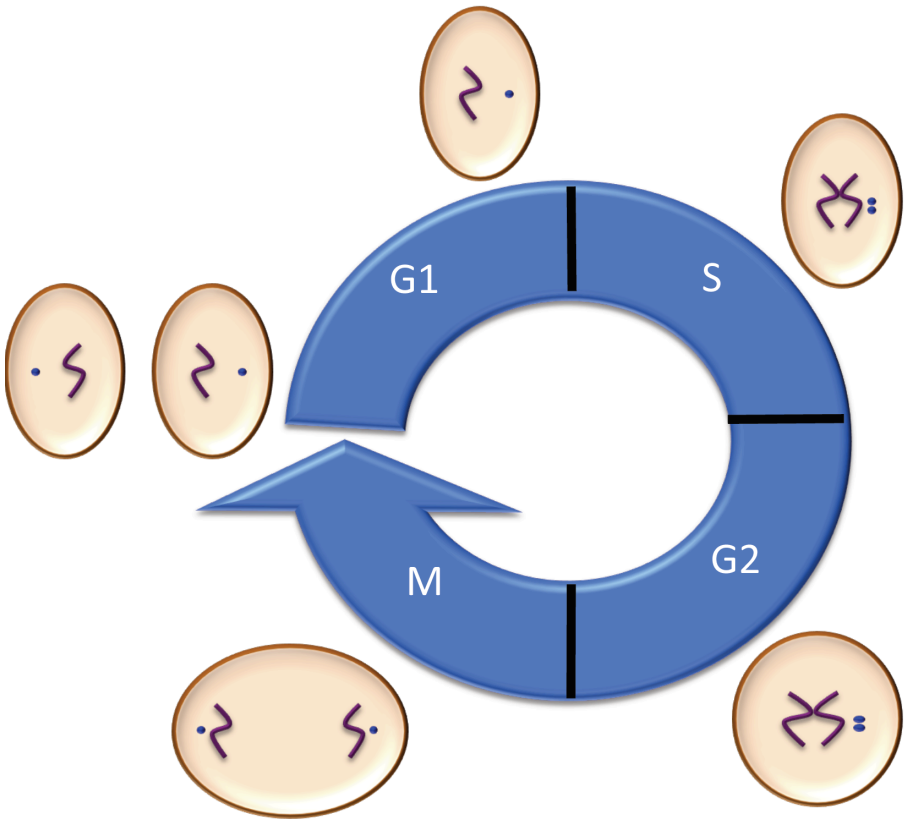

Figure 1. A schematic of the eukaryotic somatic cell cycle. Once triggered to commit cellular division, a cell begins what is known as the cell cycle. The cell cycle is split into four ordered and irreversible phases: G1, S, G2 and mitosis (M) (blue arrow). During the cell cycle, a cell performs numerous tasks necessary to undergo cellular division, like the duplication of the centrosome/spindle pole body (blue circle). Most importantly is the proper duplication of the chromosomes (purple) during S phase, and equal division of said chromosomes during mitosis.

divided between new daughter cells). Thus, commitment and progression through the cell cycle is highly governed by cell cycle checkpoints that ensure the previous stage of the cell cycle has completed successfully before continuing on.

Cyclin dependent kinases (Cdks) are a highly conserved family of protein kinases that control commitment and progression through the eukaryotic cell cycle (Enserink and Kolodner, 2010; Morgan, 1997). Combined with their activating partners, the cyclins, Cdks control the order of cell cycle events by triggering biochemical switches through phosphorylation of specific cellular substrates. This order and specificity is controlled through oscillating production of specific cyclin partners (Figure 2). Upon successful completion of chromosomal division, mitotic Cdk activity is abolished to allow for cytokinesis and resetting of the cell for another round of division. This inactivation of Cdk activity occurs in the late stages of mitosis, from anaphase to telophase, also known as mitotic exit.

More specifically, Cdk1, bound to its cyclin B partner, controls entry into and progression through mitosis (Morgan, 1997). For proper mitotic exit and cytokinesis, a reduction in Cdk1 activity, as well as dephosphorylation of its substrates must occur. This happens through two mechanisms. One mechanism is the activation of the anaphase promoting complex/cyclosome (APC/C). The APC/C, along with its two co-activators, Cdc20 and/or Cdh1, ubiquitylate specific substrates, including S and M phase cyclins, which targets them for degradation by the proteosome (Manchado et al., 2010). This targeted destruction of S and M phase cyclins abolishes Cdk1 activity until the next committed round of cellular division, and ensures that nuclear division occurs only once.

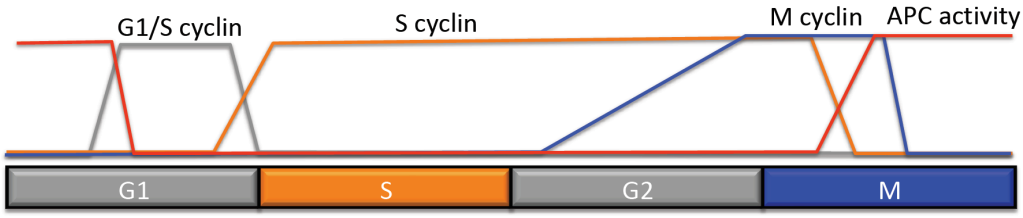

Figure 2. Schematic of cyclin oscillation. Cdks associated with specific cyclin partners drive the cell cycle. Though the Cdk subunit provides the catalytic activity, oscillatory changes in cyclins and their levels specify the order of cell cycle. This system functions to ensure that certain events associated with particular phases of the cell cycle occur only once. Late in mitosis Cdk-cyclin activity needs to be turned off to reset the cell for another round of division. This is accomplished by the anaphase promoting complex/cyclosome, which targets cyclins for degradation. (Adapted from Morgan, 2007)

The second mechanism cells utilize to promote mitotic exit and cytokinesis involves mitotic phosphatases (e.g. the Cdc14 phosphatase family, which will be the subject of the next section), which function to reverse mitotic phosphorylation events, particularly Cdk phosphorylation, on substrates that are not targeted for degradation by the APC/C (Wurzenberger and Gerlich, 2011). Antagonism of mitotic phosphorylation is important to reverse particular mitotic events (e.g. condensation of chromosomes), and prevent further rounds of nuclear division. Additionally, mitotic phosphatases control activation of cytokinesis, the physical process of cell division.

During mitosis, cells are primed to undergo cytokinesis, however, this process does not occur until chromosomes have been appropriately segregated. To preserve the order of these cellular processes, high Cdk1-cyclin B activity drives early mitotic events necessary for chromosome segregation, while inhibiting the cytokinetic machinery. Once cells complete chromosome segregation, triggering the APC^{Cdh1} complex to degrade cyclin B, mitotic phosphatases reverse inhibitory Cdk1-cyclin B phosphorylation on cytokinetic machinery and physical cell division occurs. Thus, mitotic phosphatases that antagonize Cdk phosphorylation play a key role in controlling mitotic exit and cytokinesis.

Cdc14 phosphatases

The Cdc14 phosphatases are a well conserved family (Mocciaro and Schiebel, 2010) of proline directed ser/thr phosphatases, classified as dual specificity phosphatases (Gray et al., 2003), that function to antagonize Cdk phosphorylation during mitotic exit (Mocciaro and Schiebel, 2010; Queralt and Uhlmann, 2008; Stegmeier and Amon, 2004).

The founding family member, Cdc14, was identified in *Saccharomyces cerevisiae* as an essential cell cycle phosphatase necessary for Cdk1 inactivation during mitotic exit (Stegmeier and Amon, 2004). Further studies on Cdc14 orthologs from yeast to humans have characterized additional roles for this enzyme family in cytokinesis (Clifford *et al*., 2008a), chromosome segregation (Clemente-Blanco et al., 2011; Mocciaro and Schiebel, 2010; Stegmeier and Amon, 2004), transcription (Clemente-Blanco et al., 2009; Clemente-Blanco et al., 2011; Guillamot et al., 2011; Papadopoulou et al., 2010), centrosome duplication (Mocciaro and Schiebel, 2010), and ciliogenesis (Clement et al., 2011, 2012). Therefore, proper regulation of the Cdc14 phosphatase family is important to ensure the fidelity of multiple cellular processes.

Localization of the Cdc14 phosphatases

In line with their multiple roles during anaphase, Cdc14 phosphatases undergo dynamic subcellular localization changes that are dependent on the cell cycle (Mocciaro and Schiebel, 2010; Stegmeier and Amon, 2004). During interphase, Cdc14 phosphatases are sequestered within the nucleolus and/or at the spindle pole body (SPB)/centrosome until mitosis when they are released from these locales (Figure 3). This release from sequestration during mitosis is considered a major activation step allowing Cdc14 phosphatases access to their substrates localized at specific mitotic structures. In *Schizosaccharomyces pombe,* the Cdc14 phosphatase ortholog, Clp1/Flp1 (hereafter refer to as Clp1), relocalizes to the kinetochores, along the mitotic spindle, and to the contractile ring (Figure 4). The inability of Clp1 to localize to these specific sites

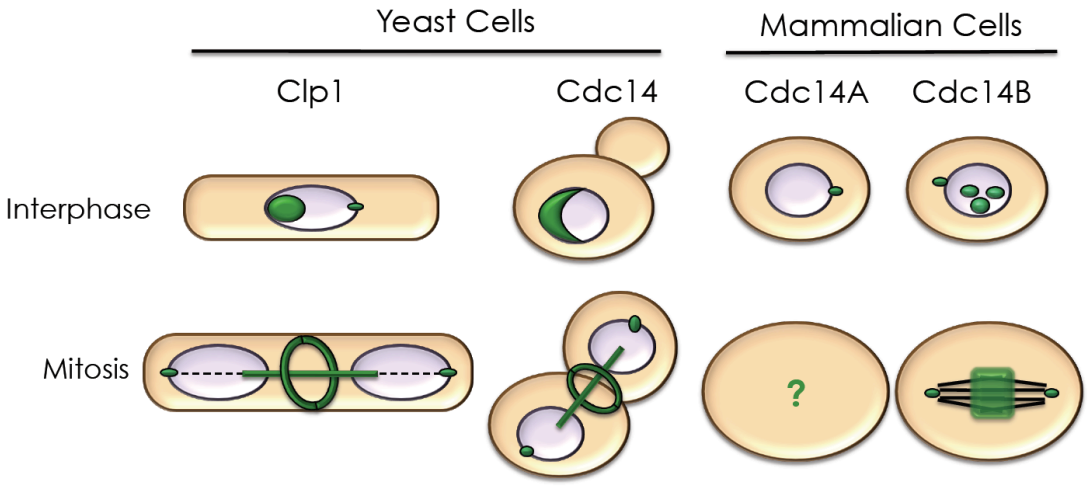

Figure 3. Schematic indicating the intracellular localization of Cdc14 phosphatases. In each representation, purple signifies the nucleus, and green is Cdc14 for either interphase or mitotic cells as indicated. The *S. pombe* ortholog, Clp1, localizes to the nucleolus and SPB during interphase, and first transitions to the kinetochores (not shown) and the contractile ring during early mitosis. Later in mitosis, Clp1 relocalizes along the mitotic spindle, and remains at the contractile ring until the completion of cytokinesis. In *S. cerevisiae*, Cdc14 is localized to the nucleolus during interphase, and redistributes to the SPB, mitotic spindle and contractile ring after the metaphase-anaphase transition. Mammalian cells have two Cdc14 orthologs denoted Cdc14A and Cdc14B. During interphase Cdc14A is localized to the centrosome, and Cdc14B is localized to centrioles as well as sequestered within the nucleolus. During mitosis, Cdc14A leaves the centrosomes and is presumed to be diffuse throughout the cell, while Cdc14B is released from the nucleolus and relocalizes to the spindle midzone and midbody, but remains at centrioles.

increases errors in chromosome segregation and cytokinetic failure (Chen et al., 2008; Clifford et al., 2008b; Mishra et al., 2005; Trautmann and McCollum, 2005; Trautmann et al., 2004). Additionally, failure to release Cdc14 from nucleolar sequestration arrests cells in late mitosis with high levels of Cdk1 activity (Stegmeier and Amon, 2004). Thus identifying mechanisms that facilitate Cdc14 phosphatase family nucleolar release is important to understanding Cdc14 phosphatase regulation, and this is the subject of chapter III.

It is equally important to understand how Cdc14 phosphatases localize to specific sites once released from interphase sequestration. Currently, Mid1, an anillin like protein, is the only Clp1 tether identified, being necessary for Clp1 to localize to the contractile ring during cytokinesis (Clifford et al., 2008b). Cells that lack *mid1*$^{+}$ or contain a mutant form of *mid1* that is unable interact with Clp1, display cytokinetic defects in line with Clp1's role in cytokinesis (Clifford et al., 2008b). Additionally, Clp1 localizes to both nuclear and cytoplasmic addresses throughout the cell cycle (Stegmeier and Amon, 2004), and its occupancy to both cellular compartments is critical for Clp1's functions (Mishra et al., 2005; Trautmann and McCollum, 2005), yet how this is maintained is not understood. Chapter IV focuses on addressing these issues.

Cdc14 phosphatase nucleolar release

Most studies that focus on understanding nucleolar release of Cdc14 phosphatases have been preformed in *S. cerevisiae*. In *S. cerevisiae*, Net1/Cfi1 (hereafter referred to as Net1) tethers and inhibits Cdc14 within the nucleolus throughout interphase (Shou et al., 1999; Visintin et al., 1999). During anaphase, Cdc14 is triggered to exit the nucleolus by

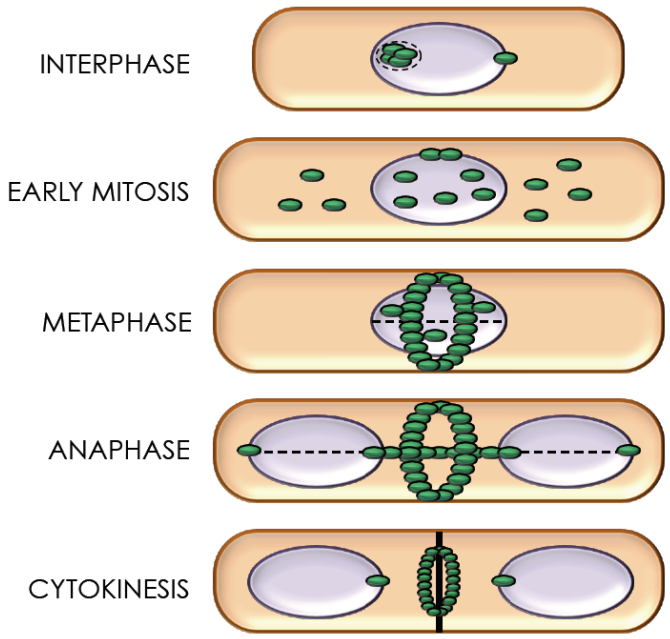

Figure 4. A schematic of Clp1 localization during the cell cycle. The *S. pombe* Cdc14 ortholog, Clp1, localizes at the SPB and is sequestered within the nucleolus during interphase. Early in mitosis, Clp1 is relieved of its nucleolar sequestration and redistributes to both nuclear and cytoplasmic localizations. More specifically, during metaphase, Clp1 is localized to the kinetochores and contractile ring. During anaphase, Clp1 relocalizes along the mitotic spindle and remains at the constricting contractile ring until completion of mitosis.

two networks, the Cdc Fourteen Early Anaphase Release (FEAR) network and the Mitotic Exit Network (MEN) (Liang et al., 2009; Manzoni et al., 2010; Mohl et al., 2009; Rock and Amon, 2009; Stegmeier and Amon, 2004; Tomson et al., 2009; Waples et al., 2009). The FEAR network controls the initial transient nucleolar release of Cdc14 during anaphase (Stegmeier et al., 2002; Sullivan and Uhlmann, 2003; Yoshida and Toh-e, 2002). Although not essential, FEAR-dependent release is important for proper chromosome segregation, spindle stability and progression through anaphase (Rock and Amon, 2009). After the initial FEAR-dependent Cdc14 release, the MEN promotes full activation and maintains the release of Cdc14 until the completion of mitotic exit (Stegmeier and Amon, 2004). In contrast to FEAR-dependent release, MEN-dependent Cdc14 nucleolar release is essential for Cdc14 inactivation of Cdk1 (Rock and Amon, 2009; Stegmeier and Amon, 2004).

Unlike *S. cerevisiae* Cdc14, the fission yeast ortholog, Clp1, and mammalian Cdc14B exit the nucleolus prior to anaphase (Berdougo et al., 2008; Cho et al., 2005; Cueille et al., 2001; Kaiser et al., 2002; Nalepa and Harper, 2004; Trautmann et al., 2001). Additionally, *S. pombe* orthologs of FEAR network or MEN (known as the Septation Initiation Network (SIN) in *S. pombe*) components are not required for Clp1 nucleolar release (Chen *et al*., 2006). These observations suggest that the mechanisms controlling Clp1 and Cdc14B nucleolar release may differ from those controlling nucleolar release of *S. cerevisiae* Cdc14. However, the SIN does prohibit return of Clp1 to the nucleolus until the completion of cytokinesis (Chen et al., 2008; Mishra et al., 2005). This is due to direct phosphorylation on RxxS (x can be any amino acid) sites by the SIN kinase, Sid2, and consequent association between Clp1 and the *S. pombe* 14-3-3

proteins, Rad24 and Rad25 (Figure 5) (Chen et al., 2008; Mishra et al., 2005). A Clp1 mutant that cannot be phosphorylated by Sid2 returns to the nucleolus before the completion of cytokinesis, which results in increased cytokinetic failure (Chen *et al*., 2008).

Phosphoregulation of Cdc14 phosphatases

Beyond being regulated through changes in localization, the Cdc14 phosphatases are also controlled through phosphorylation. Most of our understanding of Cdc14 phosphatase family phosphoregulation comes from studies of the *S. pombe* ortholog, where Clp1 was shown to be highly phosphorylated during mitosis (Wolfe et al., 2006). Above, I described one mechanism of phosphoregulation in which Sid2 directly phosphorylates Clp1 on RxxS sites to prevent its nucleolar sequestration (Chen et al., 2008; Mishra et al., 2005). Interestingly, upon DNA replication stress the same RxxS sites are phosphorylated by the checkpoint effector kinase, Cds1 (Diaz-Cuervo and Bueno, 2008). This will be further discussed in the section below.

Cdk1, the cell cycle kinase, also contributes to Clp1 mitotic phosphorylation by directly phosphorylating Clp1 on TP sites early in mitosis (Figure 5). This phosphorylation was shown to inhibit the catalytic activity of Clp1, preventing it from prematurely dephosphorylating Cdk1 substrates before mitotic exit (Wolfe et al., 2006). When Cdk1 activity is attenuated during anaphase, Clp1 autodephosphorylates, activating itself to dephosphorylate Cdk1 substrates and promote cytokinesis (Wolfe et al., 2006).

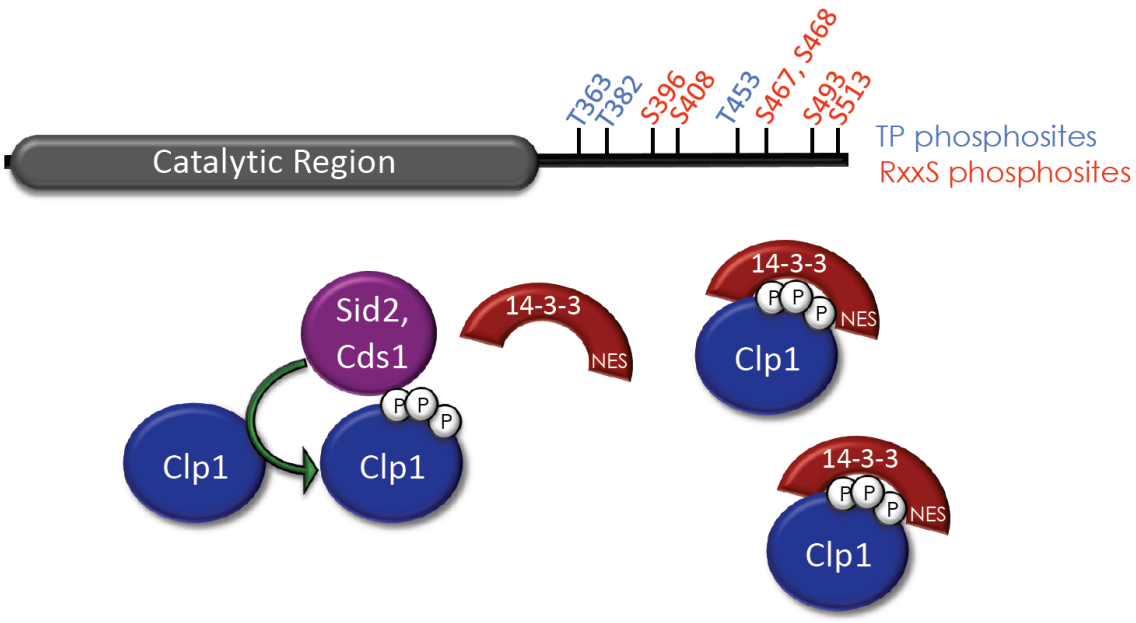

Figure 5. Schematic of Clp1 phosphoregulation. Above, a cartoon schematic of Clp1 with both TP, those phosphorylated by Cdk1 (Wolfe et al., 2006), and RxxS, those phosphorylated by Sid2 and/or Cds1 (Chen et al., 2008; Diaz-Cuervo and Bueno, 2008), phosphosites indicated. Phosphorylation of Clp1 TP sites inhibits the catalytic activity of Clp1, while RxxS site phosphorylation prevents Clp1 from being resequestered within the nucleolus. The mechanism in which Clp1 RxxS phosphorylation prevents nucleolar sequestration is cartooned below. Sid2 and/or Cds1 phosphorylate Clp1 on RxxS sites, which allows Clp1 to bind the *S. pombe* 14-3-3 proteins, Rad24 and Rad25, and this association prevents Clp1 from being resequestered within the nucleolus.

Cdc14 phosphatases and the DNA damage response

While the cellular genomic material is under constant assault, the conservation of the genomic code is critical for cell survival. To ensure cells do not pass along damaged DNA, cells utilize the DNA damage checkpoint to halt the cell cycle, allowing cells time to repair damaged DNA before cellular division. If left unrepaired, cells can face detrimental consequences, or in the case of disease, exhibit cancer phenotypes. Therefore, a comprehensive understanding of the DNA damage checkpoints, as well as DNA repair has been one major focus of cell cycle research.

In addition to roles in mitosis, one mammalian Cdc14 phosphatase ortholog, Cdc14B, is thought to participate in the DNA damage checkpoint by dephosphorylating Cdh1, an activator of the APC/C; premature APC/C activation promotes a cell cycle delay at the G2/M transition (Bassermann *et al*., 2008; Takahashi *et al*., 2012). Other reports indicate that when Cdc14A, a second mammalian ortholog, or Cdc14B genes are deleted, repair of DNA double strand breaks is prolonged suggesting that Cdc14 phosphatases play a role in DNA damage repair but not checkpoint arrest (Mocciaro *et al*., 2010; Wei *et al*., 2011b). Despite the lack of clarity regarding Cdc14 phosphatase function in response to genotoxic stress, there is agreement that this stimulus results in nucleolar release of Cdc14B during interphase (Bassermann *et al*., 2008; Mocciaro *et al*., 2010; Takahashi *et al*., 2012). Similarly, Clp1 moves out of the nucleolus during interphase when DNA replication is blocked by treatment with hydroxyurea (HU), and Clp1 nucleolar release is required for a normal checkpoint response to HU (Diaz-Cuervo and Bueno, 2008).

Interphase nucleolar release of Clp1 and Cdc14B in cells treated with HU or DNA damaging agents, respectively, depends on checkpoint effector kinases (Diaz-Cuervo and Bueno, 2008; Peddibhotla *et al*., 2011), suggesting that relocalization of these two phosphatases may occur through a conserved mechanism. In *S. pombe*, the ATR ortholog, Rad3, is the major sensor of genotoxic insults, and is essential for arresting the cell cycle in response to stalled replication forks or DNA damage (Langerak and Russell, 2011). Rad3 activates two downstream effector kinases, Cds1 in response to genotoxic insults during DNA replication, or Chk1 in response to genotoxic insults during G2 (Brondello et al., 1999; Lindsay et al., 1998; Martinho et al., 1998; Walworth and Bernards, 1996). Interestingly, in response to HU treatment, Cds1 phosphorylates Clp1 on the same sites as does Sid2 during mitosis (Figure 5), and a Clp1 phosphomutant which abolishes these RxxS phosphosites prevents HU-induced nucleoplasmic relocalization (Diaz-Cuervo and Bueno, 2008). However, the RxxS phosphomutant does exit the nucleolus normally during mitosis (Chen *et al*., 2008; Diaz-Cuervo and Bueno, 2008).

Summary

The conserved family of Cdc14 phosphatases function to antagonize Cdk phosphorylation to promote mitotic exit and cytokinesis, and likely contributes to the DNA damage response. Due to the important cell cycle processes that Cdc14 phosphatases influence, a comprehensive understanding of their functions and regulations are essential to our understanding of the cell cycle. In this work, I examined whether interphase nucleolar release of *S. pombe* Clp1 is a general response to cellular stress, and

further investigated the mechanism in which these processes occur (chapter III). I found that Clp1 interphase nucleolar release occurs in response to hydrogen peroxide as well as hydroxyurea suggesting it is a specific response to genotoxic stress. Upon investigating the pathways that trigger interphase nucleolar release after these treatments, I found that in addition to Chk1 and Cds1, the cell wall integrity mitogen-activated protein kinase (MAPK), Pmk1, and the cell cycle kinase, Cdk1, are involved although the relative effect of these kinases on Clp1 localization is dependent on the type of genotoxic insult. Accordingly, a Clp1 phosphomutant that abolishes Chk1, Cds1, Pmk1 and Cdk1 phosphosites prevented interphase nucleoplasmic accumulation of Clp1 upon either type of genotoxic stress. Reciprocally, a Clp1 mutant that is constitutively phosphorylated on TP sites cannot be retained in the nucleolus.

Additionally, I investigated how Clp1 is localized throughout the cell cycle (chapter IV). I identified both *cis* and *trans* factors that facilitate localization of Clp1 to both nuclear and cytoplasmic compartments of the cell. Furthermore, I present data that suggests Clp1 localizes to both nuclear and cytoplasmic localizations, specifically the nucleolus and SPB, with very dynamic behavior. These studies advance our understanding of Clp1 phosphatase regulation, and may provide insight into the mechanisms controlling Cdc14B localization in higher eukaryotes.

CHAPTER II

METHODS

Strains and General Yeast Methods

S. pombe strains used in this study (Table 1) were grown in yeast extract (YE) or Edinburgh minimal media (EMM) plus supplements. For induction from the *nmt41* promoter (Maundrell, 1993), cells were first grown overnight in media containing 5 μg/ml thiamine, then washed three times with media lacking thiamine and allowed to grow for 18 hr in thiamine-free media.

clp1^{+}, *clp1(D257A), clp1(6A), clp1(3A), clp1(6A3A), sal3*$^{+}$, *gar2*$^{+}$, and *rad24*$^{+}$ were tagged endogenously at the 3' end of their open reading frames with *GFP:kan*^{R}, *MYC13:kan*^{R}, *Flag3:kan*^{R}, *mRFP:kan*^{R}, *mCherry:kan*^{R}, or *HA3-TAP:kan*^{R} cassettes as previously described (Bahler *et al*., 1998). The *clp1(D257A), clp1(6A), clp1(3A)* and *clp1(6A3A)* mutants were constructed by site-directed mutagenesis of *clp1*$^{+}$ cDNA flanked by *clp1*$^{+}$ 5' and 3' genomic sequences in the pIRT2 vector. Correct mutations were verified by DNA sequencing (GenHunter, Nashville, TN). To construct the *clp1(1-500)* mutant I introduced a GFP::kan^{R} cassette following amino acid 500 in the endogenous *clp1*$^{+}$ open reading frame, eliminating the last 37 amino acids of the protein. The *clp1NLSmut* mutant was constructed by introducing ala substituions at R524, K527, K529, K532 and R534 within the tagging PCR. Integration of phosphosite mutants at the *clp1*$^{+}$ endogenous locus and subsequent tag insertions were done by lithium acetate transformation (Keeney and Boeke, 1994), and correct integrations were confirmed by

Table 1 ***S. pombe*** **strains used in this study**

Strain	Genotype	Source
Figure 6		
KGY14062	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *rad3*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14063	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *chk1*::*ura4 cds1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14064	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *pmk1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14065	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *rad3*::*ura4 pmk1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14066	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G ade6*-M21X *ura4*-D18 *leu1*-32 h?	This study
KGY14067	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G rad3*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14068	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G pmk1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14069	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G pmk1*::*ura4 rad3*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14072	*clp1*::*3A-GFP*:kan^R *gar2-mCherry*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14073	*clp1*::*6A-GFP*:kan^R *gar2-mCherry*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14074	*clp1*::*6A3A-GFP*:kan^R *gar2-mCherry*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
Figure 7		
KGY12132	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
Figure 8		
KGY14062	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *rad3*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14063	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *chk1*::*ura4 cds1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14064	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *pmk1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14065	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *rad3*::*ura4 pmk1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14066	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G ade6*-M21X *ura4*-D18 *leu1*-32 h?	This study
KGY14067	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G rad3*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14068	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G pmk1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14069	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *cdc2-F84G pmk1*::*ura4 rad3*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
Figure 9		
KGY12132	*clp1-GFP*:kan^R *gar2-mCherry*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study

Figure 10		
KGY861	*nda3-km311, h*$^{-}$	Lab stock
KGY5809	*nda3-KM311 clp1-MYC13:kan*^{R} *ura4-D18 leu1-32 h-*	Lab stock
KGY5832	*nda3-KM311 clp1(3A)-MYC13:kan*^{R} *ade6*-M21x *ura4*-D18 *leu1*-32 h$^{?}$	Lab stock
KGY6236	*clp1-MYC13*:kan^{R} *ade6*-M21x *ura4*-D18 *leu1*-32 h^{+}	Lab stock
KGY13249	*clp1-MYC13*:*kan*^{R} *rad3*::*ura4+ pmk1*::*ura4+ cdc2-as ade6-M210 leu1-32 ura4-D18 h*$^{?}$	This study
KGY6406	*pmk1*::*ura4 ura4*-D18 *leu1*-32 h-	Lab stock
Figure 11		
KGY14070	*clp1-MYC13*:*kan*^{R} *rad24-HA3-TAP ade6*-M21x *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY14071	*clp1-MYC13*:*kan*^{R} *rad24-HA3-TAP rad3*::*ura4*$^{+}$ *pmk1*::*ura4+ cdc2-as ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY6236	*clp1-MYC13*:*kan*^{R} *ura4-D18 ade6-M21X leu1-32 h*$^{+}$	Lab stock
KGY13251	*clp1-MYC13*:kan^{R} *cdc2-F84G pmk1*::*ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
Figure 12		
KGY14072	*clp1*::*3A-GFP*:kan^{R} *gar2-mCherry*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY14073	*clp1*::*6A-GFP*:kan^{R} *gar2-mCherry*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY14074	*clp1*::*6A3A-GFP*:kan^{R} *gar2-mCherry*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY13655	*clp1*::*6A3A-MYC13*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY14075	*clp1(D257A)-GFP*:*kan*^{R} *gar2-mCherry*:*kan*^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
Figure 13		
KGY12126	*clp1*::*clp1(6A3A)-GFP*:*Kan*^{R} *ura4*-D18 *leu1*-32 *ade6*-M216 *h*$^{+}$	Lab stock
Figure 14		
KGY246	*ade6*-M210 *ura4*-D18 *leu1*-32 h^{-}	Lab stock
KGY8856	*sty1*::*ura4 ura4*-D18 *leu1*-32 h^{-}	Lab stock
KGY3381	*clp1*::*ura4* ade6-M210 *ura4*-D18 *leu1*-32 h^{-}	Lab stock
KGY8840	*rad3*::*ura4 ura4*-D18 *leu1*-32 h^{-}	Lab stock
KGY12837	*leu1*::*pJK148-nmt41-clp1-TAP*:*kan*^{R} *ade6*-M21x *leu1*-32 *ura4*-D18 *h*$^{?}$	This study
KGY6406	*pmk1*::*ura4 ura4*-D18 *leu1*-32 *h*$^{-}$	Lab stock
Figure 16		
KGY6236	*clp1-MYCx13*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h^{+}	Lab stock
KGY6340	*sal3-FLAGx3*:hyg^{R} *ade6*-M210 *ura4*-D18 *leu1*-32 h^{-}	This study
KGY14147	*clp1-MYCx13*:kan^{R} *sal3-FLAGx3*:hyg^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY8019	*clp1-GFP*:kan^{R} *sid4-mRFP*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h^{-}	Lab stock
KGY12113	*clp1-GFP*:kan^{R} *sid4-mRFP*:kan^{R} *sal3::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h^{-}	This study
Figure 18		
KGY8019	*clp1-GFP*:kan^{R} *sid4-mRFP*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h^{-}	Lab stock

KGY12113	*clp1-GFP*:kan^R *sid4-mRFP*:kan^R *sal3::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^-$	This study
KGY9728	*clp1NLSmut-GFP*:kan^R *sid4-mRFP*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY10743	*clp1(1-500)-GFP*:kan^R *sid4-mRFP*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^-$	This study
KGY14211	*rpn11-mCherry*:kan^R *ade6*-M210, *ura4*-D18, *leu1*-32 *h*$^+$	This study
Figure 19		
KGY14213	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *kap104::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
KGY14214	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *kap111::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
KGY14215	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *kap113::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
KGY14216	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *kap114::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
KGY14217	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *kap123::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
KGY14218	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *imp1::*clonat *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
KGY14219	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *cut15-122 ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
KGY14220	*clp1-GFP*:kan^R *nog1-mCherry*:kan^R *cut15-122 imp1:*clonat *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This Study
Figure 20		
KGY10743	*clp1(1-500)-GFP*:kan^R *sid4-mRFP*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^-$	This study
KGY9728	*clp1NLSmut-GFP*:kan^R *sid4-mRFP*:kan^R *ade6*-M21X *ura4*-D18 *leu1*-32 h$^?$	This study
KGY14211	*rpn11-mCherry*:kan^R *ade6*-M210, *ura4*-D18, *leu1*-32 *h*$^+$	This study
KGY14212	*rpn11-mCherry*:kan^R *sal3::ura4 ade6*-M210, *ura4*-D18, *leu1*-32 *h*$^+$	This study
Figure 21		
KGY246	*ade6*-M210 *ura4*-D18 *leu1*-32 h$^-$	Lab stock
KGY19	*cdc2-33 leu1*-32 h$^-$	Lab stock
KGY3381	*clp1*::*ura4* ade6-M210 *ura4*-D18 *leu1*-32 h$^-$	Lab stock
KGY5960	*clp1*::*ura4 cdc2-33 ade6*-M210 *ura4*-D18 *leu1*-32 h$^-$	Lab stock
KGY12100	*clp1NLSmut-GFP*:*kan^R ade6*-M210 *ura4*-D18 *leu1*-32 h$^-$	This study
KGY14642	*clp1NLSmut-GFP*:*kan^R cdc2-33* h$^-$	This study
KGY349	*cdc3-124 ade6*-M216 *ura4*-D18 *leu1*-32 h$^-$	Lab stock
KGY648	*cdc3-124* clp1::*ura4 ade6*-M21x *ura4*-D18 *leu1*-32 h$^-$	Lab stock
KGY12091	*clp1NSLmut-GFP*:*kan*$^+$ *cdc3-124 ade6*-M21x *ura4*-D18 *leu1*-32 h$^-$	This study
Figure 22		
KGY3374	*clp1-GFP:kan^R ade6*-M216 *ura4*-D18 *leu1*-32 h$^-$	Lab stock
Figure 23		
KGY3374	*clp1-GFP:kan^R ade6*-M216 *ura4*-D18 *leu1*-32 h$^-$	Lab stock
KGY7411	*clp1-GFP:kan^R nsk1::ura4*$^+$ *ade6*-M21X *ura4*-D18 *leu1*-32 h$^+$	Lab stock

KGY7412	*clp1-GFP:kan*^{R} *dnt11::ura4*$^{+}$ *ade6*-M21X *ura4*-D18 *leu1*-32 h^{+}	Lab stock
KGY7413	*clp1-GFP:kan*^{R} *nsk1::ura4*$^{+}$ *dnt1::Kan*^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	Lab stock
Figure 24		
KGY3374	*clp1-GFP:kan*^{R} *ade6*-M216 *ura4*-D18 *leu1*-32 h^{-}	Lab stock
KGY6112	*clp1-GFP*:kan^{R} *sal3::ura4 ade6*-M21X *ura4*-D18 *leu1*-32 h^{-}	This study
Figure 25		
KGY12132	*clp1-GFP*:kan^{R} *gar2-mCherry*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
Figure 27		
KGY12132	*clp1-GFP*:kan^{R} *gar2-mCherry*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study
KGY14801	*clp1(1-372)-GFP*:kan^{R} *gar2-mCherry*:kan^{R} *ade6*-M21X *ura4*-D18 *leu1*-32 h$^{?}$	This study

whole-cell PCR and immunoblot or fluorescence microscopy when appropriate. Introduction of various *clp1* alleles into other genetic backgrounds was accomplished using standard *S. pombe* mating, sporulation and tetrad dissection techniques.

For spot assays, cells were grown to mid-log phase at 32˚C, 8 million cells were resuspended in 1 ml of water and 1:10 serial dilutions were made. Then 2.5 μl of each dilution were plated on YE, YE plus 1.2 M sorbitol, 1 mM H_2O_2, or 5 mM HU, or EMM+supplements, EMM+supplements plus 1.2 M sorbitol, 1 mM H_2O_2, or 5 mM HU. Plates were incubated at 32˚C for 3-4 days.

To inhibit Crm1 dependent export, cells were grown in YE at 25˚C overnight. Cultures were then split in half and treated with either 100 ng/ml leptomycin B (LMB) or methanol (mock treatment) for 3 hr prior to microscopy.

Microscopy

All images were taken using a personal DeltaVision microscopy system (Applied Precision, Issaquah, WA). This system includes an IX71 microscope (Olympus, Center Valley, PA), 60x NA 1.42 PlanApo objective, a CoolSnap HQ2 camera (Photometrics, Tucson, AZ), and softWoRx imaging software. Z series optical sections were taken at 0.5 μm steps.

Time point analyses of *clp1-GFP* localization was performed on asynchronous cultures grown in YE at 30˚C with constant shaking. To induce cellular stress, cultures were treated with either of the following conditions: cultures were shifted cultures from 30˚C to 40˚C for thermal, addition of sorbitol (Sigma, St. Louis, MO) to a final concentration of 1.2 M for osmotic, addition of H_2O_2 (Sigma) to a final concentration of 1

mM for oxidative or addition of HU (Sigma) to a final concentration of 12 mM. H_2O was used as mock treatment. Images were taken at 5, 15 or 30 min intervals. Nuclei with nucleoplasmic Clp1-GFP were determined by comparing Clp1-GFP localization to Gar2-mCherry, a known nucleolar marker. Cells with Clp1 localized to mitotic spindles were not considered. Line scans 5 μm in length were made using SoftWoRx imaging software. Experiments were repeated at least four times per strain, and done on at least two separate days. In addition, at least one experiment for each mutant strain was done at the same time as the wild type *clp1-GFP gar2-mCherry* strain (Figure 6).

To inhibit Cdk1 kinase activity, strains containing an analog sensitive allele of Cdk1 (*cdc2-F84G*) were treated with 5 μM 4-Amino-1-*Tert*-Butyl-3-(1'-Napthylmethyl)Pyrazolo(3,4-d)Pyrimidine (1NM-PP1) (EMD Biosciences, San Diego, CA) for 30 min prior to the addition of stress agents, and DMSO (Sigma) addition served as mock treatment.

For live-cell microscopy of leptomycin B (LMB) or mock (DMSO) treated cells, asynchronous cultures were grown at 25°C to midlog phase, either 250 μM LBM or DMSO was added to the culture for 3 Hr and then the cells were imaged. All live images were acquired using a spinning disk confocal microscope (Ultraview LCI; PerkinElmer) equipped with a 100x NA 1.40 Plan-Apochromat oil immersion objective and a 488-nm argon ion laser. Images were taken via a charge-coupled device camera (Orca-ER; Hamamatsu Photonics) and subsequently processed using Metamorph 7.1 software (MDS Analytical Technologies). Z-sections were taken with 11 slices at 0.5 μm each.

Fluorescence recovery after photobleaching (FRAP) of Clp1-GFP in the nucleolus was performed on a Zeiss LSM 510 inverted confocal microscope in the Cell Imaging

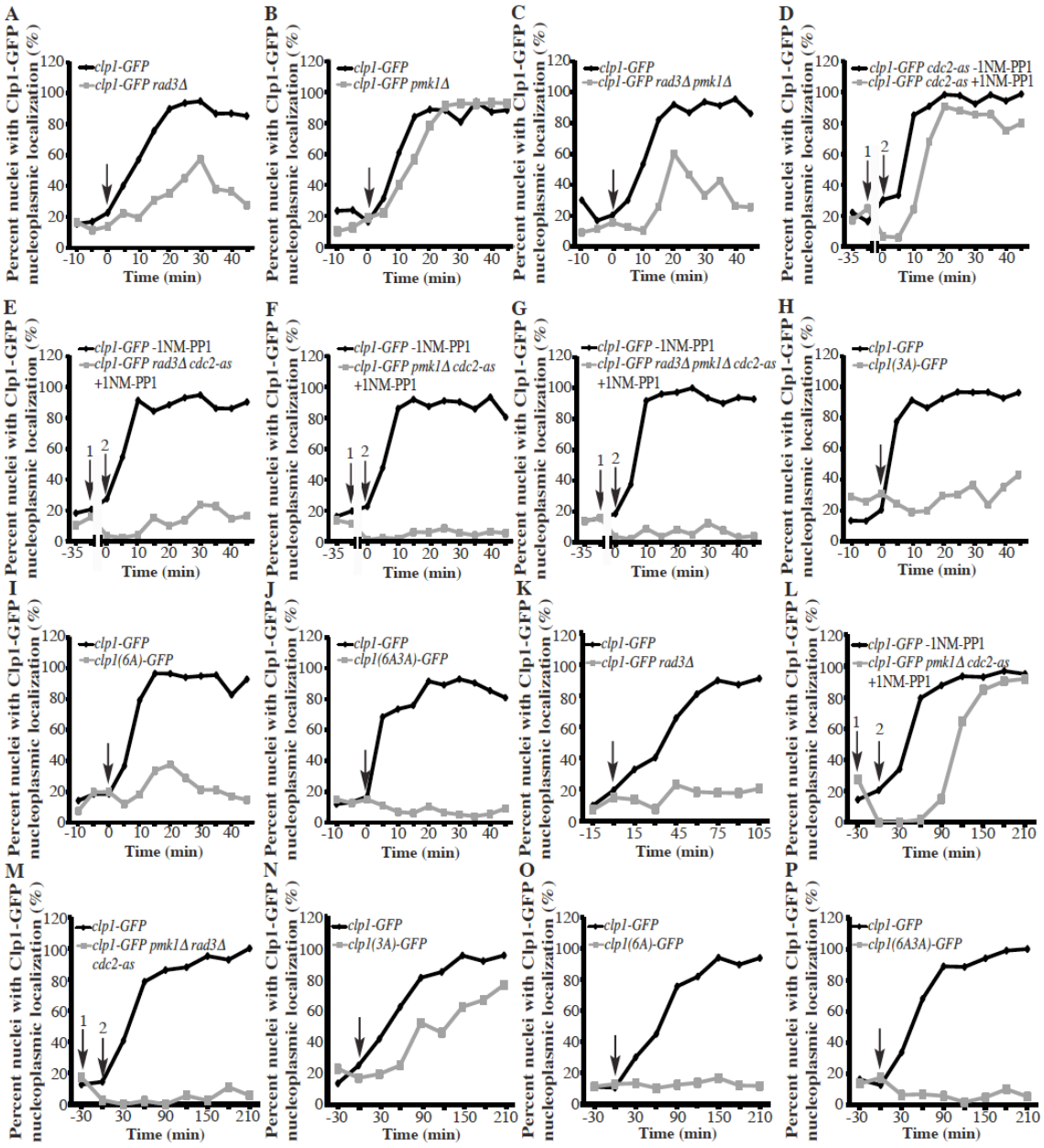

Figure 6. Stress-induced Clp1-GFP nucleoplasmic accumulation experiments performed on the same day. (A-P) Same as in Figure 2, except each line represents a single experiment in which *clp1-GFP gar2-mCherry* and the indicated kinase mutant strains were performed at the same time. (A-J) Stress was induced by addition of 1 mM H_2O_2. (K-P) Stress was induced by addition of 12 mM HU.

Shared Resource (CISR) center (Vanderbilt University). This microscope was equipped with a 63x/1.40 Plan-APOCHROMAT oil immersion objective and 488nm laser. After 6 initial images were taken, a 0.2 μm^2 region was bleached with a sequence of 13 high intensity (100%) laser iterations. Images were taken every second. Fluorescence intensity was analyzed by Zeiss LSM software version 4.2. FRAP of Clp1-GFP at the spindle pole body (SPB) in *sal3Δ* cells was performed on the same system as nucleolar Clp1-GFP FRAP. However, the SPB was bleached with 10 high intensity (100%) laser iterations. Images were taken every 3 seconds. FRAP of Clp1-GFP at the SPB in *wildtype* cells was preformed on a Zeiss LSM 710 META inverted confocal microscope in the CISR center. This microscope was equipped with a 63x/1.40 Plan-APOCHROMAT oil immersion objective and 488nm laser. The FRAP experiment were preformed as described above. Fluorescence intensity was analyzed by Zeiss Zen 2011 software version 7.0.

Protein methods

Whole-cell lysates were prepared in NP-40 buffer as previously described (Gould *et al*., 1991). Lysates were subjected to immunoprecipitation with either anti-MYC (9E10), anti-Flag (Sigma), Novagen GST-bind™ Resin (EMD Chemicals), protein G sepharose (GE healthcare, Uppsala, SE) or IgG sepharose (GE healthcare). Phospho-p44/42 XP™ rabbit mAB (Cell Signaling Technology, Danvers, MA) was used to detect activated Pmk1. Polyclonal antibodies for the Clp1 T453 phosphorylated residue, anti-T453p, were raised against a phosphorylated peptide (DETRTVGpTPTETISV) coupled to keyhole limpet hemocyanin in rabbits using the premium protocol by Invitrogen

(Carlsbad, CA). Immunoblot analysis and λ-phosphatase assays were performed as previously described (Tasto *et al*., 2003), except primary antibodies were detected by secondary antibodies coupled to IRDye 680LT or IRDye 800CW (LI-COR Biosciences, Lincoln, NE) and visualized using an Odyssey scanner (LI-COR Biosciences). Relative protein abundance was determined on an Odyssey Infrared Imaging System (LI-COR Biosciences, NE).

Protein kinase assays

in vitro protein kinase assays were preformed as previously described (Yoon *et al*., 2006), with the exception that the kinase buffer consisted of 10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl and 2 mM DTT supplemented with 100 μM cold ATP and 5 μCi γ-[^{32}P]ATP (GE Healthcare, Piscataway, NJ). For Pmk1 kinase assays, GST-Pmk1 on GST-bind resin was washed three times in 1 ml NP-40 buffer, and then three times in 1 ml kinase buffer. For Cdk1, approximately 50 ng of recombinant Cdk1-Cdc13 purified from baculovirus-infected insect cells was used as previously described (Yoon *et al*., 2002). One μg of recombinant MBP-fusion substrates were used in all experiments, reactions were for 30 min at 30˚C and were terminated by the addition of 5x SDS-PAGE sample buffer and boiling. Phosphorylation was analyzed by separation on SDS-PAGE and visualization by Coomassie blue staining, autoradiography, and/or immunobloting.

CHAPTER III

Clp1 NUCLEOLAR RELEASE IS INFLUENCED BY MULTIPLE PROTEIN KINASES UPON GENOTOXIC STRESS

Introduction

Within their native environment, cells experience numerous forms of environmental stresses (e.g. ultraviolet light, ionizing radiation, reactive oxygen species, high salt, extreme temperatures). In order for cells to survive during stressful conditions, they have devised signaling networks that respond to specific cellular conditions to promote cell survival, and ensure that the genomic material is replicated and divided equally without error (Alao and Sunnerhagen, 2008; Smith et al., 2010). Failure in a cell's ability to response to changing environmental conditions can be detrimental to cell survival.

As discussed in chapter I, in addition to mitotic nucleolar release, Clp1 and Cdc14B are triggered for nucleolar release in interphase when cells are treated with DNA damaging agents (Bassermann et al., 2008; Diaz-Cuervo and Bueno, 2008; Mocciaro et al., 2010; Takahashi et al., 2012). Nucleolar release of Cdc14 phosphatases is a major activation step, at least during mitosis, allowing Cdc14 phosphatases access to their substrates. This prompted me to determine whether interphase nucleolar release was a specific response to DNA damage or whether additional cellular stresses could induce the same response. In this chapter I detail my work showing that interphase nucleolar release of Clp1 is a specific response to genotoxic stress. Additionally, I utilized genotoxic stress

as an inducible trigger of nucleolar release to further investigate mechanistically how Clp1 is released from the nucleolus.

Results

Clp1 relocalizes to the nucleoplasm following genotoxic stress

Because Clp1 relocalizes from the nucleolus to the nucleoplasm during interphase when cells encounter a block to DNA replication (Diaz-Cuervo and Bueno, 2008), I explored whether other cellular stresses would have the same effect. To answer this question, asynchronously growing *clp1-GFP gar2-mCherry* cells (Gar2 is a nucleolar marker (Sicard *et al*., 1998)) were treated with various cellular stresses for 1 hour. As previously reported, HU induced relocalization of Clp1-GFP to the nucleoplasm (Figure 7A and B) (Diaz-Cuervo and Bueno, 2008). This could be readily seen in line scans measuring the relative fluorescence intensity of Clp1-GFP and Gar2-mCherry through the nucleus. Cells treated with HU had a lower, broader peak of Clp1-GFP than those of untreated cells where Clp1-GFP intensity mirrored that of Gar2-mCherry (Figure 7C). In contrast, Clp1-GFP did not relocalize to the nucleoplasm in either thermal- or osmotically-stressed cells (Figure 7A-C) suggesting that nucleolar release is not a general response to cellular stress. Although not the topic of this study, I note that thermal stress caused release of Clp1 from SPBs (Figure 7A). The addition of 1 mM H_2O_2, however, did cause Clp1-GFP to accumulate within the nucleoplasm at the expense of nucleolar localization (Figure 7A-C). Because H_2O_2 can cause DNA damage (Cadet *et al*., 2010), Clp1 nucleoplasmic accumulation may be a common response to different forms of genotoxic stress.

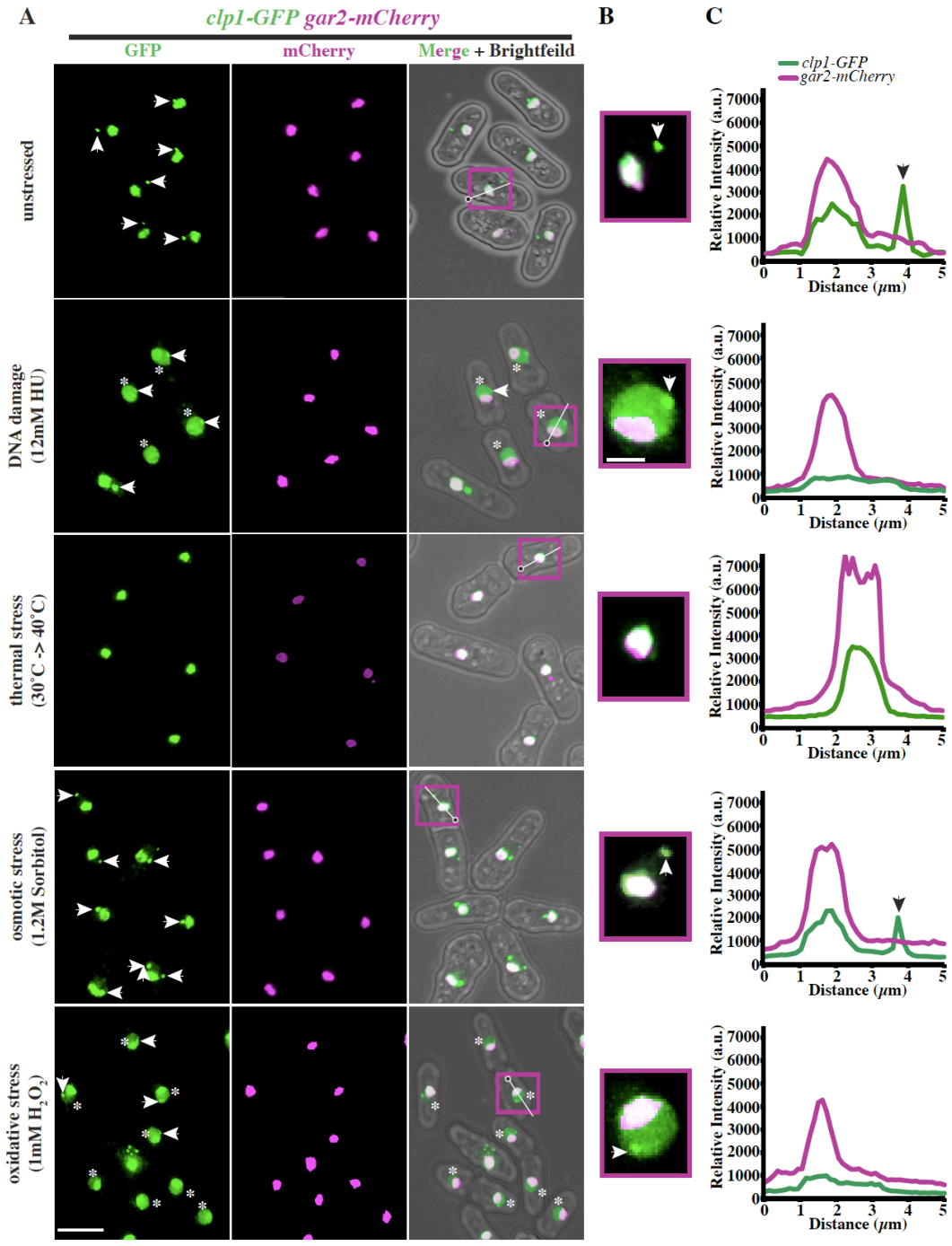

Figure 7. Genotoxic stress induces Clp1-GFP nucleoplasmic accumulation. (A) Live cell images of *clp1-GFP gar2-mCherry* cells after treatment with the specified stress for one hour. Scale bar = 5 µm. Asterisks indicate nuclei with Clp1-GFP relocalized from the nucleolus to the nucleoplasm. Arrowheads point to spindle pole bodies. (B) Zoomed views of the boxed nuclei from the merged images in (A). Scale bar = 1.5 µm. Arrowheads point to spindle pole bodies. (C) Line scans of Clp1-GFP and Gar2-mCherry fluorescence, each spanning a distance of 5 µm, in which the paths are represented in (A) by a white line with a black circle to indicate the origin of the line scan. Arrowheads point to spindle pole bodies.

Release of Clp1 from the nucleolus upon replication stress depends on the checkpoint effector kinases, Chk1 and Cds1 (Diaz-Cuervo and Bueno, 2008) that are activated by Rad3 (Brondello et al., 1999; Lindsay et al., 1998; Martinho et al., 1998; Walworth and Bernards, 1996). To determine if the same mechanism contributed to H_2O_2-induced Clp1-GFP relocalization, I examined *clp1-GFP gar2-mCherry* or *clp1-GFP gar2-mCherry rad3Δ* cells after the addition of 12 mM HU or 1 mM H_2O_2 over time (Figure 8A and B). Since asynchronous cells were used in this analysis, some cells have Clp1 released from the nucleolus prior to treatment because they are in mitosis (Cueille *et al*., 2001; Trautmann *et al*., 2001). Late mitotic cells (e.g. cells with Clp1 on the mitotic spindle) were excluded from my analysis, however residual cells with Clp1 released from the nucleolus at time 0 are likely early mitotic cells (Figure 9). As expected, Clp1-GFP accumulated throughout the nucleoplasm of cells following HU treatment. Nearly 100% of these cells had nucleoplasmic Clp1 by 105 min, while mock treated cells did not relocalize Clp1-GFP (Figure 8A). HU-induced Clp1 nucleoplasmic accumulation was not observed in cells lacking *rad3*$^+$, consistent with previously reported results (Diaz-Cuervo and Bueno, 2008) (Figure 8A). In response to H_2O_2, Clp1-GFP also accumulated within the nucleoplasm and to the same extent per cell (Figure 7 and data not shown), however the response was faster (Figure 8B). The largest percentage of cells showing nucleoplasmic Clp1 occurred at 20 min post H_2O_2 addition (Figure 8B). Unlike HU-treated cells, H_2O_2-induced relocalization of Clp1-GFP was not abolished in the absence of *rad3*$^+$, although the response was altered both in terms of timing and the percentage of cells responding at any given time-point (Figure 8B). Interestingly, after the peak accumulation of nucleoplasmic Clp1-GFP, the percentage of cells with nucleoplasmic

Clp1-GFP decreased, suggesting that the maintenance of Clp1 nucleoplasmic distribution was affected (Figure 8B). To examine whether these changes in the response to H_2O_2 were due to loss of checkpoint effector kinase activation, I examined Clp1 localization in a *chk1Δ cds1Δ* strain. As in *rad3Δ* cells, the appearance of Clp1-GFP in the nucleoplasm was delayed relative to *wild type* cells; only 68% of the nuclei simultaneously accumulated Clp1-GFP in the nucleoplasm during the time course (Figure 8B and C). Additionally, after the peak, the percentage of nuclei containing nucleoplasmic Clp1-GFP decreased (Figure 8B and C). I therefore conclude that the relocalization defects seen in *rad3Δ* cells are due to loss of Cds1 and/or Chk1 activities. These results indicate that while the Rad3 pathway contributes to Clp1-GFP relocalization induced by oxidative stress, additional signaling pathways must contribute.

Multiple protein kinases contribute to Clp1 nucleoplasmic relocalization

Clp1 is a highly phosphoregulated protein (Chen et al., 2008; Diaz-Cuervo and Bueno, 2008; Wolfe et al., 2006). Since H_2O_2-induced Clp1-GFP relocalization was still observed in the absence of *rad3*$^+$ (Figure 8B and C), I reasoned that additional protein kinases might contribute to oxidative stress-induced relocalization of Clp1-GFP.

First, I looked at the possible influence of MAPKs, which become activated by various environmental stresses (Roux and Blenis, 2004). In *S. pombe,* there are three MAPKs: Spk1, Sty1 and Pmk1. Spk1 and Sty1 are involved in the mating response and stress sensing, respectively, whereas Pmk1 functions in the cell wall integrity pathway (Toda *et al*., 1996). Of the three MAPKs, only *pmk1*$^+$ loss affected H_2O_2-induced Clp1-GFP relocalization, delaying nucleolar release by 5 min compared to *pmk1*$^+$ cells

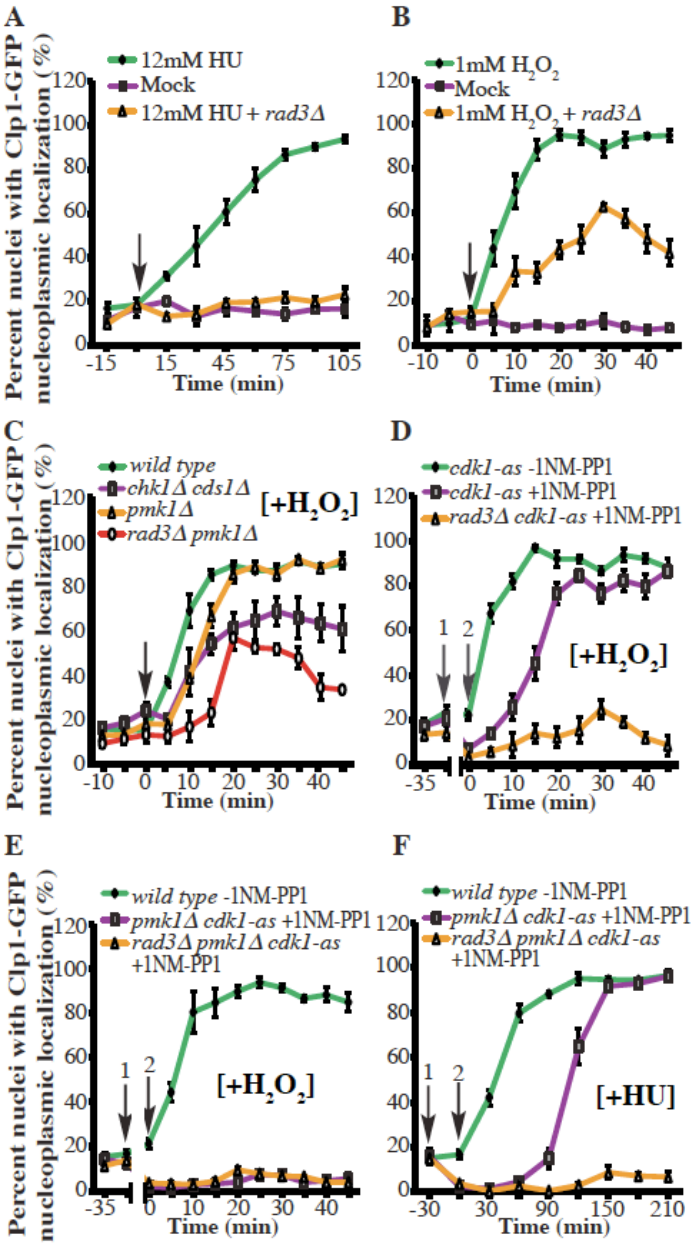

Figure 8. Multiple kinases affect genotoxic stress-induced Clp1-GFP nucleoplasmic accumulation. (A) The graphs show the percentage of nuclei with Clp1-GFP detected in the nucleoplasm of *clp1-GFP gar2-mCherry* or *clp1-GFP gar2-mCherry rad3*Δ cells as judged by live cell microscopy after the addition of 12 mM HU or H_2O (mock treatment) at t = 0 min, indicated by the arrow. (B) Same as (A) except 1 mM H_2O_2 was used to induce cellular stress. (C) Similar to (B), the graph shows the percentage of nuclei with Clp1-GFP detected in the nucleoplasm following oxidative stress (1 mM H_2O_2) in the indicated strains. (D-E) Same as in (B-C) except 5 µM 1NM-PP1 was added at t = -30 min, indicated by arrow 1, to asynchronous cultures prior to H_2O_2 addition at t = 0 min, indicated by arrow 2. DMSO was added instead of 1NM-PP1 for mock treated cells. (F) As in (D-E) except 12 mM HU was used to induce stress at t = 0 min. In (A-F), each curve represents the average of 4 experiments, and each time point represents ≥132 cells. Standard error of the mean is shown for each time point.

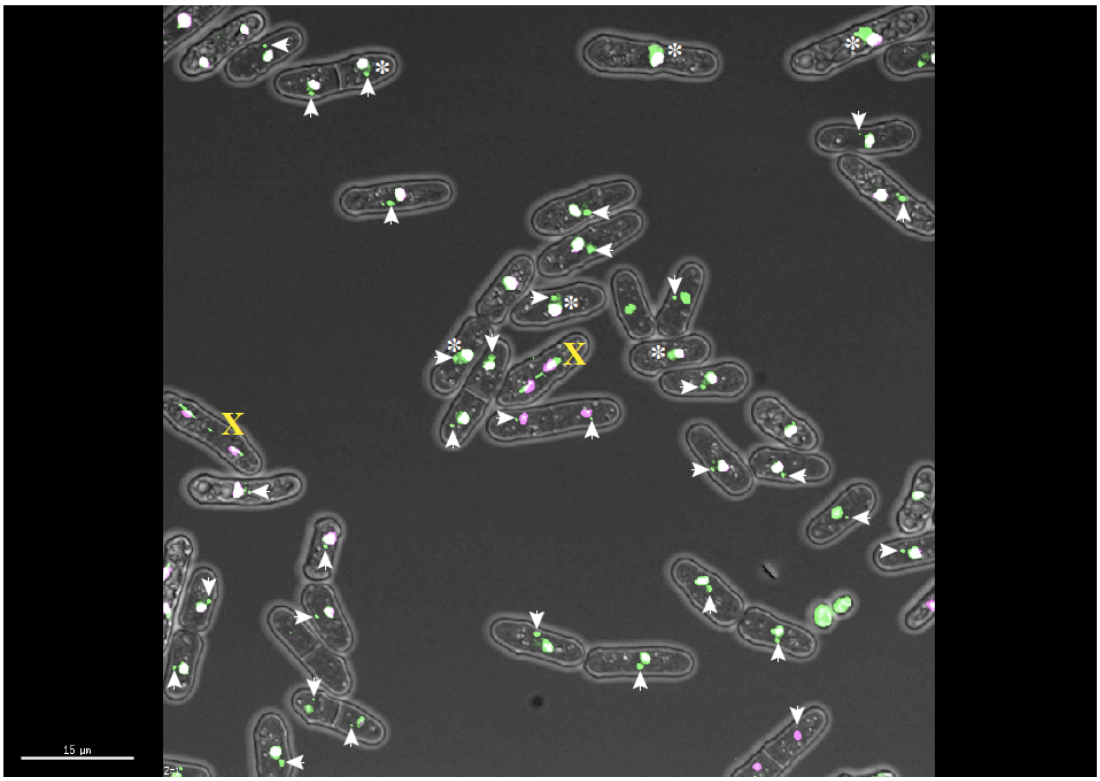

Figure 9. Clp1 relocalizes to the nucleoplasm following H_2O_2 induced stress. Live cell image of *clp1-GFP gar2-mCherry* cells taken at t=0 min, the point of stress addition. Nuclei of 6 cells, indicated with asterisks, showed nucleoplasmic Clp1-GFP of a total of 44 (14%). Arrowheads point to SPBs. Xs mark cells that were not considered because Clp1 was clearly visible on the spindle.

(Figure 8C and data not shown). However, *pmk1Δ* cells did reach the same high level of Clp1 release as *wild type* cells, suggesting that Pmk1 has a likely role in timing Clp1-GFP nucleoplasmic accumulation during oxidative stress but not its maintenance within the nucleoplasm (Figure 8C). To test if the Rad3 and cell wall integrity pathways act in concert to influence Clp1 localization, I examined Clp1-GFP in *rad3Δ pmk1Δ* cells. Clp1 redistribution was delayed by 10-15 min with a decreased percentage of Clp1-GFP nucleoplasmic localized nuclei in *rad3Δ pmk1Δ* cells, though the peak was still reached at 20 min post H_2O_2 addition (Figure 8C). Also, similar to other *rad3Δ* cells examined, the percentage of nuclei that contained nucleoplasmic Clp1-GFP was not maintained. Together, these results suggest that while both Rad3 and Pmk1 contribute to Clp1 relocalization from the nucleolus to the nucleoplasm during oxidative stress, they do so independently of each other, and still additional pathways or factors contribute.

I next examined whether Cdk activity had a role in Clp1 relocalization since Cdk1 had previously been shown to directly phosphorylate Clp1 to modulate the phosphatase's activity, but the effect on Clp1 localization was not examined (Wolfe *et al*., 2006). Because Cdk1 (Cdc2 in *S. pombe*) is an essential kinase, I utilized an analog sensitive mutant, *cdc2-F84G* (*cdk1-as*), which is inhibited by the ATP analog, 1NM-PP1 (Dischinger *et al*., 2008). Inactivation of Cdk1-as by 1NM-PP1 resulted in a drop in the number of cells with Clp1-GFP localized to the nucleoplasm at t = 0 min due to the inability of cells to enter mitosis (Figure 8D). After the addition of 1 mM H_2O_2 there was a 5 min delay in Clp1-GFP nucleoplasmic accumulation similar to *pmk1Δ* cells (Figure 8C and D) indicating a potential role for Cdk1 in regulating Clp1 relocalization.

To assess the collective roles of the Rad3 pathway, the cell wall integrity pathway and Cdk1, I examined Clp1-GFP nucleoplasmic relocalization in strains with combinations of the kinase mutants. While combining *rad3Δ* and *cdk1-as* had a significant effect on Clp1-GFP relocalization to the nucleoplasm, it did not completely eliminate it (Figure 8D). In both *pmk1Δ cdk1-as* and *rad3Δ pmk1Δ cdk1-as* cells, however, the ability of Clp1-GFP to relocalize from the nucleolus to the nucleoplasm upon oxidative stress was abolished (Figure 8E). These results indicate that Pmk1 and Cdk1 cooperate to promote Clp1-GFP relocalization during oxidative stress, and suggest that the Rad3 pathway is important for maintenance of the nucleoplasmic Clp1 pool.

Various forms of genotoxic stress including HU activate the *S. cerevisiae* ortholog of Pmk1, Slt2, and the closely related Erk1/2 MAPKs of higher eukaryotes; this activation is important to cope with genotoxic insults (Queralt and Igual, 2005; Soriano-Carot et al., 2012; Wei et al., 2011a). Additionally, *pmk1Δ* cells are sensitive to HU (Han *et al*., 2010). Since Pmk1 and Cdk1 both modulate Clp1's behavior in response to H_2O_2, I investigated the potential role of Pmk1 and Cdk1 in HU-induced Clp1-GFP relocalization. To examine whether Pmk1 and/or Cdk1 affected HU-induced Clp1 nucleoplasmic accumulation, I used *pmk1Δ cdk1-as* and *rad3Δ pmk1Δ cdk1-as* strains. Compared to *wild type* cells, in which Clp1-GFP began to rapidly relocalize in some cells, *pmk1Δ cdk1-as* cells did not start to relocalize Clp1-GFP to the nucleoplasm until 90 min post HU addition. Once relocalization began, however, it quickly reached the same penetrance as in *wild type* cells (Figure 8F). As in *rad3Δ* cells, Clp1-GFP did not accumulate in the nucleoplasm in *rad3Δ pmk1Δ cdk1-as* cells under replicative stress, suggesting that Pmk1, Cdk1 or both kinases contribute to release under these

circumstances, but Rad3 plays a more dominant role (Figure 8E and F). Collectively, these results suggest that HU and H_2O_2 activate the Rad3 pathway, cell wall integrity pathway and Cdk1, although to different extents and with possibly different kinetics, to redistribute Clp1 from the nucleolus into the nucleoplasm.

Cds1 and/or Chk1, Pmk1 and Cdk1 phosphorylate Clp1 upon HU and H_2O_2 stress treatment

As introduced above, Cds1 and Sid2 directly phosphorylate Clp1 on its multiple RxxS sites. This promotes the association of Clp1 with the two *S. pombe* 14-3-3 proteins, Rad24 and Rad25, which, at least in the case of Sid2, prevents the return of Clp1 to the nucleolus until the completion of cytokinesis (Chen et al., 2008; Diaz-Cuervo and Bueno, 2008; Mishra et al., 2005). Additionally, Cdk1 directly phosphorylates Clp1 on TP sites primarily in early mitosis and inhibits Clp1 catalytic activity (Wolfe *et al*., 2006). To determine whether phosphorylation on Clp1 RxxS and/or TP sites occurred during genotoxic stress, I first generated a phospho-specific antibody to one Cdk1 phosphosite, T453. To test the specificity of anti-T453p, I analyzed lysates of *clp1*$^+$, *clp1-MYC13,* or *clp1(3A)-MYC13* cells arrested in mitosis, when Clp1 is highly phosphorylated by Cdk1 (Figure 3A) (Wolfe *et al*., 2006). The Clp1(3A) mutant abolishes three Cdk1 phosphorylation sites including T453 (Figure 12A) (Wolfe *et al*., 2006). Clp1 and Clp1-MYC13 were detected by anti-T453p, however Clp1(3A)-MYC13 was not (Figure 10A), indicating that anti-T453p is specific for Clp1 phosphorylated at T453.

Next, I used anti-T453p to probe immunoblots of immunoprecipitated Clp1-MYC13 protein from asynchronous cultures of either *wild type* or *rad3Δ pmk1Δ cdk1-as*

cells treated with 1 mM H_2O_2, 12 mM HU or H_2O (mock). Clp1-MYC13 phosphorylation at T453 in *wild type* cells increased over time after the induction of stress by either H_2O_2 or HU compared to mock treated cells, however the H_2O_2-treated cells show a more robust phosphorylation at T453 than HU-treated cells (Figure 10B-E). In contrast, Clp1-MYC13 immunoprecipitated from *rad3Δ pmk1Δ cdk1-as* cells first treated with 5 μM 1NM-PP1, and then with H_2O_2 or HU did not show an increase in T453 phosphorylation (Figure 10B-E). These results suggest a correlation between genotoxic stress mediated Clp1 nucleoplasmic relocalization and accumulation of Clp1 T453 phosphorylation.

MAPKs, like Cdk1, are proline directed kinases (Roux and Blenis, 2004). Since deleting *pmk1*$^+$ affected Clp1-GFP nucleoplasmic relocalization (Figure 8C), I investigated whether Pmk1 could phosphorylate Clp1, and if it did so at sites overlapping with Cdk1. GST or GST-Pmk1 was produced in *S. pombe* cells, activated by treatment with 1 mM H_2O_2 and then purified. Activation of the kinase was confirmed by immunoblotting with anti-p44/p42 (Figure 10F) (Barba *et al*., 2008). GST-Pmk1, but not GST, phosphorylated MBP-Clp1, but not MBP alone, indicating that Pmk1 can directly phosphorylate Clp1 *in vitro* (Figure 10G). To test whether Pmk1 phosphorylates the same residues as Cdk1, I incubated recombinant MBP, MBP-Clp1, or MBP-Clp1(3A) *in vitro* with recombinant Cdk1-Cdc13/cyclinB or activated GST-Pmk1 (Figure 10H). While anti-T453p did not recognize MBP, it detected MBP-Clp1 and MBP-Clp1(3A) (likely due to the high concentration of recombinant protein used and that the serum was not affinity purified); however, the phospho-shifted form was only recognized in MBP-Clp1 and not MBP-Clp1(3A) for both kinases (Figure 10H). These results indicate that

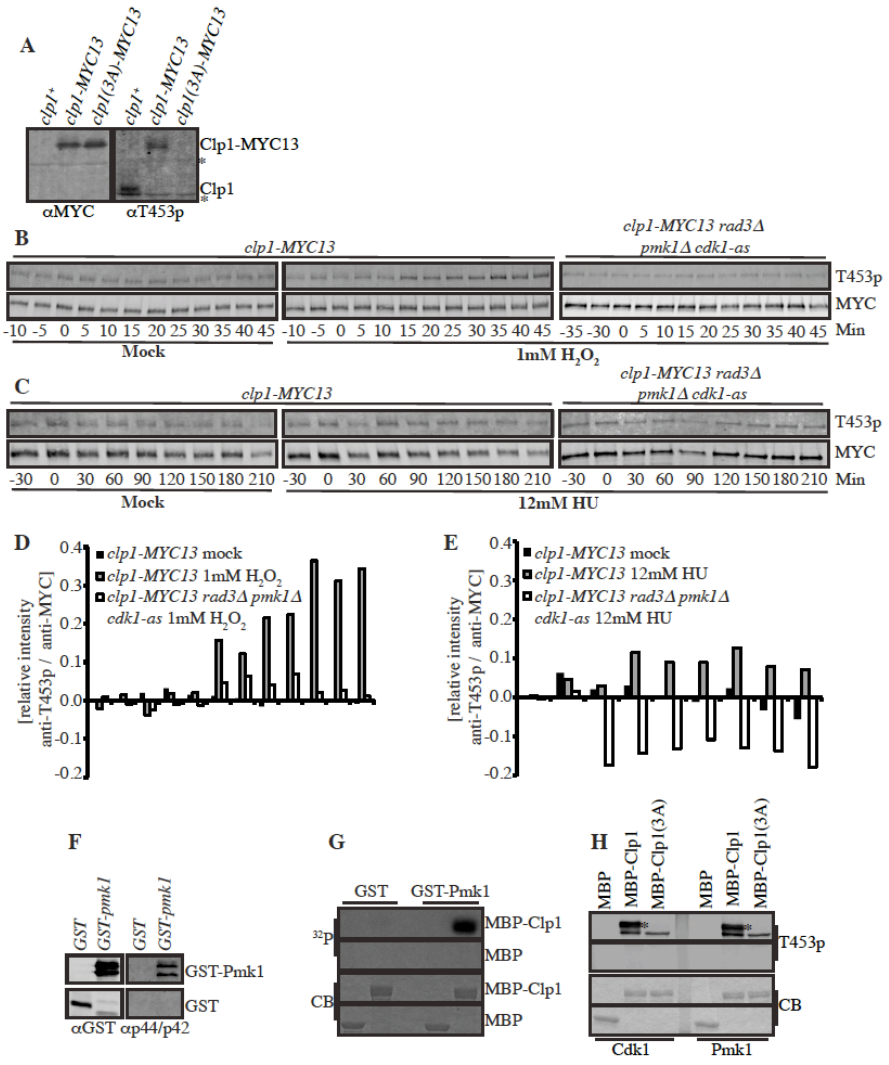

Figure 10. Clp1 is phosphorylated at TP sites upon genotoxic stress. (A) Immunoblots of lysates from the indicated strains arrested in prometaphase with the cold sensitive β-tubulin mutant, *nda3-km311*. Anti-MYC or anti-T453p were used for immunobloting. Asterisks indicate non-specific bands. (B) Immunoblots of immunoprecipitated Clp1-MYC13 from the indicated strains over time treated at t = 0 min with 1 mM H_2O_2 or H_2O (mock treatment). Immunoblots were probed with anti-MYC or anti-T453p. To inhibit Cdk1-as, cells were treated with 5 μM 1NM-PP1 at t = -30 min, and then at t = 0 min with either 1 mM H_2O_2 or H_2O (mock treatment). (C) Same as (B) except 12 mM HU was used to stress cells. (D and E) The ratios of the relative intensities of anti-T453p to that of anti-MYC signals normalized to the pre-stress time points. Intensities were calculated using an Odyssey instrument from the immunoblots in B and C. (F) Immunoblot analysis of GST or GST-Pmk1 purified from cells treated with 1 mM H_2O_2 for 30 min with anti-GST or anti-P44/P42. (G) MBP or MBP-Clp1 was incubated with GST or GST-Pmk1 in an *in vitro* kinase reaction. The inputs, detected by Coomassie blue (CB) staining, and the corresponding autoradiographs are shown. (H) Recombinantly produced MBP, MBP-Clp1 or MBP-Clp1(3A) were incubated with either recombinantly produced Cdk1-Cdc13/cyclinB or activated and immunoprecipitated GST-Pmk1. The inputs, detected by CB staining, and the corresponding autoradiographs are shown. The asterisks indicate a phospho-shifted form of MBP-Clp1.

both Pmk1 and Cdk1 can phosphorylate Clp1 at T453 *in vitro*.

To observe Clp1 RxxS phosphorylation, I followed the RxxS phospho-dependent association between Rad24 and Clp1 as an indirect measure of Clp1 phosphorylation at RxxS sites (Chen *et al*., 2008; Diaz-Cuervo and Bueno, 2008). In accord with Clp1 phosphorylation correlating with nucleoplasmic accumulation, I saw an increase in the Rad24-Clp1 association after the addition of 1 mM H_2O_2 or 12 mM HU suggesting that Clp1 is phosphorylated at RxxS sites after induction of genotoxic stress (Figure 11A-D). Interestingly, HU-treatment promoted a more robust association between Rad24 and Clp1 than H_2O_2 treatment, the converse of what I observed with Clp1 T453 phosphorylation (Figure 11A-D and Figure 10B-E). The H_2O_2 and/or HU-induced Rad24-Clp1 association was disrupted in *rad3Δ pmk1Δ cdk1-as* cells (Figure 11A-D). These results suggest a correlation between genotoxic stress induced Clp1 relocalization, and the accumulation of RxxS phosphorylation. Furthermore, the timing of T453 phosphorylation and Clp1-Rad24 interactions accumulate directly parallels Clp1 nucleoplasmic accumulation upon H_2O_2 or HU stress suggesting a direct relationship.

To examine the overall phosphostate of Clp1, I analyzed the SDS-PAGE mobility of Clp1-MYC13 in *wild type, rad3Δ, pmk1Δ cdk1-as* and *rad3Δ pmk1Δ cdk1-as* cells first treated with 5 μM 1NM-PP1, and then with 1 mM H_2O_2, 12 mM HU or H_2O (mock). Clp1-MYC13 immunoprecipitated from mock treated cells had a slower mobility than its lambda phosphatase collapsed counterpart (Figure 11E). This is likely due to the mitotic fraction of cells within the asynchronous culture in which Clp1 is known to be phosphorylated (Wolfe *et al*., 2006). Clp1-MYC13 immunoprecipitated from either H_2O_2 or HU treated cells had an even slower mobility compared to Clp1-MYC13 from mock

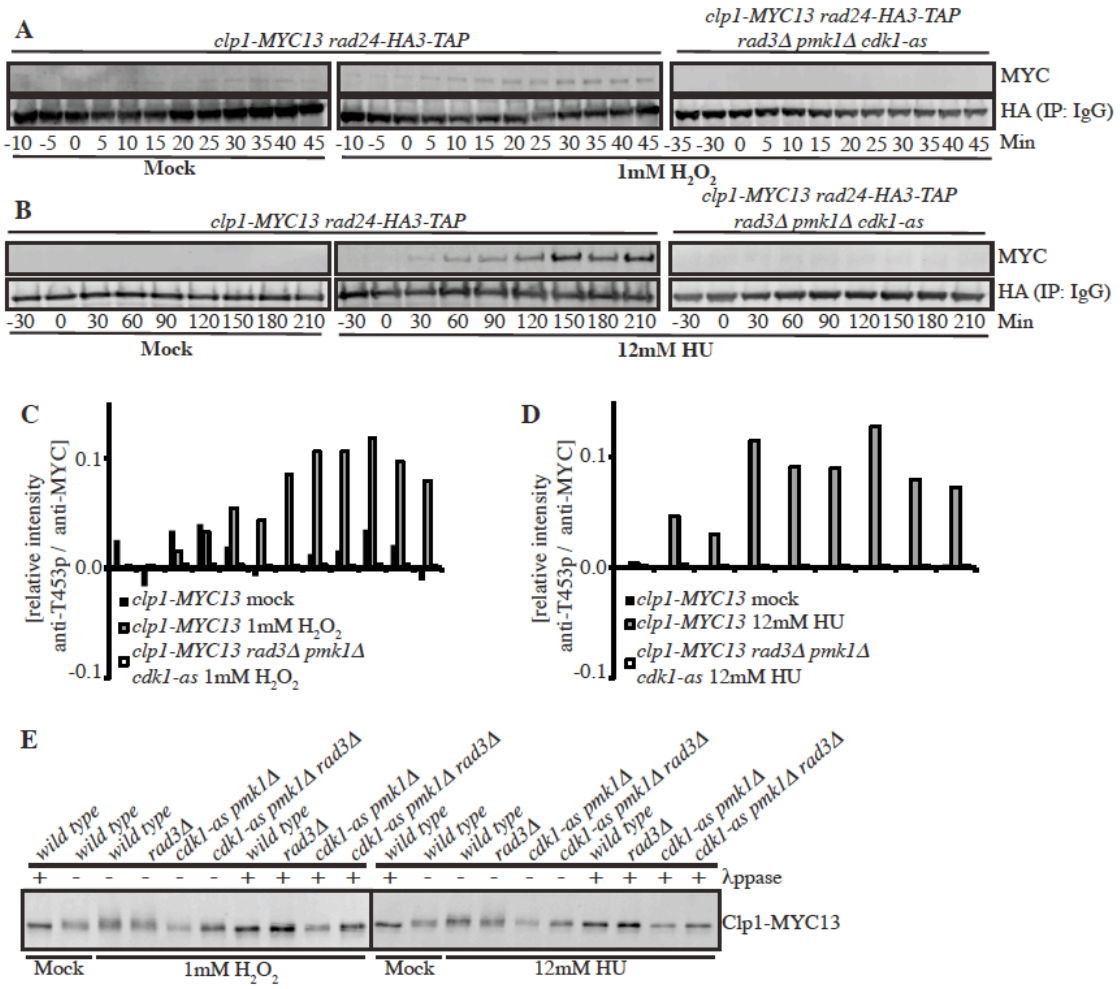

Figure 11. Clp1 is phosphorylated at RxxS sites upon genotoxic stress. (A) Clp1-MYC13 was captured by Rad24-HA3-TAP at different times after stress induction by the addition of 1 mM H_2O_2 or H_2O (mock treatment) at t = 0 min to asynchronous cultures of the indicated genotypes. Anti-MYC or anti-HA antibodies were used to detect Clp1-MYC13 or Rad24-HA3-TAP respectively. Cdk1-as was inhibited by first incubating cells with 5 μM 1NM-PP1 at t = -30 min before the addition of H_2O_2 at t = 0 min. (B) Same as in (A) except 12 mM HU was used to induce stress. (C and D) The ratios of the relative intensities of anti-MYC to that of anti-HA signals, normalized to the pre-stress time points, calculated using an Odyssey instrument from the immunoblots in A and B, respectively. (E) Cells were treated with 5 μM 1NM-PP1 for 30 min to inhibit Cdk1-as, and then with 1 mM H_2O_2 for 45 min or 12 mM HU for 210 min. Clp1-MYC13 immunoprecipitates from the indicated strains were treated with (+) or without (-) λ-phosphatase and then examined by anti-MYC immunoblotting.

treated cells (Figure 11E), indicating that the population of Clp1 is more heavily phosphorylated in genotoxicly stressed cells. Additionally, while Clp1 from genotoxicly stressed *rad3Δ* cells had a similar mobility compared to stressed *wild type* cells, Clp1-MYC13 immunoprecipitated from either *pmk1Δ cdk1-as* or *rad3Δ pmk1Δ cdk1-as* cells migrated significantly faster compared to *wild type* cells (Figure 11E). Together with the Rad24 binding experiments, these results indicate that inhibiting the Rad3 pathway, the cell wall integrity pathway and Cdk1 affects the phosphostate of Clp1-MYC13 during genotoxic stress and indicate a correlation between genotoxic stress-mediated Clp1 nucleoplasmic relocalization, and the accumulation of Clp1 phospho-modifications mediated by Cds1 and/or Chk1, Pmk1 and Cdk1 kinases.

Clp1 phosphosite mutants do not relocalize properly in response to genotoxic stress

Given the above results, I expected that abolishing Clp1 TP and/or RxxS phosphosites would prevent Clp1 relocalization during genotoxic stress. To test this, I used previously characterized Clp1 phosphomutants that were integrated at the *clp1*$^{+}$ endogenous locus and tagged at their C-terminus with GFP (Figure 12A) (Chen et al., 2008; Wolfe et al., 2006).

Since Cdk1 and Pmk1 likely phosphorylate the same Clp1 phosphosites, I anticipated that Clp1(3A)-GFP (Figure 12A) would act similarly to Clp1-GFP in *pmk1Δ cdk1-as* cells after stress induction. Indeed, although Clp1(3A)-GFP still relocalized to the nucleoplasm upon H_2O_2 treatment, its ability to relocalize was severely hindered

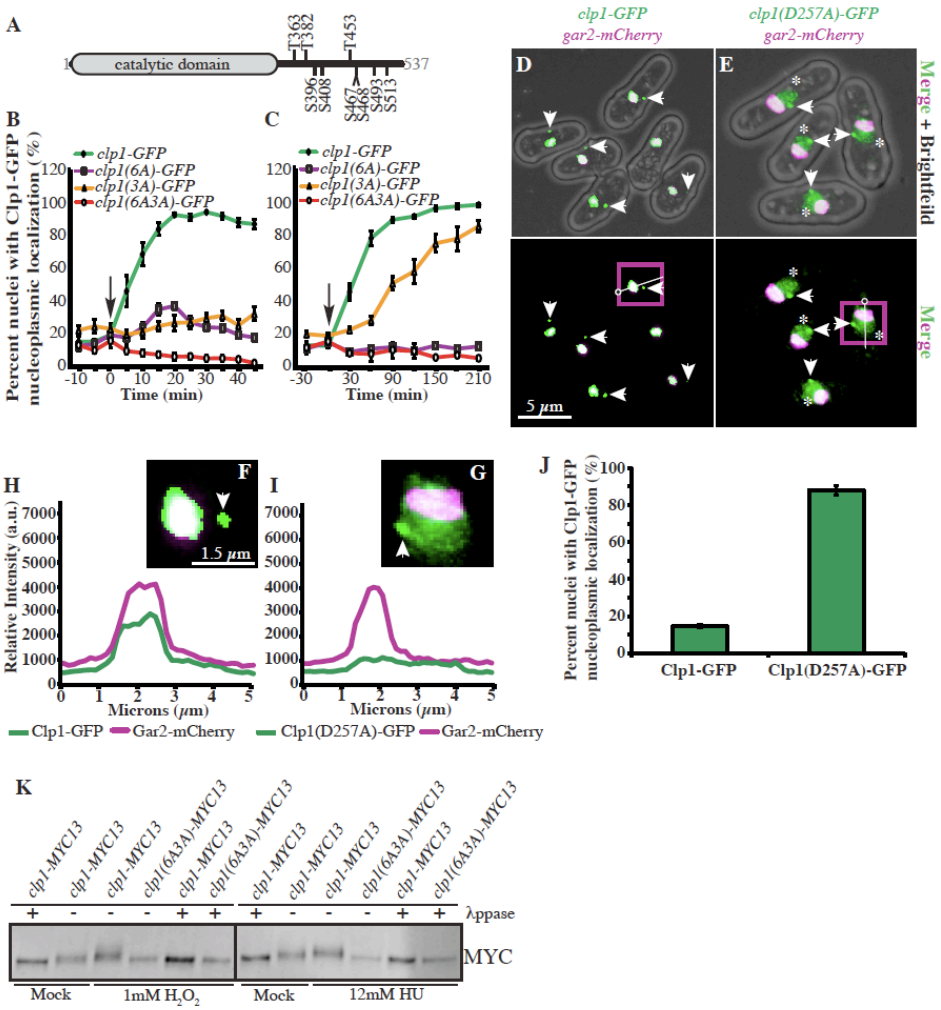

Figure 12. Mutation of RxxS and TP phosphosites abolishes Clp1 nucleoplasmic accumulation due to genotoxic stress. (A) A schematic of Clp1 with its TP (above) and RxxS (below) phosphosites indicated. (B) Same as in Fig 2 (B and C) except the indicated Clp1 phosphomutants were examined for their ability to accumulate within the nucleoplasm following genotoxic stress induced at t = 0 min (indicated by arrow) by the addition of 1 mM H_2O_2. (C) Same as in (B) except 12 mM HU was used to induce stress at t = 0 min (indicated by arrow). (D and E) A live cell image of unstressed *clp1-GFP gar2-mCherry* (D) or *clp1(D257A)-GFP gar2-mCherry* (E) cells. Scale bar = 5 µm. Asterisks indicate nuclei with Clp1-GFP relocalized from the nucleolus to the nucleoplasm. Arrowheads point to spindle pole bodies. (F and G) Zoomed views of the boxed nuclei from the merged images in (D) and (E), respectively. Scale bar = 1.5 µm. Arrowheads point to SPBs. (H and I) Line scans of either Clp1-GFP (H) or Clp1(D257A)-GFP (I) and Gar2-mCherry fluorescence, each spanning a distance of 5 µm, in which the paths are represented in (D) and (E), respectively, by a white line with a black circle to indicate the origin of the line scan. (J) The percent of nuclei with Clp1-GFP or Clp1(D257A)-GFP localized to the nucleoplasm was determined in asynchronous cultures of the indicated strains. The experiment was repeated three times and standard error of the mean is shown. $n \geq 429$. (K) Immunoblot of Clp1-MYC13 and Clp1(6A3A)-MYC13 immunoprecipitates from cells treated with either 1 mM H_2O_2, 12 mM HU or H_2O (mock). Immunoprecipitates were treated with (+) or without (-) λ-phosphatase.

(Figure 12B). Additionally, in HU-stressed cells, Clp1(3A)-GFP was delayed between 60-90 min in the ability to accumulate within the nucleoplasm (Figure 12C). These results support the idea that Pmk1 and Cdk1 cooperate to phosphorylate the same TP sites in order to promote Clp1 relocalization to the nucleoplasm upon genotoxic stress.

It is known that Clp1 auto-dephosphorylates its Cdk1 phosphorylations (Wolfe *et al*., 2006), so I next considered whether Clp1 activity contributed to its nucleolar sequestration. To test this idea, I examined the distribution of a catalytically compromised Clp1 mutant, *clp1(D257A),* which is constitutively phosphorylated on multiple Cdk1 sites due to its inability to auto-dephosphorylate (Wolfe et al., 2006). Upon examining unstressed *clp1(D257A)-GFP gar2-mCherry* cells, I observed that Clp1(D257A)-GFP was present in the nucleoplasm in a much higher percentage of cells compared to unstressed *clp1*$^{+}$ cells (Figure 12 D-J). This is consistent with Clp1 hyperphosphorylation on Cdk1 sites promoting nucleoplasmic accumulation.

A second mutant, *clp1(6A)-GFP*, has 6 Ser residues essential for the phospho-dependent association of Rad24 with Clp1 mutated to Ala (Figure 12A) (Chen *et al*., 2008). After H_2O_2 treatment, much like Clp1-GFP in *rad3Δ* cells, Clp1(6A)-GFP showed a delay in release, fewer nuclei contained nucleoplasmic Clp1 at the peak of release, and there was a decrease in the percentage of nuclei with nucleoplasmic Clp1 after the peak (Figure 12B). However, the decrease in percent nuclei accumulating Clp1(6A)-GFP was greater than Clp1-GFP in *rad3Δ* cells treated with H_2O_2. Among other possibilities, this difference may reflect modulation of Clp1 phosphorylation at TP sites and/or Clp1 conformation resulting from the six mutations. In HU-treated cells,

Clp1(6A)-GFP never accumulated in the nucleoplasm, a result similar to Clp1-GFP in *rad3Δ* cells (Figure 12C and 8A).

Though mutating the 6 RxxS sites abolished HU-induced Clp1 nucleoplasmic accumulation, Clp1(6A)-GFP and Clp1(3A)-GFP still re-localized in response to peroxide treatment in a population of cells. Thus, I reasoned that combining the phosphosite mutants to make *clp1(6A3A)* might effect this response more significantly. Indeed, Clp1(6A3A)-GFP did not relocalize to the nucleoplasm in response to either H_2O_2 or HU (Figure 12B and C). Additionally, Clp1(6A3A)-MYC13 immunoprecipitated from cells treated with 1 mM H_2O_2 for 45 min or 12 mM HU for 210 min had a faster mobility than wild type Clp1-MYC13 (Figure 12K). These data further support the model that direct phosphorylation of Clp1 RxxS and TP sites by Cds1 and/or Chk1, Pmk1 and Cdk1 upon genotoxic stress facilitates the relocalization of Clp1 from the nucleolus to the nucleoplasm during interphase.

As mentioned previously, Clp1 is also released from the nucleolus upon mitosis. Curious to see if the Clp1(6A3A) mutant maintained nucleolar sequestration during mitosis I examined the localization of Clp1(6A3A)-GFP in an untreated asynchronous population. Clp1(6A3A)-GFP was able to localize to all previously described Clp1 localizations including the mitotic spindle and contractile ring (Figure 13). These data suggests that the Clp1(6A3A)-GFP mutant is still released from the nucleolus upon mitotic entry, and that the regulation of mitotically released Clp1 is more complex than that of genotoxic stress induced Clp1 redistribution.

While strict regulation of Clp1 nucleolar release during mitosis is important for

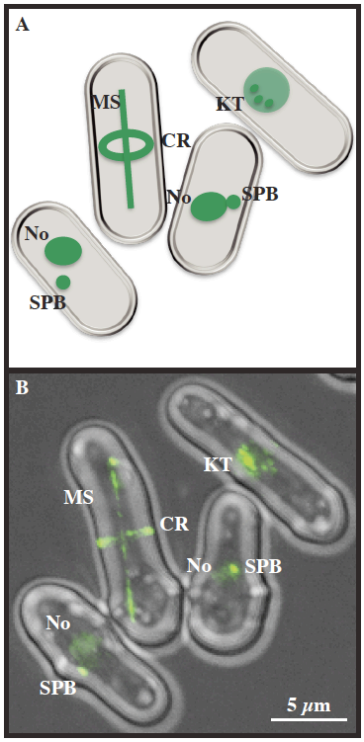

Figure 13. The Clp1(6A3A)-GFP mutant localizes to all typical Clp1 cell cycle localizations. (A) A cartoon representation of typical Clp1 localizations. (B) Live cell image of *clp1(6A3A)-GFP* cells. No=nucleolus, SPB=Spindle pole body, KT=Kinetochores, MS=Mitotic spindle and CR=Contractile Ring. Scale bar = 5 μm.

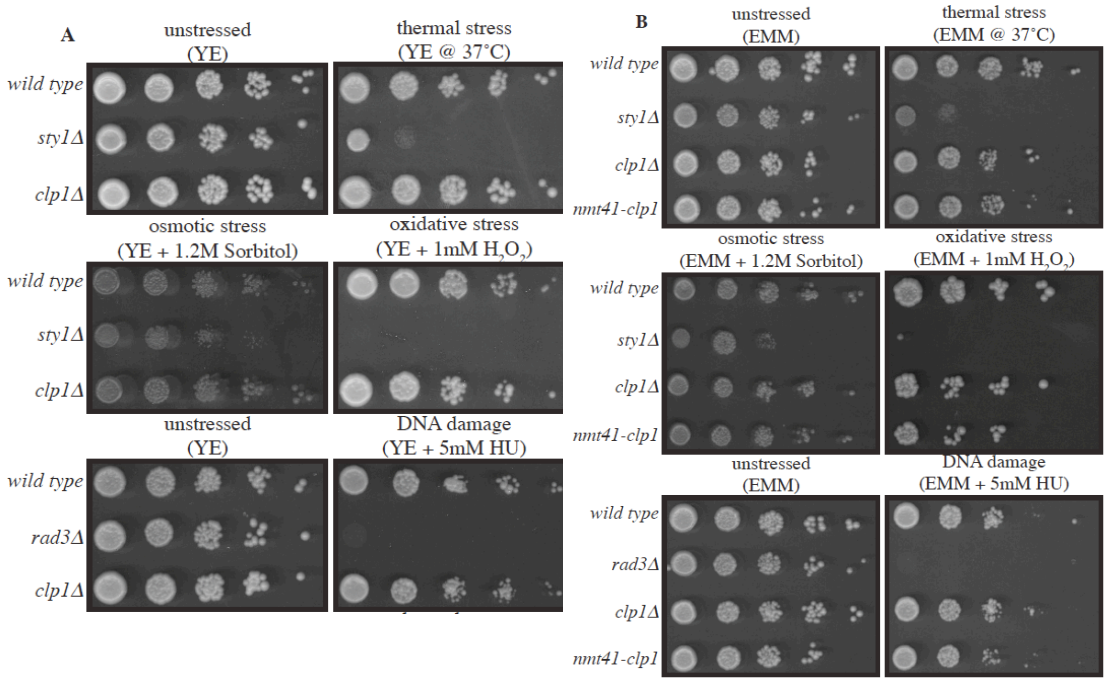

Figure 14. Deletion or overexpression of *clp1*$^{+}$ does not sensitize cells to various forms of cellular stress. (A) Spot assays examining the growth sensitivity of the indicated strains upon various forms of chronic stress exposure. Sty1 is a stress activated MAPK sensitive to various cellular stresses (Smith *et al*., 2010). (B) Same as (A) except thiamine was absent from the media to induce overexpression of Clp1.

the fidelity and timing of mitotic events (Chen et al., 2008; Clifford et al., 2008b; Cueille et al., 2001; Mishra et al., 2004; Trautmann and McCollum, 2005; Trautmann et al., 2004; Trautmann et al., 2001), it remains unclear why such complex regulation of genotoxic stress-induced Clp1 nucleolar release exists. To investigate cell growth effects I performed growth assays in which *clp1*$^{+}$, *clp1Δ* or *nmt41-clp1* (clp1 over-expression) strains were grown in the presence of various stress conditions. In all conditions I tested, *clp1Δ* and *nmt41-clp1* cells grew similar to *clp1*$^{+}$ cells (Figure 14) suggesting that Clp1 is not necessary for growth fidelity under conditions of cellular stress.

Summary

Nucleolar release of the Cdc14 phosphatases is a major activation step allowing access to substrates. It was previously reported that *S. pombe* Clp1 undergoes a redistribution from exclusively nucleolar to nucleoplasmic following HU treatment of interphase cells (Diaz-Cuervo and Bueno, 2008). Here, I extended this observation by showing that Clp1 also accumulates in the nucleoplasm upon genotoxic stress induced by H_2O_2 treatment of interphase cells. Using these treatments as assays for Clp1 redistribution, I was able to dissect the signaling networks capable of controlling Clp1 localization during interphase. I identified multiple kinase pathways that directly phosphorylate Clp1 to regulate the nucleolar to nucleoplasmic transition, and showed that the type of genotoxic stress influences the contribution to Clp1 regulation provided by each network. Furthermore, I found that Clp1 regulates its own nucleolar sequestration by antagonizing a subset of these networks. Collectively, my results reveal unexpected complexities in the regulation of Clp1 nucleolar localization, and support a model that

upon genotoxic stress during interphase, Cds1 and/or Chk1 together with Pmk1 and Cdk1 ensure the redistribution of Clp1 throughout the nucleoplasm (Figure 15). Since mammalian Cdc14B is also mobilized from the nucleolus by direct DNA damage (Bassermann et al., 2008; Diaz-Cuervo and Bueno, 2008; Guillamot et al., 2011; Mocciaro et al., 2010; Wei et al., 2011b) or H_2O_2 treatment (D. McCollum, unpublished observations), my results may provide insight towards understanding its relocalization upon genotoxic stress, and contribute to a better understanding of Cdc14 family phosphatase regulation. It still remains unknown, however, why Cdc14 phosphatases are release from the nucleolus upon genotoxic stress under such regulated conditions. Additionally, it will be important to determine whether TP and RxxS phosphosites contribute to Clp1 mitotic nucleolar release, and if so what further complexities exist for Clp1 mitotic nucleolar release.

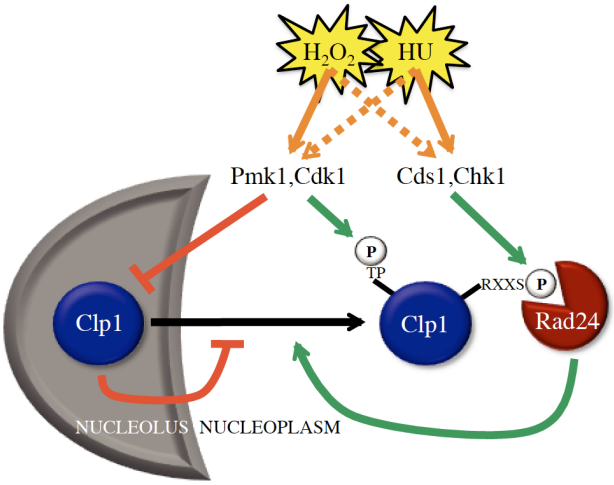

Figure 15. Model of the phosphoregulatory networks that control interphase genotoxic stress-induced redistribution of Clp1. Solid orange lines represent the more dominant network response relative to the dotted orange lines. Green lines indicate positive regulation while negative relationships are in red.

CHAPTER IV

UNDERSTANDING Clp1 LOCALIZATION

Introduction

As described in chapter I, Cdc14 phosphatases localize dynamically throughout the cell cycle; however, we lack a mechanistic understanding of how this is achieved. In chapter III, I explained how complex phosphoregulation triggers nucleolar release upon genotoxic stress, yet what sequesters Clp1 within the nucleolus is unknown. In fact, beyond Clp1's localization to the contractile ring, how Clp1 is directed and tethered to its other multiple sites of localization is unknown. Furthermore, Clp1 localizes to both nuclear and cytoplasmic addresses throughout the cell cycle (Stegmeier and Amon, 2004), and its occupancy of both cellular compartments is critical for Clp1's functions (Trautmann and McCollum, 2005). In order for Clp1 to localize to both the nucleus and cytoplasm, Clp1 must be actively transported by karyopherin family members through nuclear pores (Mosammaparast and Pemberton, 2004). However, neither the machinery at the nuclear pore, nor the sequences motifs within Clp1 that regulate its nuclear-cytoplasmic distribution have been defined. Here, I present my work identifying *trans* and *cis* factors impacting Clp1's intracellular distribution, and I present data suggesting that Clp1 localization to the nucleolus and SPB may not involve high affinity molecular tethers.

Results

Identification of the karyopherin, Sal3, as the mediator of Clp1 nuclear import

To identify Clp1 substrates and/or interacting partners, tandem affinity purifications (TAPs) (Gould et al., 2004; Puig et al., 2001) of Clp1 were performed and the resultant complexes were analyzed by 2D-LC tandem mass spectrometry (MS/MS) (McDonald et al., 2002). Because interaction between Clp1 and its substrates might be transient, a catalytically inactive mutant of Clp1, Clp1(C286S), (Wolfe and Gould, 2004; Wolfe et al., 2006) and wild type Clp1, were used as bait in the purifications. The Clp1(C286S) mutant should act as a substrate trap, able to bind its substrates but unable to dephosphorylate them (Tonks and Neel, 1996). To enhance the chances of identifying the largest number of interacting proteins, Clp1 was purified from asynchronously growing cells in which Clp1 is primarily at the SPB and nucleolus, and mitotically arrested cells in which Clp1 has spread throughout the cell, decorating the mitotic spindle, the kinetochores, and the contractile ring. To enrich for cells in mitosis, a ß-tubulin mutant, *nda3-KM311,* was used to arrest cells in prometaphase (Hiraoka et al., 1984), and a thirty minute release of this arrest was used to capture cells in anaphase. Each TAP-LC-MS/MS analysis was performed in duplicate.

Proteins identified as Clp1 interactors were then classified into three groups: general Clp1 interacting partners that associated with wild type Clp1-TAP and Clp1(C286S)-TAP equally, Clp1 substrates that were more than two-fold enrichment within Clp1(C286S)-TAP samples and Clp1 putative regulators/co-factors that associated with more than two-fold enrichment in wild type Clp1-TAP relative to Clp1(C286S)-TAP complexes (Chen *et al*., submitted). Within this analysis, the importin-β3, Sal3 (Chua et al., 2002), was prominent among those proteins with no apparent preference for either Clp1-TAP or Clp1(C286S)-TAP (Chen *et al*., submitted). Validating the MS

results, I found that Clp1-MYC13 could be co-immunoprecipitated with Sal3-FLAG3 (Figure 16A). Since the regulation of Clp1 nucleoplasmic shuttling is critical for its function (Mishra et al., 2005; Trautmann and McCollum, 2005), I further pursued the relevance of this interaction.

Like other importins, Sal3 facilitates cargo transport through nuclear pore complexes (Figure 17) (Chook and Blobel, 2001; Mosammaparast and Pemberton, 2004). *sal3*$^{+}$ is not an essential gene so it was possible to evaluate Clp1-GFP localization in *sal3Δ* cells, even though the cells are highly elongated due to a defect in Cdc25 nuclear import (Chua et al., 2002). Clp1-GFP was produced at normal levels in *sal3Δ* cells (Figure 18) and was detected normally at SPBs during interphase (n=186) and the cytokinetic ring during mitosis (n=32) (Figure 16B and C). However, Clp1-GFP was not detected in the nucleus at any stage in the cell cycle as compared to *wild type* cells (Figure 16B and C). Thus, Sal3 is required for Clp1 nuclear import.

To test whether Sal3 is the only importin affecting Clp1 nuclear import, Clp1-GFP localization was systematically examined in mutations of all other known *S. pombe* importins (Chen et al., 2004). There are 7 importin-βs (Kap95, Kap104, Kap111, Kap113, Kap114, Kap123, and Sal3) and 2 importin-αs (Imp1 and Cut15) in *S. pombe* (Chen et al., 2004; Umeda et al., 2005). Unlike in *sal3Δ* cells, Clp1-GFP localized normally to the nucleus in the single deletions of the 6 other non-essential importins (Figure 19A), suggesting that none of them are required for Clp1 nucleocytoplasmic shuttling. *kap95*$^{+}$ and *cut15*$^{+}$ are the two essential importin genes in *S. pombe*. I found that Clp1-GFP localized normally in a temperature-sensitive mutant of Cut15 upon shift to 36˚C for 3 hours (Figure 19B). Lastly, Kap95 depends on both importin-αs as

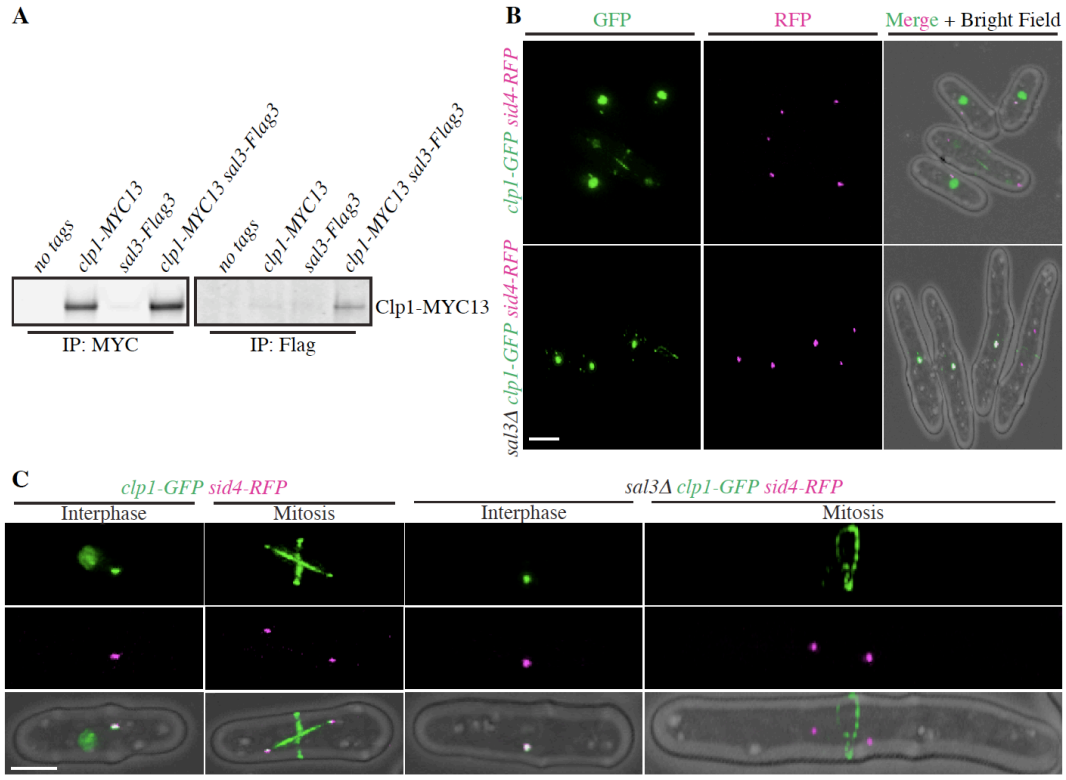

Figure 16. Characterization of Sal3 dependent Clp1 nuclear import. (A) Strains with the indicated genotypes were immunoprecipitated with either anti-MYC or anti-Flag. Immunoprecipitates were then immunoblotted for MYC. (B-C) Live cell images of *clp1-GFP sid4-RFP* (Sid4 is a SPB marker (Chang and Gould, 2000)) or *sal3Δ clp1-GFP sid4-RFP* from an asynchronous population (B) or cells in either interphase or mitosis (C). Scale bar in (B) = 5 μm. Scale bar in (C) = 3 μM

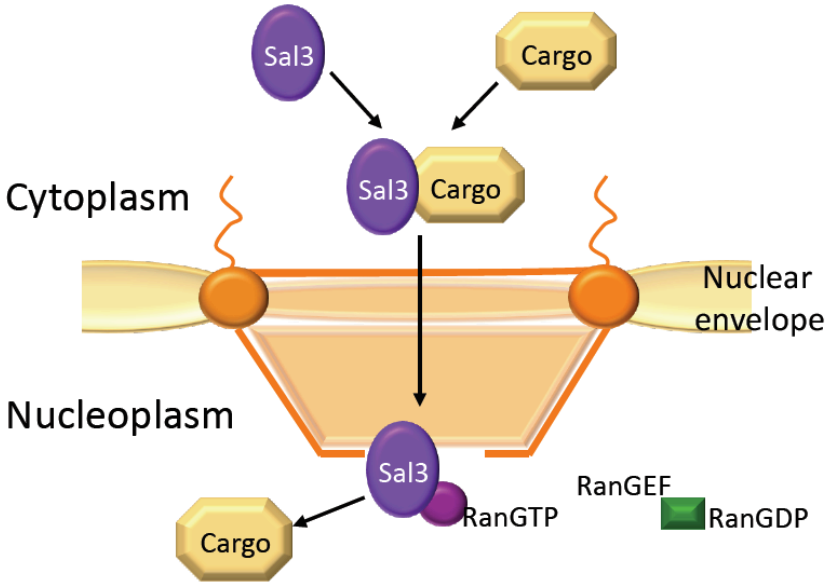

Figure 17. Schematic of Sal3 nuclear import. Sal3 binds specific cargo in the cytoplasm and transports it into the nucleus via nuclear pore complexes (represented in orange). In this case Clp1 would be the specific cargo. Once inside the nucleoplasm, RanGTP binds to Sal3, which causes release of the cargo into the nucleoplasm.

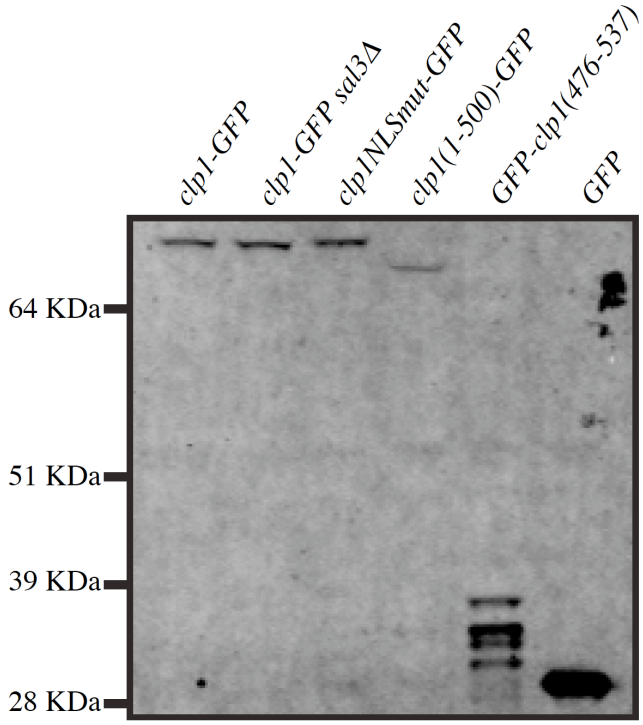

Figure 18. Expression of Clp1 and Clp1 mutants. *clp1*$^{+}$, *clp1NLSmut*, and *clp1(1-500)* were expressed from the *clp1*$^{+}$endogenous locus and C-terminally tagged with GFP. *GFP-clp1(476-537)* and *GFP* were expressed in *wild type* cells from the *nmt81* inducible promoter. These proteins were immunoprecipitated from cell lysates and then analyzed by immunoblot analysis with anti-GFP.

adaptors for cargo selection (Chook and Blobel, 2001). To examine the consequence of abolishing Kap95 nuclear import on Clp1-GFP localization, I constructed a double mutant, *imp1Δ cut15-122,* abolishing the activity of both importin-αs upon temperature shift. Clp1-GFP localized normally in this strain at the non-permissive temperature (Figure 19C). Together, these data indicate that Sal3 alone controls Clp1's access to the nucleus.

Identification of a Clp1 Nuclear Localization Sequence

The importin-β3 family binds its cargo directly, without the use of adaptor proteins, via nuclear localization sequences (NLSs) (Mosammaparast and Pemberton, 2004). Thus, I next sought to identify the NLS that allowed for Clp1 recognition by Sal3. The NLS for *S. cerevisiae* Cdc14 was identified at the protein's C-terminal end (Mohl et al., 2009). To determine if this was conserved in Clp1 I introduced a GFP::kan^R cassette following amino acid 500 in the endogenous *clp1*$^+$ open reading frame, eliminating the last 37 amino acids of the protein (Figure 20A). Like Clp1-GFP in *sal3Δ* cells, Clp1(1-500)-GFP was absent from the nucleus at all cell cycle stages (n=134 interphase and 42 mitotic cells) although it maintained localization to SPBs during interphase and the cytokinetic ring during mitosis (Figure 20B). This result suggested that a NLS exists within the last 37 amino acids of Clp1.

A typical NLS contains a string of basically charged amino acids (Lange et al., 2007) and I identified such a motif within amino acids 500-537 of Clp1 (Figure 20A). To determine whether the five basic residues in this region functioned as the Clp1 NLS, I replaced *clp1*$^+$ with a mutant version, *clp1NLSmut-GFP*, in which R524, K527, K529,

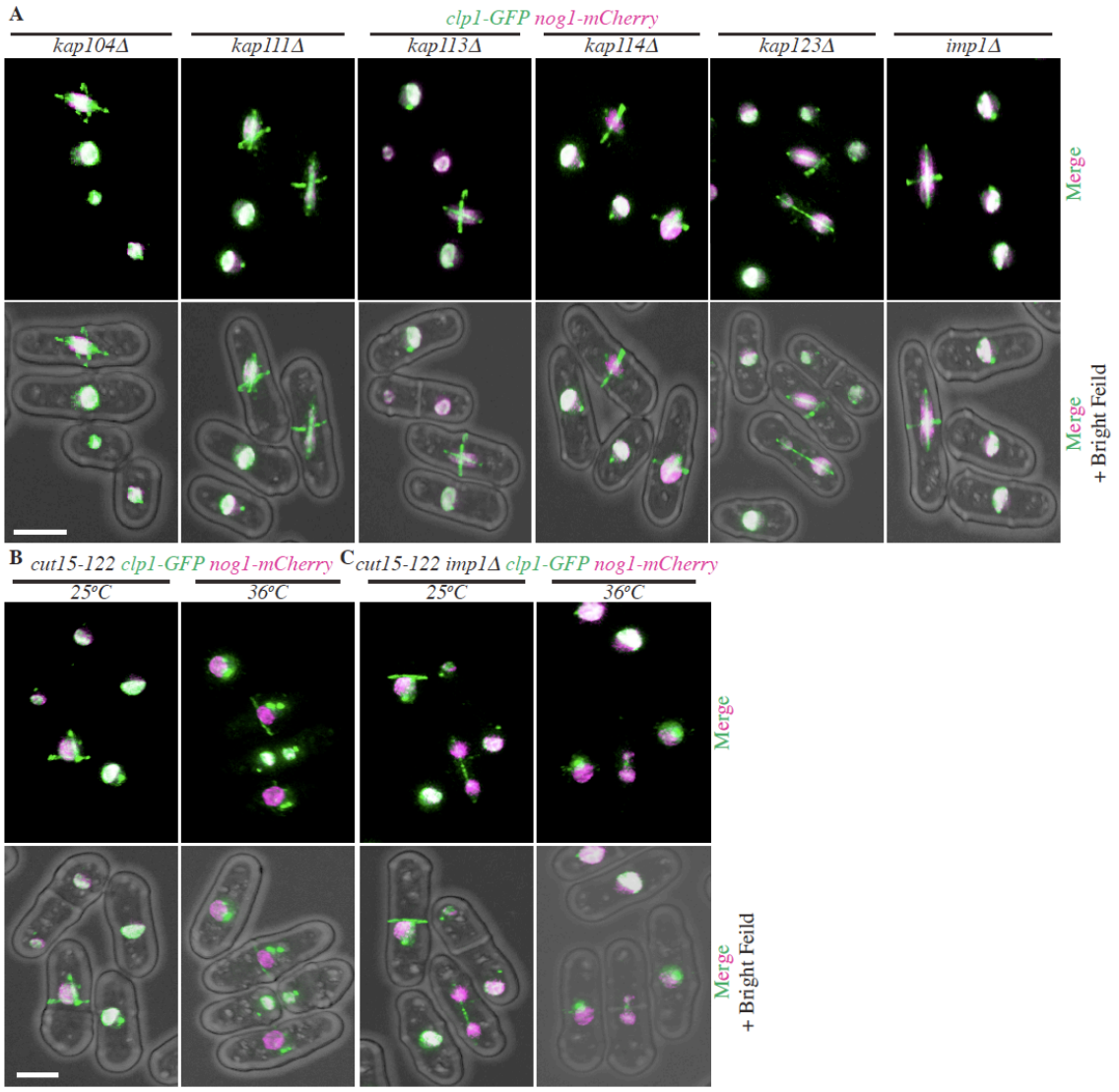

Figure 19. Clp1 is imported normally to the nucleus in the absence of 8 of 9 *S. pombe* importins. (A) Live cell images of the indicated importin deletion strains containing *clp1-GFP nog1-mCherry* (Nog1 is a nucleolar marker (Matsuyama et al., 2006)). (B) Live cell images of *cut15-22 clp1-GFP nog1-mCherry* cells grown at either 25˚C or 36˚C. (C) Same as (B) but with *cut15-22 imp1Δ clp1-GFP nog1-mCherry* cells. Scale bars = 5 μm.

K532 and R534 were mutated to alanines. Clp1NLSmut-GFP localized to the SPBs during interphase and the cytokinetic ring during mitosis, but was not observed in the nucleus (n=117 interphase and 38 mitotic cells) (Figure 20C), indicating that these basic amino acids are a crucial element of the Clp1 NLS. I next sought to determine if this NLS mutant was catalytically active. Like *clp1*$^{+}$ and unlike *clp1Δ* cells, cells containing *clp1NLSmut-GFP* opposed the function of *cdc2-33* indicating that the mutant is active. Moreover, unlike *clp1Δ, clp1NLSmut-GFP* did not show negative interactions with the cytokinesis mutant, *cdc3-124,* indicating that its cytoplasmic functions were intact (Figure 21).

Finally, to determine if the C-terminus of Clp1 was sufficient for nuclear localization, I fused GFP to the C-terminal amino acids of Clp1 (476-537) (Figure 20A), and expressed this fusion in cells from the inducible *nmt81* promoter. While GFP alone localized diffusely throughout the cell, GFP-(476-537) accumulated within the nucleus (Figure 20D), indicating that the C-terminus of Clp1 is both necessary and sufficient for nuclear localization. GFP-(476-537), however, localized diffusely in the cytoplasm when it was expressed in *sal3Δ* cells (Figure 20E) consistent with the requirement of Sal3 for Clp1 nuclear import.

Crm1 is the conserved Cdc14 phosphatase nuclear exportin

Crm1 is a member of the karyopherin family known to export numerous cargoes from the nucleus (Kutay and Guttinger, 2005). *S. cerevisiae* Cdc14 and the higher eukaryotic Cdc14 phosphatases are among its targets (Bembenek et al., 2005). I therefore tested whether Clp1 similarly depended on Crm1 for nuclear export. *clp1-GFP* cells

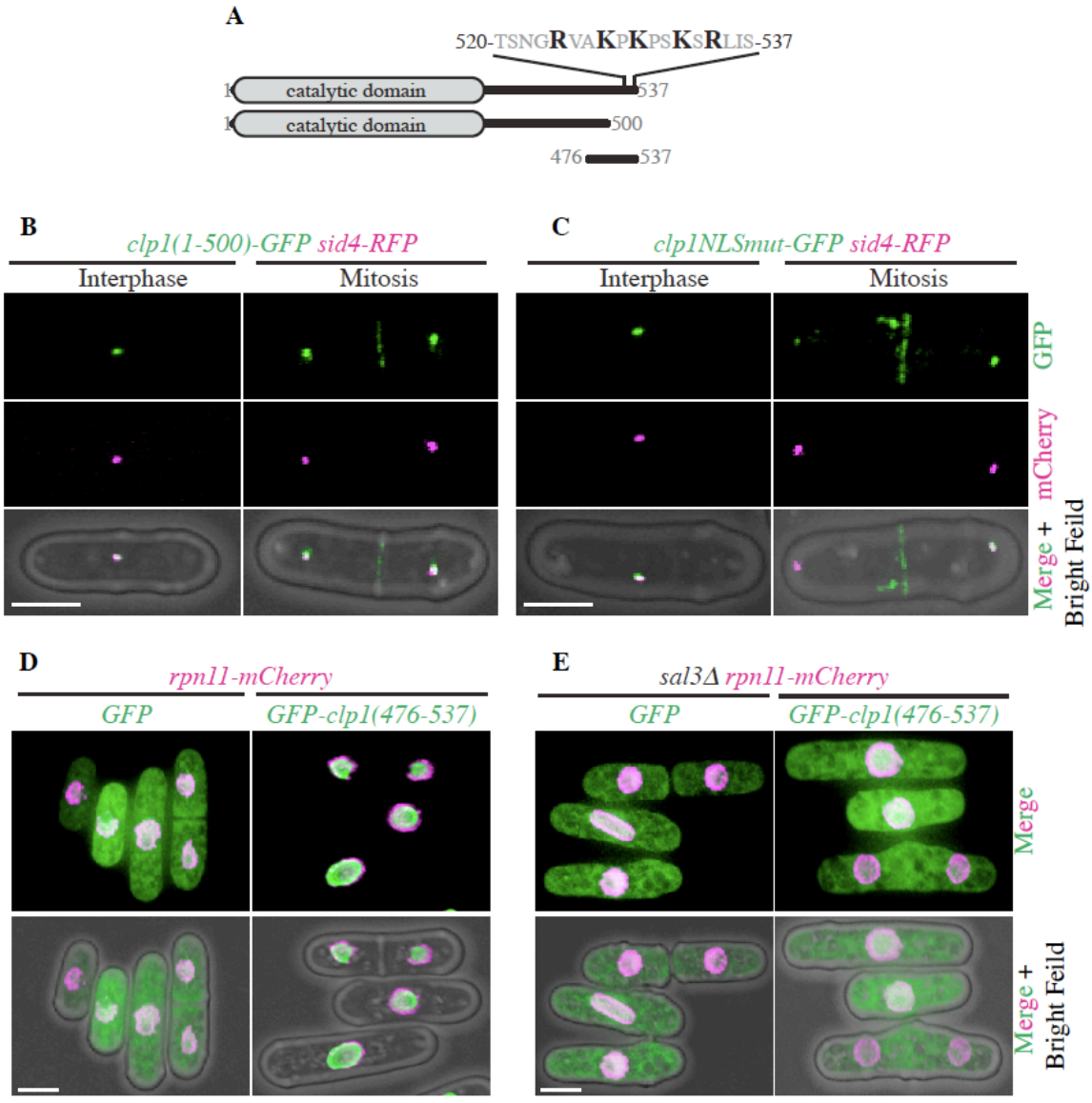

Fig 20. Identification of a functional Clp1 NLS. (A) Schematic of Clp1, Clp1 truncations, and Clp1 mutations. The C-terminal 17 amino acids that contain the stretch of basic residues mutated to alanines (highlighted in black) in Clp1NLSmut are shown above full length Clp1. (B) Live cell images of *clp1(1-500)-GFP sid4-RFP* cells in either interphase or mitosis. Scale bar = 4 μm (C) Same as (B) but images are of *clp1NLSmut-GFP sid4-RFP* cells. (D-E) Live cells images of *rpn11-mCherry* (Rpn11 is a marker of the nuclear envelope (Kouranti et al., 2010)) (D) or *sal3Δ rpn11-mCherry* (E) cells expressing *GFP* or *GFP-clp1(476-537)*. Scale bar = 5 μm.

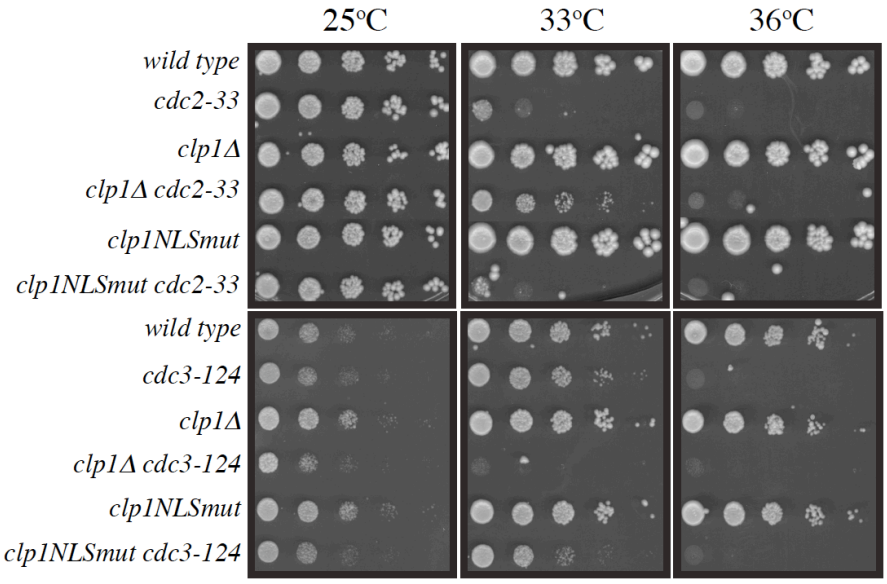

Figure 21. Clp1NLSmut is a functional mutant. The indicated strains were grown to mid-log phase at 25˚C in YE, then spotted in 10-fold serial dilutions on YE plates and incubated at the indicated temperatures.

were either treated with Leptomycin B (LMB), a known Crm1 inhibitor (Kudo et al., 1999) or vehicle (1.4% methanol) for 3hr and the localization of Clp1-GFP was examined. Mock treated cells were able to localize Clp1-GFP to cytoplasmic targets, the SPB and/or the contractile ring, where as cells treated with LMB showed exclusively nuclear Clp1-GFP localization (Figure 22). These data indicate that the mechanism of nuclear export for Cdc14 phosphatases is conserved throughout evolution, relying on Crm1.

Clp1 localizes dynamically to the nucleolus and/or spindle pole body

As determined by fluorescence recovery after photobleaching (FRAP) analysis of nucleolar localized Cdc14-GFP, *S. cerevisiae* Cdc14 is tightly anchored within the nucleolus and lacks the ability to exchange readily with other cellular compartments (Tomson et al., 2009). This behavior correlates with Cdc14 having a high-affinity nucleolar binding partner, Net1, which serves as its nucleolar tether (Shou et al., 1999; Traverso et al., 2001; Visintin et al., 1999). However, nucleolar tethers have not been identified for any other Cdc14 phosphatase family member suggesting that their mechanisms of localization to the nucleolus may be different than that of *S. cerevisiae* Cdc14.

In support of this, Jun-Song Chen's proteomic study identified Mid1, the Clp1 contractile ring tether, as a Clp1 interacting protein (Chen et al., submitted). Interestingly, and consistent with its function to tether Clp1 at the contractile ring, Mid1

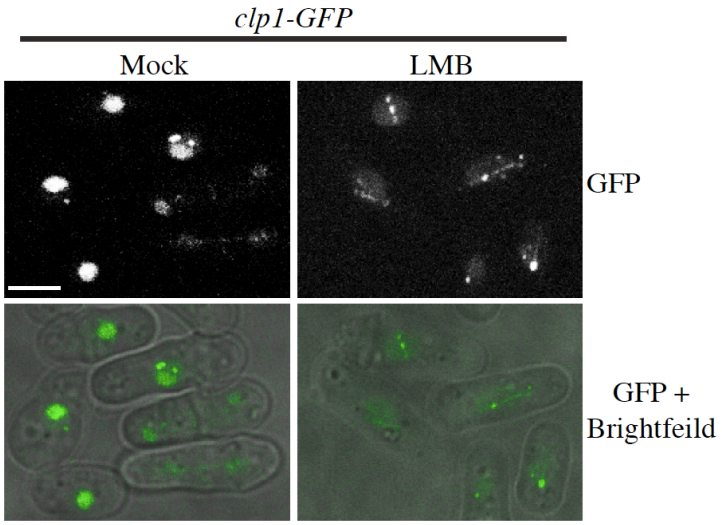

Figure 22. Clp1 nuclear export depends on the exportin Crm1. Live cell images of *clp1-GFP* cells after treatment with vehicle (1.4% methanol) or treatment with leptomycin B (LMB) for 3 hr. Scale bar = 5 μm.

was identified with more than two-fold enrichment in wild type Clp1-TAPs relative to Clp1(C286S)-TAPs. This proteomic study, however, did not identify a similar interaction between Clp1 and a protein(s) localized within the nucleolus or at SPB. Furthermore, core structural/scaffold components of the SPB were not identified as Clp1 interacting partners, leading me to hypothesize that Clp1 may localize to these sites through numerous low-affinity transient interactions.

To test my hypothesis, I analyzed Clp1-GFP dynamics at the nucleolus and SPB by FRAP. In contrast to *S. cerevisiae* Cdc14-GFP, Clp1-GFP was very dynamic within the nucleolus (Figure 23), exhibiting a rapid half time of recovery ($T_{1/2}$), 1.41 $\pm$ 0.50 sec, and a mobile fraction (M_f) of 98 $\pm$ 15% (Figure 23). These data suggest that high-affinity tethers for Clp1 do not exist as they do for *S. cerevisiae* Cdc14 within this cellular compartment, and that Clp1 may be retained by numerous low-affinity interactions.

Dnt1 and Nsk1 are two known Clp1 interacting proteins that localize to the nucleolus during interphase (Chen et al., 2011a; Jin et al., 2007). To determine if either of them influences Clp1 nucleolar dynamicity, FRAP analyses of Clp1-GFP was performed in the absence of Nsk1, Dnt1, or both proteins. In each case, there was no apparent change in Clp1's dynamicity (Figure 23). Additionally, the nucleolar intensity of Clp1-GFP, as judged by fluorescence intensity, was unaffected by loss of *nsk1*, *dnt1* or both genes. These data support the hypothesis that Clp1 localizes to the nucleolus through numerous low-affinity interactions, and that removal of only a few interacting proteins does not significantly affect the localization or dynamic behavior of Clp1 within the nucleolus.

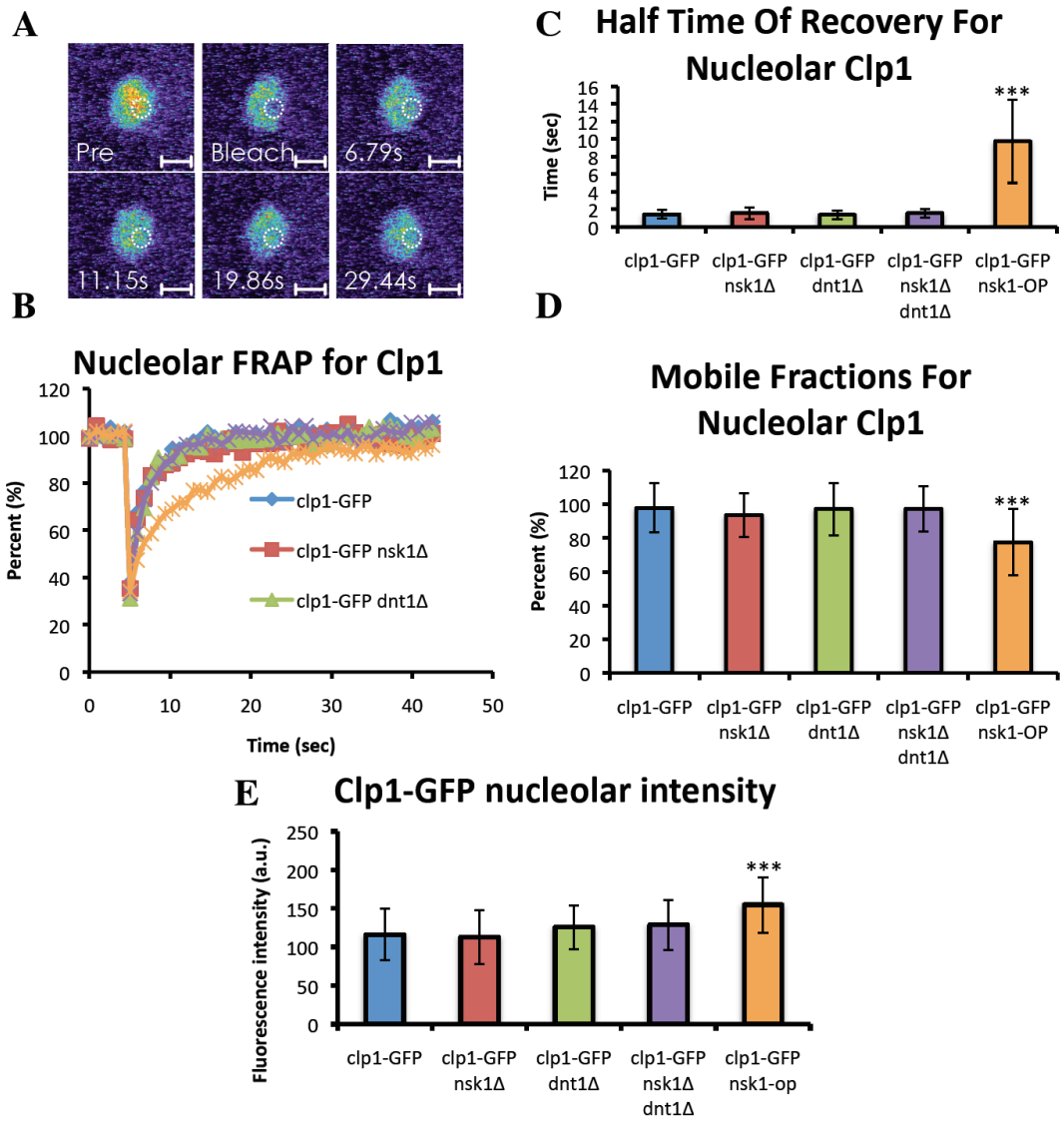

Figure 23. Clp1 is dynamic within the nucleolus. (A) Representative images of Clp1-GFP localized within the nucleolus throughout the FRAP experiment. The colors represent fluorescence intensity with red being the most intense and black least intense. Pre-bleach Clp1 localization is in the upper left panel denoted "Pre." "Bleach," indicates the nucleolus directly after bleaching. S, seconds after bleaching. The dotted circle represents the bleached area within the nucleolus. Scale bar = 1 μm (B) Fluorescence recovery curves for the indicated genotypes. Each line is an average of greater than or equal to 31 nucleoli. (C) A graphical representation of the $T_{1/2}$ generated from fluorescence recovery curves in (B). Standard deviation is shown. Asterisks indicate statistically significant as determined by student's *t* test ($p < 0.0001$). (D) A graphical representation of the M_f generated from fluorescence recovery curves in (B). Standard deviation is shown. Asterisks indicate statistically significant as determined by student's *t* test ($p < 0.0001$). (E) The graph represents the average fluorescence intensity for Clp1-GFP within the nucleolus for the indicated genotype. Standard deviation is shown. $n \geq 85$ nucleoli. Asterisks indicate statistically significant as determined by student's *t* test ($p < 0.0001$).

Further evidence that multiple interactions may stabilize Clp1 within the nucleolus was provided by an over-expression experiment. In cells over-expressing *nsk1*$^{+}$, which causes an accumulation of Nsk1 in the nucleolus, Clp1's $T_{1/2}$ slowed modestly to 9.73 $\pm$ 4.73 sec and the M_f dropped to 78 $\pm$ 19.% (Figure 23). Also, the intensity of Clp1-GFP within the nucleolus increased 32% when Nsk1 was over-produced, suggesting that increased Nsk1-Clp1 interactions within the nucleolus can increase Clp1's nucleolar interaction.

To test the hypothesis that Clp1 also does not have a high-affinity interaction partner at the SPB, I analyzed Clp1-GFP by FRAP at the SPB in 25 cells expressing *clp1-GFP* from the endogenous *clp1*$^{+}$ promoter. At the SPB Clp1 had a $T_{1/2}$ of 28.64 $\pm$ 8.57 sec (Figure 24); this is a very fast recovery time when considering the $T_{1/2}$ of two known *S. pombe* SPB scaffold proteins, Sid4 and Cdc11, which recover on the order of mins (Feoktistova et al., 2012; Morrell et al., 2004). The M_f of Clp1 at the SPB was 43 $\pm$ 19% (Figure 24). This M_f of Clp1-GFP at the SPB might suggest that there is a stable population of Clp1 attached to the SPB. However, the interpretation of these data is complicated by the following consideration. In a typical FRAP experiment, only a portion of a particular localization site is bleached. However, the SPB is so small that its entirety is bleached in these experiments. Recovery of Clp1-GFP at the SPB must then on rely another pool within the cell, with the most probable source being nucleolar-localized Clp1-GFP. To investigate whether SPB recovery following FRAP required a nucleolar pool, I preformed FRAP analysis of Clp1-GFP at the SPB in *sal3Δ* cells.

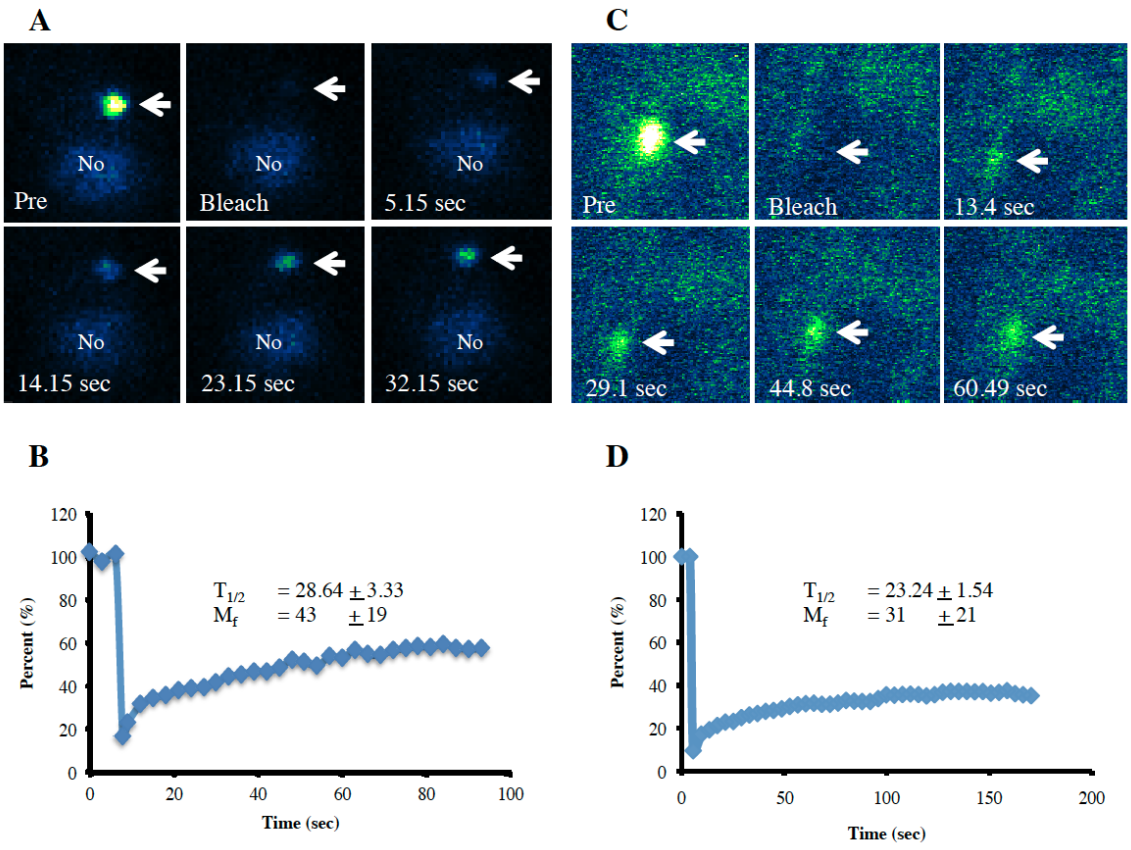

Figure 24. Clp1-GFP localizes dynamically at the spindle pole body. (A) Representative images of Clp1-GFP throughout the FRAP experiment. The colors represent fluorescence intensity with being yellow most intense and black least intense. Pre-bleach Clp1 localization is represented in the upper left panel denoted "Pre." "Bleach," indicates directly after bleaching. Sec, seconds after bleaching. The arrowhead points to the SPB. No indicates Clp1's nucleolar localization. Scale bar = 1 μm (B) Fluorescence recovery curves of Clp1-GFP at the SPB. The line represents the best-fit curve of 25 SPBs. The calculated $T_{1/2}$ and M_f are indicated with their standard deviation. (C) Same as in (A) except cells lack *sal3*$^+$. (D) Same as in (B) except cells lack *sal3*$^+$. n = 37.

Sal3 is responsible for all nuclear import of Clp1. In *sal3Δ* cells, Clp1-GFP is only cytoplasmic and during interphase it is concentrated solely at the SPB (Figure 24). The M_f for Clp1-GFP at the SPB in *sal3Δ* cells dropped to 31 $\pm$ 21% (Figure 24), suggesting that SPB recovery of Clp1-GFP following bleaching is due to exchange with its nucleolar localized pool. Additionally, since the M_f for Clp1-GFP in *sal3Δ* cells is not 0%, the SPB localized Clp1-GFP likely exchanges with a cytoplasmic population of Clp1-GFP. In *sal3Δ* cells, the $T_{1/2}$ of Clp1-GFP at the SPB was 23.24 $\pm$ 1.54 sec, which is comparable to that of *wild type* cells (Figure 24). Overall, these results suggest that Clp1-GFP localizes very dynamically to the SPB and is able to quickly exchange with non-SPB localized Clp1-GFP.

Summary

It has previously been shown that Cdc14 phosphatases localize to multiple changing addresses within both the nucleoplasm and cytoplasm throughout the cell cycle. These changes in localization serve to regulate substrate access spatially and temporally for the Cdc14 phosphatases. We lack the understanding, however, of how Cdc14 phosphatases localize to specific sites. Work presented within this chapter expands our understanding of what controls the ability of Clp1 to localize to both nuclear and cytoplasmic locations, and the specific behavior of Clp1 within the nucleolus and at the SPB. I show that Clp1 is actively imported into the nucleus by the importin-β3, Sal3, and that Sal3 recognizes Clp1 for import by a C-terminal NLS. Additionally, conserved with budding yeast Cdc14 and human Cdc14A (Bembenek et al., 2005), I found that Crm1, an exportin, actively transports Clp1 out of the nucleus and into the cytoplasm. I also found

that Clp1 localizes to both nuclear and cytoplasmic sites with very dynamic behavior. This dynamic localization within the nucleolus and at the SPB suggests that Clp1 is in constant flux, and may explain why I have been unable to identify tethers that hold Clp1 in any particular localization. This information helps explain why Net1 was easily found as the nucleolar tether for *S. cerevisiae* Cdc14, while nucleolar binding partners for other Cdc14 phosphatase family members are still largely unknown and are unlikely to be found in biochemical approaches that rely on high affinity interactions. Additionally, these data support the idea that nucleolar localization of budding yeast Cdc14 is fundamentally different than that of other Cdc14 family members.

CHAPTER V

DISCUSSION

Summary

Cdc14 phosphatase family members function to antagonize Cdk1 phosphorylation. They contribute to the fidelity of mitotic exit and cytokinesis, and likely in the cellular response to genotoxic insults. These roles are regulated through changes in Cdc14 localization throughout the cell cycle and upon genotoxic stress, affecting access of the Cdc14 phosphatases to their substrates. Factors that contribute toward directing localization of Cdc14 phosphatases are mostly unknown. Here I show, at least upon genotoxic stress, that Clp1 is released from nucleolar sequestration through direct phosphorylation by multiple kinase pathways, and that Clp1 controls it own nucleolar sequestration through autodephosphorylation. Additionally, I presented data suggesting that Clp1 localizes within the nucleolus and at the SPB through numerous low-affinity transient interactions based on its dynamicity at these sites. Lastly, I identify both *cis* and *trans* factors that are responsible for nucleocytoplasmic transport of Clp1, allowing Clp1 to access both nuclear and cytoplasmic compartments.

Complexity of genotoxic stress-induced Clp1 nucleolar release

At the single cell level, the response of Clp1 to genotoxic stress appeared to be all-or-none; Clp1-GFP was either obviously restricted to the nucleolus or clearly diffuse within the nucleus in each cell (Figure 7 and 25). All-or-none responses are typical

outputs produced by phosphoregulated systems, and have been extensively study within the context of MAPK networks (Ferrell, 2002; Ferrell *et al*., 2009; Kholodenko *et al*., 2010). In multiple eukaryotic systems, MAPK signaling networks turn graded inputs into switch-like outputs (Bagowski et al., 2003; Mackeigan et al., 2005; Malleshaiah et al., 2010; Melen et al., 2005). This setup allows MAPKs to filter out signaling noise and generate decisive cellular responses. The Raf-MEK-ERK network, which is most closely related to the cell wall integrity pathway, also exhibits negative feedback control and has been proposed to act similarly to a negative feedback amplifier (Sturm *et al*., 2010), to limit the duration of responses and to produce more graded responses to a broad range of inputs (Birtwistle and Kolch, 2011). When Clp1 was released from the nucleolus in a Pmk1-dependent manner, an increasing number of cells responded to H_2O_2 for 30 min; however, afterwards Clp1 began to transition back to the nucleolus. This indicates that Pmk1 signaling may be turned off in cells after 30 min even though H_2O_2 is still present. Indeed, Pmk1 activation, as determined by the activating phosphorylation on Pmk1, decreases after 30 min even though an environmental stress remains (Madrid *et al*., 2006). Additionally, examples of negative feedback loops within Pmk1 signaling have already been described (Madrid *et al*., 2007; Takada *et al*., 2010). These observations suggest that the cell wall integrity pathway likely functions similarly to the Raf-MEK-ERK pathway in terms of limiting its affect on Clp1 localization.

Cdk1 dependent redistribution of Clp1 upon H_2O_2 addition also stops responding after 20 min. However, when Cds1/Chk1 signaling is functional along with either Pmk1 or Cdk1, cells accumulated Clp1 within the nucleoplasm to levels similar to *wild type* cells, and this response was maintained in the presence of the genotoxic agent.

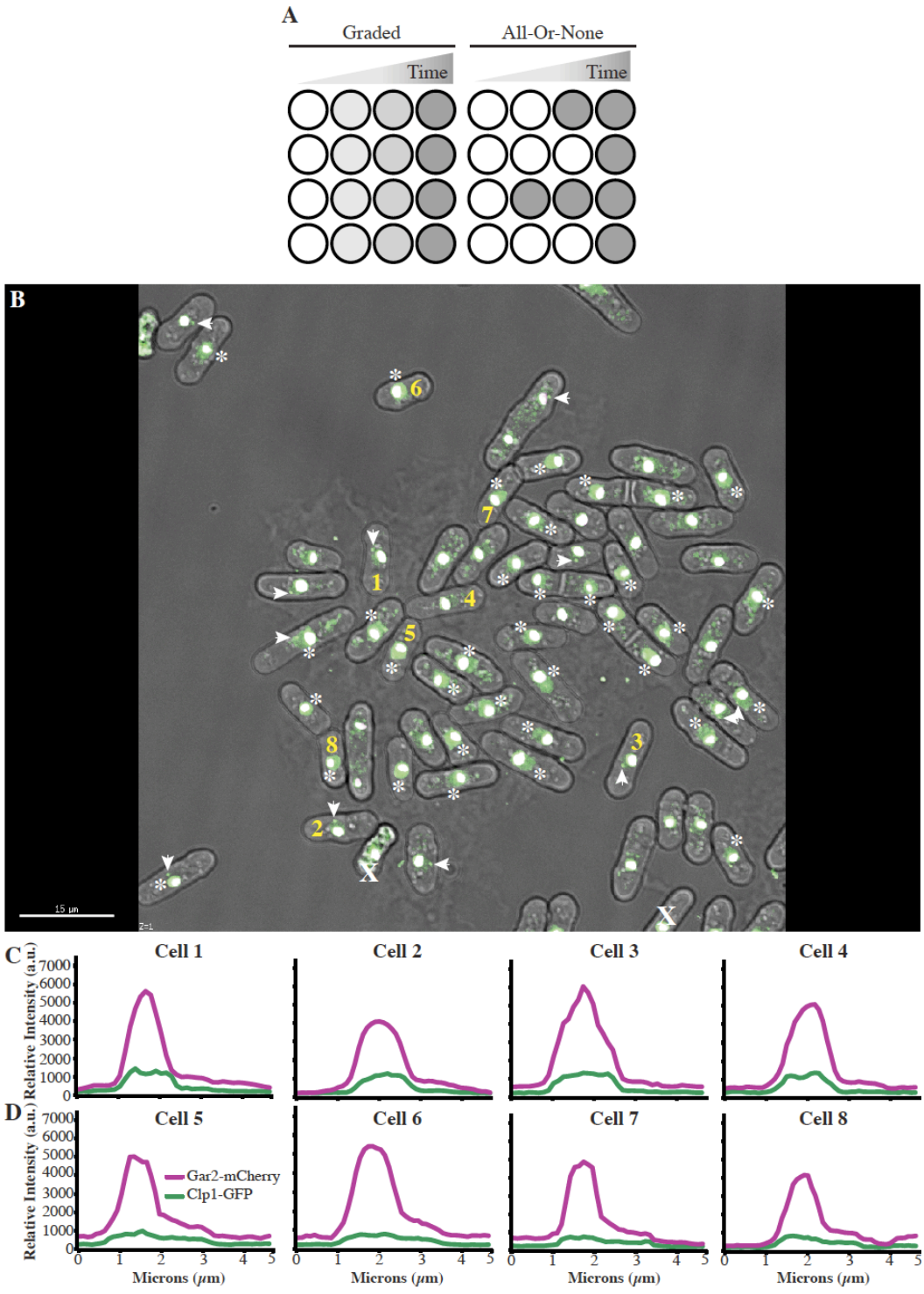

Figure 25. Release of Clp1-GFP from the nucleolus is an all-or-none response. (A) A schematic representing the difference between graded or all-or-none responses, as it corresponds to my system. (B) Live cell image of *clp1-GFP gar2-mCherry* cells taken at t=5 min post H_2O_2 addition. (C and D) Line scans for Clp1-GFP and Gar2-mCherry fluorescence of 4 cells in which Clp1-GFP was restricted to the nucleolus (C) and 4 cells in which it had distributed throughout the nucleoplasm (D).

Therefore, it is likely that Pmk1 and Cdk1 induce the initial nucleolar release of Clp1, while activity of the checkpoint effector kinases are necessary to maintain Clp1 nucleoplasmic localization and provide robustness during persistent genotoxic stress. This is similar to the role of Sid2 in mitosis (Chen et al., 2008; Mishra et al., 2005), and indicates that cells may use overlapping strategies to localize Cdc14 phosphatases regardless of the situation.

That H_2O_2 among the tested stresses was the only one to influence Clp1 nucleolar release similar to HU treatment is not entirely surprising. Reactive oxygen species are known to cause DNA damage (Cadet *et al*., 2010). However, the difference in overall time to accumulate Clp1 within the nucleoplasm may reflect the distinct mechanisms and timeframes by which HU and H_2O_2 cause DNA damage. HU-treated cells may respond more slowly because the initial stalled replication forks do not signal Clp1 redistribution, but DNA double strand breaks that result from unmaintained replication forks after prolonged HU exposure may trigger Clp1 relocalization. More interesting was that Rad3 was not required for Clp1 nucleoplasmic accumulation upon H_2O_2 treatment as in HU treatment. This finding led me to identify Pmk1 and Cdk1 as additional kinases that function independently from Chk1 and Cds1 to facilitate Clp1 nucleoplasmic accumulation. In accord with the involvement of Cdk1 in phosphorylating Clp1 upon genotoxic insults and in promoting Clp1 redistribution, Cdk activity is involved in the DNA replication/damage checkpoint and promotes homologous recombination over non-homologous end joining repair in response to DNA double strand breaks post-replication (Cerqueira et al., 2009; Chen et al., 2011b; Enserink et al., 2009; Enserink and Kolodner, 2010; Ira et al., 2004; Langerak and Russell, 2011). Additionally, Cdk1 phosphorylates

the *S. cerevisiae* specific Cdc14 nucleolar tether, Net1 (Azzam *et al*., 2004). This event assists FEAR network dependent nucleolar release of Cdc14 during mitosis, and the loss of this phosphorylation event modulates early anaphase release of Cdc14 (Azzam *et al*., 2004). Thus, the role of Cdk1 in promoting nucleolar release of Cdc14 phosphatases appears conserved between these two yeast species. All together, my results support a model that upon genotoxic stress during interphase, Cds1 and/or Chk1 together with Pmk1 and Cdk1 ensure the redistribution of Clp1 throughout the nucleoplasm (Figure 15).

In the context of Clp1 mitotic nucleolar release, a Clp1 phosphomutant shown to abolish identified Cdk1 TP and Sid2 RxxS phosphosites, Clp1(6A3A), still localizes to the kinetochores, along the mitotic spindle and to the contractile ring during mitosis (Figure 13). This was unexpected since Clp1(6A3A)-GFP remained sequestered within the nucleolus when cells were treated with either HU or H_2O_2. While this suggests that Clp1 mitotic nucleolar release is different from its genotoxic stress induced release, I have not fully evaluated the Clp1(6A3A) phosphomutant during mitotic nucleolar release. Thus it will be interesting to investigate the effect on timing of Clp1 nucleolar release during mitosis. Additionally, with the high level of Cdk1 activity during early mitosis, it's likely that Clp1 nucleolar interacting partners are also phosphorylated. Similar to Cdk1 phosphorylated Net1 in budding yeast, phosphorylation of Clp1 nucleolar interacting proteins may facilitate Clp1 nucleolar release during mitosis, thus masking the affect of abolished direct Clp1 phosphorylation.

While strict regulation of Clp1 nucleolar release during mitosis is important for the fidelity and timing of mitotic events (Chen et al., 2008; Clifford et al., 2008b; Cueille

et al., 2001; Mishra et al., 2004; Trautmann and McCollum, 2005; Trautmann et al., 2004; Trautmann et al., 2001), it is still unclear why such complex regulation of genotoxic stress-induced Clp1 nucleolar release exists. Clp1 contributes modestly to Cds1 activation (Diaz-Cuervo and Bueno, 2008); however, Clp1 is not important for cell viability in response to genotoxic stress (Figure 14). Since Cdk1 phosphorylation promotes Clp1 nucleoplasmic accumulation upon genotoxic stress and Cdk1 phosphorylation inhibits Clp1 activity (Wolfe *et al*., 2006), Clp1 may only be primed by its nucleolar release but not actually active under these circumstances. Alternatively, Clp1 may be involved in resetting the phosphostate of DNA replication/damage checkpoint substrates after resolution of a genotoxic insult in combination with other phosphatases and its role may be masked by their presence. Since mammalian Cdc14B is also mobilized from the nucleolus by direct DNA damage (Bassermann et al., 2008; Diaz-Cuervo and Bueno, 2008; Guillamot et al., 2011; Mocciaro et al., 2010; Wei et al., 2011b) or H_2O_2 treatment (D. McCollum, unpublished observations), my results may provide insight towards understanding its relocalization upon genotoxic stress, and contribute to a better understanding of Cdc14 family phosphatase regulation.

Nucleolar sequestration and SPB localization of Cdc14 phosphatases

S. cerevisiae Cdc14 is stably bound within the nucleolus by its high-affinity interaction with Net1 (Shou et al., 1999; Tomson et al., 2009; Traverso et al., 2001; Visintin et al., 1999). In marked contrast, my FRAP analysis indicated that Clp1 localization within the nucleolus is very dynamic, suggesting that Clp1 is held within the nucleolus through numerous low-affinity transient interactions. Interestingly, as

determined by FRAP analysis, the human nucleolar localized ortholog, Cdc14B, also localizes within the nucleolus dynamically (Dan McCollum – unpublished results). This suggests that localization within the nucleolus through low-affinity transient interaction may be conserved between Clp1 and higher eukaryotes, while nucleolar localization of Cdc14 may be fundamentally different.

This dynamic behavior is not unusual for proteins localized within the nucleolus or nucleoplasm, as proteins localized within these compartments tend to be in constant flux (Hernandez-Verdun, 2006; Phair and Misteli, 2000). Numerous low-affinity transient interactions would provide enough residence time to concentrate particular proteins within non-membranous organelles, like the nucleolus, while allowing them to behave dynamically (Dundr and Misteli, 2010; Emmott and Hiscox, 2009). Because the nucleolus is not bound by a membrane, and components within it exhibit constant flux, it is suggested that nucleolar proteins are brought together by mutual interest (Misteli, 2001); in the case of Clp1 and other non-ribosomal biogenesis proteins this interest is likely sequestration until needed. In line with this, the ability to be sequestered by low-affinity transient interactions likely allows for a quick response to changes within the cell. An example would be the quick response changes that occur upon cellular stress, which the nucleolus is known to contribute to (Boulon et al., 2010). This theme is in line with my observation that Clp1 is quickly redistributed from the nucleolus when cells encounter a peroxide stress.

Consistent with its dynamicity within the nucleolus, my data also suggest that nucleolar Clp1 can exchange with its other interphase localization, the SPB. Human Cdc14B, although predominately localized within the nucleolus during interphase,

shuttles between the nucleolus and cytoplasm (Bembenek et al., 2005), where it is localized to centrioles within the centrosome (the SPB equivalent) (Wu et al., 2008). Although it has not been directly tested, it is likely that nucleolar Cdc14B exchanges between these two sites of localization. Interestingly, *S. cerevisiae* Cdc14 is solely localized within the nucleolus during interphase and transitions to the SPB only after its initial nucleolar release (Stegmeier and Amon, 2004). This difference in Cdc14 localization may reflect the contrast in dynamicity and type of high- versus low-affinity nucleolar interactions.

The idea that Clp1 interacts with multiple partners is supported by my FRAP analysis of *dnt1Δ, nsk1Δ* and *dnt1Δ nsk1Δ* strains, in which the dynamicity and nucleolar intensity of Clp1-GFP were unaffected compared to *wild type* cells. This again is in stark contrast to *S. cerevisiae* Cdc14, in which deletion or mutants of *net1* completely disrupt the nucleolar localization of Cdc14 (Bloom et al., 2011; Shou et al., 1999; Visintin et al., 1999). Additionally, over-expression of the Clp1 nucleolar interacting partner, Nsk1, did not significantly affect Clp1's dynamicity. Though, over-productions of Nsk1 did slow the $T_{1/2}$ and lower Clp1's M_f compared to *wild type* cells, Clp1's nucleolar localization still remained very dynamic; this is especially true when comparing Clp1's $T_{1/2}$ to that Cdc14 or ribosomal proteins, which have no recovery or a $T_{1/2}$ greater than a minute, respectively (Chen and Huang, 2001; Tomson et al., 2009). The affects seen can likely be attributed to two scenarios: first, the large increase in available Nsk1-Clp1 interactions within the nucleolus could slightly slow Clp1's dynamicity within the nucleolus, or second, Nsk1 over-production may be causing molecular crowding within the nucleolus, globally slowing the dynamicity of nucleolar proteins. Both situations, and the fact that

Clp1 remains dynamic within the nucleolus, suggest that Clp1 localizes within the nucleolus through numerous low-affinity transient interacting partners, and that affects to the global nucleolar environment contribute to Clp1's behavior within it. Furthermore, Cdc14 nucleolar localization appears to be fundamentally different than that of Clp1 or higher eukaryotes.

FRAP analysis of Clp1-GFP localized at the SPB, showed that Clp1 also exhibits dynamic behavior at this cellular structure. This dynamicity at the SPB/centrosome was previously reported for *S. cerevisiae* Cdc14 and human Cdc14A. These studies however, analyzed very few cells ($n \leq 4$). Additionally, Cdc14A was over-expressed from a plasmid, and unlike both Clp1 and Cdc14A, which localize to the SPB throughout interphase, Cdc14 only localizes to the SPB after it is released from nucleolar sequestration during mitosis. My analysis however, examines *clp1-GFP* expressed from the endogenous $clp1^{+}$ promoter in a larger population size (n=38), providing a more thorough and convincing analysis. The fact that Clp1, Cdc14, and Cdc14A all exhibit dynamic localization behavior at the SPB/centrosome, suggests that their means of localization to this cellular structure is conserved, and it's likely that they localize through low-affinity transient interactions, as was suggested for Clp1's nucleolar localization. Thus, localization of Cdc14 phosphatases through low-affinity transient interactions may be a common theme, however, further investigation is needed.

Clp1 nucleocytoplasmic transport

During mitosis, Cdc14 phosphatases localize to multiple addresses, occupying both the nucleus and cytoplasm (Stegmeier and Amon, 2004). Experiments that affect

the nuclear-to-cytoplasmic equilibrium of Cdc14 phosphatases, either forcing sole cytoplasmic or nuclear localization, cause errors in chromosome segregation or cytokinesis, respectively, suggesting nucleocytoplasmic transport is important for the functions of Cdc14 phosphatases (Bembenek et al., 2005; Trautmann and McCollum, 2005). While Cdc14 phosphastases may be able to freely flux between the nucleoplasm and cytoplasm, evidence that this process is regulated exists.

In line with this, the proper localization of human Cdc14A to the centrosome during interphase depends on both the *cis* and *trans* factors for Crm1 dependent nuclear export (Bembenek et al., 2005; Mailand et al., 2002). Additionally, upon genotoxic stress, Clp1-GFP releases from the nucleolus, but is restricted to the nucleoplasm. This suggests that Clp1 nuclear export can be directly regulated, or perhaps the general process of nucleocytoplasmic transport is regulated upon genotoxic stress (e.g. Crm1 activity is inhibited upon genotoxic stress). Either way, these examples suggest a means of regulating the nucleocytoplasmic transport equilibrium of Cdc14 phosphatases exist, and that further investigation is needed to more comprehensively understand Cdc14 phosphatase regulation.

One likely mechanism has been postulated in *S. cerevisiae* for Cdc14 though phosphorylation by the MEN kinase, Dbf2. As described in chapter I, the MEN and SIN function to keep their respective Cdc14 phosphatases from being sequestered within the nucleolus. To prevent nucleolar sequestration, the homologous downstream kinases of both networks phosphorylate their respective Cdc14 phosphatases on RxxS sites (Chen et al., 2008; Mohl et al., 2009). Interestingly, in addition to prevention of nucleolar sequestration, phosphorylation of Cdc14 at RxxS sites by Dbf2 in *S. cerevisiae* was also

shown to inhibit the C-terminal NLS of Cdc14, allowing the phosphatase to accumulate within the cytoplasm (Mohl et al., 2009). Consistently, abolishment of Cdc14 RxxS phosphosites shifted the nuclear-to-cytoplasmic equilibrium of Cdc14-GFP toward the nucleoplasm (Mohl et al., 2009), suggesting that Dbf2 phosphorylation may influence the equilibrium of Cdc14 nucleocytoplasmic transport. While abolishing RxxS phosphorylation sites in Clp1 does not inhibit its cytoplasmic localization (Chen et al., 2008), detailed analysis has not been done to determine if the nuclear-to-cytoplasmic equilibrium of Clp1 localization has been affected. However, since the mechanism and major function of the MEN and SIN seem highly conserved regarding Cdc14 phosphatases, it is likely their control of nucleocytoplasmic transport may also be conserved.

In line with this being a conserved means of regulation, in my studies, I identified a C-terminal NLS containing a string of basic residues within Clp1, similar to that of *S. cerevisiae*, Cdc14 (Mohl et al., 2009). While a putative NLS has been predicted within the catalytic region of Cdc14B and Cdc14A outside of the C-terminus (Kaiser et al., 2004), the functionality has never been tested. Interestingly, a C-terminal fragment of Cdc14B was shown to localize within the nucleus suggesting a putative NLS exits (Nalepa and Harper, 2004). Additionally, a string of basic residues exist at the very C-terminus of both Cdc14B and Cdc14A (Figure 26), suggesting that a C-terminal NLS may be conserved throughout the Cdc14 phosphatase family.

Furthermore, I report that Clp1 is actively transported into the nucleus by the importin-β3, Sal3, via Clp1's C-terminal NLS. Since both Clp1 and Cdc14 have a conserved C-terminal NLS, and exportin, Crm1, it is likely that the importin may be

Clp1	- 517 -	AQRSVSMSSLNNTSNGRVAKPKPSKSRLIS	- 537
Cdc14	- 521 -	LLPKNRRVTSGRRTTSAAGGIRKISGSIKK	- 551
Cdc14B	- 468 -	AGITKRTTRSASRKSSVKSLSISRTKTVLR	- 498
Cdc14A	- 593 -	GPQNPECNFCALPSQPRLPPKKFNSAKEAF	- 623

Figure 26. A C-terminal NLS may be conserved throughout the Cdc14 phosphatase family. Protein sequence alignment for the last 30 C-terminal residues of the indicated Cdc14 phosphatase family members. Basically charged residues are highlighted in red.

conserved as well. Consistent with this idea, and in line with the essential nature of Cdc14, the budding yeast Sal3 ortholog, Pse1, is an essential importin (Seedorf and Silver, 1997).

Future Directions

How Cdc14 phosphatases are triggered to leave the nucleolus during mitosis remains a major outstanding question. In chapter III I've shown that upon genotoxic stress multiple kinases function to phosphorylate Clp1 on TP and RxxS sites triggering Clp1 nucleolar release. A Clp1 phosphomutant, Clp1(6A3A), which abolishes both TP and RxxS site phosphorylation, does not exit the nucleolus upon genotoxic stress, but is still released from the nucleolus during mitosis. To further understand the process of mitotic driven nucleolar release, strains could be created containing three fluorescent markers, including Clp1 or Clp1(6A3A)-GFP, Gar2-mCherry as a nucleolar marker and a fluorescent timing component. As a molecular timer, Sid4-CFP could be used to mark the SPBs, and the length of SPB separation would provide a reference to examine the timing of Clp1 mitotic nucleolar release. Additionally, the timing of septation and/or contractile ring formation and constriction can be followed and used as molecular timers. These strains should allow the question of whether phosphorylation of Clp1 TP and RxxS sites contributes to mitotic nucleolar release, though further complexity must exist, or whether Clp1 mitotic nucleolar release occurs though an entirely separate mechanism.

It would also be of great interest to determine how phosphorylation events at TP and RxxS sites function to control Clp1 nucleolar release. One possibility is that phosphorylation affects the association of Clp1 with its nucleolar-binding partners. In support of this hypothesis, a Clp1 C-terminal truncation mutant that lacks the TP and

RxxS phosphorylated region, Clp1(1-372)-GFP, is unable to localize within the nucleolus, and is localized diffusely throughout the nucleoplasm (Figure 27). One approach to answer this question could be a comparative proteomics approach. Though mass spectrometry did not identify any high-affinity nucleolar-binding partners, it may have identified specific low-affinity binding partner that were dismissible by our cut-off criteria. To better analyze these putative low-affinity binding partners, mass spectrometry could be utilized to identifying binding partners of Clp1-TAP and Clp1(6A3A)-TAP from cells treated or not treated with genotoxic stress. Proteins identified in untreated Clp1-TAP samples, that are absent in treated Clp1-TAP samples, would likely represent Clp1 nucleolar associations. Proteins identified in the nucleolar sequestered Clp1(6A3A)-TAP samples, both treated and untreated, versus treated Clp1-TAP, will also represent nucleolar associations. Alternatively, comparisons can be made between Clp1-TAP and Clp1(1-372)-TAP samples. Proteins identified as putative Clp1 nucleolar-binding partners could then be further investigated for their interaction and role in localizing Clp1 to the nucleolus.

While the proteomic approach above will likely yield useful information, it may not give a clear-cut answer. The fact that Clp1 likely localizes to the nucleolus through numerous low-affinity transient interactions may make the proteomic data complicated to decipher. Therefore it will also be helpful to understand how Clp1 interacts with its binding partners. The proteomic screen, in which the importin, Sal3, was identified, has identified numerous Clp1 interacting partner and substrates beyond those currently described within the literature (Chen *et al*., submitted). Using this more comprehensive list, how Clp1 interacts with both substrates and interacting partners could be elucidated.

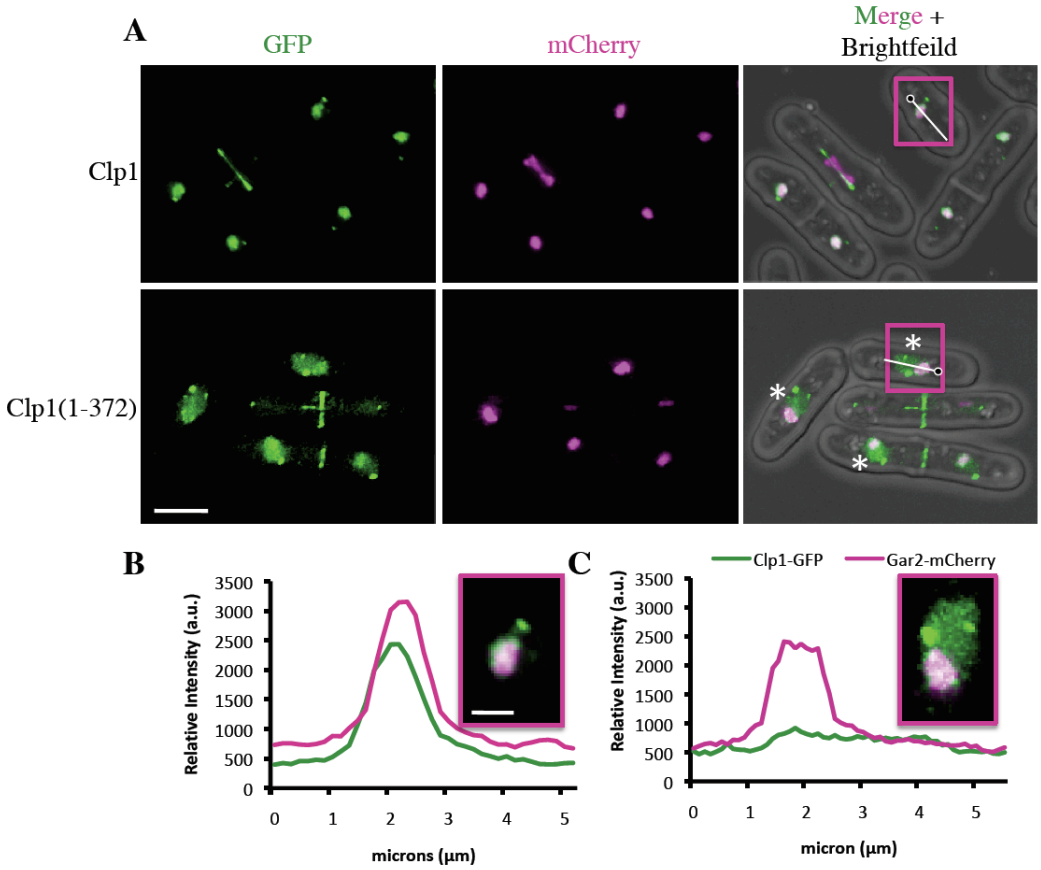

Figure 27. Truncation of the Clp1 C-terminus disrupts its nucleolar localization. (A) Live cell images of *clp1-GFP* or *clp1(1-372)-GFP gar2-mCherry* cells. Scale bar = 5 µm. Asterisks indicate nuclei with Clp1-GFP or Clp1(1-372)-GFP relocalized from the nucleolus to the nucleoplasm. (B) Line scans of Clp1-GFP and Gar2-mCherry fluorescence, each spanning a distance of 5 µm, in which the paths are represented in (A) by a white line with a black circle to indicate the origin of the line scan. Also, a zoomed view of the boxed nuclei from the merged images in (A) for Clp1-GFP. Scale bar = 1.5 µm. (C) Same as (B) except the line scan and zoomed nuclei represent the indicated Clp1(1-372)-GFP and Gar2-mCherry cell.

This would be done utilizing two-hybrid analysis and *in vitro* binding assays to map specific Clp1 binding platforms. Not only would this information help determine if TP and/or RxxS site phosphorylation directly influences Clp1 binding partner selectivity, but it would also contribute to the global understanding of Cdc14 phosphatases.

Another important question to address is how Clp1 is directed to localize to specific targets once released from the nucleolus. Currently, Mid1, an anillin like protein, is the only known molecular tether necessary to direct Clp1's localization. In cells that lack *mid1*$^{+}$ or contain a mutant *mid1* that disrupts the Clp1 interaction site, Clp1 is unable to localize to the contractile ring (Clifford et al., 2008b). What tethers Clp1 to the SPB, kinetochores and along the mitotic spindle remains a mystery. In chapter IV, I have presented work suggesting that Clp1 localizes within the nucleolus and to the SPB in a dynamic nature, and suggest that Clp1 may localize to these sites though multiple low-affinity transient interactions. This theme may also exist for Clp1 localization at both the kinetochores and the mitotic spindle, given that proteomic studies have not identified high-affinity binding partner for Clp1 at theses sites. Experiments investigating how Clp1 interacts with specific binding partners and substrates will hopefully shed light on how Clp1 localizes to specific sites.

Beyond localization, it will be also interesting to examine how binding interactions influence Clp1's catalytic activity. As described in chapter IV, the proteomic study preformed by Jun-Song Chen identified Clp1 interacting partners in three different classes: those that generally bound Clp1, those that bound the Clp1 substrate trap more efficiently than wild type Clp1 suggesting they are putative substrates, and those that bound wild type Clp1 preferentially. Mid1, the Clp1 contractile ring tether, was

identified and classified within this last group. The only other localization tether characterized for the Cdc14 phosphatases is Net1, which inhibits the catalytic activity of *S. cerevisiae* Cdc14 (Shou et al., 1999; Visintin et al., 1999). It would be interesting to evaluate whether Mid1, like Net1, can inhibit the catalytic activity of Clp1. This could be analyzed through *in vitro* phosphatase assays that incorporate recombinant proteins of both Clp1 and Mid1.

Additionally, it will be important to further investigate mechanisms of Clp1 phosphoregulation. Phosphorylation controls both the activity and nucleolar localization of Clp1 (Chen et al., 2008; Mishra et al., 2005; Wolfe et al., 2006). It seems reasonable that phosphorylation may also direct Clp1 localization after nucleolar release. Beyond previously characterized TP and RxxS phosphosites, proteomic studies have identified additional phosphorylation sites within Clp1's C-terminus (data not shown). Furthermore, at least *in vitro*, the polo like kinase, Plo1, the PAK-related kinase, Shk1, and Casein kinase II can phosphorylate Clp1, yet the consequence of their phosphorylation has not been determined (data now shown). To analyze the function of these sites, Clp1 mutants that abolish or attempt to mimic phosphorylation could be constructed. These mutants would then be integrated at the *clp1*$^{+}$ endogenous locus and tagged with GFP to examine localization. These mutants could also be examined for effects on catalytic activity by measuring cell size and examining known genetic relationships. Additionally, *in vitro* activity could be measured utilizing recombinantly produced substrates and/or small molecule reporters (e.g. 6,8-difluoro-4-methylumbelliferyl phosphate – DiFMUP). Phosphosites that affect Clp1 localization or

catalytic activity would then be further characterized to identify what particular kinase(s) phosphorylates said sites, and during what point within the cell cycle.

Though Cdc14 phosphatases respond to DNA damage, their contribution is not fully understood. One interesting finding in my work presented in Chapter III is that upon genotoxic stress, Cdk is active and contributes to Clp1 nucleolar release. Also at this time, Cdk phosphorylation influences the cellular decision to repair DNA double strand breaks through homologous recombination over non-homologous end joining (Cerqueira et al., 2009; Chen et al., 2011b; Enserink et al., 2009; Enserink and Kolodner, 2010; Ira et al., 2004; Langerak and Russell, 2011). Considering the mitotic role of Clp1 to antagonize Cdk1 phosphorylation (Mocciaro and Schiebel, 2010; Queralt and Uhlmann, 2008; Stegmeier and Amon, 2004), it's possible that Clp1 functions similarly upon DNA damage. To address this question, the phosphostate of known Cdk targets that contribute to homologous recombination and/or non-homologous end joining in strains expressing *clp1*$^{+}$, *clp1(6A3A)* or lack *clp1*$^{+}$ after treatment with genotoxic stress could be investigated. Furthermore, the genetic relationships between Clp1 and proteins involved in homologous recombination and non-homologous end joining could be examined to determine the impact of Clp1 activity on these processes. These experiments will help us further identify the role Cdc14 phosphatases play in the cellular response to genotoxic stress.

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