SUMO-Targeted Ubiquitin Ligase (STUbL) Slx5 regulates proteolysis of centromeric histone H3 variant Cse4 and prevents its mislocalization to euchromatin

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Running Title: Slx5 regulates Cse4 proteolysis

Abbreviations used: SUMO, small ubiquitin-related modifier; STUbL, SUMO-targeted ubiquitin ligase; CATD, centromere-targeting domain; FACT, facilitates chromatin transcription/transactions; PTM, post-translational modification; CTF, chromosome transmission fidelity; CHX, cycloheximide; FRAP, fluorescence recovery after photobleaching.

Key Words: Slx5, Cse4, Psh1, sumoylation, ubiquitination

Abstract

Centromeric histone H3, CENP-A^{Cse4}, is essential for faithful chromosome segregation. Stringent regulation of cellular levels of CENP-A^{Cse4} restricts its localization to centromeres. Mislocalization of CENP-A^{Cse4} is associated with aneuploidy in yeast, flies and tumorigenesis in human cells; thus, defining pathways that regulate CENP-A levels is critical for understanding how mislocalization of CENP-A contributes to aneuploidy in human cancers. Previous work in budding yeast has shown that ubiquitination of overexpressed Cse4 by Psh1, an E3 ligase, partially contributes to proteolysis of Cse4. Here, we provide the first evidence that Cse4 is sumoylated by E3 ligases Siz1 and Siz2 *in vivo* and *in vitro.* Ubiquitination of Cse4 by Small Ubiquitin-related Modifier (SUMO)-Targeted Ubiquitin Ligase (STUbL) Slx5 plays a critical role in proteolysis of Cse4 and prevents mislocalization of Cse4 to euchromatin under normal physiological conditions. Accumulation of sumoylated Cse4 species and increased stability of Cse4 in *slx5*Δ strains suggest that sumoylation precedes ubiquitin-mediated proteolysis of Cse4. Slx5-mediated Cse4 proteolysis is independent of Psh1 since *slx5*Δ *psh1*Δ strains exhibit higher levels of Cse4 stability and mislocalization compared to either *slx5*Δ or *psh1*Δ strains. Our results demonstrate a role for Slx5 in ubiquitin-mediated proteolysis of Cse4 to prevent its mislocalization and maintain genome stability.

Introduction

Centromeres are specialized chromosomal loci that are essential for faithful chromosome segregation. The kinetochore (centromeric DNA and associated proteins) provides an attachment site for microtubules for segregation of sister chromatids during mitosis. Despite the wide divergence of centromere DNA sequences, kinetochore proteins such as centromeric histone H3 variant are evolutionarily conserved from yeast to humans (Cse4 in *Saccharomyces cerevisiae*, Cnp1 in *Schizosaccharomyces pombe*, CID in *Drosophila*, and CENP-A in mammals) and are essential for chromosome segregation (Kitagawa and Hieter, 2001; Smith, 2002; Biggins, 2013). The function of CENP-A is also evolutionarily conserved as budding yeast Cse4 can rescue a depletion of mammalian CENP-A (Wieland *et al.*, 2004).

Stringent regulation of CENP-A expression is essential for genome stability. Overexpression of CENP-A causes ectopic mislocalization to chromosome arms and promotes aneuploidy in humans, flies, and yeast (Scott and Sullivan, 2014). Overexpression and mislocalization of CENP-A are observed in many cancers and contribute to tumorigenesis in human cells (Tomonaga *et al.*, 2003; Amato *et al.*, 2009; Hu *et al.*, 2010; Li *et al.*, 2011; Wu *et al.*, 2012; Lacoste *et al.*, 2014; Athwal *et al.*, 2015). In flies, mislocalization of CID causes formation of ectopic kinetochores and leads to mitotic delays, anaphase bridges, chromosome fragmentation, aneuploidy and lethality (Heun *et al.*, 2006). In fission yeast, overexpression of Cnp1 leads to indiscriminate deposition of Cnp1 at non-centromeric regions resulting in growth defects and severe chromosome missegregation during mitosis and meiosis (Choi *et al.*, 2012; Castillo *et al.*, 2013; Gonzalez *et al.*, 2014). In budding yeast, mislocalization of Cse4 to euchromatin leads to chromosome segregation defects and the extent of Cse4 mislocalization directly correlates with the level of chromosome loss (Au *et al.*, 2008). Furthermore, various

pathways involving kinetochore protein Spt4 (Crotti and Basrai, 2004), histone chaperones Cac1 and Hir1 (Sharp *et al.*, 2002; Lopes da Rosa *et al.*, 2011), and chromatin remodeler Snf2 (Gkikopoulos *et al.*, 2011) act to prevent the mislocalization of Cse4.

Protein post-translational modifications (PTMs), such as ubiquitination (Kerscher *et al.*, 2006), are important for regulating steady-state levels and preventing mislocalization. For example, proteolysis of CID prevents its mislocalization to ectopic regions in flies (Heun *et al.*, 2006; Moreno-Moreno *et al.*, 2011). Proteolysis of CENP-A has also been observed in senescent human cells or upon infection with herpes simplex virus 1 (Lomonte *et al.*, 2001; Maehara *et al.*, 2010). In fission yeast, the N-terminus of Cnp1 regulates ubiquitin-mediated proteolysis of Cnp1 and prevents its mislocalization to ectopic loci (Gonzalez *et al.*, 2014). In budding yeast, ubiquitin-mediated proteolysis of Cse4 by E3 ubiquitin ligase Psh1 (Hewawasam *et al.*, 2010; Ranjitkar *et al.*, 2010) and proline isomerase Fpr3 (Ohkuni *et al.*, 2014) regulate cellular levels of Cse4. Both the N-terminus of Cse4 and the centromere-targeting domain (CATD) in the C-terminus of Cse4 (that interacts with Psh1) are required for proteolysis of overexpressed Cse4 (Hewawasam *et al.*, 2010; Ranjitkar *et al.*, 2010; Au *et al.*, 2013). Psh1-mediated proteolysis of Cse4 is also affected by the FACT (facilitates chromatin transcription/transactions) complex (Deyter and Biggins, 2014) and phosphorylation of Psh1 by casein kinase 2 (CK2) (Hewawasam *et al.*, 2014). Given that Cse4 is not completely stabilized in the *psh1*Δ mutant, we hypothesize that additional mechanisms regulate proteolysis of Cse4. Identification of cellular pathways for CENP-A proteolysis is critical for understanding mechanisms that prevent mislocalization of CENP-A and aneuploidy in human cancers.

In addition to ubiquitination, sumoylation is also a critical modifier of chromatin proteins, such as histone H3 (Nathan *et al.*, 2006). Sumoylation affects several biological processes

including transcription, signal transduction, and genome integrity by regulating protein-protein or protein-DNA interactions or by localization and stability of the interacting proteins (Ulrich, 2009; Gareau and Lima, 2010; Everett *et al.*, 2013; Flotho and Melchior, 2013; Jentsch and Psakhye, 2013). A recent study showed that the interaction of CDC48/p97 with sumoylated CENP-A activates ribosomal RNA genes in *Arabidopsis thaliana* (Merai *et al.*, 2014). However, these studies did not define the sumoylation enzymes or a functional role for sumoylated CENP-A in chromosome segregation. In budding yeast, the covalent attachment of Smt3, a Small Ubiquitin-related Modifier (SUMO), to target lysines requires the activity of E3 SUMO ligases such as Siz1 and Siz2 (Johnson and Gupta, 2001; Takahashi *et al.*, 2001). SUMO-targeted ubiquitin ligases (STUbLs) link SUMO and ubiquitin modification pathways and facilitate proteolysis of the substrate. Slx5 and Slx8, two of at least four STUbLs proteins in *S. cerevisiae*, form a heterodimer to mediate SUMO-targeted degradation of several proteins, including Mot1 and MATα 2 (Wang and Prelich, 2009; Xie *et al.*, 2010), nuclear Siz1 during mitosis (Westerbeck *et al.*, 2014), and proteins involved in the DNA damage response and genome maintenance (Cook *et al.*, 2009; Hickey *et al.*, 2012; Garza and Pillus, 2013; Sriramachandran and Dohmen, 2014). Slx5, but not Slx8, has been shown to associate with centromeres, and *slx5*Δ and *slx8*Δ mutants show defects in chromosome segregation (van de Pasch *et al.*, 2013). Depletion of the human homolog of Slx5/8, RNF4, also leads to chromosome segregation errors (van de Pasch *et al.*, 2013), suggesting that the role of Slx5/8 is evolutionarily conserved. The kinetochore substrates targeted and modified by Slx5/8 have not been identified, and therefore the role of STUbL proteins in chromosome segregation and genome stability is not well understood.

Here, we provide evidence that Cse4 is sumoylated by SUMO E3 ligases Siz1 and Siz2 *in vivo* and *in vitro* and that SUMO modification of Cse4 regulates its proteolysis. Slx5 plays a critical role in ubiquitin-mediated proteolysis of Cse4 and prevents mislocalization of Cse4 under normal physiological conditions. Mislocalized Cse4 is highly stable and is not efficiently degraded in *psh1*Δ and *slx5*Δ strains. Our results show that Slx5 regulates ubiquitin-mediated proteolysis of Cse4 to prevent its mislocalization in a Psh1-independent manner.

Results

Cse4 is sumoylated *in vitro* and *in vivo*.

Protein post-translational modifications (PTMs) such as ubiquitination and sumoylation are important for regulating steady-state levels of cellular proteins (Kerscher *et al.*, 2006; Everett *et al.*, 2013). Although canonical histones are sumoylated (Nathan *et al.*, 2006), there is no evidence for sumoylation of Cse4. Optimization of the biochemical purification of Cse4 allowed us to detect sumoylation of Cse4 *in vivo*. We performed a pull down of 8His-hemagglutinin (HA)-tagged Cse4 using Ni-NTA agarose beads and detected SUMO-modified Cse4 by western blot analysis with an anti-Smt3 antibody (Figure 1A). Protein levels of cse4 16KR, in which all lysine (K) residues are mutated to arginine (R), were greatly increased due to its stabilization, as reported previously (Collins *et al.*, 2004). At least three high molecular weight bands were observed after transient overexpression of 8His-HA-Cse4 in wild-type cells (Figure 1A, long exposure, denoted by arrows). In contrast, these SUMO-modified Cse4 species that are visible on wild-type 8His-HA-Cse4 were not detected with vector alone or 8His-HA-cse4 16KR. These results show that Cse4 is sumoylated *in vivo*.

To identify the SUMO E3 ligase responsible for Cse4 sumoylation, we tested the role of two functionally redundant SUMO E3 ligases, Siz1 and Siz2, that are responsible for sumoylation of a majority of substrates (Johnson and Gupta, 2001; Takahashi *et al.*, 2001; Johnson, 2004; Montpetit *et al.*, 2006; Reindle *et al.*, 2006) including the kinetochore protein Ndc10 and histones H2B and H4 in *S. cerevisiae* (Montpetit *et al.*, 2006; Nathan *et al.*, 2006). *In vitro* sumoylation assays using purified Cse4 (Supplemental Figure S1) revealed that Siz1 serves as an E3 for Cse4 sumoylation (Figure 1B). We tested a *siz1*Δ *siz2*Δ mutant to determine the role of Siz1 and Siz2 in sumoylation of Cse4 *in vivo* (Figure 1C). We failed to detect SUMO-modified Cse4 species in the *siz1*Δ *siz2*Δ strain when compared to the wild-type strain (Figure 1C, Pull down). The lower levels of input Cse4 in the *siz1*Δ *siz2*Δ strain may be due to their slow growth and/or a defect in transcriptional induction from the *GAL* promoter (Figure 1C, Input); however, SUMO-modified Cse4 species were not detected in the *siz1*Δ *siz2*Δ strain even upon a longer exposure (Figure 1C, long exposure). Our results show that SUMO E3 ligases Siz1 and Siz2 sumoylate Cse4 *in vivo*.

STUbL, Slx5, regulates ubiquitin-mediated proteolysis of Cse4.

Previous studies have shown that STUbLs link SUMO and ubiquitin modification pathways to facilitate proteolysis of cellular substrates (Garza and Pillus, 2013; Sriramachandran and Dohmen, 2014). Slx5, one of four STUbL proteins (Slx5, Slx8, Uls1, Rad18) in *S. cerevisiae*, forms a complex with Siz1 (Westerbeck *et al.*, 2014) and *slx5*Δ strains exhibit chromosome segregation defects (van de Pasch *et al.*, 2013). Hence, we investigated the role of Slx5 in Cse4 proteolysis. To investigate if Slx5 interacts with Cse4 *in vivo*, we performed a GST

pull down assay using a strain expressing glutathione S-transferase (GST)-tagged Slx5 and HA-tagged Cse4. Slx5-GST was affinity purified on glutathione sepharose, and copurifying HA-Cse4 was detected by western blot analysis with anti-HA antibody (Figure 2A). Our results show that GST-Slx5 interacts with HA-Cse4 *in vivo*.

To determine if Slx5 ubiquitinates Cse4 *in vivo*, we performed an affinity pull down assay using agarose with tandem ubiquitin-binding entities (Ub^+) from a strain expressing HA-tagged Cse4 (Figure 2B). Ubiquitinated Cse4 was detected as a laddering pattern in wild-type cells expressing HA-Cse4 but was absent in strains with vector alone. Faster migrating Cse4 species (Figure 2B, denoted by an asterisk) similar in size to those in input lane were observed from both wild-type and *slx5*Δ strains. These species were also observed in experiments with wild-type Cse4 and *cse4 16KR* mutant in which all lysines are mutated to arginines (Au *et al.*, 2013; Hewawasam *et al.*, 2014). Since cse4 16KR cannot be ubiquitinated, this faster migrating species represents unmodified Cse4, which likely interacts with ubiquitinated proteins such as canonical histones. The laddering pattern of higher molecular weight forms of Cse4 was greatly reduced in an *slx5*Δ strain (Figure 2B). Quantification of ubiquitinated Cse4 showed a five-fold reduction in high molecular weight forms of Cse4 when normalized to input Cse4 in the *slx5*Δ strain (Figure 2C). We next investigated if defects in Cse4 ubiquitination result in increased protein stability *in vivo*. Overexpressed HA-tagged Cse4 was transiently induced from a *GAL* promoter by the addition of galactose, and cells were shifted to glucose medium containing cycloheximide (CHX) to inhibit translation. Western blot analysis with protein extracts from different time points was used to measure levels of Cse4 after CHX treatment (Figure 2D). HA-Cse4 was rapidly degraded in the wild type strain ($t_{1/2}$ = 39.0 min), yet was stabilized in the *slx5*Δ strain ($t_{1/2}$ = 73.2 min). Consistent with this observation, we detected a

similar stabilization of Cse4 in the *siz1*Δ *siz2*Δ strain ($t_{1/2}$ = 74.8 min vs. 35.8 min in wild-type, Supplemental Figure S2). Thus, we conclude that Slx5 is required for ubiquitination and proteolysis of Cse4 *in vivo*.

Since *slx5*Δ strains show defects in Cse4 ubiquitination and increased stability of Cse4, we examined if sumoylated Cse4 accumulates in these strains. We first constructed a strain expressing His-Flag-tagged Smt3 (HF-Smt3) and Myc-tagged Cse4. HF-Smt3 was purified from cell extracts using Ni-NTA agarose beads and the level of sumoylated proteins and sumoylated Cse4 was determined using anti-Flag (Smt3) and anti-Myc (Cse4) antibodies, respectively. When Myc-Cse4 was transiently overexpressed, SUMO-modified Cse4 species were barely detectable in the wild-type strain (Figure 2E, α-Myc). In contrast, the *slx5*Δ strain showed substantial levels of sumoylated Cse4, especially high molecular weight SUMO modified Cse4 (Figure 2E, α-Myc). Therefore, reduced STUbL activity in *slx5*Δ strain contributes to the accumulation of sumoylated Cse4 species, suggesting that Slx5-mediated proteolysis is a downstream consequence of Cse4 sumoylation.

We next examined if higher levels of sumoylated Cse4 species accumulate in the *slx5*Δ strain under normal physiological conditions. Even though sumoylated Cse4 species were detected in wild-type cells when Cse4 is overexpressed (Figure 1A), we failed to detect sumoylated Cse4 species in the wild-type strain when Cse4 is expressed from its own promoter (Figure 2F). The failure to detect sumoylated Cse4 species may be due to low Cse4 expression and/or ongoing proteolysis of sumoylated Cse4. In contrast, higher levels of sumoylated Cse4 species were clearly observed in the *slx5*Δ mutant (Figure 2F, denoted by arrows). A defect in STUbL activity may in part contribute to accumulation of polysumoylated (high molecular weight) Cse4 species in the *slx5*Δ strain. Sumoylated Cse4 does not accumulate in strains deleted

for *PSH1*, an E3-ubiquitin ligase targeting Cse4, nor in *SIZ1/SIZ2* deleted strains. These results indicate that lack of STUbL activity in *slx5*Δ strains contributes to the accumulation of sumoylated Cse4 species under normal physiological conditions.

Slx5 regulates ubiquitin-mediated proteolysis of Cse4 in a Psh1-independent manner.

Previous studies have shown that Psh1 interacts with Cse4 and *CSE4* overexpression causes growth inhibition in a *psh1*Δ strain (Hewawasam *et al.*, 2010; Ranjitkar *et al.*, 2010). Similar to the growth defect observed for *psh1*Δ strains, *slx5*Δ and *siz1*Δ *siz2*Δ strains also showed growth inhibition with *GAL-CSE4* on galactose media (Supplemental Figure S3). Given that Slx5 and Psh1 are E3 ligases that ubiquitinate Cse4 and that deletion of *SLX5* results in accumulation of sumoylated Cse4 species, we examined if Slx5-mediated proteolysis of Cse4 is dependent on Psh1. We constructed *psh1*Δ *slx5*Δ strains using standard yeast mating and sporulation. The *psh1*Δ *slx5*Δ strains do not exhibit growth defects at 30°C, but exhibit a slow growth phenotype at low (22°C, 25°C) and high (37°C) temperatures, compared to each single mutant (Supplemental Figure S4A). The *psh1*Δ *slx5*Δ strains also exhibit sensitivity to growth on benomyl (microtubule depolymerizing agent) containing plates. Furthermore, *psh1*Δ *slx5*Δ strains exhibit defects in segregation of a reporter chromosome in a colony color assay to measure chromosome transmission fidelity (CTF) (Supplemental Figure S4B).

We next analyzed the stability of Cse4 after transient overexpression of HA-Cse4 in wild-type, *psh1*Δ, *slx5*Δ, and *psh1*Δ *slx5*Δ strains (Figure 3A). As expected, deletion of *PSH1* or *SLX5* moderately stabilized HA-Cse4 protein levels. In contrast, the double deletion mutant, *psh1*Δ *slx5*Δ, showed a dramatic increase in HA-Cse4 protein stability. The half-life of HA-Cse4 in *psh1*Δ *slx5*Δ ($t_{1/2}$ = 138.6 min) is twice that of *psh1*Δ ($t_{1/2}$ = 77.0 min) or *slx5*Δ ($t_{1/2}$ = 69.3 min)

strains (Figure 3B). We next analyzed Cse4 stability in strains expressing HA-Cse4 from its own promoter. Protein stability assays showed that Cse4 is rapidly degraded in wild type cells ($t_{1/2}$ = 34.7 min), modestly stable in *psh1*Δ ($t_{1/2}$ = 46.2 min) and *slx5*Δ ($t_{1/2}$ = 53.3 min) single mutants, and highly stable in *psh1*Δ *slx5*Δ strain ($t_{1/2}$ = 77.0 min) (Figure 3C and 3D). We conclude that Slx5 regulates ubiquitin-mediated proteolysis of Cse4 independently of Psh1.

Slx5 prevents mislocalization of Cse4 in a Psh1-independent manner.

We investigated the physiological consequence of defects in STUbL activity by analyzing Cse4 localization in strains expressing Cse4 from its endogenous promoter. Subcellular fractionation and chromosome spreads were used to examine if Cse4 was mislocalized in the *slx5*Δ strain. These approaches were previously used to show that defects in ubiquitin-mediated proteolysis of Cse4 in a *psh1*Δ strain led to the enrichment of Cse4 in chromatin and mislocalization to euchromatin when Cse4 was overexpressed (Hewawasam *et al.*, 2010; Ranjitkar *et al.*, 2010; Deyter and Biggins, 2014). Subcellular fractionation of whole cell lysates was performed with *psh1*Δ, *slx5*Δ, and *psh1*Δ *slx5*Δ strains. Cse4 was barely detectable in the chromatin fraction in a wild-type strain, as its localization is restricted to centromeres (Figure 4A). In contrast, Cse4 was enriched in chromatin in *psh1*Δ and *slx5*Δ strains and this enrichment was further enhanced in *psh1*Δ *slx5*Δ strains. Similar results were observed for chromatin enrichment of Cse4 transiently overexpressed from a *GAL* promoter in *psh1*Δ and *slx5*Δ strains with maximum enrichment in the *psh1*Δ *slx5*Δ strain (Supplemental Figure S5). These results suggest that Slx5 regulates localization of Cse4 independently of Psh1.

We next used chromosome spreads, a technique that removes soluble material to visualize localization of chromatin-bound Cse4 in wild-type, *psh1*Δ, *slx5*Δ, and *psh1*Δ *slx5*Δ strains expressing Cse4 from own promoter (Figure 4B). In wild-type cells, Cse4 foci were restricted to one or two dots, which correspond to kinetochore clusters. In contrast, diffused or multiple foci of Cse4 that overlapped with the DAPI-stained nucleus were observed in *psh1*Δ and *slx5*Δ strains and this was further exacerbated in *psh1*Δ *slx5*Δ cells (Figure 4B and 4C). Based on these results, we conclude that Slx5 and Psh1 prevent mislocalization of Cse4 under normal physiological conditions, and that Slx5 regulates localization of Cse4 in a Psh1-independent manner.

Fluorescence recovery after photobleaching (FRAP) analysis shows mislocalized Cse4 is highly stable in *psh1*Δ and *slx5*Δ strains.

To determine if the euchromatic pool of mislocalized Cse4 observed in chromosome spreads of *slx5*Δ and *psh1*Δ strains is stably incorporated, we performed FRAP in both mutants. In wild-type strains, Cse4-GFP signal is restricted to the cluster of 16 kinetochores proximal to the spindle pole body, and Cse4 incorporated into kinetochores does not recover after photobleaching (Pearson *et al.*, 2004). In addition to the kinetochore signal (Foci), the nuclei of *slx5*Δ and *psh1*Δ strains show a diffuse fluorescence emanating from non-centromeric regions (Haze), consistent with the increased levels of Cse4 in chromatin fractions (Figure 4A) and mislocalization to euchromatin (Figure 4B). Similar to wild-type cells, the centromere Cse4-GFP foci in both *slx5*Δ and *psh1*Δ cells did not exhibit recovery after photobleaching (Figure 5A and 5B). We next examined FRAP of the Cse4-GFP haze in *slx5*Δ and *psh1*Δ strains. For *psh1*Δ strains, we imaged Cse4-GFP haze every 30 seconds post-bleach (Figure 5B and 5D); however,

since the Cse4-GFP haze was dimmer in *slx5*Δ, we used only two Z-series (immediately post-bleach, and 5 minutes post-bleach) after laser bleaching to minimize photobleaching from imaging (Figure 5A and 5C). Similar to the centromere Cse4-GFP foci, the Cse4-GFP haze did not exhibit recovery after photobleaching in either *slx5*Δ or *psh1*Δ cells (Figure 5C and 5D). We conclude that the ectopically localized Cse4 present in *slx5*Δ and *psh1*Δ cells, once deposited, is stable.

Discussion

In this study, we have shown that sumoylation and ubiquitination of Cse4 regulate its proteolysis and prevent its mislocalization. We provide the first evidence for sumoylation of Cse4, by SUMO E3 ligases Siz1 and Siz2 *in vivo* and *in vitro*, and define a role for Slx5 in ubiquitin-mediated proteolysis of Cse4. Slx5-mediated proteolysis of Cse4 is independent of Psh1 and Cse4 is mislocalized to euchromatin in both *psh1*Δ and *slx5*Δ strains under normal physiological conditions. Consistent with these results, Cse4 is highly enriched in the chromatin fraction and stably incorporated into euchromatin in *psh1*Δ and *slx5*Δ strains. Taken together, our results support a role for Slx5 in ubiquitination of sumoylated Cse4 to regulate its proteolysis and localization.

Several lines of evidence support the role of Slx5 in proteolysis of Cse4. Overexpression of *CSE4* results in growth inhibition in *slx5*Δ and *siz1*Δ *siz2*Δ strains, similar to *psh1*Δ strain (Hewawasam *et al.*, 2010; Ranjitkar *et al.*, 2010; Au *et al.*, 2013). Second, defects in Cse4 ubiquitination observed in *slx5*Δ strains correlate with an increased half-life of Cse4 in these strains. Higher stability of Cse4 is also observed in an *slx8*Δ strain (data not shown), suggesting

that the heterodimeric Slx5/8 STUbL complex is important for Cse4 proteolysis. Third, deletion of *SLX5* leads to accumulation of higher molecular weight species of sumoylated Cse4. Similar results for higher levels of sumoylated Mot1, a regulator of TATA-binding protein and defects in proteolysis, have been observed previously when *SLX5* and/or *SLX8* are deleted (Wang and Prelich, 2009). Accumulation of sumoylated Cse4 species and increased stability of Cse4 in the *slx5*Δ strain suggests that sumoylation precedes ubiquitin-mediated proteolysis of Cse4. These phenotypes are not limited to cases where Cse4 is overexpressed (e.g., using the *GAL* promoter), higher levels of sumoylated Cse4 and increased stability of Cse4 are also observed under physiological conditions when Cse4 is expressed from its own promoter.

The increased stability of Cse4 in the *psh1*Δ *slx5*Δ double mutant, compared to either the *slx5*Δ or *psh1*Δ single mutant, shows that Slx5-mediated proteolysis of Cse4 is independent of Psh1 under normal physiological conditions. The residual proteolysis of Cse4 observed in *psh1*Δ *slx5*Δ strains suggests that additional pathways/regulators that have yet to be identified also mediate Cse4 proteolysis. This is perhaps not surprising given that degradation of excess of histone H3 is also regulated by at least five E3 ubiquitin ligases (Singh *et al.*, 2012). Additionally, non-ubiquitin mediated pathways partially contribute to Cse4 proteolysis because mutant cse4 16KR in which all lysines are changed to arginine is still degraded (Collins *et al.*, 2004; Au *et al.*, 2013).

Endogenously expressed Cse4 is enriched in chromatin fractions and mislocalized to euchromatin in *slx5*Δ and *psh1*Δ strains, and these phenotypes are further exacerbated in the *slx5*Δ *psh1*Δ double mutant. Previous studies have only examined Cse4 turnover in the context of the kinetochore, where it is stably incorporated into chromatin (Pearson *et al.*, 2004). Though the signal is low, the increased levels of non-centromeric Cse4 present in *slx5*Δ and *psh1*Δ strains

(the “haze”) enable analysis by FRAP. The apparent stability of mislocalized Cse4 observed by FRAP suggests that ectopically localized Cse4 is stably incorporated in the euchromatin; however, it is also possible that the ectopic Cse4-containing nucleosomes are dynamic, in equilibrium with (non-fluorescently tagged) H3 in the nucleus (Verdaasdonk *et al.*, 2012). The latter explanation would require that the exchange mechanism utilizes a different Cse4/H3 pool than that under which the Cse4 was initially misincorporated.

We propose a model (Figure 6) in which sumoylation and ubiquitination regulate Cse4 proteolysis to prevent its stable incorporation into euchromatin. At least two independent pathways regulate Cse4 proteolysis. One of these pathways is dependent on the interaction of Psh1 with Cse4, which is potentiated by the nucleosome-destabilizing activity of the FACT complex. This suggests that Psh1 is primarily responsible for removing nucleosomal Cse4 at non-centromeric chromatin, even though the interaction of Psh1 and soluble Cse4 is also reduced in the absence of FACT (Deyter and Biggins, 2014). The second pathway, identified here, requires sumoylation of Cse4 by Siz1/Siz2 and subsequent ubiquitination of Cse4 by Slx5 to regulate cellular levels of Cse4 and prevent its mislocalization to euchromatin. While we do not yet know if the Siz1/2-Slx5 pathway acts on soluble Cse4 or chromatin-bound Cse4, together the two pathways act to regulate cellular levels of Cse4 and prevent its mislocalization to euchromatin. Given that mislocalization of Cse4 leads to chromosome segregation defects, it is not surprising that cells utilize multiple ubiquitination pathways for proteolysis of high levels of Cse4.

Unlike Psh1, Slx5/8 are evolutionarily conserved, and depletion of the human STUbL ortholog, RNF4, results in defects in chromosome segregation (van de Pasch *et al.*, 2013). Similar to Slx5, it is possible that RNF4 also regulates the localization of CENP-A and that

defects in this pathway lead to chromosome missegregation. Previous studies have shown that mislocalization of centromeric histone H3 variants Cse4, Cnp1 and CID contribute to chromosome segregation defects in flies and budding/fission yeast (Heun *et al.*, 2006; Au *et al.*, 2008; Gonzalez *et al.*, 2014). Thus, we propose that the mislocalization of CENP-A contributes to chromosome segregation defects in *slx5*Δ and RNF4-depleted cells. These studies are important, as we do not fully understand how overexpression and mislocalization of CENP-A observed in many cancers contribute to tumorigenesis. Our studies on the role of STUbL's in ubiquitin-mediated proteolysis of Cse4 provide mechanistic insights into pathways that prevent mislocalization of CENP-A and aneuploidy in human cancers.

Materials and Methods

Yeast strains and plasmids

Supplemental Table S1 and S2 describe the genotype of yeast strains and plasmids used for this study, respectively.

Sumoylation assay *in vivo* and *in vitro*

In vivo sumoylation was assayed in crude yeast extracts using Ni-NTA agarose beads to pull down His-HA-tagged Cse4 or His-Flag tagged Smt3 (HF-Smt3) under denaturing condition, as described previously (Ohkuni *et al.*, 2015). *In vitro* sumoylation assays were carried out as described previously (Takahashi *et al.*, 2003). Briefly, the components of the conjugation reaction: Smt3gg, GST–Uba2, GST–Aos1, Ubc9, and Siz1–Δ440 proteins were expressed and purified from *E. coli* and then used in the reaction mixture containing Cse4 as a substrate. Cse4 was produced in *E. coli* and purified by Sephacryl-S200 chromatography as described (Luger *et al.*, 1997). Substrate (Cse4), E1 (GST-Uba2, GST-Aos1), E2 (Ubc9), SUMO (Smt3gg) were incubated in a total volume 20 µl for 60 min in the presence of 10 mM ATP, 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, and 2 mM dithiothreitol at 37˚C. Reaction was stopped by adding 2x Laemmli buffer.

Protein stability and Ubiquitin pull down assays

Protein stability assay was performed as described previously (Ohkuni *et al.*, 2014) with some modifications. Cells were grown to logarithmic phase of growth in a 2% raffinose synthetic complete medium at 25°C. Galactose was added to the media to a final concentration of 2% to induce Cse4 expression from the *GAL* promoter for 2 hrs at 25°C. Cycloheximide (CHX) and

glucose were then added to final concentrations of 10 μg/ml and 2%, respectively. Samples were taken at the indicated time points and levels of Cse4 were quantified by western blot analysis. Protein levels at each time point were quantified using Gene Tools software (version 3.8.8.0) from SynGene. Ubiquitin-pull down assays were performed by inducing HA-tagged Cse4 from a *GAL* promoter by the addition of galactose (2%) for 2 hrs, as described previously (Au *et al.*, 2013).

GST pull down assay

The *ubc4*Δ *ubc6*Δ (YOK 2501) strain was transformed with *pGAL-GST-SLX5* (BOK 629, Open Biosystems Yeast GST Collection YSC4515202484078), *pGAL-3HA-CSE4* (pMB 1515), or both *pGAL-GST-SLX5* and *pGAL-3HA-CSE4*. Transformants were grown in appropriate selective media with proline as nitrogen source and 2% sucrose to logarithmic phase, then 2% galactose and 0.003% SDS were added to the cultures and incubation continued for another 6 hours. 75 μM MG132 was added half an hour before harvesting the cells. 200 OD units of yeast cells were assayed as described previously (Westerbeck *et al.*, 2014). Whole-cell extracts (2 OD) and pull-down (20 OD) were analyzed by western blot analysis.

Subcellular fractionation and chromosome spreads

Subcellular fractionation to assay chromatin enrichment of Cse4 was performed as described previously (Au *et al.*, 2008). Cells were grown to logarithmic phase of growth in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) at 25°C. Chromosome spreads were performed as described previously (Collins *et al.*, 2004; Crotti and Basrai, 2004) with some modifications. 16B12 Mouse anti-HA antibody (Covance, Babco; MMS-101P) was used as primary antibody at

1:2500 dilution. Cy3 conjugated Goat anti mouse (Jackson ImmunoResearch Laboratories, Inc., 115165003) was used as secondary antibody at 1:5000 dilution. Cells were visualized by DAPI staining (1 μg/ml in PBS) mounted in antifade mountant (Molecular Probes, P36935). Cells were observed under an Axioskop 2 (Zeiss) fluorescence microscope equipped with a Plan-APOCHROMAT 100X (Zeiss) oil immersion lens. Image acquisition and processing were performed with the IP Lab version 3.9.9 r3 software (Scanalytics, Inc.).

Antibodies

Antibodies for experiments were as follows: Rabbit polyclonal anti-Cse4 (gift from Alexander Strunnikov), anti-Tub2 antibodies (Basrai laboratory), anti-HA (12CA5, Roche), anti-HA (ab9110, Abcam), anti-myc (A-14, Santa Cruz Biotechnology, Inc.), anti-GST (ab6613, Abcam), anti-Smt3 (y-84, Santa Cruz Biotechnology, Inc.), and anti-H3 (ab1791, Abcam). Anti-Cse4 was used at a dilution of 1:1,000, anti-HA was used at a dilution from 1:1,000 to 1:10,000, anti-Tub2 and anti-Smt3 were used at 1:3,000, anti-GST and anti-H3 were used at 1:5,000.

Fluorescent recovery after photobleaching

Strains YMB9430 (Cse4-GFP, *psh1*Δ) and YMB9429 (Cse4-GFP, *slx5*Δ) were grown in YPD to mid logarithmic growth phase prior to imaging. Both YMB9430 and YMB9429 were grown at 24° C, but YMB9429 was shifted to 37° C 6 hours prior to imaging. Cells were imaged using a Nikon Eclipse Ti wide-field inverted microscope with a 100x Apo TIRF 1.49 NA objective (Nikon, Melville, New York, USA) and Andor Clara CCD camera (Andor, South Windsor, Connecticut, USA) using Nikon NIS Elements imaging software (Nikon, Melville, New York, USA). Photobleaching was performed with a Sapphire 488-50 CDRH laser (Coherent, Santa

Clara, CA, USA). A 7-step Z-series with 200 nm step size with 600 ms exposure time was taken prior to a 300 ms exposure from the laser. Immediately after the laser exposure, a 5 minute timelapse with 30 second intervals with the same settings as the first Z-series was initiated. The Z-series were compiled into single images using maximum projection, and the integrated intensity of the bleached area was measured using MetaMorph 7.7 imaging software (Molecular Devices, Sunnyvale, CA, USA). The integrated intensity of the bleached area had the integrated intensity of the cell background subtracted at each timepoint and photobleaching was corrected for by determining the average bleaching rate of a nearby Cse4-GFP signal and adding back the average signal loss per Z-series. Photobleaching and background subtraction was performed using Excel (Microsoft, Redmond, WA, USA).

Chromosome transmission fidelity (CTF)

The CTF assay was performed as described previously (Spencer *et al.*, 1990; Ohkuni *et al.*, 2008). Strains were plated on synthetic medium with limiting adenine and incubated at 25°C for 4 days. Loss of the reporter chromosome results in red sectors in an otherwise a white colony. Colonies that are at least half red indicate loss of the reporter chromosome in the first cell division.

Acknowledgments

We would like to thank members of the Basrai laboratory for helpful discussions and comments on the manuscript. We gratefully acknowledge Charlie Boone, Frank Holstege, and Sue Biggins for reagents and advise, Tatiana Karpova (Fluorescent Imaging Facility in NCI) for assistance with cell biology experiments, Kathy McKinnon (Vaccine Branch FACS Core in NCI) for assistance with FACS, Anita Corbett, Ian Cheeseman, Michael Lichten, Tom Misteli and Peter Kaiser for comments on the manuscript. The National Institutes of Health Intramural Research Program to M. Basrai, NSF grant MBC #1051970 to O. Kerscher, and National Institutes of Health R37 Grant GM32238 to K. Bloom supported this work.

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Figure Legends

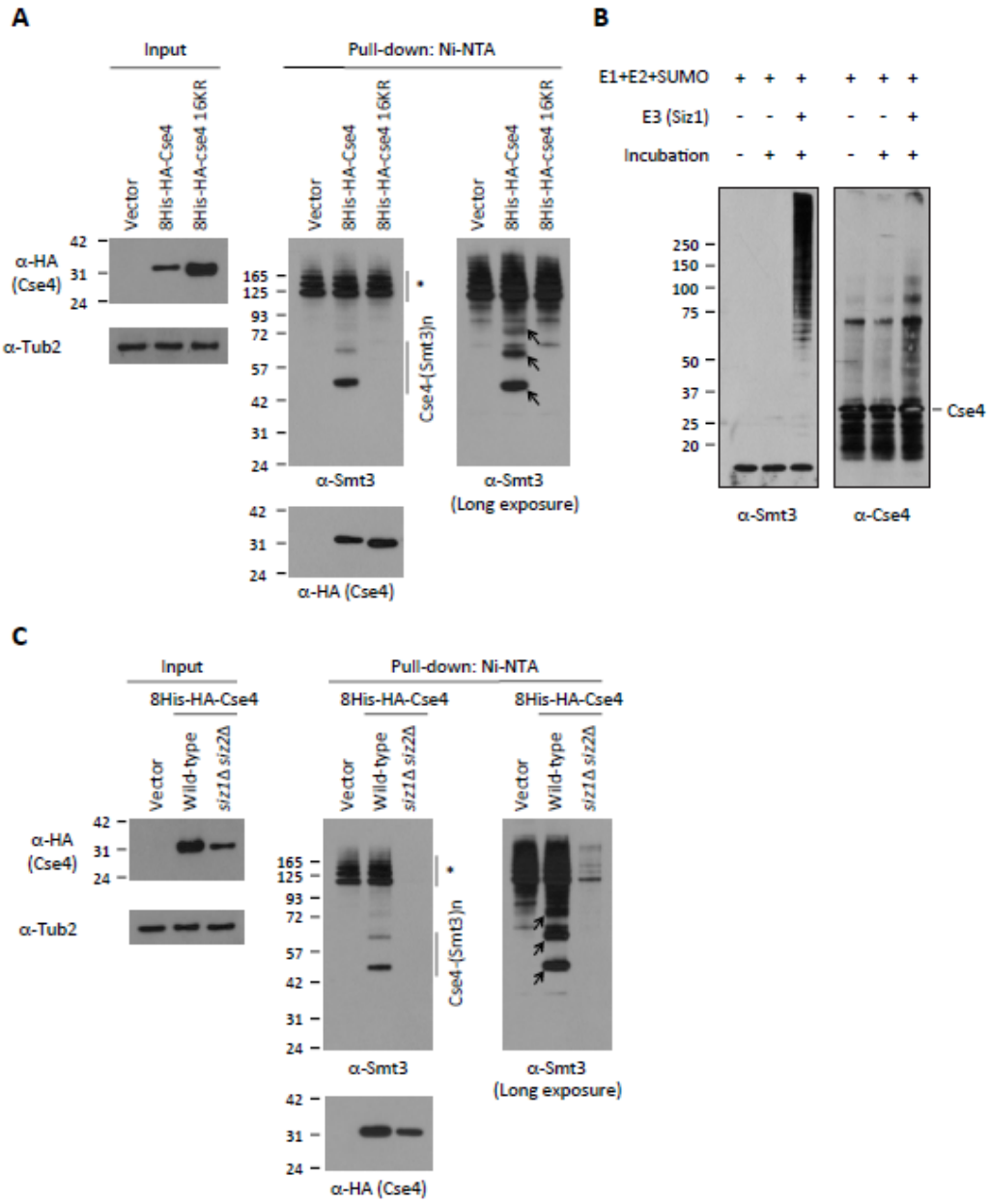

Figure 1

Figure 1. Cse4 is sumoylated by Siz1/2 *in vitro* and *in vivo*. (A) Cse4 is sumoylated *in vivo*. Wild-type strain (BY4741) transformed with vector (pYES2), *pGAL-8His-HA-CSE4* (pMB1345) or *pGAL-8His-HA-cse4 16KR* (pMB1344) was grown in raffinose/galactose (2%) for 4 hrs. to induce expression of Cse4. Levels of sumoylated proteins and sumoylated Cse4 were detected using Ni-NTA pull-down followed by western blot analysis with anti-Smt3 and anti-HA (Cse4) antibodies, respectively. At least three high molecular weights of 8His-HA-Cse4 (arrows) were detected (Long exposure). Input samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Asterisk shows non-specific sumoylated proteins that bind to the beads. The mutations of lysine to arginine in 8His-HA-cse4 16KR slightly affect its mobility when compared to wild type 8His-HA-Cse4. (B) *In vitro* assay for Cse4 sumoylation. E1 (GST-Uba2/GST-Aos1), E2 (Ubc9), Smt3gg and ATP were incubated with or without Siz1Δ440. After the reaction, SUMO and SUMO conjugated Cse4 were detected by western blot analysis with anti-Smt3 and anti-Cse4 antibodies, respectively. (C) SUMO E3 ligases Siz1 and Siz2 sumoylate Cse4 *in vivo*. Wild-type (BY4741) and *siz1*Δ *siz2*Δ (YMB7277) strains expressing *pGAL-8His-HA-CSE4* (pMB1345) were assayed as described in (A). High molecular weight species of 8His-HA-Cse4 and non-specific sumoylated proteins are marked with arrows and an asterisk, respectively.

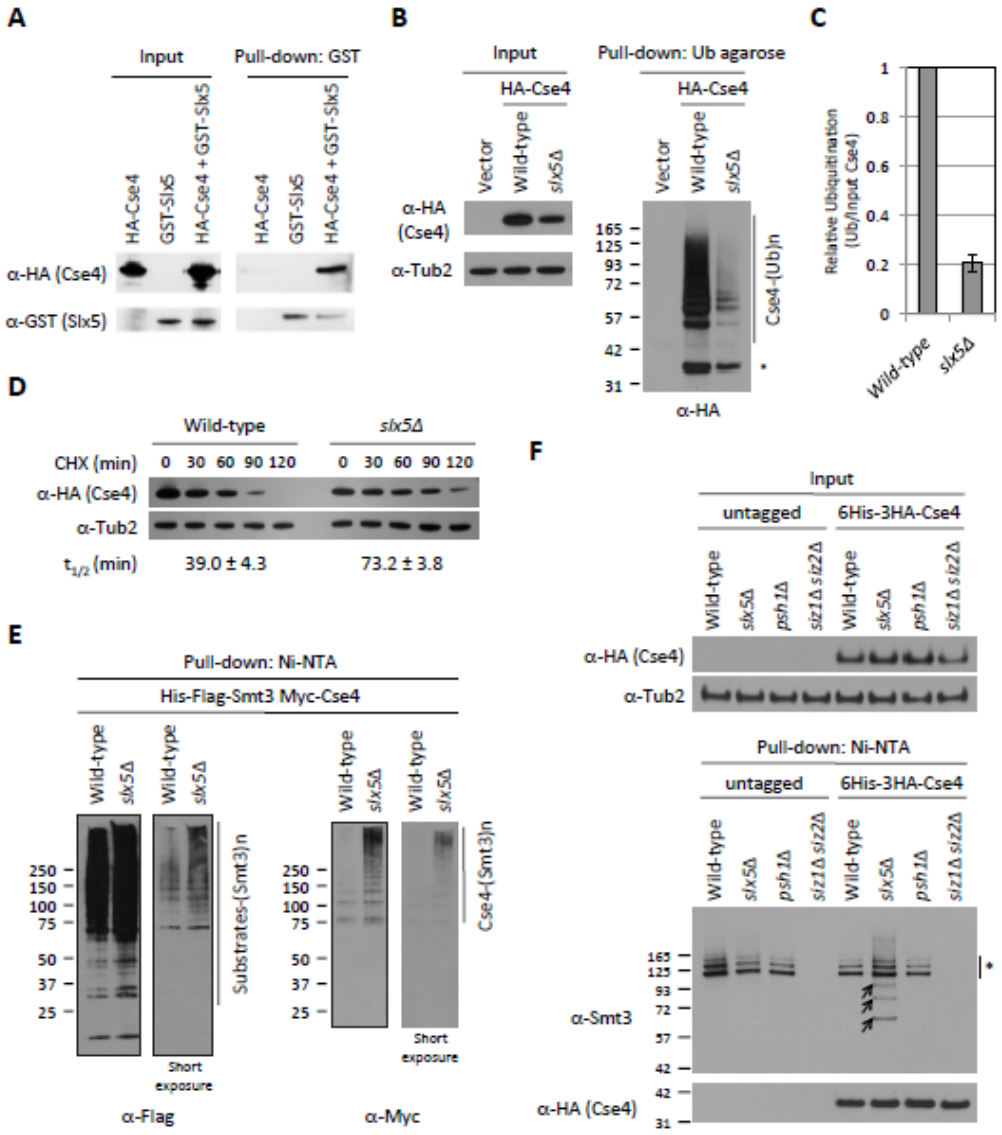

Figure 2. The STUbL Slx5 interacts with Cse4 and regulates ubiquitin-mediated proteolysis of Cse4. (A) Slx5 associates with Cse4. Expression of *pGAL-3HA-CSE4* (pMB1515) and/or *pGAL-GST-SLX5* (BOK629) in *ubc4*Δ *ubc6*Δ (YOK2501) was induced by the addition of galactose (2%) for 6 hrs. Glutathione sepharose beads were used for GST-Slx5 pull down and the eluate was analyzed by western blot analysis with anti-HA (Cse4) and anti-GST (Slx5) antibodies. (B) Slx5 regulates Cse4 ubiquitination. Wild-type (BY4741) and *slx5*Δ (YMB9035) strains expressing *pGAL-3HA-CSE4* (pMB1597) were grown in raffinose/galactose (2%) for 2

hrs. Agarose-TUBE1 was used for pull down with tandem ubiquitin binding entities. Ubiquitination levels of Cse4 were detected by western blot analysis with anti-HA antibody and input samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Wild-type (BY4741) strain transformed with vector (pMB433) was used as negative control. Asterisk shows non-modified Cse4. (C) The graph represents the relative ubiquitination of Cse4 with average deviation of two biological repeats. Cse4 was normalized using input Cse4 levels. (D) Increased stability of Cse4 in *slx5*Δ strain. Cse4 expression from *pGAL-6His-3HA-CSE4* (pMB1458) in wild-type (BY4741) and *slx5*Δ (YMB9035) strains was induced by the addition of galactose (2%) for 2 hrs. Glucose (2%) containing cycloheximide (CHX, 10 µg/ml) was added and cells were collected at the indicated time points. Blots were probed with anti-HA (Cse4) or anti-Tub2 (loading control) antibody. Cse4 protein half life ($t_{1/2}$) represents the mean of two biological repeats with average deviation. (E) Deletion of *SLX5* shows an accumulation of sumoylated Cse4 species. Wild-type (YMB7278) and *slx5*Δ (YMB7875) strains expressing *pGAL-13Myc-CSE4* (pSB816) were grown in raffinose/galactose (2%) for 4hrs. His-Flag-tagged Smt3 (HF-Smt3) was pulled down by Ni-NTA agarose beads. Cellular levels of sumoylated proteins and sumoylated Cse4 were detected by western blot analysis with anti-Flag (Smt3) and anti-Myc (Cse4) antibodies, respectively. Two different exposures are shown. (F) Deletion of *SLX5*, but not *PSH1*, shows an accumulation of sumoylated Cse4 expressed from its own promoter. Protein extracts were prepared from cells grown to logarithmic phase in YPD. Sumoylation levels of Cse4 and non-modified Cse4 were detected using Ni-NTA pull down followed by western blot analysis with anti-Smt3 and anti-HA (Cse4) antibodies, respectively. Input samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. At least three high molecular weights of 6His-3HA-Cse4 (Arrows) were detected in the *slx5*Δ strain. Asterisk shows

non-specific sumoylated proteins that bind to the beads. Isogenic yeast strains used are wild-type (YMB7290), *slx5*Δ (YMB7588), *psh1*Δ (YMB7393), *siz1*Δ *siz2*Δ (YMB7611) and untagged strains (BY4742, YMB9034, YMB9035, YMB7277).

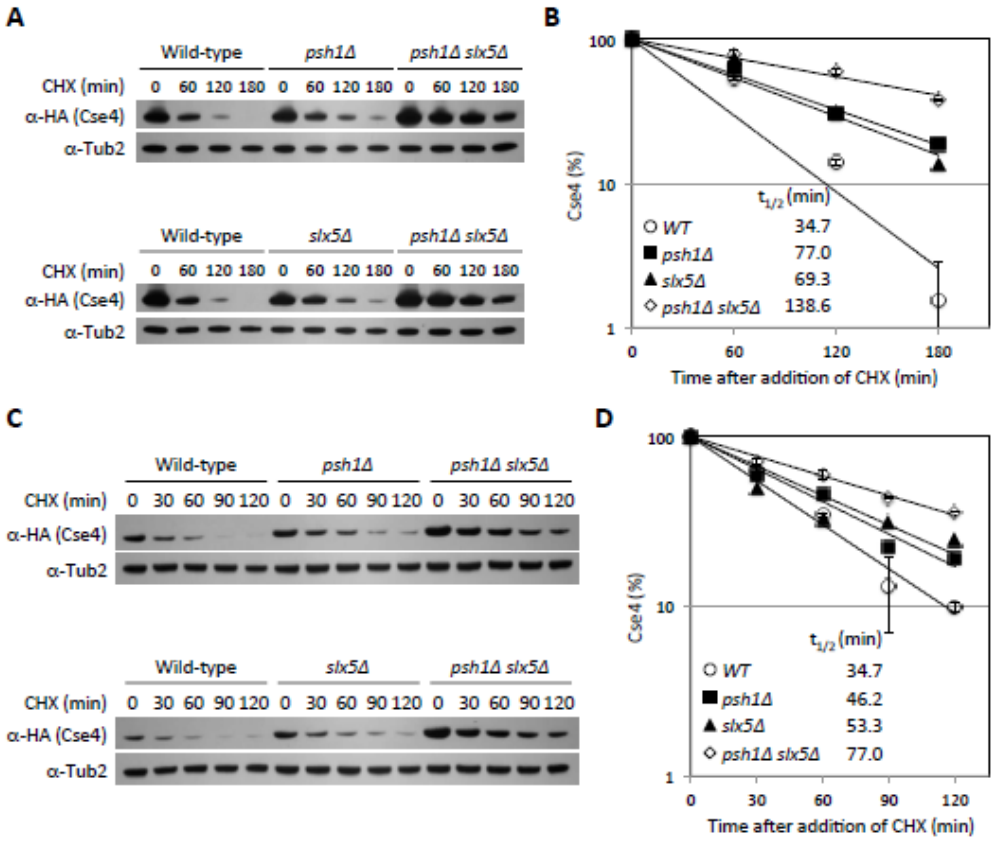

Figure 3. Slx5 regulates Cse4 proteolysis in a Psh1-independent manner. (A) Cse4 expressed from galactose inducible promoter is highly stable in *psh1*Δ *slx5*Δ strain. Wild-type (BY4741), *psh1*Δ (YMB9034), *slx5*Δ (YMB9035), and *psh1*Δ *slx5*Δ (YMB9040) expressing *pGAL-6His-3HA-CSE4* (pMB1458) were assayed as described in Figure 2D. (B) The graph shows the kinetics of turnover from (A). Cse4 protein half-life ($t_{1/2}$) is indicated. Error bars in wild-type and *psh1*Δ *slx5*Δ represent average deviation of two replicates. (C) Cse4 expressed from its own promoter is moderately stabilized in *psh1*Δ and *slx5*Δ strains and highly stable in *psh1*Δ *slx5*Δ strain. Protein extracts were prepared from cells grown to logarithmic phase in YPD and treated

with cycloheximide (CHX, 20 µg/ml) for various time points, and analyzed by western blot analysis with anti-HA (Cse4) or anti-Tub2 (loading control) antibody. Isogenic yeast strains used are wild-type (YMB7290), *psh1*Δ (YMB7393), *slx5*Δ (YMB7588), and *psh1*Δ *slx5*Δ (YMB7607). (D) The graph shows the kinetics of turnover from (C). Cse4 protein half-life ($t_{1/2}$) is indicated and error bars in wild-type and *psh1*Δ *slx5*Δ represent average deviation of two replicates.

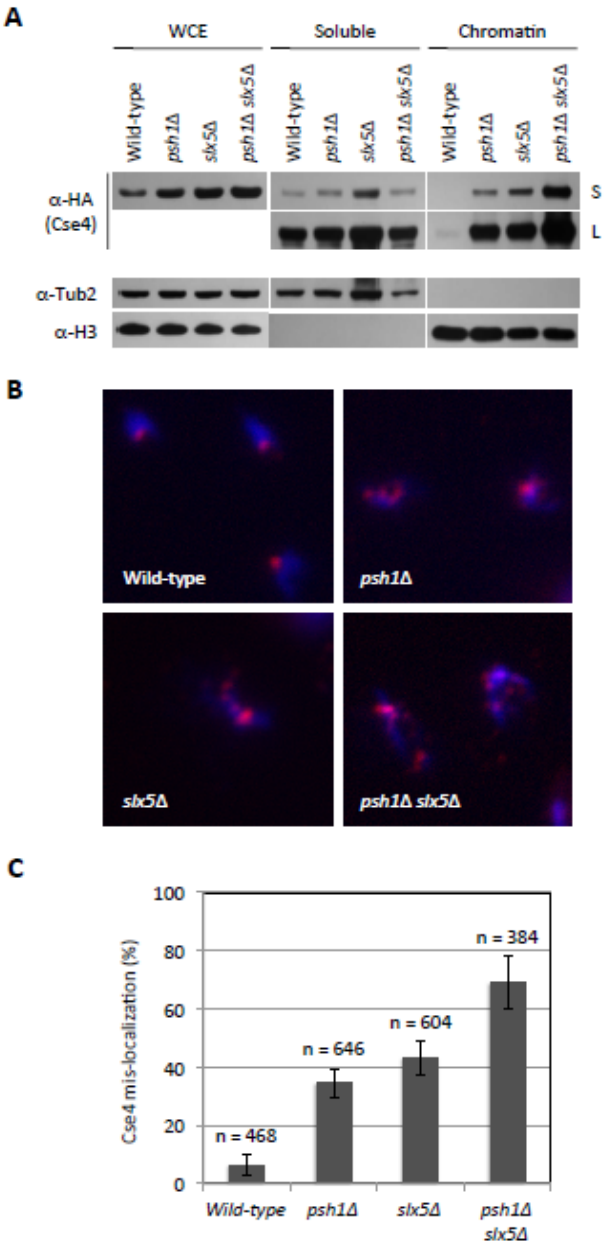

Figure 4. Slx5 prevents mislocalization of Cse4 to euchromatin. (A) Cse4 expressed from its own promoter is increased in chromatin fraction in *psh1*Δ, *slx5*Δ, and *psh1*Δ *slx5*Δ strains. Whole cell extracts (WCE) prepared from equal number of logarithmically growing cells in YPD were fractionated into soluble and chromatin fraction and assayed by western blot analysis. Tub2 and histone H3 were used as markers for soluble and chromatin fractions, respectively. Two blots shown for Cse4 are indicated (S: short exposure, L: long exposure). (B and C) Cse4 expressed from its own promoter is mislocalized in *psh1*Δ, *slx5*Δ, and *psh1*Δ *slx5*Δ strains. Chromosome spreads were done by logarithmically growing cells in YPD. DAPI (blue) and α-HA (red) staining were used to visualize DNA and Cse4 localization, respectively. In wild-type strains, Cse4 is predominantly localized to one to two kinetochore clusters. In mutant strains, mislocalization of Cse4 is observed as multiple foci or diffused localization throughout the nucleus. The graph quantifies the number of cells exhibiting Cse4 mislocalization and error bars are average deviation of two independent experiments. The number of cells used is indicated (n). Isogenic yeast strains used in (A-C) are wild-type (YMB7290), *psh1*Δ (YMB7393), *slx5*Δ (YMB7588), and *psh1*Δ *slx5*Δ (YMB7607).

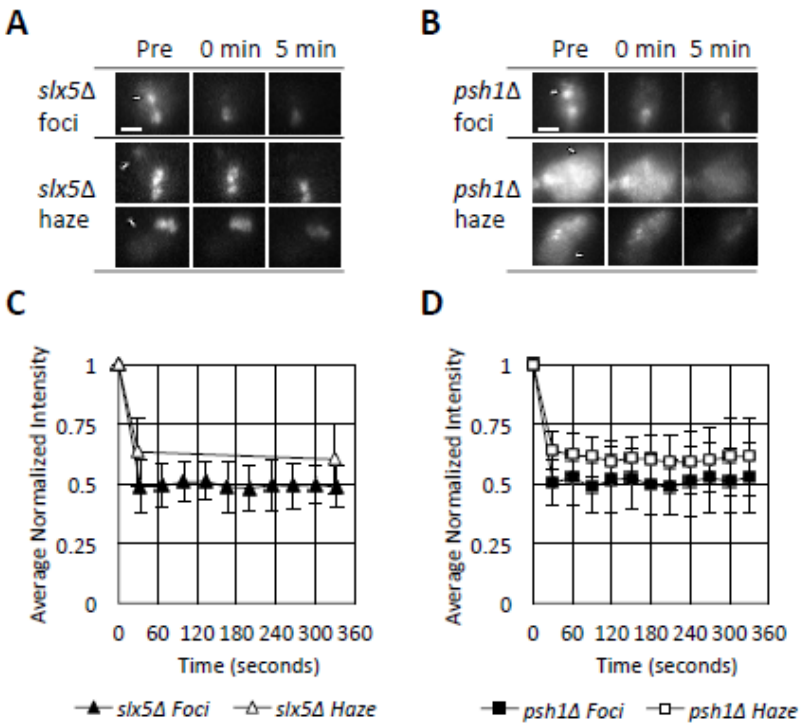

Figure 5. Cse4 is stable in euchromatin in *slx5*Δ and *psh1*Δ strains. Representative images of *slx5*Δ (A) and *psh1*Δ (B) cells containing Cse4-GFP before (Pre), immediately after (0 min), and 5 minutes after photobleaching. Foci correspond to Cse4-GFP enrichment at centromeres. Haze corresponds to Cse4-GFP at euchromatin. Arrows indicate bleached area. Images were compiled using maximum projection. Scale bar = 1 µm. Plots of average normalized intensity of the bleached area over time for *slx5*Δ (C) and *psh1*Δ (D). Integrated intensity was background subtracted and corrected for photobleaching. Error bars show standard deviation. Bleaching was set at t=30 seconds. For *slx5*Δ n=9 cells each for foci and haze, *psh1*Δ n=8 cells for each foci and haze. Isogenic yeast strains used are *slx5*Δ (YMB9429), and *psh1*Δ (YMB9430).

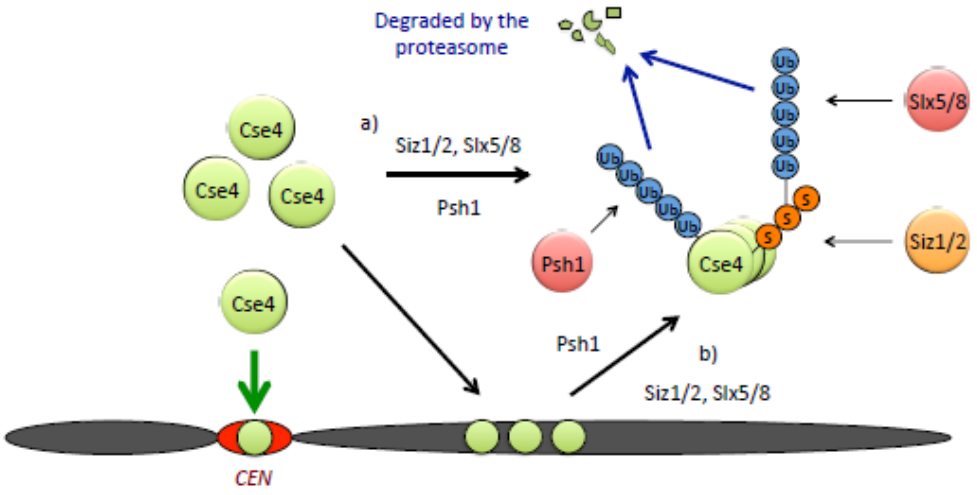

Figure 6. Model for how Slx5 regulates proteolysis of Cse4 and prevents its mislocalization to euchromatin. Restricting the localization of Cse4 to centromeric DNA is essential for faithful chromosome segregation. At least two independent pathways prevent the stable incorporation of Cse4 into euchromatin. One of these pathways is dependent on the interaction of Psh1 with Cse4. The second pathway requires sumoylation of Cse4 by Siz1/Siz2 and ubiquitination of sumoylated Cse4 by Slx5. The two pathways may regulate: a) soluble pools of Cse4 to prevent its mislocalization and/or b) facilitate proteolysis of chromatin bound Cse4.