Regulation of the Centromeric Histone H3 Variant Cse4 by the E3 Ubiquitin Ligase, Psh1

Prerana Ranjitkar

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Susan Biggins

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Abstract

Regulation of the Centromeric Histone H3 variant Cse4 by the E3 Ubiquitin Ligase, Psh1

Prerana Ranjitkar

Chair of the Supervisory Committee:
Professor, Sue Biggins
Division of Basic Sciences, FHCRC

Aneuploidy, one of the most common hallmarks of cancer, results from defects in chromosome segregation during cell division. Once cells replicate their genome, sister chromatids must be accurately segregated into daughter cells during mitosis in order to ensure genomic stability.

Chromosome segregation is directed by the kinetochore, a multi-protein complex that assembles onto specialized chromosomal locus called the centromere. Because centromere DNA is not conserved, there are epigenetic mechanisms that ensure its propagation. Cenp-A, a conserved histone H3 variant appears to be the epigenetic mark that specifies centromere identity and is essential for kinetochore assembly and function. Exclusive localization of Cenp-A to the centromere is critical to prevent formation of di-centric chromosome that can be broken during segregation. Proteolysis is one of the mechanisms that cells utilize to regulate Cenp-A levels and prevent its mislocalization to non-centromeric loci in budding yeast and flies. However, the molecular machinery and the underlying mechanisms are not known. In my research, I focused on identifying proteins involved in degradation of Cenp-A and elucidate the

mechanism by which the proteolytic machinery prevents ectopic localization of Cenp-A using budding yeast as a model system.

Cse4 is the Cenp-A homolog in budding yeast. To identify Cse4 degradation machinery, I focused on known and novel Cse4 interacting proteins that were previously identified in the lab. Of the various candidates, I focused on Psh1, a protein of unknown function. Psh1 contains a RING domain, a hallmark of all RING type E3 ligases. I demonstrated that Psh1 is a bonafide E3 ligase *in vitro* and functions to ubiquitinate and degrade Cse4 *in vivo*. Moreover, Psh1 mediated degradation of Cse4 prevents mislocalization of Cse4 to non-centromeric loci. Consistent with this, overexpression of Cse4 is toxic to *psh1*Δ cells and leads to mislocaliztion of Cse4 to the euchromatin. The toxicity of Cse4 overexpression is correlated with the levels of Cse4 protein and can be partly attributed to the activation of the spindle checkpoint.

Since Cse4 is about 60% identical to the canonical H3, I also investigated if Psh1 is a specific E3 ligase for Cse4 and how it is able to discriminate between the two histones. Psh1 co-purifies with Cse4 but fails to interact with histone H3. Consistent with a specific function of Psh1 for Cse4 proteolysis, overexpression of H3 had no effect on the viability of *psh1*Δ cells. Domain swap experiments reveal the centromere targeting domain (CATD) in Cse4, which is absent in H3, to be the recognition motif by which Psh1 distinguishes Cse4 from H3. In addition, this domain appears to be required and sufficient for Psh1 mediated degradation. Taken together, my work has shown that the CATD has a previously unknown role in maintaining the exclusive localization of Cse4 by preventing its mislocalization to euchromatin via Psh1-mediated degradation.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

Alpha factor (αF)

Galactose (GAL)

Glucose (GLU)

Green fluorescent protein (GFP)

Immunoprecipitation (IP)

Minutes (min)

Polymerase chain reaction (PCR)

Wild-type (WT)

LIST OF FIGURES

LIST OF TABLES

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To my family,

without whose love, support and sacrifices,
I would not have achieved this.

CHAPTER 1:
Introduction

Chromosome Segregation

Proper execution of a number of coordinated events is crucial for ensuring accurate segregation of chromsomes and hence the maintainence of genomic stability during each cell division. Defects in this process lead to cancer and various human diseases. Understanding the mechanism of chromosome segregation will therefore give insight into the molecular basis of these diseases.

Cell division begins with the replication of the genome during S-phase. The duplicated sister chromatids are held together by a protein complex called cohesin. During mitosis, microtubules emanating from opposite poles of the cell make attachments to the sister chromatids via the kinetochore, a large protein complex that assembles onto the centromere (Fig 1.1 and 1.2) During metaphase, tension across bioriented chromosomes is generated by the pulling forces of the microtubules that are opposed by the linkage between the sister chromatids. In the event that there is even a single pair of sister chromatids that have improper attachments, cells activate the spindle checkpoint, which halts the metaphase to anaphase transition, allowing for correction of these errors. Once all the sister kinetochores have achieved biorientation during metaphase, the spindle checkpoint is satisfied and cohesin is released allowing microtubules to pull the sister chromatids to opposite poles, resulting in two daughter cells, each containing the

correct complement of the genetic material (Fig 1.1) (for reviews, see (Bouck et al., 2008; Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009)).

The Centromeric Chromatin and the kinetochore

The centromere is the specialized chromosomal locus onto which the kinetochore is assembled. Therefore, centromere function is essential for proper chromosome segregation and thus for genomic stability. Despite this essential and highly conserved function of the centromere, the sequence composition and size of the centromere are poorly conserved throughout the eukaryotes. The budding yeast *S. cerevisiae* contains a defined centromere sequence of ~ 125bps, which constitutes three DNA elements termed CDEI, CDEII and CDEIII, that are conserved across all chromosomes. In contrast, the fission yeast *S. pombe* centromere contains a non-repetitive central core, flanked by inverted repeat regions while centromeres in metazoans such as *D. melanogaster* and *H. sapiens* are composed of highly repetitive DNA sequences that lack sequence specificity (Verdaasdonk and Bloom, 2011). Because centromeric DNA is not conserved, there are epigenetic mechanisms that ensure its propagation. A hallmark of all eukaryotic centromere is the presence of the histone H3 variant CENP-A.

The fundamental unit of the chromatin is the nucleosome, which contains two copies of each histones H2A, H2B, H3 and H4 wrapped around DNA. While the bulk chromatin is packaged into these canonical nucleosomes, at the centromeric nucleosome, histone H3 is replaced by its variant called CENP-A. The presence of CENP-A at the centromere is absolutely essential for kinetochore formation and this function cannot be

replaced by the canonical H3.

The kinetochore is the macromolecular structure that is essential to direct chromosome segregation. The best-characterzied kinetochore is the budding yeast kinetochore, which comprises greater than 65 constitutive components, many of which are highly conserved. These components are found in biochemically distinct complexes and are divided into the inner, central and outer domains. The centromeric histone H3 variant Cse4, DNA binding proteins Mif2 and Cbf1 and the CBF3 complex together comprises the inner kinetochore. The CBF3 complex binds directly to the centromeric DNA and is required for the localization of all other kinetochore proteins including Cse4. The central kinetochore includes the MTW1 and the CTF19/COMA complexes. Finally, the NDC80 and DAM1/DDD/DASH complex constitutes the outer kinetochore where microtubule attachments are made. These proteins are conserved across species and serve similar function in higher organisms as well (Fig 1.2)

Centromeric histone H3 variant, CENP-A

All centromeres are characterized by the presence of the essential histone H3 variant CENP-A, which localizes exclusively to the centromere (Earnshaw and Rothfield, 1985); (Palmer et al., 1987). Because it is present at all active centromeres, it is an excellent candidate to epigenetically specify centromere identity (Amor and Choo, 2002; Warburton et al., 1997). In most organisms, centromeres contain arrays of CENP-A nucleosomes interspersed with histone H3 nucleosomes. In contrast, a single CENP-A nucleosome exists at the budding yeast centromere, consistent with a single microtubule

binding site (Furuyama and Biggins, 2007a; Winey et al., 1995).

The centromeric histone H3 variant CENP-A is highly conserved throughout eukaryotes. CENP-As contain a unique N-terminal tail and a conserved histone fold domain. The homology between H3 and its variant CENP-A is limited to this histone fold domain, which also contains residues that are important for targeting of CENP-A to the centromere. Loop 1 and α2 helix of CENP-A comprises the centromere targeting domain (CATD) which is necessary and sufficient to target an H3 chimera to the centromere (Black et al., 2004; Vermaak et al., 2002b) (Black et al., 2007). This domain also binds to the chaperones Scm3 in budding yeast (Shivaraju et al., 2011; Zhou et al., 2011) and HJURP in humans, which are required for CENP-A deposition at the centromere, suggesting a potential mechanism to explain the role of the CATD in CENP-A localization (Dunleavy et al., 2009; Foltz et al., 2009). In addition to the CATD, a whole host of other proteins have been shown to be important for CENP-A loading in various organisms. For instance in humans, CENP-A incorporation is thought to occur in a step-wise fashion, starting with the licensing of the centromere by the recruitment of Mis18 complex, CENP-H, CENP-I, RbAp46/48, followed by recruitment of loading factors KNL2 and HJURP that loads new CENP-A and finally proper spacing between CENP-A nucleosomes is maintained by remodeling factors such as the RSF complex (for review see (Verdaasdonk and Bloom, 2011)).

CENP-A is highly enriched at functional centromeres, but it can also be incorporated into the euchromatin. Low levels of endogenous CENP-A can be detected in highly transcribed euchromatic regions of the yeast genome (Camahort et al., 2009;

Lefrancois et al., 2009) and overexpression of CENP-A leads to euchromatic localization in some organisms (Heun et al., 2006b; Tomonaga et al., 2003; Van Hooser et al., 2001). In addition, CENP-A can be detected at sites of DNA double strand breaks prior to removal at the time of DNA repair (Zeitlin et al., 2009). These data suggest that CENP-A can localize to euchromatin but is not stably maintained outside of centromeres. However, ectopic incorporation of CENP-A can lead to genomic instability (Amato et al., 2009; Au et al., 2008; Heun et al., 2006b)) and has been shown to occur in primary colorectal cancer tissues (Tomonaga et al., 2003). However, whether mistargeting of CENP-A to euchromatin is sufficient to assemble a kinetochore varies among eukaryotes. In *Drosophila* ectopic CENP-A is sufficient for kinetochore formation (Heun et al., 2006a) whereas in humans CENP-A alone is not sufficient to assemble a kinetochore (Van Hooser et al., 2001) and requires the constitutive DNA-binding kinetochore components, CENP-C and CENP-T (Cheeseman and Desai, 2008). Taken together, these data suggest that there are multiple controls over both CENP-A localization and kinetochore formation that are critical for genomic stability.

Insights into mechanisms that ensure exclusive centromeric localization of CENP-A have come from studies of Cse4, the budding yeast homolog and CID, the homolog in flies. The levels of Cse4 are regulated by proteasome dependent ubiquitin-mediated proteolysis that specifically degrades the euchromatic CENP-A while the centromere-bound CENP-A appears to be protected from degradation (Collins et al., 2004b). CID is also proteolytically regulated, although whether it is also ubiquitin-mediated is not known (Moreno-Moreno et al., 2006). CENP-A proteolysis has also been detected in human

cells undergoing senescence or infection with herpes simplex virus 1 (Lomonte et al., 2000; Maehara et al., 2010) suggesting that CENP-A proteolysis is a conserved mechanism. However, the molecular machinery involved in CENP-A degradation has not been identified in any organism and the underlying mechanism is unclear. Similarly, how the centromere bound CENP-A is protected from degradation is not known.

The Ubiqutin-Proteasome Pathway

Ubiquitination is a multi-step process that results in conjugation of a substrate protein with ubiquitin monomers via an isopeptide bond between the C terminus of ubiquitin and typically a lysine residue of the target protein. This process starts with the activation of the ubiquitin monomer with an E1 activating enzyme, followed by the transfer of the activated ubiquitin to an E2 conjugating enzyme and finally to the target protein via the E3 ligase enzyme which brings the substrate and the E2 enzyme in close proximity enabling the transfer of the ubiquitin monomer onto the substrate protein. The substrate can be monoubiquitinated at multiple sites or additional ubiquitins can be conjugated to any of the seven lysine residues of ubiquitin to form a polyubiquitin chain. The number of ubiquitin monomers assembled on a single chain dictates the fate of the ubiquitinated protein. Conjugation of a chain containing four or more ubiquitin monomers targets the substrate for degradation by the proteasome. The E3 ligase enzymes, which are defined by the presence of either the HECT domain or the RING domain, impart specificity in this enzymatic cascade by pairing the E2 enzyme and the target protein (for review see (Deshaies and Joazeiro, 2009).

Description of Dissertation

A large number of studies have revealed how the essential histone H3 variant CENP-A is recruited and maintained at the centromere. However, a critical question in centromere biology of how CENP-A is maintained exclusively at the centromere and prevented from being stably incorporated into the euchromatin is not very well understood.

I used the budding yeast, *S. cerevisiae* to address key questions about the mechanisms that regulate CENP-A localization and functions. Unlike higher organisms, budding yeast has a single CENP-A nucleosome at the centromere that is sufficient to assemble a kinetochore (Furuyama and Biggins, 2007b). However, despite this variation in the number of CENP-A nucleosomes at centromeres, the budding yeast homolog Cse4 can rescue a depletion of mammalian CENP-A (Allshire and Karpen, 2008; Wieland et al., 2004), suggesting functional conservation. Although budding yeast is the only organism with a defined centromeric DNA sequence that is sufficient to mediate kinetochore assembly (Fitzgerald-Hayes et al., 1982), epigenetic components also contribute to yeast kinetochore assembly (Mythreye and Bloom, 2003; Tanaka et al., 1999), so to varying degrees all eukaryotic organisms propagate kinetochores using epigenetic components. Therefore, budding yeast is an excellent model system to elucidate mechanisms underlying CENP-A localization and hence kinetochore assembly.

Previous work in the lab revealed that ubiquitin-mediated proteolysis specifically degrades euchromatin-bound but not centromere-bound Cse4 (Collins et al., 2004a).

However, the molecular machinery is not known and the underlying mechanism is not well understood. For example, it is not known whether Cse4 that mis-incorporates into chromatin is directly degraded, or whether it is removed from chromatin creating a soluble pool that is targeted for degradation. Moreover, it is unclear how the degradation machinery distinguishes Cse4 from H3 nucleosomes. These questions must be addressed in order to understand how cells maintain a single Cse4 nucleosome at the centromere, yet prevent euchromatin incorporation to ensure high fidelity chromosome segregation.

In order to identify the molecular machinery involved in Cse4 degradation, we took advantage of a mutant Cse4 protein, which cannot be ubiquitinated, thus accumulating at higher cellular levels than its WT counterpart. This facilitated the identification of a large number of known and novel Cse4 interacting proteins. In chapter 2, I present the characterization of one of the novel interactors, Psh1, as an E3 ligase enzyme that degrades Cse4. Psh1-mediated degradation of Cse4 appears to be important for preventing accumulation of Cse4 in the euchromatin, consistent with the observation that overexpression of Cse4 is toxic to *psh1Δ* cells. Interestingly, overexpression of H3 has no effect on the viability of *psh1Δ* cells, leading to the hypothesis that Psh1 may be specific to Cse4 and not H3. In chapter 3, I describe experiments showing that Psh1 is an E3 ligase that specifically recognizes Cse4. By performing domain swap experiments, I was able to identify the centromere targeting domain of Cse4 to be the recognition motif of Psh1 through which it distinguishes Cse4 from H3. In addition, this domain also regulates Cse4 stability via its binding to the E3 ubiquitin ligase. I have also included some data that implicates FACT, a chromatin remodeling complex in the degradation of

Cse4

In Appendix A, I have summarized the results that were obtained from an e-map screen to identify positive and negative genetic interactors of Psh1. Because Cse4 is not completely stabilized in *psh1Δ* mutants, there must be alternate pathways of Cse4 degradation. We predict that genes involved in this parallel pathway will display negative genetic interaction with *psh1Δ* mutants. In Appendix B, I have also included some interesting observations that could be followed up on for future direction. In Appendix C, I present some preliminary data demonstrating that Cse4 is sumoylated.

Taken together the work presented in this dissertation sheds insight into a largely unexplored area in centromere biology of how cells prevent CENP-A from mis-incorporating at non-centromeric loci and provides a molecular basis for understanding this question.

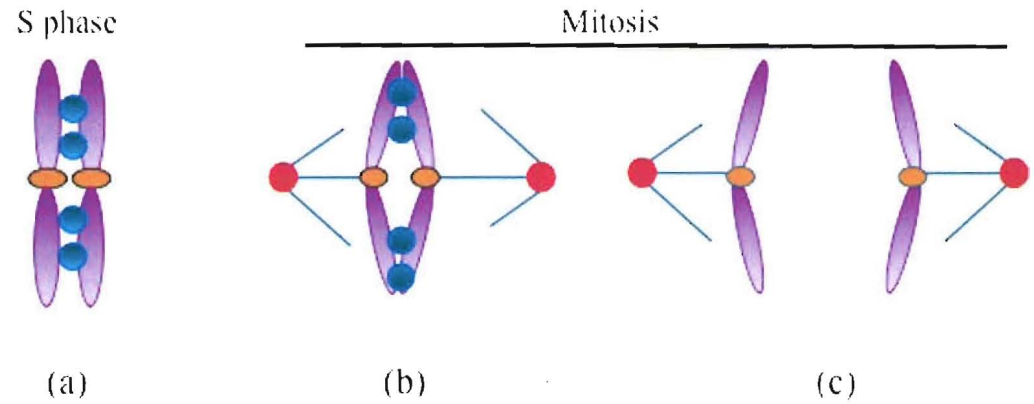
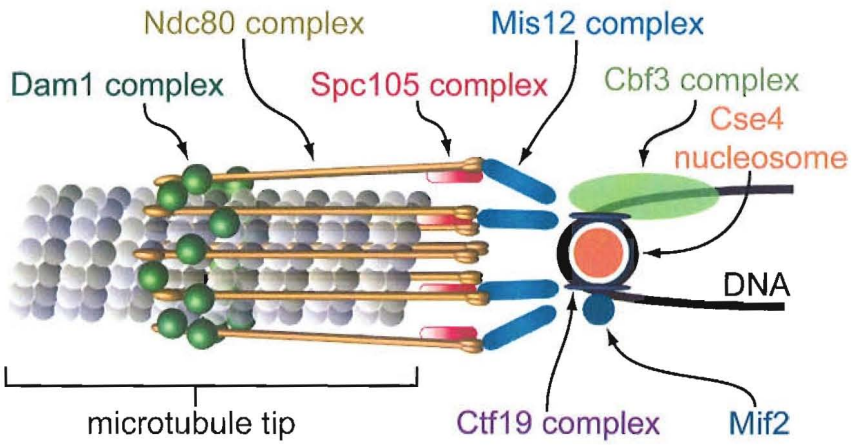


Fig 1.1. Events during chromosome segregation.
(a) Genome replication occurs during S phase, where the replicated sister chromatids are held together by the cohesin complex (shown as blue circles). (b) The sister chromatids are pulled by microtubules emanating from opposite poles (shown in red) via attachment to the kinetochore (shown in orange). The pulling forces of the microtubules are opposed by the cohesin linkage between the sisters chromatids, thereby generating tension and establishing proper bipolar attachments. (c) Once all pairs of sister chromatids have achieved proper attachment, cohesin is released and the sisters are pulled to the opposite poles.



Adapted from Joglekar et al (2009)

Fig 1.2. Schematic representation of the Kinetochore.

CHAPTER 2:

Identification of an E3 ligase that mediates Cse4 degradation and prevents ectopic localization of Cse4.

Summary

The budding yeast centromeric histone H3 variant, Cse4 is regulated by ubiquitin mediated proteasome proteolysis. However, the proteins that mediate Cse4 degradation, namely the E3 ligase and E2 conjugating enzymes, are not known. Here, I identified Psh1 as an E3 ligase that ubiquitinates Cse4 both *in vivo* and *in vitro* and mediates its degradation *in vivo*. Psh1-mediated degradation of Cse4 appears to be important to prevent ectopic chromatin localization of Cse4. Consistent with this, overexpression of Cse4 leads to euchromatic localization resulting in decreased viability of *psh1Δ* cells. The toxicity of Cse4 overexpression is correlated with the levels of Cse4 protein and can be partly attributed to the activation of the spindle checkpoint. Thus, we have identified part of the molecular machinery responsible for regulating Cse4.

Introduction

A hallmark of all active centromeres is the presence of the histone H3 variant CENP-A that creates a specialized chromatin structure for the assembly of the kinetochore for directing chromosome segregation. Therefore, it is extremely critical that CENP-A is exclusively localized to the centromere to ensure the fidelity of chromosome segregation. Studies from different organisms have identified a host of proteins that are

important for centromeric localization of CENP-A. In budding yeast, Ndc10, a component of the CBF3 complex that directly binds to the centromeric DNA, is required for localization of Cse4 (Goh and Kilmartin, 1993). In addition, recent studies have implicated Scm3 in the deposition and assembly of Cse4 nucleosomes (Camahort et al., 2007; Shivaraju et al., 2011; Stoler et al., 2007). Similarly, work in *S. pombe* has also shown Scm3, together with Mis16 and Mis18, to function in the assembly of CENP-A nucleosome at the centromere. In humans, the histone chaperones RbAp46/48, the centromere proteins CENP-H and CENP-I, and the loading factors KNL2 and HJURP, are responsible for loading new CENP-A at the centromere in a multi-step process (Verdaasdonk and Bloom, 2011). These studies have advanced our understanding of how CENP-A is localized and maintained at the centromere.

Although CENP-A is highly enriched at functional centromeres, studies have shown that it can also incorporate into euchromatin. Low levels of endogenous Cse4 can be detected in highly transcribed euchromatic regions of the yeast genome (Camahort et al., 2009) (Lefrancois et al., 2009) and overexpression of CENP-A leads to euchromatic localization in some organisms (Van Hooser et al., 2001) and CENP-A can be detected at sites of DNA double strand breaks prior to removal at the time of DNA repair (Zeitlin et al., 2009). Taken together, these data suggest that CENP-A can localize to euchromatin but is not stably maintained outside of the centromeres. However, the mechanisms that are responsible for preventing CENP-A mislocalization or removing ectopically localized CENP-A are not very well understod.

Ubiquitin-mediated proteolysis has emerged as one mechanism that regulates CENP-A localization in budding yeast and flies. This mechanism appears to specifically degrade euchromatic but not centromere-bound CENP-A (Collins et al., 2004b). Although it has not been directly tested if CENP-A degradation occurs in humans, cells undergoing senescence or infection with herpes simplex virus 1 (Lomonte et al., 2000; Maehara et al., 2010) appear to degrade CENP-A. In addition, CENP-A levels are destabilized when its chaperone HJURP is down regulated (Dunleavy et al., 2009). However, the key machinery involved in CENP-A destruction has not been identified in any organism to date.

Ubiquitin-mediated proteolysis requires the covalent conjugation of ubiquitin monomers onto a substrate protein (for review, see (Deshaies and Joazeiro, 2009)). After E1 activation, ubiquitin is transferred to an E2 conjugating enzyme and subsequently conjugated to a substrate via an E3 ligase. The specificity of substrate recognition is largely dictated by the E3 ligases, consistent with the large number of predicted E3 relative to E1 and E2 enzymes throughout eukaryotes. Budding yeast has only 1 E1 activating enzyme, 12 known E2 conjugating enzyme and greater than 30 predicted E3 ligases. Here, I have identified and characterized an E3 ligase that regulates the budding yeast Cse4.

Result

Psh1 is an E3 ligase that mediates Cse4 ubiquitination

To identify proteins involved in CENP-A degradation, we purified a lysine-free mutant of the budding yeast Cse4 protein that is not ubiquitinated *in vivo* (Collins et al., 2004b). 3xFLAG-Cse4^{16R} was overexpressed as the sole genomic copy and affinity purified by anti-FLAG immunoprecipitation. The material was eluted with FLAG peptide and subjected to anion exchange followed by mass spectrometry (LC-MS/MS) on two of the peak fractions containing Cse4. As expected (Camahort et al., 2009; Westermann et al., 2007) we detected Cse4 and histones H2A, H2B and H4, as well as six kinetochore proteins (Fig 2.1A). We also identified a large number of novel interacting proteins including Psh1 (Pob3/Spt16/histone binding protein;(Ranjitkar et al., 2010)), a putative E3 ubiquitin ligase that was first isolated via its interaction with the FACT complex (Pob3/Spt16) (Krogan et al., 2002).

Because Psh1 contains a RING domain that is a hallmark of many E3 enzymes (Fig 2.1B, (Deshaies and Joazeiro, 2009)) I tested whether it is a ubiquitin ligase. Recombinant GST-Psh1 exhibited robust autoubiquitination activity *in vitro* when incubated with ubiquitin, ATP, E1 and E2 enzymes (Fig 2.2A). The activity is specific to Psh1 because it requires the conserved catalytic cysteines C45 and C50 in the RING domain. I next tested whether Psh1 could facilitate the addition of ubiquitin to the Cse4 protein. When Cse4 octamers were incubated with Psh1 in a ubiquitin reaction *in vitro*, we detected a ladder of higher molecular weight conjugates on Cse4 (Fig 2.2B). Taken together, these data show that Psh1 is a ubiquitin ligase that can mediate Cse4 polyubiquitination *in vitro*.

Psh1 interacts with non-centromeric Cse4

Because Psh1 was identified via its interaction with an overexpressed Cse4^{16R} mutant protein, I tested whether it also binds to the endogenous wild-type Cse4 protein. In addition, I asked whether the interaction can be detected in the absence of centromere-bound Cse4 by abolishing its centromeric localization using the *ndc10-1* temperature sensitive mutant (Goh and Kilmartin, 1993). Psh1-FLAG was immunoprecipitated from wild-type and *ndc10-1* mutant cells and equivalent amounts of Cse4 were detected (Fig 2.3A). Although this does not exclude an interaction between Psh1 and Cse4 at the centromere, it shows that Cse4 does not need to associate with centromeres for the proteins to interact. Cse4 localizes to some non-centromeric loci at low abundance (Camahort et al., 2009; Lefrancois et al., 2009) so I tested whether Psh1 and Cse4 interact in euchromatin. Yeast extracts were fractioned and Psh1-FLAG was immunoprecipitated from the soluble and chromatin fractions. Psh1 and Cse4 were associated in both chromatin and soluble fractions, and their interaction was not altered in *ndc10-1* mutant cells where all chromatin-bound Cse4 is euchromatic (Fig 2.3B). Taken together, these data show that Psh1 and Cse4 can bind independently of the centromere.

Psh1 is required for Cse4 ubiquitination and degradation in vivo

Because Psh1 is an E3 ligase that associates with Cse4 and can ubiquitinate Cse4 octamers *in vitro*, I asked whether it targets Cse4 for ubiquitin-mediated proteolysis *in vivo*. First, I analyzed ubiquitin conjugates on Cse4 in the presence and absence of Psh1. Similar to the Cse4^{16R} protein that cannot be ubiquitinated, I found that the slower-

migrating forms of Cse4 that were previously shown to be ubiquitin conjugates are no longer detectable on either overexpressed or endogenous Cse4 protein in *psh1* mutant cells (Fig 2.4A, 2.4B and (Collins et al., 2004b)). I therefore analyzed Cse4 stability at various cell cycle stages in wild-type and *psh1Δ* cells by arresting cells in G1-, S- and M-phases and monitoring the endogenous Cse4 protein levels after repressing translation. At all cell cycle stages, Cse4 has a short half-life, which is extended when Psh1 is deleted (Fig 2.4C). Quantification confirmed that the Cse4 half-life increases in *psh1Δ* cells (data not shown), although we cannot determine the precise change because there are soluble, euchromatic and centromere-bound pools of Cse4, each with unique half-lives (Collins et al., 2004b). Similar to previous observations on $Cse4^{16R}$ (Collins et al., 2004b) the total levels of Cse4 eventually decrease in *psh1Δ* cells. The residual degradation is not due to the Tom1-mediated histone degradation pathway (Singh et al., 2009) (Fig 2.4D) and it is unclear whether the mechanism is ubiquitin-dependent.

Cse4 accumulates in euchromatin in psh1Δ cells

Because Psh1 associates with both soluble and chromatin-bound Cse4 (Fig 2.3B), I tested whether Psh1 specifically affects one of these Cse4 pools. Although there was more Cse4 in the soluble fraction prepared from *psh1Δ* cell extracts, there was a more substantial increase in chromatin-associated Cse4 (Fig 2.5A). I confirmed this by localizing Cse4 in *psh1Δ* cells using chromosome spreads, a technique that removes soluble material and allows the chromatin-bound Cse4 to be specifically visualized (Loidl et al., 1998). In contrast to wild-type cells where discrete kinetochore foci are observed,

transient overexpression of Cse4 in *psh1Δ* cells results in overall euchromatic localization in 100% of the DAPI masses examined. When I analyzed the kinetochore protein Mtw1-3GFP, there was a discrete signal in 95% of the cells, suggesting that the kinetochore is largely intact in these cells (Fig 2.5B). Taken together, these data are consistent with Psh1-mediated degradation preventing Cse4 from accumulating in euchromatin.

Cse4 overexpression is toxic to psh1Δ cells

Although *psh1Δ* cells have a genomic instability phenotype (Yuen et al., 2007) and display a very mild sensitivity to benomyl, the cells grow well and do not exhibit temperature sensitivity (Fig 2.6A and 2.6B). In addition, I did not detect a delay in cell cycle progression in *psh1* mutants (Fig 2.7). I considered the possibility that cells are viable without Psh1 because they maintain low cellular levels of Cse4 due to additional sources of regulation, such as transcriptional and post-transcriptional controls that regulate the major histone (Osley, 1991). I therefore tested the effect of Cse4 overexpression on *psh1Δ* mutant cells. A Myc-Cse4 construct had a moderate effect on the growth of *psh1* mutant cells when overexpressed (Fig 2.8A), while a FLAG-Cse4 construct strongly inhibited growth (Fig 2.8B). The difference in growth inhibition is correlated with protein expression differences between the two contructs (Fig 2.8C), and it is specific to Cse4 because H3 overexpression had no effect on *psh1Δ* cell viability (Fig 2.8B). I further analyzed the phenotype by releasing *psh1Δ* mutant cells from G1 into conditions that overexpress FLAG-Cse4 and found that they die within a single cell cycle (Fig 2.8D).

To determine whether the growth defect is due to altered kinetochore function, I analyzed cell cycle progression in wild-type and *psh1Δ* cells overexpressing FLAG-Cse4. The anaphase inhibitor Pds1 cycled normally in the control wild-type and *psh1Δ* mutant cells released from G1 (Fig 2.7). However, although Pds1 cycled normally in wild-type cells overexpressing Cse4, there was a transient delay in Pds1 destruction when Cse4 was overexpressed in *psh1Δ* mutant cells released from G1 (Fig 2.9). The delay was mediated by the spindle checkpoint because it was eliminated when the Mad2 checkpoint protein was deleted, suggesting that Cse4 overexpression in *psh1Δ* mutant cells alters kinetochore function. Taken together, these data suggest there is an effect on kinetochore function when Cse4 is overexpressed in *psh1Δ* cells, although the transient checkpoint activation is unlikely to solely account for the complete lack of viability in a single cell cycle. The high level of Cse4 accumulation in euchromatin in the absence of Psh1-mediated degradation likely disrupts one or more chromatin-based processes in addition to altering kinetochore function.

Discussion

I report here the first E3 ubiquitin ligase identified for CENP-A in any organism. Psh1 polyubiquitinates and partially mediates Cse4 degradation *in vivo*. In the absence of Psh1, Cse4 overexpression leads to euchromatic mis-incorporation and lethality.

Psh1 mediated degradation of Cse4

Although the endogenous Cse4 protein can be detected in euchromatin at highly transcribed genes, its abundance is much lower than at centromeres (Camahort et al., 2009; Lefrancois et al., 2009). These data suggest that Cse4 transiently localizes to euchromatin but there are mechanisms that ensure it is not stably maintained. Consistent with this, the overexpression of Cse4 does not lead to detectable euchromatin localization or have growth consequences in WT cells (Collins et al., 2004b). In contrast, Cse4 overexpression is toxic and leads to its accumulation in euchromatin in the absence of Psh1. These data strongly suggest that Psh1-mediated degradation of Cse4 is a key mechanism that prevents Cse4 from stably incorporating into euchromatin.

Psh1 is present in both the soluble and chromatin fractions and interacts with Cse4 in both fractions. The Psh1 and Cse4 interaction can also be detected in the chromatin in the *ndc10-1* mutant, suggesting that the two proteins are able to associate with each other in the euchromatin, independent of the centromere. Although an additional role for Psh1 at the centromere cannot be ruled out, I did not detect any defects in cell cycle progression in *psh1Δ* cells. However, a recent study detected Psh1 at the centromere (Hewawasam et al., 2010) although it is not yet clear what its functional role at the centromere is. Nevertheless, the previously detected genomic instability in *psh1Δ* mutant cells is just as consistent with a function in preventing ectopic Cse4 localization as in regulating centromere function (Collins et al., 2007; Yuen et al., 2007). I hypothesize that the cellular levels of Cse4 are kept low by additional mechanisms, such as transcriptional and post-transcriptional controls that regulate the major histones (Osley, 1991) such that Cse4 mis-incorporation in the absence of Psh1 is not sufficient to

manifest a gross defect under normal conditions. Consistent with this, Cse4 levels are only partially stabilized in the absence of Psh1 so additional pathways of Cse4 degradation exist that also need to be identified in the future.

The toxicity of Cse4 overexpression in psh1Δ mutants.

My data suggest that the toxicity caused by Cse4 overexpression in *psh1Δ* mutant cells may be a result of multiple defects. The spindle checkpoint is eventually satisfied in these cells, yet they die within a single cell cycle. It is not possible to determine if ectopic kinetochores form *de novo* in budding yeast because they cannot be visualized due to the limit of resolution of the light microscope. In addition, traditional chromatin immunoprecipitation techniques cannot identify ectopic kinetochores unless the site of assembly is similar throughout the population. I favor the possibility that the toxicity is due to the mislocalization of one or more kinetochore proteins as well as the disruption of chromatin-based processes such as transcription when Cse4 accumulates in euchromatin. Consistent with this, it was previously shown that the relative stoichiometry of H3 and Cse4 is important for the proper localization of each protein (Au et al., 2008). I was not able to directly test this due to technical difficulty in achieving similar levels of Cse4 expression when H3 was simultaneously overexpressed. Regardless, my data show that Psh1 mediates Cse4 degradation and contributes to the fidelity of its cellular localization.

Experimental Procedures

Strain construction and Microbial techniques

Media and microbial techniques are as described (Rose et al., 1990; Sherman et al., 1974). Yeast strains were constructed by standard genetic techniques and are listed in table 2.1.

Plasmid Construction

The *pGAL-Myc-CSE4* construct used (pSB816) was previously published (Collins et al., 2004b). GST-Psh1 (pSB1535) was constructed by amplifying genomic *PSH1* using primers SB2248 and SB2249 with engineered BamHI and EcoRI sites and cloned into the same sites of the pGEX-2T vector (Pharmacia Biotech). C45S and C50S mutations were introduced into pSB1535 by site directed mutagenesis using primers SB2346 and SB2437 to create pSB1541. Construction of *pCUP1-3XFLAG-CSE4*^{16R} was carried out as follows: *CSE4*^{16R} was PCR amplified using primers SB790 and SB70 with HindIII and SacII sites engineered and cloned into *pGAL*, 2μ vector (pSB17) to generate *pGAL-CSE4*^{16R} (pSB779). pSB779 was subsequently digested with XhoI and HindIII to insert 3X FLAG sequence and obtain *pGAL-3XFLAG-CSE4*^{16R} (pSB840). The *pGAL* promoter was replaced with *pCUP1* promoter by digesting pSB840 with XhoI and KpnI and ligating in *pCUP1* at the same sites to create *pCUP1-3XFLAG-CSE4*^{16R} (pSB1034). pSB1665 was constructed as follows: Cse4 + 500 bp downstream region was cloned into a pGAL vector (pSB761). pSB761 was subsequently digested with XhoI and HindIII to insert 3X FLAG sequence and obtain *pGAL-3XFLAG-CSE4* (pSB839). pSB839 was then digested with SacI and KpnI to isolate the *pGAL-3XFLAG-CSE4* fragment and ligated

into a URA integrating vector (pSB147) at the same sites to generate pSB1665. HHT1 was PCR amplified with SB 2771 and 2772 with SpeI and SacI sites engineered. The resulting fragment was digested with the above enzymes and cloned into the same sites in pSB209 to generate *pGAL-Myc*-HHT1 (pSB1704).

Protein and Immunological techniques

Protein purification

Plasmids containing either GST-Psh1 (pSB1535) or the GST-Psh1 RING mutant (pSB1541) were transformed into BL-21 cells. Protein was purified from 2 L of cells after inducing expression with 1 mM IPTG for 2 hrs. GST purification was carried out as described (Kellogg et al., 1995; Kellogg and Murray, 1995) .

For Cse416R purification, 500 mls of mid-log (OD600~ 0.6) cells carrying pCUP1-3XFLAG-CSE416R were induced with CuSO4 to a final concentration of 5μM for 30 minutes. Lysates were prepared in Buffer H/0.1M KCl (25 mM HEPES pH 8.0, 2 mM MgCl 2, 0.1mM EDTA pH 8.0, 0.5 mM EGTA pH 8.0, 0.1 % NP-40, 15% glycerol and 100 mM KCl) supplemented with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin, and 0.2 mM PMSF) and phosphatase inhibitors (1 mM sodium pyrophosphate, 2 mM Na-beta-glycerophosphate, 0.1 mM NA 3VO4, 5 mM NaF, 100 nM microcystin-LR). Cells were lysed with glass beads in a beater (Biospec Products, Inc.) for 30 seconds, three times, with 1 min on ice in between and then centrifuged for 90 minutes at 27,000 rpm at 4°C. The supernatant was incubated with 60 μl of M2 anti-FLAG agarose beads (Sigma) for 3 hrs at 4°C. The beads were washed

with buffer H/0.3 M KCl 3 times, followed by 3 more washes with buffer H/0.1 M KCl. Proteins were eluted with 60µl of 0.5mg/ml FLAG peptide. The eluate from the purification was subject to anion exchange (0.2 ml of Source Q column) with a starting salt gradient of 0.1M KCl and final concentration of 1M KCl.

Mass Spectromerty (MS)

Mass Spectrometry (MS) analysis was carried out on fractions 14 and 15 that contained a peakof Cse4 protein. Each ion exchange fraction was reduced, alkylated with iodoacetamide, and digested with trypsin as described previously(Klammer and MacCoss, 2006). Each peptide mixture was loaded onto a microcapillary LC column (75um x 25 cm) packed with C12 reversed phase chromatography material (Phenomenex, Jupiter 4µ Proteo 90Å) and interfaced with an LTQ mass spectrometer (Thermo Fisher) using an HPLC and an autosampler (Agilent 1100) in a nanoflow configuration as described previously (Klammer and MacCoss, 2006). A cycle of one full-scan mass spectrum (400-1400 m/z) followed by five data-dependent MS/MS spectra was collected throughout the reversed phase gradient.

Each MS/MS spectrum was searched against a protein sequence database containing the *S. cerevisiae* open reading frames downloaded from SGD and common contaminants using SEQUEST(Eng et al., 2008). The spectra were also searched a second time using a decoy database. The results from the two database searches were then analyzed and assigned q-values and posterior error probabilities (Kall et al., 2008a, b) using the post-processing algorithm Percolator (Kall et al., 2007). An in house

implementation of the IDPicker algorithm (Zhang et al., 2007) was used to filter peptide spectrum matches with a q-value ≤ 0.001 and assemble the resulting peptides into the parsimonious list of protein identifications.

Immunoprecipitation

For immunoprecipitations, 50 ml cultures were harvested at midlog phase and lysates were prepared as described except that cell extracts were (Akiyoshi et al., 2009)prepared with glass beads in a beater (Biospec Products, Inc.) for 35 sec, three times, with 1 min on ice in between and then centrifuged for 30 minutes. The supernatant was incubated with 10 µl of protein G dynabeads (Invitrogen) and either 0.5 µl of M2anti-Flag (sigma) or 4 µl of A-14 anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for 2 hrs at 4° C. The beads were washed four times with lysis buffer and the immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting as described below.

For immunoblotting, protein extracts were made and analyzed as described (Minshull et al., 1996). 9E10 anti-Myc antibodies were obtained from Covance (Richmond, CA) and used at a 1:10,000 dilution. Cse4 antibodies were used at 1:500 (Collins et al., 2004). Tubulin antibodies were obtained from Accurate Chemical and Scientific (Westbury, NY) and used at 1:1000. Pgk1 antibodies obtained from Invitrogen were used at 1:10,000. K4-Me2 H3 antibodies were obtained from Upstate Cell Signaling Solutions (NY, gift from Rich Gardner) and used at 1:3000. Quantitative immunoblotting was performed using IR Dye 800CW obtained from LI-COR (gift from Steve Hahn,

FHCRC). Note that due to an increase in Cse4 levels in psh1Δ mutant cells and Cse416R, different exposures were used to detect Cse4 ubiquitin conjugates. However, conjugates were not detected in the psh1 mutant cells even when exposure times were increased.

Stability assays

For endogenous Cse4 stability assays, cells were grown in YEPD and subsequently arrested with 1 μg/ml α-factor (G1), 0.023 g/ml hydroxyurea (S) or 15 μg/ml nocodozole (M) for 3 hrs. Samples were taken for analysis after repressing protein synthesis with 50 μg/ml of cycloheximide. For all stability assays, cells were grown in 2% raffinose media, a timepoint (-) was taken, and then cultures were induced with 2% galactose for 2.5 hrs at 23°C. Timepoints were taken after inhibiting transcription and translation by adding glucose to 2% and cycloheximide to a final concentration of 50μg/ml respectively. Extracts were prepared and protein levels were assayed using Myc antibodies or quantified as described above.

Ubiquitination Assay

Ubiquitination assays were performed as described (Gard(Gardner et al., 2005)ner et al., 2005). 15 μl reactions containing 0.4 μg ubiquitin activating enzyme (yeast Uba1), 0.4 μg ubiquitin conjugating enzyme (human UbcH5a), 36 ng purified GST-Psh1 (or C45S, C50S mutant), 2.5 μg ubiquitin, 2 mM Mg-ATP, 50 mM Tris-HCl (pH7.5), 2.5 mM MgCl 2, and 0.5 mM DTT were incubated at 23°C for 30 min. Reactions were stopped by adding sample buffer and boiling for 3 minutes. Uba1, UbcH5a, ubiquitin and

Mg-ATP were obtained from Boston Biochemicals. Ubiquitin conjugates were detected using anti-ubiquitin antibodies (gift from D. Gottschling, FHCRC). Cse4 and core histones were produced in E. coli (Luger et al., 1997). Histone octamers were refolded and purified by Superdex S-200 gel-filtration chromatography as described(Dyer et al., 2004). 1μg of Cse4 octamers were used in ubiquitination assays and probed for Cse4 ubiquitin conjugates with anti-Cse4 antibodies (gift from Carl Wu, NIH).

Chromatin Fractionation assays

Chromatin Fractionation experiments were performed as described (Liang and Stillman, 1997) with the following changes. 30 Units of micrococcal nuclease (Worthington Biochemical Corporation) per 50 ml of mid-log culture were used to solubilize the chromatin. The micrococcal nuclease digestion was carried out at 37°C for 15 minutes and analyzed after stopping the reaction with 1mM EDTA. For fractionation followed by immunoprecipitation, the same protocol was used to prepare soluble and chromatin fractions and immunoprecipitations were carried out as described above.

Immunofluorescence

Cells were grown in lactic acid, followed by nocodazole arrest for 3hrs. For the last hour of arrest, FLAG-Cse4 expression was induced with 2% galactose. Chromosome spreads were performed as described previously (Collins et al., 2004b). DAPI was obtained from Molecular Probes (Eugene, OR) and used at a 1μg/ml final concentration. Lipsol was obtained from Fisher. Anti-Cse4 antibodies were used at 1:250

dilution(Collins et al., 2004b), Cy3 and Cy5 secondary antibodies from Jackson Immunoresearch (West Grove, PA) were used at a 1:1000 dilution, and Alexafluor-GFP antibodies obtained from Invitrogen were used at a 1:250 dilution.

Viability and Pds1 assay

To assay viability and Pds1 levels, cells were grown in YEP + lactic acid media and arrested in G1 with 1 μg/ml of α- factor for 3.5 hrs. Cse4 overexpression was induced with 2% galactose during the last 30 min of the arrest and then the cells were washed and released into 2% galactose. α- factor was added back when cells were small-budded to prevent cells from entering the second cell cycle. To assay viability, cells were plated on YPD at 23 °C after diluting 10-4 and > 100 cells were counted for each strain. To assay Pds1 levels, lysates were made as described above.

A

Proteins	% Coverage	Unique Peptides	Total Peptides
Histones			
Hta1, Hta2	56.8	7	16
Htb1, Htb2	36.6	6	7
Hhf1, Hhf2	60.2	13	20
Cse4	20.1	8	22
Kinetochore			
Ame1	11.1	3	3
Ctf19	17.9	5	5
Mcm21	16.8	4	4
Mif2	21.1	8	8
Nkp1	18.9	3	3
Okp1	17.5	6	6
Other			
Psh1	36.2	17	25

B

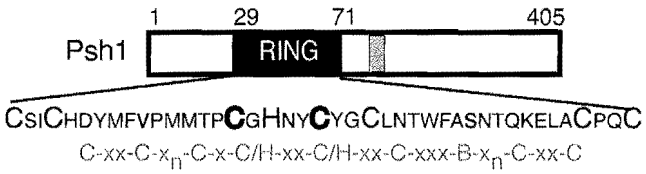


Fig 2.1. Psh1 is an E3 ligase that co-purifies with Cse4.
(A) List of histone and kinetochore proteins identified by MS after purification of 3xFLAG-Cse416R (SBY5442). (B) Psh1 has a consensus RING domain (residues 29-71, black) and a pair of C4-type zinc finger motifs (residues 150-171, gray). RING domain cysteine and histidine residues predicted to bind zinc are in large typeface and the mutants are indicated in bold. "B" is a bulky residue in the RING domain

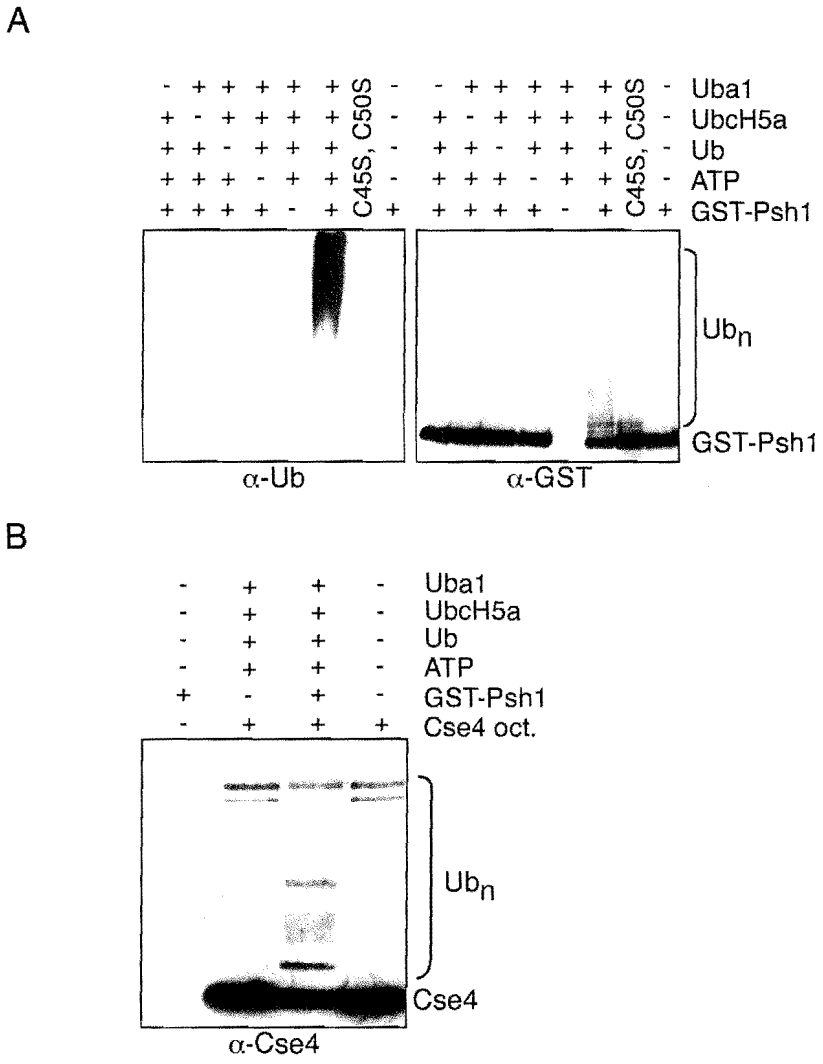


Fig 2.2. Psh1 ubiquitinates Cse4 *in vitro*.
(A) Psh1 autoubiquitinates *in vitro*. Recombinant GST-Psh1 was added to reactions in the presence (+) or absence (-) of ubiquitin-activating enzyme (Uba1), ubiquitin-conjugating enzyme (UbcH5a), ubiquitin (Ub), and ATP (lanes 1-6). A recombinant GST-Psh1 RING mutant with C45S and C50S mutations was used in a complete reaction instead of WT protein in lane 7. Lane 8 contains GST-Psh1 alone. The reactions were run on two gels and immunoblotted with either anti-ubiquitin antibody (left) or anti-GST antibody (right). (B) Recombinant Cse4 octamers were added to reactions containing ubiquitin-activating enzyme (Uba1), ubiquitin-conjugating enzyme (UbcH5a), ubiquitin (Ub), and ATP in the absence (-) or presence (+) of GST-Psh1(lanes 2 and 3). GST-Psh1 (lane 1) or Cse4 octamers (lane 4) alone were also included in the immunoblot as controls. The blot was probed with anti-Cse4 antibodies.

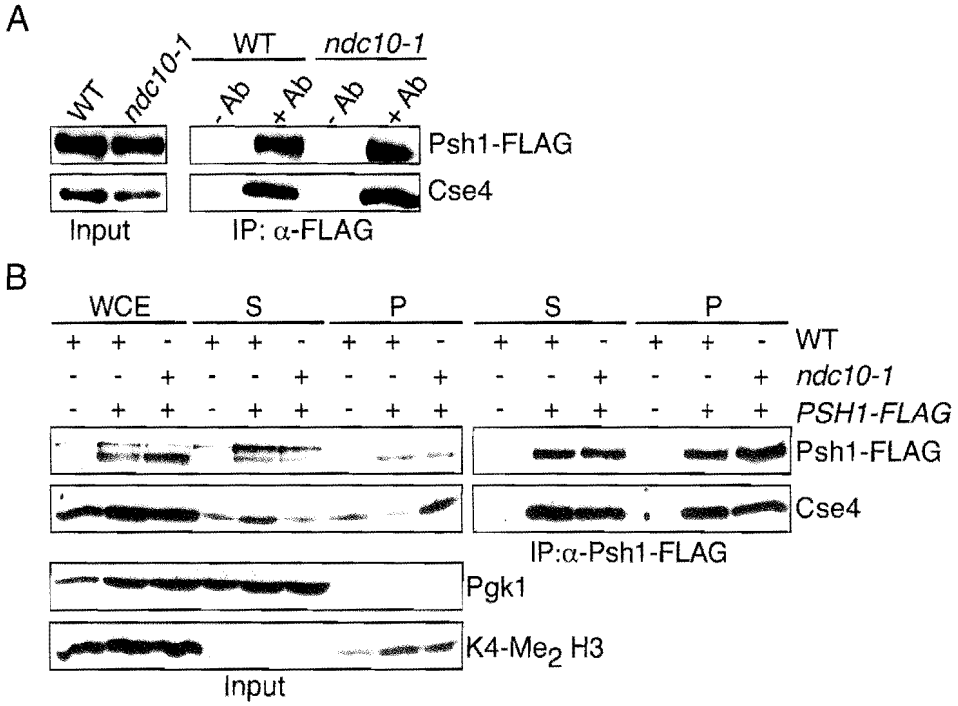
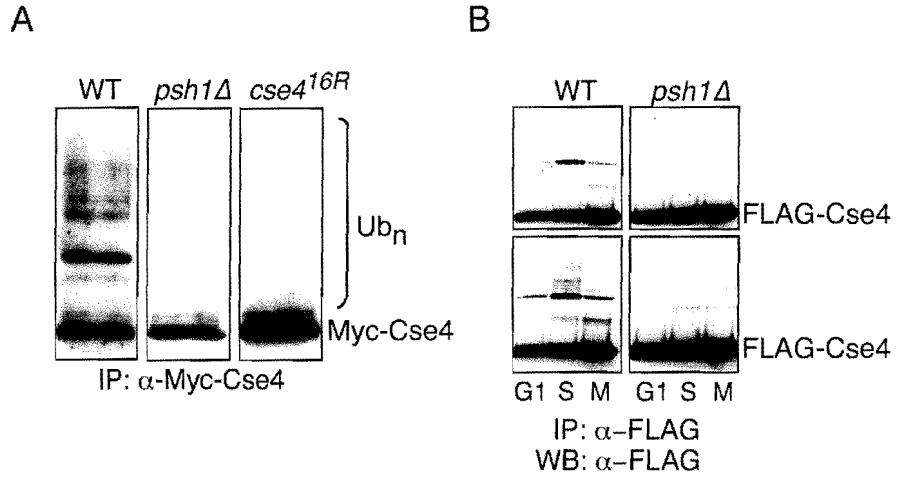
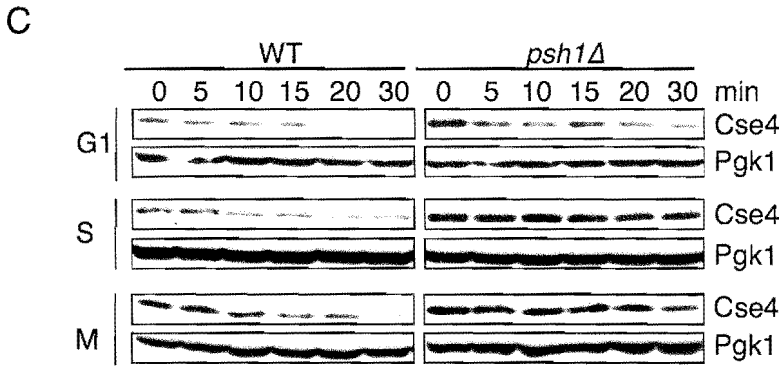
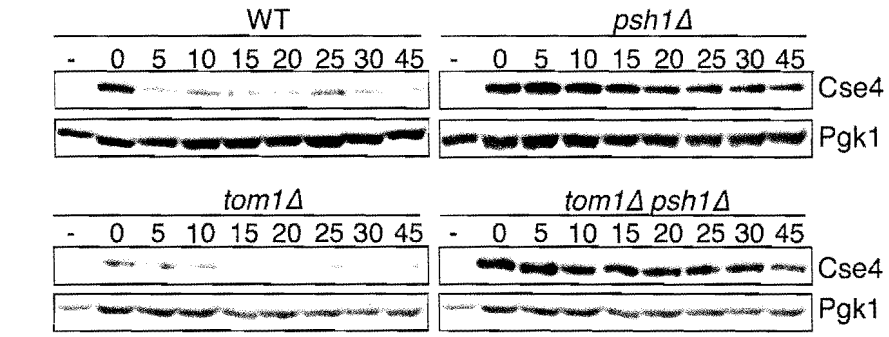


Fig 2.3. Psh1 and Cse4 can associate independently of the centromere.
(A) Wild-type (SBY8281) or *ndc10-1* mutant (SBY8282) cells containing Psh1-FLAG were shifted to 37 °C for 3 hrs and Psh1-FLAG was immunoprecipitated. The corresponding immunoblot was probed with anti-FLAG and anti-Cse4 antibodies.
(B) WT (SBY8281) or *ndc10-1* mutant (SBY8282) cells were grown as in (A). Psh1-FLAG was immunoprecipitated from the soluble (S) and chromatin (P) fractions and the resulting immunoblots were probed with anti-FLAG and anti-Cse4 antibodies. Pgk1 and K4-Me_2 H3 are shown as markers for S and P fractions, respectively. Note that there is loss of material during the procedure so the levels between soluble and chromatin fractions cannot be compared.


A
WT
psh1Δ
cse4^16R
Ub_n
Myc-Cse4
IP: α-Myc-Cse4
B
WT
psh1Δ
FLAG-Cse4
FLAG-Cse4
G1 S M G1 S M
IP: α–FLAG
WB: α–FLAG


C
WT
psh1Δ
0 5 10 15 20 30 0 5 10 15 20 30 min
G1
Cse4
Pgk1
S
Cse4
Pgk1
M
Cse4
Pgk1


D
WT
psh1Δ
- 0 5 10 15 20 25 30 45 - 0 5 10 15 20 25 30 45
Cse4
Pgk1
tom1Δ
tom1Δ psh1Δ
- 0 5 10 15 20 25 30 45 - 0 5 10 15 20 25 30 45
Cse4
Pgk1

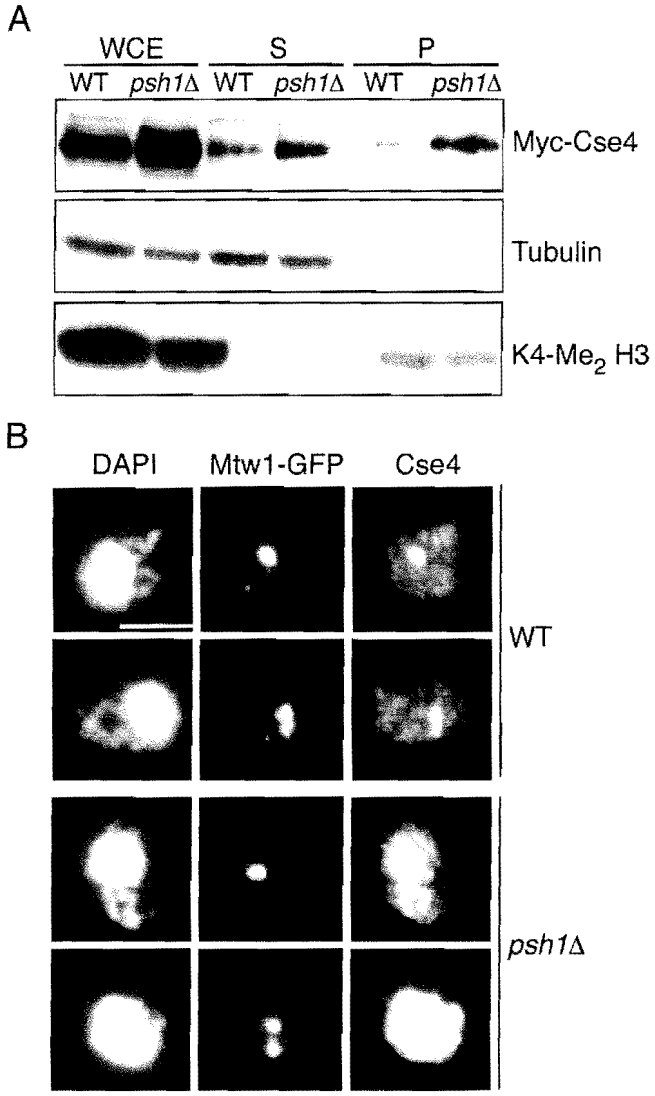


Fig 2.5 Psh1 prevents Cse4 from accumulating in euchromatin.
Extracts (WCE) from wild-type (SBY3570) and *psh1*Δ (SBY8355) cells expressing pGAL-Myc-CSE4 were fractionated into soluble (S) and chromatin (P) fractions. Cse4 levels were assayed in each fraction using anti-Myc antibodies. Tubulin and K4-Me2 H3 are markers for S and P fractions, respectively. Note that there is loss of material during the procedure so the levels between soluble and chromatin fractions cannot be compared. (B) FLAG-Cse4 was transiently overexpressed for 1 hour in wild-type (SBY8918) and *psh1*Δ (SBY8917) cells and its chromatin localization was assayed by immunofluorescence on chromosome spreads. DAPI, Alexafluor-GFP and anti-Cse4 staining recognize the DNA, Mtw1, and Cse4, respectively. Scale bar = 5 μm.

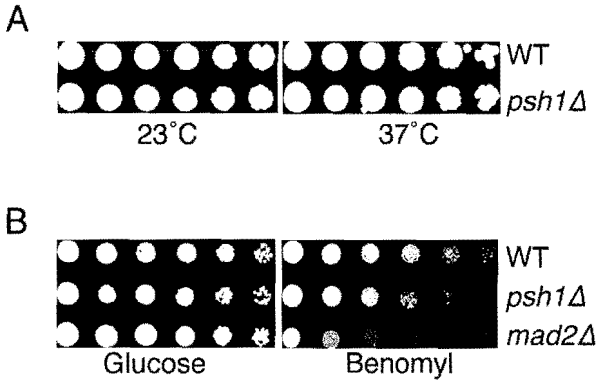


Fig 2.6. Psh1 mutants do not show sensitivity to higher temperature or to benomyl. (A) 5-fold serial dilutions of WT (SBY3), *psh1*Δ (SBY8336) and *mad2*Δ (SBY292) cells plated on glucose or benomyl plates at 23 °C. (B) 5-fold serial dilutions of WT (SBY3) and *psh1*Δ (SBY8336) cells grown at 23°C and 37°C.

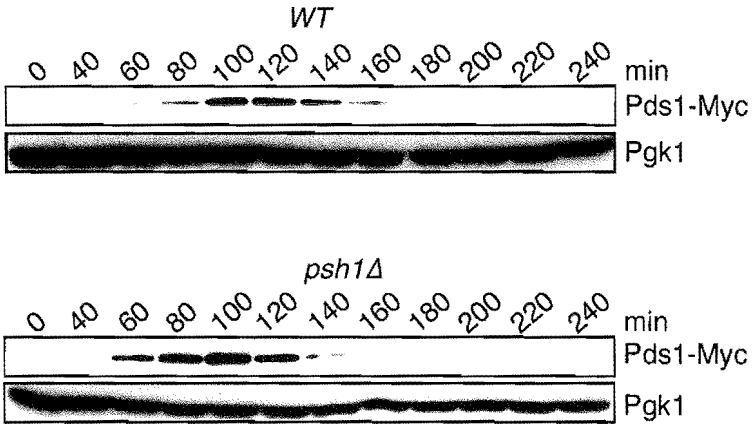
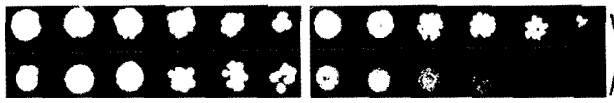


Fig 2.7. Psh1 mutants have normal cell cycle progression

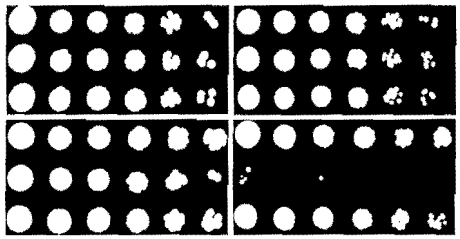
WT (SBY8738) and *psh1Δ* (SBY8737) cells were arrested in G1 in YEP + lactic acid media for 3.5 hrs. Cells were subsequently released into the cell cycle and samples were taken at the indicated timepoints. Alpha-factor was added back to analyze a single cell cycle. Pds1-Myc levels were analyzed using anti-Myc. Pgk1 is shown as a loading control.

A



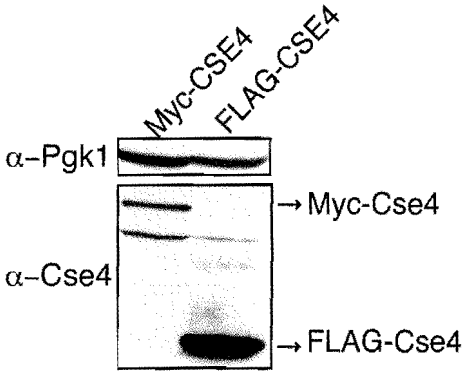
Genotype	Vector
WT	+ *pGAL-Myc-CSE4*
psh1Δ	+ *pGAL-Myc-CSE4*

B

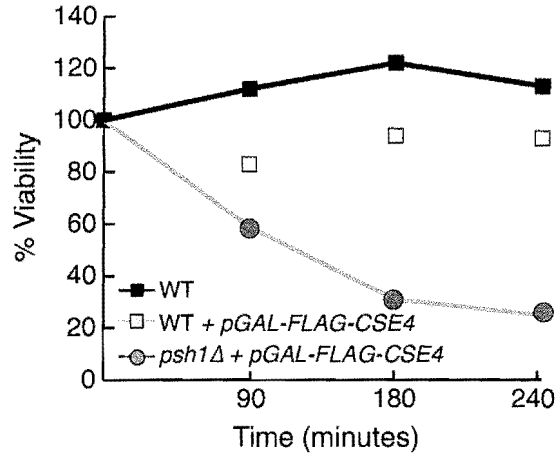


Genotype	Vector
WT	*pGAL* vector
WT	*pGAL-FLAG-CSE4*
WT	*pGAL-H3*
psh1Δ	*pGAL* vector
psh1Δ	*pGAL-FLAG-CSE4*
psh1Δ	*pGAL-H3*

C



D



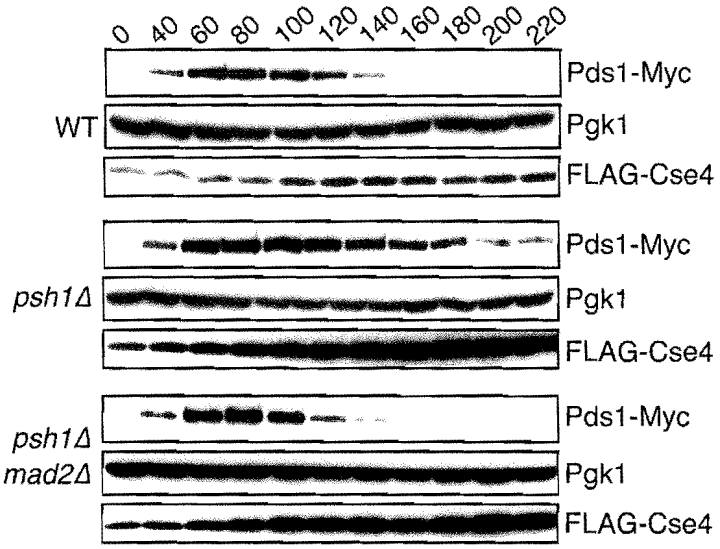


Fig 2.9. Spindle checkpoint is weakly activated in *psh1*Δ cells when Cse4 is overexpressed. Wild-type (SBY8976), *psh1*Δ (SBY 8982) and *psh1*Δ *mad2*Δ (SBY8975) cells containing p*GAL-FLAG-CSE4* were released from G1 into galactose media. Lysates were prepared at the indicated time points and analyzed for Pds1 levels by immunoblotting.

Table 2.1. Yeast strains used in this study. All strains are isogenic with the W303 background. Plasmids are indicated in brackets.

Strain	Genotype
SBY3	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1*
SBY292	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 mad2::URA*
SBY674	*MAT**a** ura3-1::pGAL::URA3 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1*
SBY3570	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 (pSB816, pGAL-13Myc-CSE4, URA3, 2μ)*
SBY3571	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 (pSB817, pGAL-13Myc-CSE4^{16R}, URA3, 2μ)*
SBY4471	*MAT**a** ura3-1 leu2,3-112 his3-11 ade2-1 can1-100 bar1-1 trp1-1::pGAL-H3::TRP1(pSB893)*
SBY5442	*MAT**a** ura3-1 leu2,3-112 his3-11 ade2-1 can1-100 bar1-1 trp1-1::256lacO::TRP1 cse4::KAN (pSB1034, pCUP1-3XFLAG-CSE4^{16R}, URA3, 2μ)*
SBY7268	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG::TRP1 ura3-1::pGAL-Myc-CSE4::URA3*
SBY8281	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG::TRP1, MTW1-3GFP::HIS3*
SBY8282	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG::TRP1 ndc10-1 MTW1-3GFP::HIS3*

SBY8336	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN*
SBY8355	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN (pSB816, pGAL-13Myc-CSE4, URA3, 2μ)*
SBY8737	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN PDS1-Myc18::LEU2*
SBY8738	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PDS1-Myc18::LEU2*
SBY8857	*MAT**a** ura3-1 leu2,3-112 his3-11 ade2-1 can1-100 bar1-1 psh1::KAN trp1-1::pGAL-H3::TRP1 (pSB893)*
SBY8903	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN ura3-1::pGAL-FLAG-CSE4::URA (pSB1665)*
SBY8904	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 ura3-1::pGAL-FLAG-CSE4::URA (pSB1665)*
SBY8917	*MAT**a** leu2,3-112 trp1-1 ade2-1 can1-100 bar1-1 his3-11,15::Mtw1-3XGFP::HIS ura3-1::pGAL-FLAG-CSE4::URA3 psh1::KAN*
SBY8918	*MAT**a** leu2,3-112 trp1-1 ade2-1 can1-100 bar1-1 his3-11,15::Mtw1-3XGFP::HIS ura3-1::pGAL-FLAG-CSE4::URA3*
SBY8975	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN mad2::KAN PDS1-Myc18:LEU2 ura3-1::pGAL-FLAG-CSE4::URA (pSB1665)*

SBY8976	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PDS1-Myc18:LEU2 ura3-1::pGAL-FLAG-CSE4::URA(pSB1665)*
SBY8982	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN PDS1-Myc18:LEU2 ura3-1::pGAL-FLAG-CSE4::URA (pSB1665)*
SBY9007	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN ura3-1::pGAL::URA3(pSB157)*

Table 2.2. Plasmids used in this study

Plasmid	Description
pSB157	*pGAL empty vector, URA3* (integrating)
pSB205	*PDS1-18Myc, LEU2* (integrating)
pSB209	*pGAL-Myc, URA3* (integrating)
pSB245	*pGAL-Myc-CSE4, URA3* (integrating)
pSB816	*pGAL-13Myc-CSE4, URA3, 2μ*
pSB817	*pGAL-13Myc-CSE4^{16R}, URA3, 2μ*
pSB893	*pGAL-HHT1, TRP* (integrating)
pSB1034	*pCUP1-3XFLAG-CSE4^{16R} +500 bp downstream, URA3, 2μ*
pSB1535	*GST-PSH1 in pGEX2T*
pSB1541	*GST-PSH1 C45S C50S in pGEX2T*
pSB1665	*pGAL-FLAG-CSE4 + 500 bp downstream, URA3 (integrating)*

CHAPTER: 3

The centromere targeting domain of Cse4 regulates its stability and allows Psh1 to distinguish it from H3.

Summary

The centromeric histone H3 variant Cse4 is regulated by proteolysis via the E3 ubiquitn ligase Psh1. Here, I present data that shows that Psh1 binds to Cse4 via the centromere targeting domain (CATD) and allows Psh1 to discriminate between Cse4 and H3. Moreover, this domain regulates the stability of Cse4. Thus, the CATD appears to have a dual role for regulating Cse4 localization by both targeting Cse4 to the centromere and preventing ectopic localization of Cse4 via degradation by Psh1.

Introduction

The centromeric histone H3 variant CENP-A is highly conserved throughout eukaryotes. All CENP-As contain a unique N-terminal tail and a conserved histone fold domain (HFD), which is involved in histone-histone interaction. The homology between H3 and its variant CENP-A is limited to this histone fold domain while the N-terminal tail has no sequence similarity (Stoler et al., 1995b). The histone fold domain of CENP-A contains residues that are important for targeting of CENP-A to the centromere. This domain, called the Centromere targeting domain (CATD) comprises Loop 1 and a2 helix, which has been demonstrated to be necessary and sufficient to target an H3 chimera to

the centromere (Black et al., 2007; Vermaak et al., 2002a). This domain also binds to the chaperones Scm3 in budding yeast (Shivaraju et al., 2011; Zhou et al., 2011) and HJURP in humans (Foltz et al., 2009) which are required for CENP-A deposition at the centromere, suggesting that this domain is required for regulating centromeric localization of CENP-A.

I found that in contrast to the overexpression of Cse4, overexpression of H3 had no effect on the viability of *psh1Δ* mutants. This led me to hypothesize that Psh1 may be specifically regulating Cse4 and not H3. Although Psh1 interacts with Cse4 both in the soluble and chromatin fractions, it is not yet clear whether Psh1 recognizes the soluble pool of Cse4 or if it recognizes ectopic Cse4 on the chromatin. Nevertheless, the ability of Psh1 to distinguish between the two histones is important in order to ensure proper regulation of the two proteins. Given the high degree of homology between Cse4 and H3, I investigated how Psh1 is able to discriminate between H3 and its variant Cse4. Here, I have shown that the CATD is required for recognition of Cse4 by Psh1. The presence of CATD is sufficient for Psh1 to recognize a chimeric H3 and destabilize it relative to H3. Thus, in addition to its known function, CATD also functions to allow Psh1 to distinguish Cse4 from H3 and regulate its stability and thereby contribute to its localization.

My preliminary data also implicates the FACT complex to be involved in Psh1 mediated degradation of Cse4. FACT (Spt16, Pob3 and Nhp6) is a highly conserved chromatin remodeling complex that disassembles nucleosomes to allow passage of the polymerase through the chromatin during transcription and replication (Reinberg and

Sims, 2006; VanDemark et al., 2006). FACT also functions as a histone chaperone to reassemble nucleosomes after polymerase passage. I found that Psh1 mediates the interaction between Spt16 and Cse4 and there is a moderate effect on Cse4 stability in an *spt16* mutant. I therefore propose that Psh1, with the help of the FACT complex, recognizes ectopically localized Cse4 on the chromatin and targets it for degradation.

Results

Psh1 recognizes Cse4 but not H3.

The lethality due to Cse4 but not H3 overexpression in *psh1Δ* mutant cells suggested that Psh1 specifically interacts with Cse4. To test this, I immuno-precipitated Psh1-Myc from cells containing T7-H3 as the sole copy of histone H3. Although the endogenous Cse4 protein co-precipitated, I did not detect H3 interacting with Psh1 despite much higher cellular protein levels (Fig 3.1A). However, Psh1 was able to ubiquitinate H3 octamers *in vitro* consistent with the possibility that the E2 enzyme used in the reaction is promiscuous *in vitro* and additional factors maybe be involved in providing specificity *in vivo* (Fig 3.1B).

Psh1 recognizes Cse4 via the CATD

I next asked how Psh1 distinguishes Cse4 from H3. The Histone Fold Domains (HFD) of Cse4 and H3 share 64% identity, whereas their N-terminal domains (NTD) bear no sequence similarity (Stoler et al., 1995a). To identify the Psh1 binding domain in Cse4, we immunoprecipitated Psh1-FLAG from strains expressing Myc-tagged full-

length Cse4, NTD-Cse4 or HFD-Cse4. While full length Cse4 and the HFD-Cse4 associate with Psh1, the NTD-Cse4 does not interact with Psh1 (Fig 3.2A). However, I reproducibly detected greater levels of full-length Cse4 associating with Psh1 compared to HFD-Cse4, suggesting that the N-terminus might contribute to the interaction *in vivo*. Because the HFD-Cse4 contains the CATD, I asked whether the CATD helps Psh1 distinguish Cse4 from H3. First, I replaced the CATD in Cse4 with loop 1 and a2 helix of H3 to generate Myc-Cse4^{-CATD} (Fig 3.2B, construct 2). The Myc-Cse4^{-CATD} no longer immunoprecipitated with Psh1-FLAG, suggesting that the CATD is required for the interaction (Fig 3.2C). Second, I tested the interaction of chimeric Myc-H3^{CATD} or Myc-H3 proteins with Psh1 in both the soluble and chromatin-bound fractions (Fig 3.2B constructs 3 and 4). Strikingly, the H3^{CATD} protein co-precipitates with Psh1-FLAG in the soluble fraction whereas H3 does not (Fig 3.2D). I also detected a robust interaction between Psh1 and H3^{CATD} in the chromatin fraction, although lower levels of H3 also co-precipitated with Psh1. This may reveal a decreased affinity of Psh1 for H3, or a transient interaction in the context of chromatin. Taken together, these data strongly suggest that the CATD helps Psh1 recognize Cse4.

The CATD is required for Psh1-mediated degradation

Given that the CATD is important for Psh1 binding to Cse4, I analyzed the stability of the chimeric proteins. First, I analyzed Myc-Cse4^{-CATD} levels in WT and *psh1* mutant cells after repressing transcription and translation. Although Myc-Cse4^{-CATD} is quickly degraded, the degradation does not depend on Psh1 (Fig 3.3A). This is consistent

with Psh1-mediated degradation requiring the CATD. Next, I assayed the stability of the Myc-$H3^{CATD}$ construct in a similar experiment and found that the chimeric $H3^{CATD}$ is also rapidly degraded (Fig 3.3B). However, in contrast to the Cse4 chimera lacking the CATD, the degradation of $H3^{CATD}$ is dependent on Psh1 (Fig 3.3C). Taken together, these data suggest that the CATD is a key regulator of Cse4 stability via the Psh1 ubiquitin ligase.

Role of the FACT complex in Cse4 regulation

Psh1 was first isolated as an interacting protein of the FACT complex and histones (Krogan et al., 2002). Interestingly, the components of the FACT complex were also identified in our Cse416R purification (Ranjitkar et al., 2010). Given the role of the FACT complex in chromatin remodeling, it raised an exciting possibility that FACT may somehow be involved in regulating Cse4. To begin to understand this question, I started out by addressing the functional relevance of these observed interactions. To test whether the interaction between the FACT complex and Cse4 is mediated by Psh1, I performed immunoprecipitation with Spt16-FLAG from either wild-type or *psh1Δ* mutants and looked at its interaction with Cse4. In wild-type cells, Cse4 co-immunoprecipitates with Spt16. In contrast, this interaction is completely abolished in the absence of Psh1, indicating that Psh1 mediates the association between these two proteins (Fig 3.4A). Moreover, because Spt16 and Cse4 interaction is intact in a catalytically inactive mutant of Psh1, I conclude that the presence of Psh1 and not the activity of Psh1 is necessary for the observed association of Spt16 and Cse4 (data not shown).

In order to test if FACT complex has any effect on Cse4 stability, I analyzed Myc-Cse4 levels in wild-type and *spt16-13* mutants. As expected, there is a modest effect on Cse4 stability in the *spt16-13* mutant relative to wild-type cells (Fig 3.4B). However, because the exact nature of the *spt16* allele is not known, this affect on Cse4 stability maybe a reflection of the pleiotropic affects of the allele. Therefore, I am currently performing domain-mapping experiments to delineate the Cse4 and Spt16 interacting domains on Psh1, which will allow us to more directly address this question both *in vivo* and *in vitro*.

Discussion

Psh1 is an E3 ligase specific for Cse4

Although Cse4 and H3 are about 60% identical in the histone fold domain, Psh1 appears to specifically recognize and degrade Cse4 *in vivo*. However, *in vitro* using a non-specific E2 enzyme, Psh1 is still able to ubiquitinate histone H3 as well as H2A and H2B (data not shown). This suggests that additional specificity must exist *in vivo* that allows Psh1 to discriminate between Cse4 and other histones. A recent study identified Ubc8 as the E2 conjugating enzyme involved with Psh1, however, whether Ubc8 contributes to specificity remains to be tested *in vivo* (Hewawasam et al., 2010)

The CATD is a key determinant of Cse4 stability

The CATD in Cse4 appears to be a key domain that directs Psh1-mediated

degradation. A CATD deletion within Cse4 prevents its association with Psh1 and abolishes Psh1-mediated degradation, and insertion of the CATD is sufficient to mediate H3 binding to Psh1. However, I reproducibly detected greater levels of full-length Cse4 associating with Psh1 compared to just the HFD of Cse4, suggesting that the N-terminus might contribute to the interaction *in vivo*. Therefore, in the future, it will be important to determine whether additional features of Cse4 are involved in mediating the interaction with Psh1, as well as to establish whether Psh1 directly recognizes Cse4 in the context of chromatin.

Mechanism of Cse4 removal from ectopic sites by Psh1

Cse4 and Psh1 interact in both the soluble and chromatin fractions, but the precise location of the ubiquitination reaction is not known. Based in my data, there are two equally likely mechanisms by which Psh1 regulates Cse4 localization. 1) Psh1 regulates the soluble pool of Cse4 thereby indirectly controlling the mis-incorporation rate of Cse4 at ectopic sites or 2) Psh1 may directly recognize the ectopically localized Cse4 on the chromatin. The 2nd possibility raises the question of how Psh1 is able to detect ectopically localized Cse4. In addition, the CATD of Cse4 that is recognized by Psh1 is buried inside the nucleosomal strucuture. I speculated that chromatin factors that disassemble nucleosomes during chromatin-based processes expose the CATD and allow Psh1 to target Cse4 for degradation. Consistent with this idea, Cse4 and Psh1 co-purify with Spt16 and Pob3, components of the budding yeast FACT complex that disassemble and reassemble nucleosomes to facilitate transcription and replication (Reinberg and Sims,

2006; VanDemark et al., 2006). Psh1 mediates the interaction between Cse4 and Spt16, suggesting that Psh1 could link Cse4 degradation to the FACT complex. In addition, mutants in the Spt4 protein that is implicated in nucleosome assembly, nucleosome stabilization, and transcription also lead to Cse4 mislocalization to euchromatin (Crotti and Basrai, 2004; Hartzog et al., 2002; Swanson and Winston, 1992). A recent study also found the SWI/SNF chromatin remodeling complex to be involved in removing Cse4 from ectopic chromosomal regions, specifically at sites normally bound by the SWI/SNF complex (Gkikopoulos et al., 2011). Hence there appears to be multiple controls over Cse4 removal from ectopic sites.

I propose the following model (Figure 3.5). Cse4 is targeted to centromeres via a chaperone-mediated interaction with the CATD, where it becomes protected from degradation by either by Scm3 or by kinetochore assembly (Collins et al., 2004b; Dunleavy et al., 2009; Foltz et al., 2009; Hewawasam et al., 2010). Cse4 also localizes to euchromatin, especially at sites of high histone turnover (Camahort et al., 2009; Lefrancois et al., 2009). Psh1 may regulate Cse4 localization by degrading Cse4 that is evicted from the chromatin when the CATD is exposed during chromatin-based processes. It is also possible that Psh1 degrades excess soluble Cse4, thereby indirectly controlling its misincorporation rate into the chromatin. Although it is not yet clear whether Psh1 homologs exist in vertebrates, CATD function is conserved throughout eukaryotes (Black et al., 2007). In addition, depletion of the HJURP chaperone that associates in a CATD-dependent manner leads to lower intracellular CENP-A levels (Dunleavy et al., 2009) (Shuaib et al., 2010) (Foltz et al., 2009) consistent with the

possibility that the proteolytic machinery targets unbound CENP-A for degradation via the CATD. In the future, it will be critical to determine whether the mechanisms that prevent the non-centromeric accumulation of CENP-A are conserved. It will also be interesting to further investigate proteolysis as a mechanism to regulate chromatin composition and ultimately maintain epigenetic states in multicellular eukaryotes.

Experimental Procedures

Plasmid construction

CSE4-NTD (residues 1-129) was PCR amplified using primers SB242 and SB245 and *CSE4-HFD* (residues 130-229) was PCR amplified using SB243 and SB244 with SpeI and SacI sites engineered and cloned into the same sites of PSB209 to create pSB378 and pSB379, respectively. pSB1667 and was constructed as follows: The $H3^{CATD}$ construct was obtained from Lars E. Jansen (Black et al., 2007). The Cse4 N-terminus (residues 1-129) present in the construct was replaced with N-terminus of H3 (residues 1- 38) using PCR overlay extension. The N-terminus of H3 was PCR amplified with primers SB2653 and SB2654. The HFD domain of the chimeric constructs were PCR amplified with primers SB2655 and SB2340. The resulting fragments were subsequently used in a PCR reaction with primers SB2653 and SB2340 with SpeI and SacI sites engineered to generate fragments containing H3 N-terminus followed by $H3^{CATD}$. The fragments were digested with SpeI and SacI and cloned into the same sites of *pGAL-Myc* vector (pSB209) to generate pSB1667. HHT1 was PCR amplified with SB

2771 and 2772 with SpeI and SacI sites engineered. The resulting fragment was digested with the above enzymes and cloned into the same sites in pSB209 to generate *pGAL-Myc*-HHT1 (pSB1704). pSB1646 was constructed by PCR overlay extension. First, Cse4 residues 1-165 were PCR amplified using primers SB 242 and SB2584, H3 residues 76-113 were amplified using SB2585 and SB2586 and Cse4 residues 207-230 were amplified using SB2587 and SB244. The three PCR fragments described above were subsequently used to produce the chimeric fragment, which was in turn amplified using SB242 and SB244, digested with SpeI and SacI and ligated into the same sites of pSB209.

Immunoprecipitation

For immunoprecipitations, 50 ml cultures were harvested at midlog phase and lysates were prepared as described (Akiyoshi et al., 2009) except that cell extracts wereprepared with glass beads in a beater (Biospec Products, Inc.) for 35 sec, three times, with 1 min on ice in between and then centrifuged for 30 minutes. The supernatant was incubated with 10 µl of protein G dynabeads (Invitrogen) and either 0.5 µl of M2anti-Flag (sigma) or 4 µl of A-14 anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies for 2 hrs at 4° C. The beads were washed four times with lysis buffer and the immunoprecipitates were separated by SDS-PAGE and analyzed by immunoblotting as described below.

For immunoblotting, protein extracts were made and analyzed as described (Minshull et al., 1996). 9E10 anti-Myc antibodies were obtained from Covance

(Richmond, CA) and used at a 1:10,000 dilution. Cse4 antibodies were used at 1:500 (Collins et al., 2004b). Pgk1 antibodies obtained from Invitrogen were used at 1:10,000. K4-Me2 H3 antibodies were obtained from Upstate Cell Signaling Solutions (NY, gift from Rich Gardner) and used at 1:3000.

Stability assays

For all stability assays, cells were grown in 2% raffinose media, a timepoint (- was taken, and then cultures were induced with 2% galactose for 2.5 hrs at 23°C. Timepoints were taken after inhibiting transcription and translation by adding glucose to 2% and cycloheximide to a final concentration of 50μg/ml respectively. Extracts were prepared and protein levels were assayed using Myc antibodies and pgk1 as a loading control.

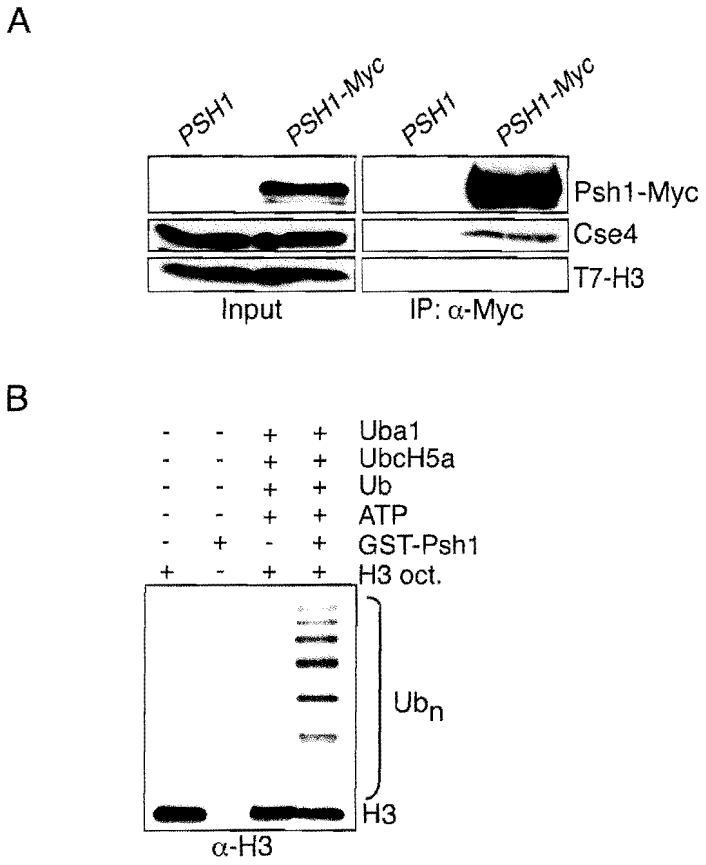
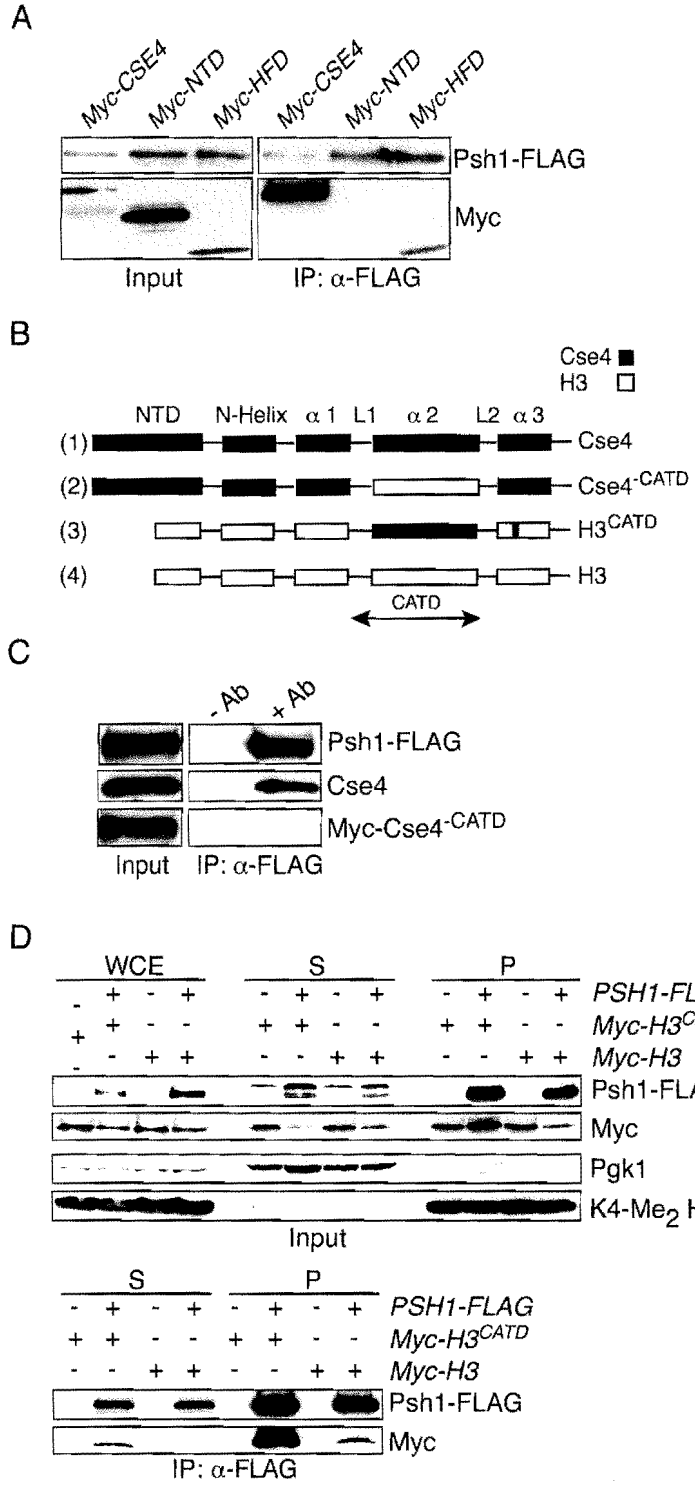


Fig 3.1 Psh1 does not associate with H3 *in vivo* but can ubiquitinate H3 *in vitro*. (A) Anti-Myc-conjugated beads were used to immunoprecipitate Psh1-Myc (SBY5364 and SBY8027). The samples were subsequently analyzed by immunoblotting with anti-Myc, anti-Cse4 or anti-T7 antibodies. (B) Recombinant H3 octamers were added to reactions containing ubiquitin-activating enzyme (Uba1), ubiquitin-conjugating enzyme (UbcH5a), ubiquitin (Ub), and ATP in the absence (-) or presence (+) of GST-Psh1 (lanes 3 and 4). GST-Psh1 (lane 2) or H3 octamers (lane 1) alone were also included in the immunoblot as controls. The blot was probed with anti-H3 antibodies.


A
Myc-CSE4
Myc-NTD
Myc-HFD
Myc-CSE4
Myc-NTD
Myc-HFD
Psh1-FLAG
Myc
Input
IP: α-FLAG
B
Cse4
H3
NTD
N-Helix
α 1
L1
α 2
L2
α 3
(1)
Cse4
(2)
Cse4-CATD
(3)
H3CATD
(4)
H3
CATD
C
- Ab
+ Ab
Psh1-FLAG
Cse4
Myc-Cse4-CATD
Input
IP: α-FLAG
D
WCE
S
P
PSH1-FLAG
Myc-H3CATD
Myc-H3
Psh1-FLAG
Myc
Pgk1
K4-Me2 H3
Input
S
P
PSH1-FLAG
Myc-H3CATD
Myc-H3
Psh1-FLAG
Myc
IP: α-FLAG

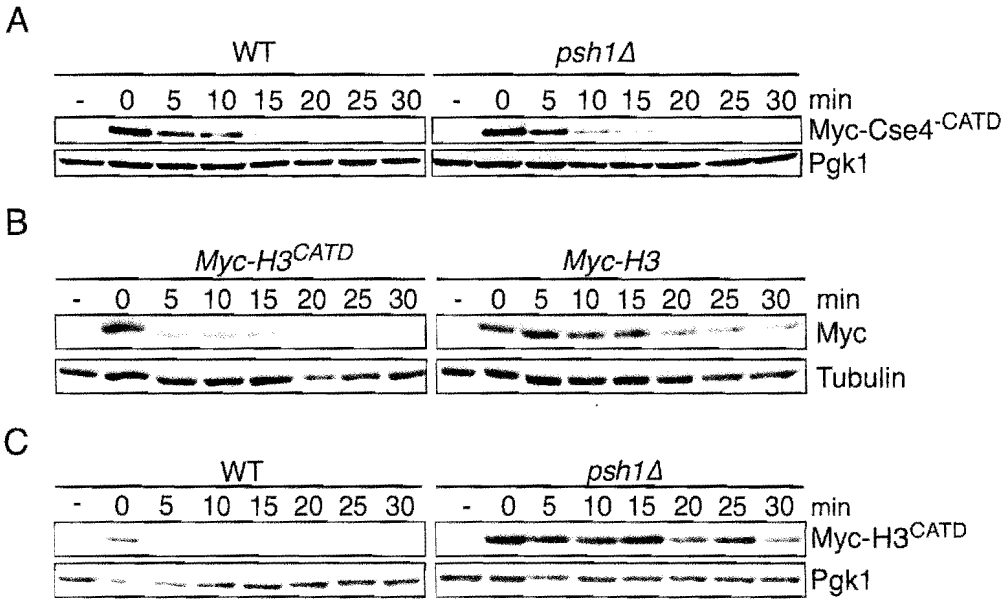


Fig 3.3. The CATD regulates Cse4 stability.
(A) The stability of Myc-Cse4-^{CATD} was assayed in WT (SBY8700) and *psh1Δ* (SBY8760) cells after repressing transcription and translation. The lysates were immublotted with anti-Myc antibodies. Pgk1 is a loading control. The (-) timepoint indicates samples taken before induction of the protein. (B) The stability of Myc-H3^{CATD} (SBY8932) and Myc-H3 (SBY 9134) in WT cells as in (A). The lysates were immunoblotted with anti-Myc antibodies. Tubulin is a loading control. (C) The stability of Myc-H3^{CATD} was assayed in wild-type (SBY8932) and *psh1Δ* (SBY8959) cells as in (A).

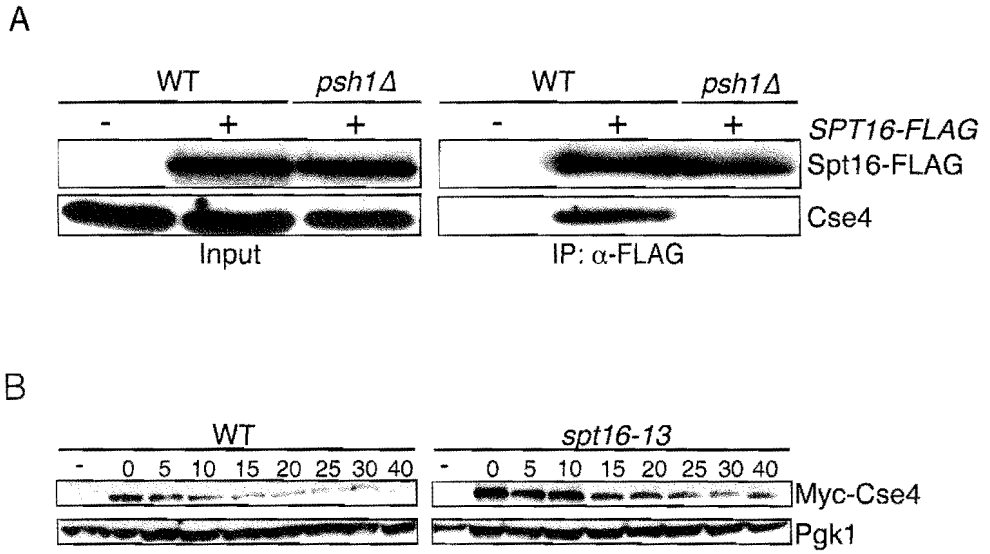


Fig 3.4. Psh1 mediates the interaction between Spt16 and Cse4.
Psh1 mediates the interaction between Spt16 and Cse4. Spt16-FLAG was immuno-precipitated from WT (SBY5647) or *psh1Δ* (SBY 8356) cells and probed for the presence of Cse4 with anti-Cse4 antibodies. Cells lacking Spt16-FLAG were used as a control (SBY3). The stability of Myc-Cse4 was assayed in wild-type (SBY3570) and *spt16-13* (SBY8662) cells after repressing transcription and protein synthesis. The blot was probed with anti-Myc. Pgk1 is a loading control.

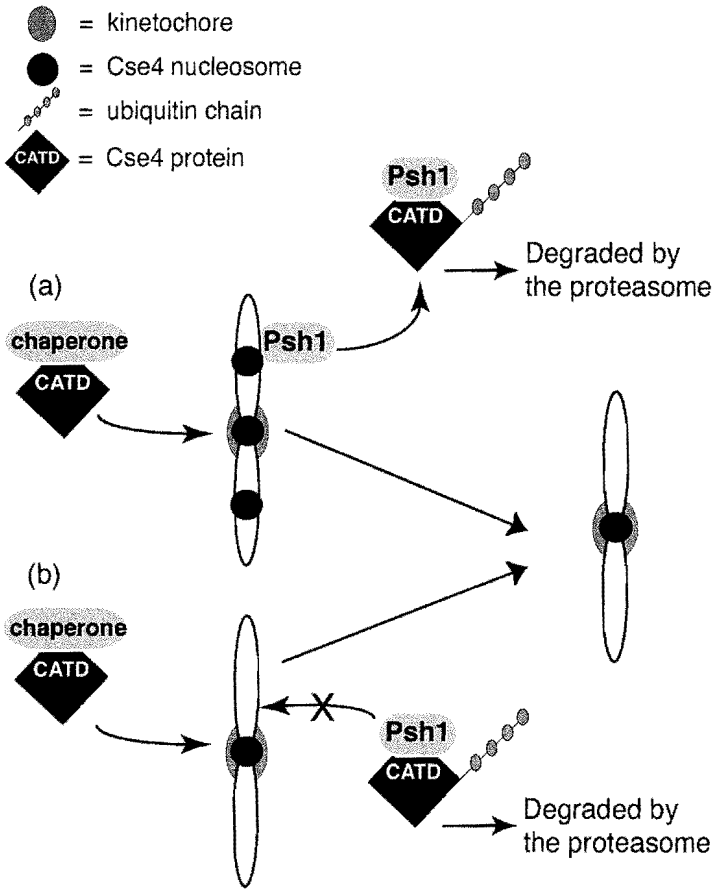


Fig 3.5. Psh1-mediated degradation of Cse4.
Cse4 recognition by its chaperone occurs via the CATD, which targets it to the centromere where it is protected from degradation by kinetochore assembly. Psh1 also recognizes Cse4 via the CATD. Psh1 prevents mislocalization of Cse4 in at least two possible ways, by (a) recognizing mis-incorporated Cse4 on the chromatin and targeting it for degradation, or by (b) by regulating the level of soluble Cse4, thereby limiting its misincorporation rate into the euchromatin. The exclusive localization of Cse4 to the centromere is therefore maintained by the CATD through a combination of targeting Cse4 to the centromere and degrading mislocalized Cse4.

Table 3.1 Yeast strains used in this study. All strains are isogenic with the W303 background. Plasmids are indicated in brackets.

Strain	Genotype
SBY3570	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 (pSB816, pGAL-13Myc-CSE4, URA3, 2μ)*
SBY5364	*MATα ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 hht1-hhf1::HYGRO hht2-hhf2::NAT (pSB977, T7-HHT1, untagged HHF1, TRP1, CEN)*
SBY5647	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1:LEU2, SPT16-3FLAG::KAN*
SBY7269	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG::TRP1 ura3-1::pGAL-13Myc-CSE4-NTD::URA3*
SBY7270	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG::TRP1 ura3-1::pGAL-13Myc-CSE4-HFD::URA3*
SBY8027	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 hht1-hhf1::HYGRO hht2-hhf2::NAT (pSB977, T7-HHT1, untagged HHF1, TRP1, CEN) PSH1-13Myc::HIS3*
SBY8356	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1:LEU2, SPT16-3FLAG::KAN, psh1::KAN*
SBY8700	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 pGAL-Myc- $Cse4^{NTD}$ –$Cse4^{Nhelix}$-$Cse4^{alpha1\ helix}$-$H3^{L1}$- $H3^{alpha2\ helix}$-$Cse4^{L2}$-$Cse4^{alpha3\ helix}$::URA3(pSB1646)*

SBY8760	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN pGAL-Myc- Cse4^{NTD} –Cse4 ^{Nhelix}-Cse4 $^{alpha1\ helix}$-H3^{L1}-H3 $^{alpha2\ helix}$ -Cse4 ^{L2}-Cse4$^{alpha3\ helix}$::URA3(pSB1646)*
SBY8932	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG::TRP1 ura3-1::pGAL-12Myc-H3^{NTD} -H3 ^{Nhelix}-H3 $^{alpha1\ helix}$-Cse4 ^{L1}- Cse4 $^{alpha2\ helix}$ -H3 ^{L2}-H3 $^{alpha3\ helix}$::URA3 (pSB1667)*
SBY8959	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 psh1::KAN ura3-1::pGAL-12Myc-H3^{NTD} -H3 ^{Nhelix}-H3 $^{alpha1\ helix}$-Cse4 ^{L1}- Cse4 $^{alpha2\ helix}$ -H3 ^{L2}-H3 $^{alpha3\ helix}$::URA3(pSB1667)*
SBY8994	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 ura3-1::pGAL-12Myc-H3^{NTD} -H3 ^{Nhelix}-H3 $^{alpha1\ helix}$-Cse4 ^{L1}- Cse4 $^{alpha2\ helix}$ -H3 ^{L2}-H3 $^{alpha3\ helix}$::URA3(pSB1667)*
SBY9126	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG:: TRP1 pGAL-Myc- Cse4^{NTD} –Cse4 ^{Nhelix}-Cse4 $^{alpha1\ helix}$-H3^{L1}- H3 $^{alpha2\ helix}$ -Cse4 ^{L2}-Cse4$^{alpha3\ helix}$::URA3(pSB1646)*
SBY9133	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 ura3-1::pGAL-Myc-HHT1::URA3 (pSB1704)*
SBY9134	*MAT**a** leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 PSH1-FLAG::TRP1 ura3-1::pGAL-Myc-HHT1::URA3 (pSB1704)*
SBY8662	*MAT**a** ura3-1 leu2,3-112 his3-11 trp1-1 ade2-1 can1-100 bar1-1 spt16-13 (pSB816, pGAL-13Myc-CSE4, URA3, 2μ)*

Table 3.2 Plasmids used in this study.

Plasmid	Description
pSB378	*pGAL-13Myc-CSE4-NTD (residues 1-129), URA3* (integrating)
pSB379	*pGAL-13Myc-CSE4-HFD (residues 130- 229), URA3* (integrating)
pSB893	*pGAL-HHT1, TRP* (integrating)
pSB1646	*pGAL-12Myc- Cse4^{NTD} –Cse4 ^{Nhelix}-Cse4 $^{alpha1\ helix}$- H3 ^{L1}- H3 $^{alpha2\ helix}$ -Cse4 ^{L2}- Cse4 $^{alpha\ 3\ helix}$, URA3* (integrating)
pSB1667	*pGAL-12Myc- H3^{NTD} -H3 ^{Nhelix}-H3 $^{alpha1\ helix}$- Cse4 ^{L1}- Cse4 $^{alpha2\ helix}$ -H3 ^{L2}- H3 $^{alpha\ 3\ helix}$, URA3* (integrating)
pSB1704	*pGAL-12Myc-HHT1, URA3* (integrating)

CHAPTER 4:

Conclusions and Perspective

In this dissertation, I have presented a detailed analysis of the role of the E3 ubiquitin ligase, Psh1 in regulating the centromeric histone H3 variant Cse4. Below, I highlight the important findings and speculate on future research.

Chapter 2: Identification of an E3 ligase that mediates Cse4 degradation and prevents ectopic localization of Cse4.

The centromeric histone H3 variant Cse4 is regulated by ubiquitin-mediated proteolysis (Collins et al., 2004b). In order to identify molecular machinery involved in the process, specifically the E3 ligase that imparts specificity of the substrate, we purified a lysine free Cse4 mutant and identified interacting partners by mass spectrometry. I identified Psh1 an E3 ligase that degrades Cse4. In contrast to wild-type cells, in the absence of Psh1, I observed mis-incoproration of overexpressed Cse4 into the euchromatin that results in lethality of these cells. I therefore propose that Psh1-mediated degradation of Cse4 prevents its misincorporation into ectopic sites, thereby ensuring exclusive localization of Cse4 to the centromere.

Although Psh1 and Cse4 can clearly associate with each other independent of the centromere, we cannot rule out the possibility of their association at the centromere. Consistent with this, a study from another group detected Psh1 at the centromere (Hewawasam et al., 2010). However, its centromeric function is not clear. Nevertheless,

the previously detected genomic instability in *psh1Δ* mutant cells is just as consistent with a function in preventing ectopic Cse4 localization as in regulating centromere function.

The presence of Psh1 at the centromere raises several interesting questions. How is the centromere bound Cse4 protected from degradation by Psh1? It was recently proposed that Scm3, the putative chaperone for Cse4, protects Cse4 from Psh1 mediated ubiquitination *in vitro* (Hewawasam et al., 2010). However, whether this protection is relevant to the centromeric pool of Cse4 is not known. In addition, the localization of Scm3 to the centromere appears to be cell cycle regulated (personal communication, Munira Basrai). Therefore, it will be important in the future to understand whether the centromeric localization of Psh1 is also cell cycle regulated. It is also possible that the activity of Psh1 maybe altered by post-translational modifications at the kinetochore or by restricting the localization of the E2 conjugating enzyme at the centromere. Interestingly, I have observed phosphorylation of Psh1 during metaphase, but the implication of this modification remains to be investigated (see Appendix B). It will also be interesting to investigate whether Psh1 has additional roles at the centromere.

In addition to the centromere, Cse4 also localizes to ectopic sites, although it is not completely clear what defines these sites. There is only a 10% correlation between ectopic sites and high histone turnover (Gkikopoulos et al., 2011), therefore additional factors must contribute to defining these sites. It will thus be informative to look at what happens to the distribution of Cse4 in the absence of proteolysis by Chip-seq experiments looking at genome wide Cse4 localization in wild-type and *psh1Δ* mutants.

Chapter 3: The centromere targeting domain of Cse4 regulates its stability and allows Psh1 to distinguish it from H3.

I observed that, in contrast to the overexpression of Cse4, overexpression of the canonical H3 had no effect on the growth of *psh1Δ* mutants. This led me to test whether Psh1 is a specific E3 ligase for Cse4 and if so, to elucidate the mechanism by which it discriminates between the two histones. Using domain swap experiments, I found that the centromere targeting domain (CATD) of Cse4 is necessary and sufficient for recognition by Psh1 and Psh1 mediated regulation of its stability. In addition, I also found that Psh1 serves as a physical link between Cse4 and FACT complex, a known chromatin remodeler. The increased stability of Cse4 in a mutant of the FACT complex indicate that FACT may cooperate with Psh1 to degrade the ectopically localized Cse4. Taken together, I propose that FACT destablilizes Cse4 nucleosomes exposing the CATD region of Cse4 for recognition by the associated Psh1 E3 ubiquitin ligase.

The underlying mechanism by which Psh1 recognizes and degrades Cse4 is not yet completely clear. My data is consistent with two possible mechanisms by which Psh1 prevents euchromatic accumulation of Cse4. Psh1 may target the soluble pool of Cse4 for degradation, thereby keeping the levels low and indirectly controlling the mis-incorporation rate of Cse4 at ectopic sites. Alternatively, Psh1 may directly recognize the ectopically localized Cse4 on the chromatin and target it for degradation. In the latter scenario, Psh1 has to be able to access the CATD of Cse4, which is not surface accessible

in the context of nucleosomal structure (Zhou et al., 2011). Therefore, FACT may provide access for Psh1 to bind to the CATD by destabilizing the nucleosome structure while degradation of Cse4 by Psh1 in turn ensures that Cse4 containing nucleosomes are not re-assembled by FACT after polymerases passage through the chromatin. This model also implicates transcription in removing ectopic Cse4, consistent with other studies where Cse4 accumulation in the euchromatin has been observed in mutants in the Spt4 protein that is involved in nucleosome assembly, nucleosome stabilization, and transcription (Crotti and Basrai, 2004; Hartzog et al., 2002; Swanson and Winston, 1992). Interestingly, *psh1* mutants also show negative genetic interactions with genes involved in transcription elongation (see appendix A). Therefore, the role of transcription in Cse4 degradation needs to be more directly tested both *in vivo* and *in vitro*.

Because the available FACT mutants have pleiotropic effects, it will be essential to first generate a separation of function allele of FACT subunit genes that specifically affect Cse4 degradation. Since Psh1 physically links Cse4 and FACT, a mutant of Psh1 that does not bind FACT can be used to directly test whether FACT cooperates with Psh1 in removing the ectopic Cse4 by performing similar experiments that we used for analyzing the role of Psh1. Similarly, Psh1 mediated *in vitro* ubiquitination experiments using Cse4 nucleosome arrays in the presence and absence of FACT should address whether destabilization of Cse4 nucleosomes by FACT is required for Cse4 ubiquitination by Psh1. This will also provide clues to the relevant form of Cse4 that acts as a substrate of Psh1. It will also be important to identify genomic sites where Cse4 accumulates both in the presence and absence of Psh1 as a first step towards directly

testing the role of transcription. Together, these experiments will give us insight into how proteolysis is coupled to transcription to specifically remove ectopic Cse4.

Reference

Akiyoshi, B., Nelson, C.R., Ranish, J.A., and Biggins, S. (2009). Quantitative proteomic analysis of purified yeast kinetochores identifies a PP1 regulatory subunit. Genes Dev *23*, 2887-2899.

Allshire, R.C., and Karpen, G.H. (2008). Epigenetic regulation of centromeric chromatin: old dogs, new tricks? Nat Rev Genet *9*, 923-937.

Amato, A., Schillaci, T., Lentini, L., and Di Leonardo, A. (2009). CENPA overexpression promotes genome instability in pRb-depleted human cells. Mol Cancer *8*, 119.

Amor, D.J., and Choo, K.H. (2002). Neocentromeres: role in human disease, evolution, and centromere study. Am J Hum Genet *71*, 695-714.

Au, W.C., Crisp, M.J., DeLuca, S.Z., Rando, O.J., and Basrai, M.A. (2008). Altered dosage and mislocalization of histone H3 and Cse4p lead to chromosome loss in Saccharomyces cerevisiae. Genetics *179*, 263-275.

Black, B.E., Foltz, D.R., Chakravarthy, S., Luger, K., Woods, V.L., Jr., and Cleveland, D.W. (2004). Structural determinants for generating centromeric chromatin. Nature *430*, 578-582.

Black, B.E., Jansen, L.E., Maddox, P.S., Foltz, D.R., Desai, A.B., Shah, J.V., and Cleveland, D.W. (2007). Centromere identity maintained by nucleosomes assembled with histone H3 containing the CENP-A targeting domain. Mol Cell *25*, 309-322.

Bouck, D.C., Joglekar, A.P., and Bloom, K.S. (2008). Design features of a mitotic spindle: balancing tension and compression at a single microtubule kinetochore interface in budding yeast. Annu Rev Genet *42*, 335-359.

Camahort, R., Li, B., Florens, L., Swanson, S.K., Washburn, M.P., and Gerton, J.L. (2007). Scm3 is essential to recruit the histone h3 variant cse4 to centromeres and to maintain a functional kinetochore. Mol Cell *26*, 853-865.

Camahort, R., Shivaraju, M., Mattingly, M., Li, B., Nakanishi, S., Zhu, D., Shilatifard, A., Workman, J.L., and Gerton, J.L. (2009). Cse4 is part of an octameric nucleosome in budding yeast. Mol Cell *35*, 794-805.

Cheeseman, I.M., and Desai, A. (2008). Molecular architecture of the kinetochore-microtubule interface. Nat Rev Mol Cell Biol *9*, 33-46.

Collins, K.A., Camahort, R., Seidel, C., Gerton, J.L., and Biggins, S. (2007). The overexpression of a Saccharomyces cerevisiae centromeric histone H3 variant mutant protein leads to a defect in kinetochore biorientation. Genetics.

Collins, K.A., Furuyama, S., and Biggins, S. (2004a). Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. Current biology : CB *14*, 1968-1972.

Crotti, L.B., and Basrai, M.A. (2004). Functional roles for evolutionarily conserved Spt4p at centromeres and heterochromatin in Saccharomyces cerevisiae. Embo J *23*, 1804-1814.

Deshaies, R.J., and Joazeiro, C.A. (2009). RING domain E3 ubiquitin ligases. Annu Rev Biochem *78*, 399-434.

Dunleavy, E.M., Roche, D., Tagami, H., Lacoste, N., Ray-Gallet, D., Nakamura, Y., Daigo, Y., Nakatani, Y., and Almouzni-Pettinotti, G. (2009). HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. Cell *137*, 485-497.

Dyer, P.N., Edayathumangalam, R.S., White, C.L., Bao, Y., Chakravarthy, S., Muthurajan, U.M., and Luger, K. (2004). Reconstitution of nucleosome core particles from recombinant histones and DNA. Methods Enzymol *375*, 23-44.

Earnshaw, W.C., and Rothfield, N. (1985). Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. Chromosoma *91*, 313-321.

Eng, J.K., Fischer, B., Grossmann, J., and Maccoss, M.J. (2008). A fast SEQUEST cross correlation algorithm. J Proteome Res *7*, 4598-4602.

Fitzgerald-Hayes, M., Clarke, L., and Carbon, J. (1982). Nucleotide sequence comparisons and functional analysis of yeast centromere DNAs. Cell *29*, 235-244.

Foltz, D.R., Jansen, L.E., Bailey, A.O., Yates, J.R., 3rd, Bassett, E.A., Wood, S., Black, B.E., and Cleveland, D.W. (2009). Centromere-specific assembly of CENP-a nucleosomes is mediated by HJURP. Cell *137*, 472-484.

Furuyama, S., and Biggins, S. (2007). Centromere identity is specified by a single centromeric nucleosome in budding yeast. Proc Natl Acad Sci U S A *104*, 14706-14711.

Gardner, R.G., Nelson, Z.W., and Gottschling, D.E. (2005). Degradation-mediated protein quality control in the nucleus. Cell *120*, 803-815.

Gkikopoulos, T., Singh, V., Tsui, K., Awad, S., Renshaw, M.J., Scholfield, P., Barton, G.J., Nislow, C., Tanaka, T.U., and Owen-Hughes, T. (2011). The SWI/SNF complex acts to constrain distribution of the centromeric histone variant Cse4. The EMBO journal.

Goh, P.Y., and Kilmartin, J.V. (1993). NDC10: a gene involved in chromosome segregation in Saccharomyces cerevisiae. J. Cell Biol. *121*, 503-512.

Hartzog, G.A., Speer, J.L., and Lindstrom, D.L. (2002). Transcript elongation on a nucleoprotein template. Biochim Biophys Acta *1577*, 276-286.

Heun, P., Erhardt, S., Blower, M.D., Weiss, S., Skora, A.D., and Karpen, G.H. (2006a). Mislocalization of the Drosophila Centromere-Specific Histone CID Promotes Formation of Functional Ectopic Kinetochores. Developmental cell *10*, 303-315.

Hewawasam, G., Shivaraju, M., Mattingly, M., Venkatesh, S., Martin-Brown, S., Florens, L., Workman, J.L., and Gerton, J.L. (2010). Psh1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. Mol Cell *40*, 444-454.

Kall, L., Canterbury, J.D., Weston, J., Noble, W.S., and MacCoss, M.J. (2007). Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat Methods *4*, 923-925.

Kall, L., Storey, J.D., MacCoss, M.J., and Noble, W.S. (2008a). Assigning significance to peptides identified by tandem mass spectrometry using decoy databases. J Proteome Res *7*, 29-34.

Kall, L., Storey, J.D., MacCoss, M.J., and Noble, W.S. (2008b). Posterior error probabilities and false discovery rates: two sides of the same coin. J Proteome Res *7*, 40-44.

Kellogg, D.R., Kikuchi, A., Fujii-Nakata, T., Turck, C.W., and Murray, A.W. (1995). Members of the NAP/SET family of proteins interact specifically with B-type cyclins. J. Cell Biol. *130*, 661-673.

Kellogg, D.R., and Murray, A.W. (1995). NAP1 acts with Clb2 to perform mitotic functions and suppress polar bud growth in budding yeast. J. Cell Biol. *130*, 675-685.

Klammer, A.A., and MacCoss, M.J. (2006). Effects of modified digestion schemes on the identification of proteins from complex mixtures. J Proteome Res *5*, 695-700.

Krogan, N.J., Kim, M., Ahn, S.H., Zhong, G., Kobor, M.S., Cagney, G., Emili, A., Shilatifard, A., Buratowski, S., and Greenblatt, J.F. (2002). RNA polymerase II elongation factors of Saccharomyces cerevisiae: a targeted proteomics approach. Mol Cell Biol *22*, 6979-6992.

Lefrancois, P., Euskirchen, G.M., Auerbach, R.K., Rozowsky, J., Gibson, T., Yellman, C.M., Gerstein, M., and Snyder, M. (2009). Efficient yeast ChIP-Seq using multiplex short-read DNA sequencing. BMC Genomics *10*, 37.

Liang, C., and Stillman, B. (1997). Persistent initiation of DNA replication and chromatin-bound MCM proteins during the cell cycle in cdc6 mutants. Genes Dev *11*, 3375-3386.

Loidl, J., Klein, F., and Engebrecht, J. (1998). Genetic and morphological approaches for the analysis of meiotic chromosomes in yeast. Methods Cell Biol *53*, 257-285.

Lomonte, P., Sullivan, K.F., and Everett, R.D. (2000). Degradation of nucleosome-associated centromeric histone H3-like protein CENP-A induced by herpes simplex virus type 1 protein ICP0. J Biol Chem *276*, 5829-5835.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251-260.

Maehara, K., Takahashi, K., and Saitoh, S. (2010). CENP-A reduction induces a p53-dependent cellular senescence response to protect cells from executing defective mitoses. Mol Cell Biol *30*, 2090-2104.

Moreno-Moreno, O., Torras-Llort, M., and Azorin, F. (2006). Proteolysis restricts localization of CID, the centromere-specific histone H3 variant of Drosophila, to centromeres. Nucleic Acids Res *34*, 6247-6255.

Mythreye, K., and Bloom, K.S. (2003). Differenctial kinetochore protein requirements for establishment versus propagation of centromere activity in *Saccharomyces cerevisiae*. J. Cell Biol. *160*, 833-843.

Osley, M.A. (1991). The regulation of histone synthesis in the cell cycle. Annu Rev Biochem *60*, 827-861.

Palmer, D.K., O'Day, K., Wener, M.H., Andrews, B.S., and Margolis, R.L. (1987). A 17-kD centromere protein (CENP-A) copurifies with nucleosome core particles and with histones. J Cell Biol *104*, 805-815.

Ranjitkar, P., Press, M.O., Yi, X., Baker, R., MacCoss, M.J., and Biggins, S. (2010). An E3 ubiquitin ligase prevents ectopic localization of the centromeric histone H3 variant via the centromere targeting domain. Mol Cell *40*, 455-464.

Reinberg, D., and Sims, R.J., 3rd (2006). de FACTo nucleosome dynamics. J Biol Chem *281*, 23297-23301.

Rose, M.D., Winston, F., and Heiter, P. (1990). Methods in yeast genetics (Cold Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press).

Santaguida, S., and Musacchio, A. (2009). The life and miracles of kinetochores. The EMBO journal *28*, 2511-2531.

Sherman, F., Fink, G., and Lawrence, C. (1974). Methods in Yeast Genetics (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Shivaraju, M., Camahort, R., Mattingly, M., and Gerton, J.L. (2011). Scm3 is a centromeric nucleosome assembly factor. J Biol Chem *286*, 12016-12023.

Shuaib, M., Ouararhni, K., Dimitrov, S., and Hamiche, A. (2010). HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. Proc Natl Acad Sci U S A *107*, 1349-1354.

Singh, R.K., Kabbaj, M.H., Paik, J., and Gunjan, A. (2009). Histone levels are regulated by phosphorylation and ubiquitylation-dependent proteolysis. Nat Cell Biol *11*, 925-933.

Stoler, S., Keith, K.C., Curnick, K.E., and Fitzgerald-Hayes, M. (1995a). A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. Genes Dev *9*, 573-586.

Stoler, S., Keith, K.C., Curnick, K.E., and Fitzgerald-Hayes, M. (1995b). A mutation in CSE4, an essential gene encoding a novel chromatin-associated protein in yeast, causes chromosome nondisjunction and cell cycle arrest at mitosis. Genes & development *9*, 573-586.

Stoler, S., Rogers, K., Weitze, S., Morey, L., Fitzgerald-Hayes, M., and Baker, R.E. (2007). Scm3, an essential Saccharomyces cerevisiae centromere protein required for G2/M progression and Cse4 localization. Proc Natl Acad Sci U S A *104*, 10571-10576.

Swanson, M.S., and Winston, F. (1992). SPT4, SPT5 and SPT6 interactions: effects on transcription and viability in Saccharomyces cerevisiae. Genetics *132*, 325-336.

Tanaka, T., Cosma, M.P., Wirth, K., and Nasmyth, K. (1999). Identification of cohesin association sites at centromeres and along chromosome arms. Cell *98*, 847-858.

Tomonaga, T., Matsushita, K., Yamaguchi, S., Oohashi, T., Shimada, H., Ochiai, T., Yoda, K., and Nomura, F. (2003). Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. Cancer Res *63*, 3511-3516.

Van Hooser, A.A., Ouspenski, II, Gregson, H.C., Starr, D.A., Yen, T.J., Goldberg, M.L., Yokomori, K., Earnshaw, W.C., Sullivan, K.F., and Brinkley, B.R. (2001). Specification of kinetochore-forming chromatin by the histone H3 variant CENP-A. J Cell Sci *114*, 3529-3542.

VanDemark, A.P., Blanksma, M., Ferris, E., Heroux, A., Hill, C.P., and Formosa, T. (2006). The structure of the yFACT Pob3-M domain, its interaction with the DNA replication factor RPA, and a potential role in nucleosome deposition. Mol Cell *22*, 363-374.

Verdaasdonk, J.S., and Bloom, K. (2011). Centromeres: unique chromatin structures that drive chromosome segregation. Nat Rev Mol Cell Biol *12*, 320-332.

Vermaak, D., Hayden, H.S., and Henikoff, S. (2002a). Centromere targeting element within the histone fold domain of Cid. Molecular and cellular biology *22*, 7553-7561.

Warburton, P.E., Cooke, C.A., Bourassa, S., Vafa, O., Sullivan, B.A., Stetten, G., Gimelli, G., Warburton, D., Tyler-Smith, C., Sullivan, K.F., *et al.* (1997). Immunolocalization of CENP-A suggests a distinct nucleosome structure at the inner kinetochore plate of active centromeres. Curr Biol *7*, 901-904.

Westermann, S., Drubin, D.G., and Barnes, G. (2007). Structures and functions of yeast kinetochore complexes. Annu Rev Biochem *76*, 563-591.

Wieland, G., Orthaus, S., Ohndorf, S., Diekmann, S., and Hemmerich, P. (2004). Functional Complementation of Human Centromere Protein A (CENP-A) by Cse4p from Saccharomyces cerevisiae. Mol Cell Biol *24*, 6620-6630.

Winey, M., Mamay, C.L., O'Toole, E.T., Mastronarde, D.N., Giddings, T.H., Jr., McDonald, K.L., and McIntosh, J.R. (1995). Three-dimensional ultrastructural analysis of the Saccharomyces cerevisiae mitotic spindle. J Cell Biol *129*, 1601-1615.

Yuen, K.W., Warren, C.D., Chen, O., Kwok, T., Hieter, P., and Spencer, F.A. (2007). Systematic genome instability screens in yeast and their potential relevance to cancer. Proc Natl Acad Sci U S A *104*, 3925-3930.

Zeitlin, S.G., Baker, N.M., Chapados, B.R., Soutoglou, E., Wang, J.Y., Berns, M.W., and Cleveland, D.W. (2009). Double-strand DNA breaks recruit the centromeric histone CENP-A. Proc Natl Acad Sci U S A *106*, 15762-15767.

Zhang, B., Chambers, M.C., and Tabb, D.L. (2007). Proteomic parsimony through bipartite graph analysis improves accuracy and transparency. J Proteome Res *6*, 3549-3557.

Zhou, Z., Feng, H., Zhou, B.R., Ghirlando, R., Hu, K., Zwolak, A., Miller Jenkins, L.M., Xiao, H., Tjandra, N., Wu, C., and Bai, Y. (2011). Structural basis for recognition of centromere histone variant CenH3 by the chaperone Scm3. Nature *472*, 234-237.

Appendix A:

Alternate pathways of Cse4 degradation

Cse4 levels are only partially stabilized in *psh1Δ* mutant cells, indicative of the existence of additional pathways that degrade Cse4 that may or may not be ubiquitin dependent. I hypothesized that any such pathway will likely show negative genetic interaction with *psh1Δ* mutants. Given that it is not clear whether there is only one or several other pathways involved, we performed an E-map screen with *psh1Δ* mutant, in collaboration with Nevan Krogan (UCSF). E-map (epistatic miniarray profile) is a quantitative measurement, which identifies both negative and positive genetic interaction between a set of proteins and the severity of such interactions. Our goal was to identify and analyze genes that showed the strongest negative interaction with Psh1. The E-map screen identified 239 genes that displayed negative interaction and 57 genes with positive genetic interaction (Table 1 and 2). I present brief discussions of different classes of mutants that were identified in the screen and focus on genes that showed negative genetic interactions.

E3 ligases

The screen identified four genes that have E3 ligase activity or have been shown to be involved in ubiquitin-mediated degradation pathway. Bre1 is an E3 ligase that mediates H2B-K123 mono-ubiquitination (Kim and Roeder, 2009). Hex3/Slx5 is a Subunit of the Slx5-Slx8 SUMO-targeted ubiquitin ligase (STUbL) complex (Xie et al.,

2007). Ubx5 and Shp1 are Ubx (ubiquitin regulatory X) domain containing proteins that interact with Cdc48 to facilitate degradation of ubiquitylated proteins (Schuberth et al., 2004). In addition, I also compiled a list of known and potential E3 ligases in budding yeast (60 genes). In order to determine whether these proteins are involved in Cse4 degradation, I looked at the effect on viability of these mutants when Cse4 is overexpressed either individually or in combination with *psh1Δ* (data not shown). However, none of the E3 ligase mutants tested, including the four identified in the e-map screen, displayed reduced viability when Cse4 is overexpressed, suggesting that the alternate pathway may be ubiquitin independent (Table 3) .

Genes involved in transcription elongation

We also identified several genes implicated in transcription elongation, consistent with our hypothesis that transcription plays a role in removal of Cse4 from ectopic sites. Two components of the Paf1 complex (Cdc73 and Ccr4) and one subunit of the elongator complex (Hap2) showed negative genetic interaction with Psh1. Interestingly, Paf1 complex has been shown to function during elongation in conjuction with Spt4/5 complex and the FACT complex (Squazzo et al., 2002). Given this, these mutants can be used to directly test the role of transcription elongation in Cse4 regulation in combination with the studies discussed in chapter 4.

Reference

Kim, J., and Roeder, R.G. (2009). Direct Bre1-Paf1 complex interactions and RING finger-independent Bre1-Rad6 interactions mediate histone H2B ubiquitylation in yeast. J Biol Chem *284*, 20582-20592.

Schuberth, C., Richly, H., Rumpf, S., and Buchberger, A. (2004). Shp1 and Ubx2 are adaptors of Cdc48 involved in ubiquitin-dependent protein degradation. EMBO Rep *5*, 818-824.

Squazzo, S.L., Costa, P.J., Lindstrom, D.L., Kumer, K.E., Simic, R., Jennings, J.L., Link, A.J., Arndt, K.M., and Hartzog, G.A. (2002). The Paf1 complex physically and functionally associates with transcription elongation factors in vivo. The EMBO journal *21*, 1764-1774.

Xie, Y., Kerscher, O., Kroetz, M.B., McConchie, H.F., Sung, P., and Hochstrasser, M. (2007). The yeast Hex3.Slx8 heterodimer is a ubiquitin ligase stimulated by substrate sumoylation. J Biol Chem *282*, 34176-34184.

Table 1: Genes that show negative genetic interaction with Psh1. Genes are listed from most negative to least as shown by the scores.

Gene	Score	Gene	Score	Gene	Score
LSM1	-19.4	MMS22	-6.1	YIL110W	-4.5
HAP2	-19.1	TFP1	-6.0	PHO88	-4.4
GSH2	-18.5	GGA2	-6.0	BIK1	-4.4
BUB3	-17.1	IES2	-5.9	BUD13	-4.4
PAM17	-16.8	DIA2	-5.9	SSN2	-4.3
NOP12	-15.4	RPL43A	-5.8	MMR1	-4.3
BUB1	-14.7	EAP1	-5.8	TEX1	-4.3
PEX15	-14.0	RAD52	-5.8	HCR1	-4.3
BIM1	-13.9	RIM21	-5.8	YER156C	-4.3
RTS1	-12.8	PDA1	-5.7	SUM1	-4.2
BEM1	-12.6	MRT4	-5.7	GCN5	-4.1
HST1	-12.5	ICE2	-5.6	PML39	-4.1
APM4	-12.1	RRP6	-5.5	PFK2	-4.1
PRS5	-12.1	VPS24	-5.5	YPR045C	-4.1
PSK2	-11.5	HTZ1	-5.5	SNF6	-4.1
MET22	-11.4	RPA34	-5.4	FPS1	-4.0
YLR426W	-10.1	REX4	-5.3	PDR16	-4.0
SIL1	-10.0	YOR006C	-5.2	IKI3	-4.0
TAT2	-9.7	RPL19B	-5.2	ARC1	-4.0
LGE1	-9.1	SIC1	-5.2	MRC1	-4.0
OPI10	-9.1	RPS16A	-5.2	YKE2	-4.0
CSM1	-9.0	MON2	-5.2	RPN10	-3.9
RPB9	-8.4	PEX17	-5.2	SNX4	-3.9
HHF1	-8.2	UBX5	-5.1	RPS4A	-3.9
STE50	-8.0	YHL029C	-5.1	ELP6	-3.9
MDM38	-7.9	RPS16B	-5.0	TYE7	-3.9
ARP6	-7.9	SAS2	-5.0	CRN1	-3.9
SRO7	-7.7	TUB3	-4.9	GET2	-3.9
RPP1B	-7.5	RTT109	-4.9	SSF1	-3.8
LSM6	-7.2	IKI1	-4.9	KTI12	-3.8
INP54	-7.2	VAM6	-4.8	MAK31	-3.8
YGL046W	-7.1	ELP3	-4.8	DEP1	-3.8
RPS28B	-6.7	BUD27	-4.8	CTF4	-3.8
PUF4	-6.6	PPZ1	-4.7	ADA2	-3.8
RTG1	-6.6	MON1	-4.7	PLB3	-3.8
ASF1	-6.6	PMP3	-4.6	HAP4	-3.8

SHP1	-6.6	TPS1	-4.6	YBR238C	-3.8
RPO41	-6.3	SWR1	-4.6	YAR1	-3.8
RPS6A	-6.1	ATG17	-4.5	LPD1	-3.7
RPL16B	-3.7	EAF6	-3.2	XRS2	-2.8
YNR004W	-3.7	GIS2	-3.2	CDC26	-2.8
VPS75	-3.7	COS6	-3.1	MET18	-2.8
HSC82	-3.7	SAM37	-3.1	CLB3	-2.8
TCO89	-3.7	IWR1	-3.1	CDC73	-2.8
RSA3	-3.6	RMI1	-3.1	PUS1	-2.8
BUD31	-3.6	SGM1	-3.1	RPS30A	-2.8
YER064C	-3.6	YAL027W	-3.0	PEX10	-2.8
YKR074W	-3.6	TOF2	-3.0	POL2	-2.8
DSS4	-3.6	MKS1	-3.0	SIF2	-2.8
RAD1	-3.5	MSH2	-3.0	GCR2	-2.8
YAF9	-3.5	SCS2	-3.0	TOP3	-2.7
TAF10	-3.5	VPS71	-3.0	SGF11	-2.7
TEP1	-3.5	GOS1	-3.0	RPS10B	-2.7
BRE1	-3.5	NGG1	-3.0	GDS1	-2.7
PEX32	-3.4	DJP1	-3.0	PEX6	-2.7
LSM7	-3.4	BST1	-3.0	WHI5	-2.7
SPT21	-3.4	URE2	-2.9	RPL11B	-2.7
RIM101	-3.4	HFI1	-2.9	VIK1	-2.7
RPL9A	-3.4	KAP120	-2.9	ASM4	-2.7
SNT2	-3.4	PAC10	-2.9	TIF2	-2.6
CCR4	-3.4	DOC1	-2.9	SIW14	-2.6
RPB7	-3.4	GIM3	-2.9	VPS41	-2.6
DBP3	-3.4	IMG2	-2.9	NKP2	-2.6
PEA2	-3.4	IXR1	-2.9	SIN3	-2.6
ARO1	-3.4	RPP2B	-3.2	GEF1	-2.6
KEM1	-3.4	YDR128W	-3.2	ERV46	-2.6
TRM10	-3.4	BUD21	-3.2	IPK1	-2.6
CBP4	-3.3	TLG2	-3.2	HEX3	-2.6
LAT1	-3.3	APE3	-3.1	PLP1	-2.6
RAD27	-3.3	NCS2	-3.1	DBF2	-2.5
SET2	-3.3	PSY2	-3.1	DPB2	-2.5
BTS1	-3.3	RPO21	-3.1	ATG21	-2.5
RPB3	-3.3	RPL23A	-3.1	HTA1	-2.5
KIP3	-3.3	RRP8	-2.9	STD1	-2.5
NUP170	-3.3	IST3	-2.9	ELP2	-2.5

YPL158C	-3.3	MNN10	-2.9	SEY1	-2.5
YEL033W	-3.3	RRM3	-2.9	MDM35	-2.5
RPS29A	-3.3	RPL34B	-2.8	VAM3	-2.5
CGR1	-3.3	MNS1	-2.8	RAD6	-2.5
RRD2	-3.3	MUS81	-2.8		

Table 2: Genes that show positive genetic interaction with Psh1. Genes are listed from most positive to least as shown by the scores.

Gene	Score	Gene	Score
RAD4	12.5	DSE3	2.6
RSC1	11.4	SLA1	2.6
YKL023W	8.9	SKI7	2.5
ASE1	6.7	RLF2	2.5
DBP7	6.1	MMS4	2.5
VPS35	3.7	LRS4	2.5
CKA1	3.6	YBL036C	2.5
PBP1	3.4	CCW12	2.5
KIN4	3.3	KTR2	2.4
KEX2	3.2	OCA1	2.4
FEN1	3.1	YGR237C	2.3
SPT23	3.1	GPB1	2.3
RBL2	3.0	CHS6	2.3
BYE1	3.0	VPS70	2.3
WSC4	3.0	YHR009C	2.3
CDC23	2.9	RPL8A	2.2
HAL5	2.8	VPS17	2.2
VPS51	2.8	SOY1	2.2
CHK1	2.8	GFD1	2.2
CBF1	2.8	MED4	2.2
ELA1	2.8	RAS2	2.2
OAF1	2.8	UBC1	2.1
RPL19A	2.7	PUF6	2.1
RBK1	2.7	MCM21	2.1
YEL007W	2.7	TAF13	2.0
CDC4	2.7	YIP5	2.0
IOC3	2.6	PHB2	2.0
CDC16	2.6	YMR102C	2.0
ERG2	2.6		

Table 3: List of E3 ligases tested with overexpression of Cse4

AIR1	HUL4	PIB2	SET3	UFD4
ASI1	HUL5	PSP5	SLX1	VPS8
ASR1	ITT1	PXL1	SLX8	YBR062C
BRE1	MAG2	PRP19	SNT2	YDR128W
CST9	MOT2	RAD5	SSM4	YDR266C
DMA1	NFI1	RAD16	STE5	YER051W
DMA2	PEP3	RAD18	TFB3	YHL010C
ECM5	PEP5	RCO1	TOM1	YIL079C
FAP1	PEX2	RIS1	TUL1	YJR119C
FAR1	PEX2	ROD1	UBR1	YKR017C
HEX3	PEX10	RKR1	UBR2	YLR247C
HRD1	PEX12	SAD1	UBX5	YMR187C
HRT1	PIB1	SAN1	UFD2	YOL138C

Appendix B:

Miscellanous data on Psh1

I. *Observations on cell cycle dependent interaction of Psh1, Cse4 and the FACT complex.*

In order to test if Psh1 and Cse4 interaction is cell cycle dependent, I performed immuno-precipitation experiments from cells arrested at various cell cycle stages. Psh1-Myc was immuno-precipitated from asynchronous, G1 (a-factor treated), S (HU treated) or M (Nocodozole) arrested cells and probed for the presence of Cse4. The interaction between Psh1 and Cse4 appears to be strongest during mitosis and weak in G1 and S phases (Fig. 1A), although Psh1 levels appear to be lower in G1. Consistent with Psh1 mediating the interaction between Cse4 and the FACT complex, the interaction between Cse4 and Spt16 also appears to be the strongest in mitosis and S phases and weak in G1 (Fig. 1B). Interestingly, Psh1 appears to be phosphorylated during mitosis, although the functional relevance or the kinase involved is not known (Fig. 1C). It will be interesting in the future to test whether phosphorylation of Psh1 is involved in regulating the interaction between these proteins and also investigate whether the cell cycle dependent interaction has relevance to Cse4 degradation or to localization of Psh1 to the centromere or the euchromatin.

II. *Co-overexpression of Mif2 exacerbates the toxicity caused by overexpression of Cse4 in psh1Δ cells.*

The severity of the toxicity of Cse4 overexpression in *psh1Δ* cells is correlated with Cse4 protein levels. Lower levels of Cse4 overexpression in *psh1Δ* cells reduces their viability but is not completely lethal. I speculated that other mechanisms that exist for Cse4 removal are able to compensate for absence of proteolysis when Cse4 is overexpressed at lower levels, perhaps by preventing stable incorporation of Cse4 in the euchromatin. We tested if Cse4 can be stabilized when a kinetochore protein is simultaneously overexpressed with Cse4. It has been shown that the human Cenp-C and Cenp-N proteins bind directly to Cenp-A nucleosomes and are good candidates to directly bind and stabilize Cenp-A nucleosomes (Carroll et al., 2010). I therefore chose to look at the yeast homolog of Cenp-C, Mif2. We found that co-overexpression of Mif2 and Cse4 exacerbates the decrease in viability of *psh1Δ* cells seen when Cse4 alone is overexpressed (Fig. 2A). In contrast, there is no effect on the viability of WT cells. However, when we looked by chromosome spreads and chromatin fractionation, we did not observe any significant increase in the level of Cse4 in the chromatin when Mif2 was simultaneously overexpressed (Fig. 2B and data not shown). A more quantitative approach such as Chip-seq may be needed to see the difference in Cse4 levels. *In vitro* experiments suggests that CENP-A/H4 tetramers are more readily destabilized relative to H3/H4 tetramers (Conde e Silva et al., 2007). Hence, it would be interesting to test if the presence of Mif2, increases the stability of Cse4/H4 tetramers in the assay. Although we still do not understand what the observed genetic interaction means, it will be interesting in the future to investigate this further.

Reference

Carroll, C.W., Milks, K.J., and Straight, A.F. (2010). Dual recognition of CENP-A nucleosomes is required for centromere assembly. J Cell Biol *189*, 1143-1155.

Conde e Silva, N., Black, B.E., Sivolob, A., Filipski, J., Cleveland, D.W., and Prunell, A. (2007). CENP-A-containing nucleosomes: easier disassembly versus exclusive centromeric localization. J Mol Biol *370*, 555-573.

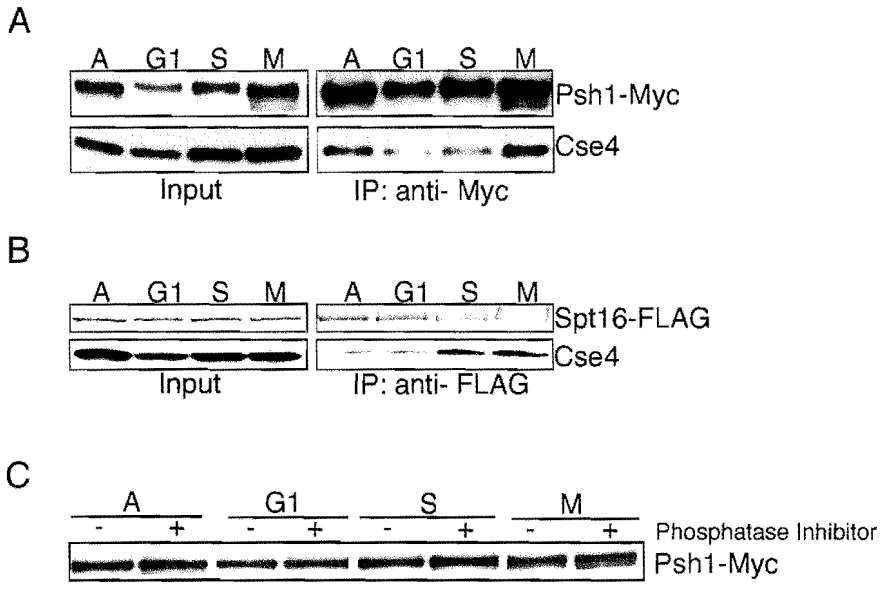


Fig. 1. Cell cycle dependent interaction of Psh1, Cse4 and Spt16.

(A) Psh1-Myc (SBY 6423) was immunoprecipitated from asynchronous (A), G1, S or M phase arrested cells and probed for the presence of Cse4. (B) Spt16-FLAG (SBY5647) was immunoprecipitated as in (A) and probed for the presence of Cse4. (C) Lysates from A, G1, S or M arrested cells were prepared (in lysis buffer) in the presence or absence of phosphatase inhibitors. Samples were then incubated at 30 °C for 1 hour before adding sample buffer. Blot was probed with anti-Myc antibodies. The phosphorylated form of Psh1 appears to migrate faster than the unphosphorylated form.

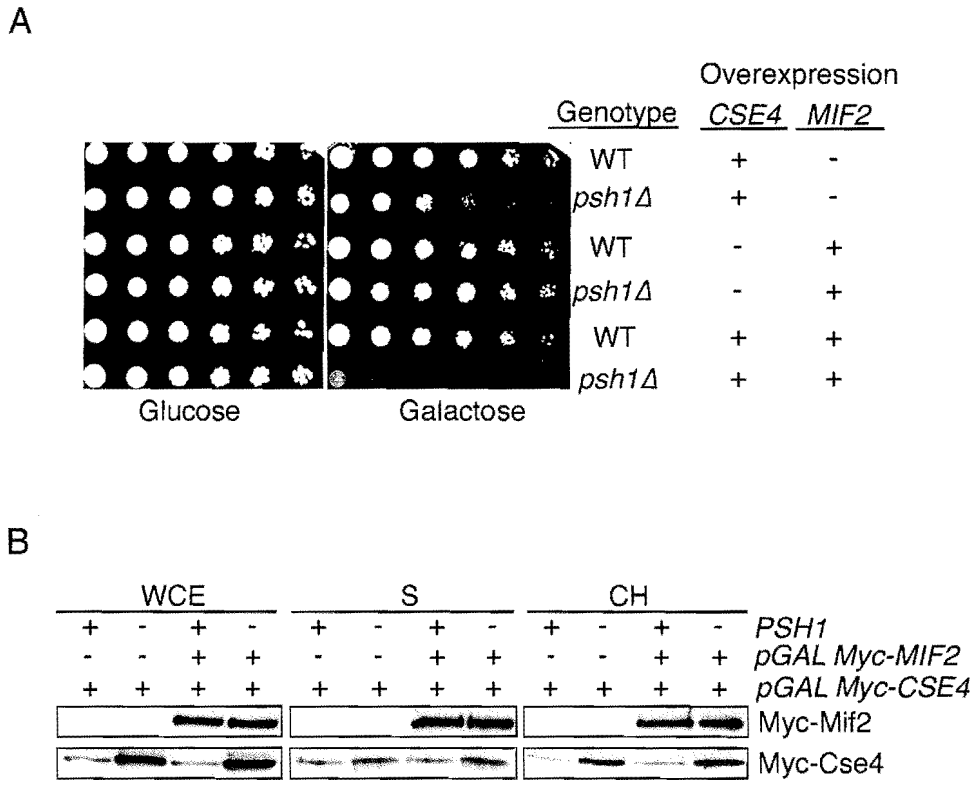


Fig. 2: Co-overexpression of Mif2 and Cse4 in *psh1*Δ.

(A) 5-fold serial dilution of WT+ *pGAL Myc-CSE4* (SBY 8783), *psh1*Δ + *pGAL Myc-CSE4* (SBY 8779), WT+ *pGAL Myc-MIF2* (SBY 8818), *psh1*Δ + *pGAL Myc-MIF2* (SBY8819), WT + *pGAL Myc-CSE4/pGAL Myc-MIF2* (SBY8768) *psh1*Δ + *pGAL Myc-CSE4/pGAL Myc-MIF2* (SBY8769) plated on glucose or galactose. (B) Chromatin fractionation of WT or *psh1*Δ cells either overexpressing Cse4 alone or with Mif2. Strains used in this experiment are the same as in (A). Blots were probed with anti -Myc antibodies.

Appendix C:

Sumoylation of Cse4

Introduction

Post-translational modifications of histones play various roles in numerous cellular processes. While a large number of post-translational modifications on canonical histones have been identified, very few are known to occur on the centromeric histone H3 variant, CENP-A to date.

Phosphorylation and ubiquitination are two modifications known to occur on CENP-A. Phosphorylation of CENP-A occurs on Ser7 and Ser50 in humans and maize respectively. Human CENP-A is phosphorylated on Ser7 by Aurora A, which appears to prime CENP-A for subsequent phosphorylation by Aurora B. Phosphorylation of CENP-A appears to be involved in localizing Aurora B to inner centromeres and is important for localizing AuroraB, INCENP and PP1from chromosomes to the spindle midzone. Because the CENP-A phosphorylation sites are present in the highly divergent N-terminal tail, it is not clear whether phosphorylation of CENP-A is conserved in other organisms (Kotwaliwale and Biggins, 2009)

Ubiquitination of CENP-A occurs in budding yeast and flies where it leads to proteasome mediated degradation of Cse4 and CID respectively. While mutation of all the ubiquitin sites on Cse4 still fails to completely stablilize the

protein, a recent study identified K163 of Cse4 to be the preferential lysine on Cse4 targeted by the Psh1 E3 ubiquitin ligase (Hewawasam et al., 2010). In addition to K163, K4, K131, K155 and K172 were also determined to be ubiquitination sites on Cse4 by mass spectrometry.

Here, I present some preliminary data demonstrating that Cse4 is sumoylated *in vivo*. Interestingly, the sumoylated form of Cse4 appears to be more prominent in the absence of ubiquitination by Psh1.

Sumoylation is a post-translational modification of a protein whereby the small ubiqutin-related modifier (SUMO) is conjugated to a substrate via a series of enzymatic reactions that are analogous to the ubiquitination reaction. SUMO is first processed into its mature form by ubiquitin-like-protein-specific proteases (Ulps) and sentrin-specific proteases (SENPs), revealing a carboxy terminal Gly-Gly motif. The mature SUMO is then activated by SUMO-activating enzyme E1 complex (Uba2/Aos1), followed by transfer onto an E2 conjugating enzyme (ubc9) and finally to the substrate either directly or via an E3 ligase (for review, see (Gareau and Lima, 2010). SUMO modification of a substrate can lead to diverse cellular fate of the substrate including degradation. SUMO-modified substrates can be recognized by SUMO dependent E3 ubiquitin ligases that can ubiquitinate the substrate and target it for degradation by the proteasome (Uzunova et al., 2007).

Results

As presented in chapter 2, when Myc-Cse4 is immunoprecipitated from wild-type cells, Cse4 ubiquitin conjugates can be detected. In contrast, in the absence of Psh1, these conjugates are completely absent. However, when FLAG-tagged Cse4 (endogeneous) was immuno-precipitated in a similar manner, I detected additional bands in the psh1 mutant that runs higher than the ubiquitin conjugates (see chapter 2 Fig 2.4B). Based on the molecular weight, I speculated that it maybe be a sumoylated form of Cse4. In order to verify whether this is indeed SUMO conjugate of Cse4, I tested whether this band becomes undetectable in the absence of sumoylation. In budding yeast, SUMO is encoded by the essential gene SMT3. I immuno-precipitated endogenous FLAG-Cse4 from *smt3-331*, a temperature sensitive mutant of SMT3, and in *psh1Δsmt3-331* double mutant, after inactivating smt3 (data not shown). However, the higher molecular weight band was still visible leading me to assay the effect on newly synthesized Cse4 upon inactivation of SMT3. When *pGAL-FLAG-CSE4* was induced in wild-type, *psh1Δ, smt3-331 and psh1Δsmt3-331* cells after inactivating SMT3 and immuno-precipitated using anti-FLAG antibodies, the higher molecular weight band that is present in *psh1Δ* was not detectable in *psh1Δsmt3-331* double mutant indicating that it is a sumoylated form of Cse4. I further verified this using antibodies specific to Smt3 (Fig. 1). Furthermore, in *smt3-331*, the ubiquitin

conjugates of Cse4 were still detectable, given that Psh1-dependent ubiquitination is still intact in these mutants. Taken together, my data suggests that sumoylation is not a priming signal for Psh1-mediated ubiquitination of Cse4 per se, rather sumoylation of Cse4 appears to occur in the absence of Psh1-mediated ubiquitination. Because, sumoylation was only detected on FLAG-Cse4 but not on Myc-Cse4, I also confirmed that it was not occurring on the FLAG tag by performing the above experiment with FLAG-Cse416R in which the only lysines available for sumoylation is in the FLAG epitope. Higher molecular weight species were not detected in either wild-type or the psh1 mutant suggesting that the FLAG epitope is not being sumoylated (Fig. 2)

Because sumoylation of a protein can lead to its ubiquitation and eventual degradation, I next investigated whether sumoylation of Cse4 also leads to its degradation. The stability of FLAG-Cse4 was assayed in WT, *psh1Δ, smt3-331 and psh1Δsmt3-331* (data not shown). As expected, Cse4 levels were partially stabilized in *psh1Δ* mutants compared to the WT. However, Cse4 stability did not increase in a double mutant of *psh1Δsmt3-331* indicating that sumoylation likely does not play a role in regulating Ce4 stability.

The consensus motif for sumoylation is ψKX(D/E), where ψ is a large hydrophobic residue. These residues are known to interact directly with the SUMO E2 and hence have a critical role in regulating the stability of the interaction of the E2 enzyme and the substrate. Cse4 has a motif in its globular

histone fold domain that closely resembles the SUMO consensus motif (MKKD). In order to test whether this is the sumoylation site on Cse4, I mutated the two lysines within the site (K215R and K216R) and tested whether this mutant is sumoylated *in vivo*. When FLAG-Cse4 (K215/216R) mutant is immuno-precipitated from wild-type and *psh1Δ* background, I was still able to detect sumoylated form of the Cse4 (K215/216R) mutant, indicating that these sites are not the SUMO sites on Cse4 or that in the absence of these sites another site is utilized (data not shown). Consistent with this, it is known that the SUMO consensus motif do not necessarily adhere to strict sequence requirements and most validated SUMO consensus sites on a substrate occur in extended loops or disordered regions of the substrate outside of its globular domain (Gareau and Lima, 2010). Therefore it will be important to identify the sites of sumoylation on Cse4 by mass spectrometry in the future to fully understand the role of this modification.

References

Kotwaliwale, C.V. and Biggins, S. (2009). Post-translational modifications that regulate kinetochore activity. In DeWulf, P.D and Earnshaw, W.C. The kinetochore. From Molecular Discoveries to Cancer Therapy (pp 293-344). New York: Springer Science+Business Media, LLC

Gareau, J.R., and Lima, C.D. (2010). The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nat Rev Mol Cell Biol *11*, 861-871.

Hewawasam, G., Shivaraju, M., Mattingly, M., Venkatesh, S., Martin-Brown, S., Florens, L., Workman, J.L., and Gerton, J.L. (2010). Psh1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. Mol Cell *40*, 444-454.

Uzunova, K., Gottsche, K., Miteva, M., Weisshaar, S.R., Glanemann, C., Schnellhardt, M., Niessen, M., Scheel, H., Hofmann, K., Johnson, E.S., *et al.* (2007). Ubiquitin-dependent proteolytic control of SUMO conjugates. J Biol Chem *282*, 34167-34175.

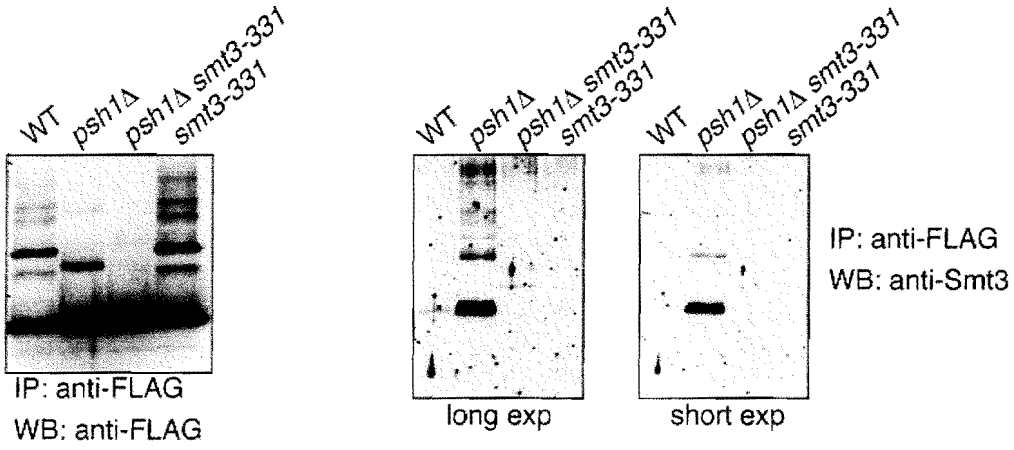


Fig.1. Sumoylation of Cse4.

WT (SBY8904), *psh1Δ* (SBY8903) , *psh1Δsmt3-331* (SBY9257) and *smt3-331* (SBY9259) cells carrying p*GAL-FLAG-CSE4* were grown in lactic acid media. The cells were shifted to 36 °C for 1.5 hours and Cse4 expression was induced for 1 hour. FLAG-Cse4 was immunopreciptated using anti-FLAG antibodies and probed with either anti-FLAG or anti-Smt3 antibodies (gift from Pam Meluh).

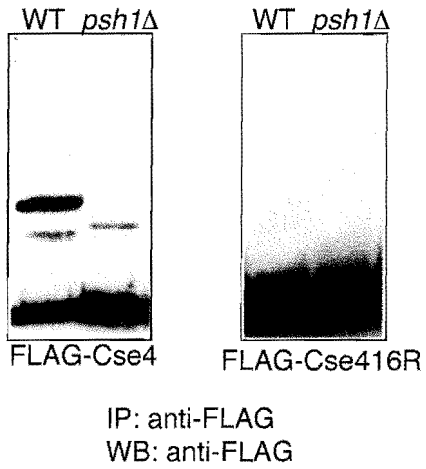


Fig. 2. Sumoylation of Cse4 is not occurring on the FLAG epitope.

WT and *psh1Δ,* cells carrying either p*GAL-FLAG-CSE4* (SBY8904 and SBY8903) or pCup-*FLAG-CSE416R* (SBY5442 and SBY9432) were grown in lactic acid media. cells Cse4 expression was induced for 1 hour. FLAG-Cse4 was immunopreciptated using anti-FLAG antibodies.

CURRICULUM VITAE

Prerana Ranjitkar

Education

2005-2011	Graduate Interdisciplinary Program: Molecular and Cellular Biology Program, University of Washington and Fred Hutchinson Cancer Research Center, Seattle. Graduate thesis work conducted in the Biggins Laboratory in the Basic Sciences Division at the Fred Hutchinson Cancer Research Center
2001-2005	2005 Bachelor of Sciences, Cum Laude with departmental honors

Professional Positions

2006-Present	Graduate Research Assistant, Fred Hutchinson Cancer Research Center, Seattle, WA, USA Advisor: Dr. Sue Biggins
2005-Present	Molecular and Cellular Biology Graduate Student, University of Washington, Seattle, WA, USA
2002-2003	Undergraduate Researcher, Institute of Neuroscience, Univ. of Oregon.
2003-2005	Undergraduate Researcher, Institute of Molecular Biology, Univ. of Oregon

Honors and Awards

2010Best	Talk by a Student or Post-Doc (2nd place), Workshop on Aneuploidy and Chromosome Segregation, Edinburgh.
2008-2011	Chromosome Metabolism and Cancer Training Grant, Fred Hutchinson Cancer Research Center
2005	Departmental Honors for Undergraduate Research, Univ. of Oregon.
2005	Graduated cum laude, Univ. of Oregon.
2005	Phi Beta Kappa Society Honors, Univ. of Oregon Chapter.

Publications

Ranjitkar P, Press MO, Xianhua Y, Baker R, MacCoss MJ and Biggins S. An E3 Ligase prevents ectopic localization of the centromeric histone H3 variant via the

centromere targeting domain. (Mol Cell. 2010 Nov 12;40(3):455-64) (Highlighted by Faculty of 1000)

Lectures and Presentations:

Talk at the Workshop on Aneuploidy and Chromosome Segregation, Edinburgh, Scotland 2010

Poster presentation at the ASCB Meeting, San Diego, USA 2009

Talk at the Seattle Mitosis Club, 2007 and 2009

Teaching and other Experience

2006 Fall term	Teaching Assistant, Biochemistry 440, Dr. Trisha Davis and Dr. Rachel Klevit, University of Washington
2007 Winter term	Teaching Assistant, Biochemistry 441, Dr. Ted Young and Dr. William Parson, University of Washington
2009 Summer	Supervised a high school summer intern.
2010	Weintraub Award Committee