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SUMO-targeted ubiquitin ligase (STUbL) Slx5 regulates proteolysis of centromeric histone H3 variant Cse4 and prevents its mislocalization to euchromatin

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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 and 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 shows 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 the 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 slx5Δ strains suggest that sumoylation precedes ubiquitin-mediated proteolysis of Cse4. Slx5-mediated Cse4 proteolysis is independent of Psh1, since slx5Δ psh1Δ strains exhibit higher levels of Cse4 stability and mislocalization than either slx5Δ or psh1Δ 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 noncentromeric regions, resulting in growth defects and severe chromosome missegregation during mitosis and meiosis (Choi et al., 2002; Lopes da Rosa et al., 2011), and chromatin remodeler Snf2 (Gikopoulos et al., 2011) act to prevent the mislocalization of Cse4.

Protein posttranslational modifications, 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 loci 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) regulates cellular levels of Cse4. Both the N-terminus of Cse4 and the centromere-targeting domain in the C-terminus of Cse4 (which 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 facilitates chromatin transcription/transactions (FACT) complex (Deyter and Biggins, 2014) and phosphorylation of Psh1 by casein kinase 2 (Hewawasam et al., 2014). Given that Cse4 is not completely stabilized in the psh1Δ 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 rRNA 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. Skn5 and Skx8, two of at least four STUbLs proteins in S. cerevisiae, form a heterodimer to mediate SUMO-targeted degradation of several proteins, including Mot1 and MATα2 (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 Pilus, 2013; Sriramachandran and Dohmen, 2014). Skx5, but not Skx8, has been shown to associate with centromeres, and skx5Δ and skx8Δ mutants show defects in chromosome segregation (van de Pasch et al., 2013). Depletion of the human homologue of Skx5/8, RNF4, also leads to chromosome segregation errors (van de Pasch et al., 2013), suggesting that the role of Skx5/8 is evolutionarily conserved. The kinetochrome substrates targeted and modified by Skx5/8 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. Skx5 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 psh1Δ and skx5Δ strains. Our results show that Skx5 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 posttranslational modifications 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 octahistidine-hemagglutinin (8His-HA)-tagged Cse4 using nickel-nitritolriacetic acid (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, which 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; Johnson, 2004; Takahashi et al., 2001; Montpetit et al., 2006; Reindle et al., 2006), including the kinetochrome 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 siz1Δ siz2Δ 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 siz1Δ siz2Δ strain compared with the wild-type strain (Figure 1C, pull down). The lower levels of input Cse4 in the siz1Δ siz2Δ 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 siz1Δ siz2Δ strain even upon a longer exposure.
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 h to induce expression of Cse4. Sumoylation levels of Cse4 and nonmodified 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 nonspecific sumoylated proteins that bind to the beads. The mutations of lysine to arginine in 8His-HA–cse4 16KR slightly affect its mobility compared with 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 Siz1∆440. 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∆ siz2∆ (YMB7277) strains expressing pGAL-8His-HA-CSE4 (pMB1345) were assayed as described in A. High–molecular weight species of 8His-HA–Cse4 and nonspecific sumoylated proteins are marked with arrows and an asterisk, respectively.
The STUbL Ssx5 regulates ubiquitin-mediated proteolysis of Cse4

Previous studies showed that STUbLs link SUMO and ubiquitin modification pathways to facilitate proteolysis of cellular substrates (Garza and Pilius, 2013; Srimachandran and Dohmen, 2014). Ssx5, one of four STUbL proteins (Ssx5, Ssx8, Us1, Rad18) in S. cerevisiae, forms a complex with Siz1 (Westerbeek et al., 2014), and sisk5A strains exhibit chromosome segregation defects (van de Pasch et al., 2013). Hence we investigated the role of Ssx5 in Cse4 proteolysis. To investigate whether Ssx5 interacts with Cse4 in vivo, we performed a glutathione S-transferase (GST) pull-down assay using a strain expressing GST-tagged Ssx5 and HA-tagged Cse4. Ssx5-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-Ssx5 interacts with HA-Cse4 in vivo.

To determine whether Ssx5 ubiquitinates Cse4 in vivo, we performed an affinity pull-down assay using agarose with tandem ubiquitin-binding entities (Ub7) 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, asterisk) similar in size to those in the input lane were observed from both wild-type and sisk5A 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). Because cse4 16KR cannot be ubiquitinated, this faster-migrating species represents unmodified Cse4, which likely interacts with ubiquitinated proteins such as canonical histones. The ladderization pattern of higher-molecular weight forms of Cse4 was greatly reduced in an sisk5A strain (Figure 2B). Quantification of ubiquitinated Cse4 showed a fivefold reduction in high-molecular weight forms of Cse4 when normalized to input Cse4 in the sisk5A strain (Figure 2C). Next we investigated whether 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 transient overexpression (Supplemental Figure S4B). The failure to detect highly stable in accumulation of sumoylated Cse4 species under normal physiological conditions. Even though sumoylated Cse4 species were detected in strains deleted for PSH1, an E3-ubiquitin ligase targeting Cse4, or in SIZ1/SIZ2-deleted strains. These results indicate that lack of STUbL activity in sisk5A strains contributes to the accumulation of sumoylated Cse4 species under normal physiological conditions.

Ssx5 regulates ubiquitin-mediated proteolysis of Cse4 in a Psh1-independent manner

Previous studies showed that Psh1 interacts with Cse4 and Cse4 overexpression causes growth inhibition in a psh1A strain (Hewawasam et al., 2010; Ranjitkar et al., 2010). Similar to the growth defect observed for psh1A strains, sisk5A and siz1A siz2A strains also showed growth inhibition with GAL-CSE4 on galactose medium (Supplemental Figure S3). Given that Ssx5 and Psh1 are E3 ligases that ubiquitinate Cse4 and that deletion of Ssx5 results in accumulation of sumoylated Cse4 species, we examined whether Ssx5-mediated proteolysis of Cse4 is dependent on Psh1. We constructed psh1A sisk5A strains using standard yeast mating and sporulation. The psh1A sisk5A strains do not exhibit growth defects at 30°C but exhibit a slow-growth phenotype at low (22, 25°C) and high (37°C) temperatures compared with single mutant (Supplemental Figure S4A). The psh1A sisk5A strains also exhibit sensitivity to growth on benomyl (microtubule-depolymerizing agent)–containing plates. Furthermore, psh1A sisk5A strains exhibit defects in segregation of a reporter chromosome in a colony color assay to measure chromosome transmission fidelity (Supplemental Figure S4B).

We next analyzed the stability of Cse4 after transient overexpression of HA-Cse4 in wild-type, psh1A, sisk5A, and psh1A sisk5A strains (Figure 3A). As expected, deletion of PSH1 or SXL5 moderately stabilized HA-Cse4 protein levels. In contrast, the double-deletion mutant psh1A sisk5A showed a dramatic increase in HA-Cse4 protein stability. The half-life of HA-Cse4 in psh1A sisk5A (t1/2 = 138.6 min) is twice that of psh1A (t1/2 = 77.0 min) or sisk5A (t1/2 = 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 (t1/2 = 34.7 min), modestly stable in psh1A (t1/2 = 46.2 min) and sisk5A (t1/2 = 53.3 min) single mutants, and highly stable in psh1A sisk5A strain (t1/2 = 77.0 min; Figure 3, C and D). We conclude that Ssx5 regulates ubiquitin-mediated proteolysis of Cse4 independently of Psh1.

Ssx5 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 whether Cse4 was
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∆ ubc6∆ (YOK2501) was induced by the addition of galactose (2%) for 6 h. 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∆ (YMB9035) strains expressing pGAL-3HA-CSE4 (pMB1597) were grown in raffinose/galactose (2%) for 2 h. 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 a negative control. Asterisk shows nonmodified Cse4. (C) 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∆ strain. Cse4 expression from pGAL-6His-3HA-CSE4 (pMB1458) in wild-type (BY4741) and slx5∆ (YMB9035) strains was induced by the addition of galactose (2%) for 2 h. Glucose (2%) containing 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∆ (YMB7875) strains expressing pGAL-13Myc-CSE4 (pSB816) were grown in raffinose/galactose (2%) for 4 h. 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
mislocalized in the slx5Δ strain. These approaches were previously used to show that defects in ubiquitin-mediated proteolysis of Cse4 in a psh1Δ 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 psh1Δ, slx5Δ, and psh1Δ slx5Δ 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 psh1Δ slx5Δ strains and this enrichment was further enhanced in psh1Δ slx5Δ strains. Similar results were observed for chromatin enrichment of Cse4 transiently overexpressed from a GAL promoter in psh1Δ and slx5Δ strains, with maximum enrichment in the psh1Δ slx5Δ strain (Supplemental Figure S5).

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 nonmodified 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 slx5Δ strain. Asterisk shows nonspecific sumoylated proteins that bind to the beads. Isogenic yeast strains used are wild type (YMB7290), psh1Δ (YMB7393), slx5Δ (YMB7588), and psh1Δ slx5Δ (YMB7607). (D) Kinetics of turnover from C. Cse4 protein half-life (t1/2) is indicated, and error bars in wild-type and psh1Δ slx5Δ strains represent average deviation of two replicates.

FIGURE 3: Slx5 regulates Cse4 proteolysis in a Psh1-independent manner. (A) Cse4 expressed from galactose-inducible promoter is highly stable in psh1Δ slx5Δ strain. Wild-type (BY4741), psh1Δ (YMB9034), slx5Δ (YMB9035), and psh1Δ slx5Δ (YMB9040) strains expressing pGAL-6His-3HA-CSE4 (pMB1458) were assayed as described in Figure 2D. (B) Kinetics of turnover from A. Cse4 protein half-life (t1/2) is indicated. Error bars in wild type and psh1Δ slx5Δ represent average deviation of two replicates. (C) Cse4 expressed from its own promoter is moderately stabilized in psh1Δ and slx5Δ strains and highly stable in psh1Δ slx5Δ strain. Protein extracts were prepared from cells grown to logarithmic phase in YPD, treated with 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), psh1Δ (YMB7393), slx5Δ (YMB7588), and psh1Δ slx5Δ (YMB7607). (D) Kinetics of turnover from C. Cse4 protein half-life (t1/2) is indicated, and error bars in wild-type and psh1Δ slx5Δ strains represent average deviation of two replicates.
Isogenic yeast strains used are wild type (YMB7290), independent experiments. The number of cells used is indicated (n). Isogenic yeast strains used are wild type (YMB7290), psh1Δ, slx5Δ, and psh1Δ slx5Δ 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 4,6-diamidino-2-phenylindole (DAPI)-stained nucleus were observed in psh1Δ and slx5Δ strains, and this was further exacerbated in psh1Δ slx5Δ cells (Figure 4, B and C). On the basis of 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 analysis shows that mislocalized Cse4 is highly stable in psh1Δ and slx5Δ strains**

To determine whether the euchromatic pool of mislocalized Cse4 observed in chromosome spreads of slx5Δ and psh1Δ strains is stably incorporated, we performed fluorescence recovery after photobleaching (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 slx5Δ and psh1Δ strains show a diffuse fluorescence emanating from noncentromeric 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 slx5Δ and psh1Δ cells did not exhibit recovery after photobleaching (Figure 5, A and B). We next examined FRAP of the Cse4-GFP haze in slx5Δ and psh1Δ strains. For psh1Δ strains, we imaged Cse4-GFP haze every 30 s postbleach (Figure 5, B and D); however, because the Cse4-GFP haze was dimmer in slx5Δ, we used only two Z-series (immediately postbleach and 5 min postbleach) after laser bleaching to minimize photobleaching from imaging (Figure 5, A and C). Similar to the centromere Cse4-GFP foci, the Cse4-GFP haze did not exhibit recovery after photobleaching in either slx5Δ or psh1Δ cells (Figure 5, C and D). We conclude that the ectopically localized Cse4 present in slx5Δ and psh1Δ cells, once deposited, is stable.

**DISCUSSION**

In this study, we showed 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 psh1Δ and slx5Δ strains under normal physiological conditions. Consistent with these results, Cse4 is highly enriched in the chromatin fraction and stably incorporated into euchromatin in psh1Δ and slx5Δ 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 slx5Δ and siz1Δ siz2Δ strains, similar to psh1Δ strain (Newawasam et al., 2010; Ranjitkar et al., 2010; Au et al., 2013). Second, defects in Cse4 ubiquitination observed in slx5Δ strains correlate with an increased half-life of Cse4 in these strains. Higher stability of Cse4 is also observed in an slx8Δ strain (unpublished data), 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, were observed when SLX5 and/or SLX8 are deleted (Wang and Prelich, 2009). Accumulation of sumoylated Cse4 species and increased stability of Cse4 in the slx5Δ strain suggest that sumoylation precedes ubiquitin-mediated proteolysis of Cse4. These phenotypes are not limited to cases in which 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 psh1Δ slx5Δ double mutant compared with either the slx5Δ or psh1Δ single mutant shows that Slx5-mediated proteolysis of Cse4 is independent of Psh1 under normal physiological conditions. The residual proteolysis of Cse4 observed in psh1Δ slx5Δ 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). In addition, 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 slx5Δ and psh1Δ strains, and these phenotypes are further exacerbated in the slx5Δ psh1Δ double mutant. Previous studies examined Cse4 turnover only in the context of the kinetochore, where it is stably incorporated into chromatin (Pearson et al., 2004). Although the signal is low, the increased levels of noncentromeric Cse4 present in slx5Δ and psh1Δ 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 uses 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 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 noncentromeric 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. Although we do not yet know whether 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 use multiple ubiquitination pathways for proteolysis of high levels of Cse4.

Unlike Psh1, Slx5 and Slx8 are evolutionarily conserved, and depletion of the human STUbL orthologue, 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 showed 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 slx5Δ 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 STUbLs in ubiquitin-mediated proteolysis of Cse4 provide mechanistic insights into pathways that prevent mislocalization of CENP-A and aneuploidy in human cancers.
assays were performed by inducing HA-tagged Cse4 from a GAL promoter for 2 h at 25°C. CHX and glucose were then added 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), and 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 MgCl2, and 2 mM dithiothreitol at 37°C. The reaction was stopped by adding 2× Laemmli buffer.

Protein stability and ubiquitin pull-down assays
Protein stability assay was performed as described previously (Ohkuni et al., 2014). 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-A440 proteins—were expressed and purified from Escherichia 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), and 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 MgCl2, and 2 mM dithiothreitol at 37°C. The reaction was stopped by adding 2× Laemmli buffer.

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 Siz5. The two pathways may a) regulate soluble pools of Cse4 to prevent its mislocalization and/or b) facilitate proteolysis of chromatin-bound Cse4.

MATERIALS AND METHODS

Yeast strains and plasmids
Supplemental Tables 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-A440 proteins—were expressed and purified from Escherichia 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), and 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 MgCl2, and 2 mM dithiothreitol at 37°C. The reaction was stopped by adding 2× 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 1% yeast extract, 2% bactopeptone, and 2% glucose (YPD) 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 (MMS-101P; Covance, Emeryville, CA) was used as primary antibody at 1:2500 dilution. Cy3-conjugated goat anti-mouse antibody (115165003; Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary antibody at 1:5000 dilution. Cells were visualized by DAPI staining (1 μg/ml in phosphate-buffered saline) mounted in antifade mountant (P36935; Molecular Probes, Eugene, OR). Cells were observed under an Axioskop 2 (Zeiss) fluorescence microscope equipped with a Plan-Apochromat 100× (Zeiss, Thornwood, NY) oil immersion lens. Image acquisition and processing were performed with the IP Lab version 3.9.9 r3 software (Scantographics, Fairfax, VA).

GST pull-down assay
The ubc6Δ ubc6Δ (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 medium with proline as nitrogen source and 2% sucrose to logarithmic phase, and then 2% galactose and 0.003% SDS were added to the cultures and incubation continued for another 6 h. MG132, 75 μM, was added ½ h before harvesting of the cells. We assayed 200 OD units of yeast cells 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 1% yeast extract, 2% bactopeptone, and 2% glucose (YPD) 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 (MMS-101P; Covance, Emeryville, CA) was used as primary antibody at 1:2500 dilution. Cy3-conjugated goat anti-mouse antibody (115165003; Jackson ImmunoResearch Laboratories, West Grove, PA) was used as secondary antibody at 1:5000 dilution. Cells were visualized by DAPI staining (1 μg/ml in phosphate-buffered saline) mounted in antifade mountant (P36935; Molecular Probes, Eugene, OR). Cells were observed under an Axioskop 2 (Zeiss) fluorescence microscope equipped with a Plan-Apochromat 100× (Zeiss, Thornwood, NY) oil immersion lens. Image acquisition and processing were performed with the IP Lab version 3.9.9 r3 software (Scantographics, Fairfax, VA).

Antibodies
Antibodies for experiments were as follows: rabbit polyclonal anti-Cse4 (Strunnikov laboratory), anti-Tub2 antibodies (Basrai laboratory), anti-HA (12CA5; Roche, Indianapolis, IN), anti-HA (ab9110; Abcam, Cambridge, MA), anti-myc (A-14; Santa Cruz Biotechnology), and anti-H3 (ab1791; Abcam). Anti-Cse4 was used at a dilution of 1:1000, anti-HA was used at a dilution from 1:1000 to 1:10,000, anti-Tub2 and anti-Smt3 were used at 1:3000, and anti-GST and anti-H3 were used at 1:5000.

FRAP
Strains YMB9430 (Cse4-GFP, psh1Δ) and YMB9429 (Cse4-GFP, slx5Δ) were grown in YPD to mid-logarithmic growth phase before imaging. Both YMB9430 and YMB9429 were grown at 24°C, but YMB9429 was shifted to 37°C 6 h before imaging. Cells were imaged using a Nikon Eclipse Ti wide-field inverted microscope with a 100×/1.49 numerical aperture Apo total internal reflection fluorescence objective (Nikon, Melville, NY) and Andor Clara charge-coupled device camera (Andor, South Windsor, CT) using Nikon NIS Elements imaging software. Photobleaching was performed with a Sapphire 488-50 CDRH laser (Coherent, Santa Clara, CA). A seven-step Z-series with
200-nm step size with 600-ms exposure time was taken before a 300-ms exposure lapse from the laser. Immediately after the laser exposure, a 5-min time lapse with 30-s intervals with the same settings as the first Z-series was initiated. The Z-series was 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). The integrated intensity of the bleached area had the integrated intensity of the cell background subtracted at each time point, 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).

Chromosome transmission fidelity
The chromosome transmission fidelity 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 d. Loss of the reporter chromosome results in red sectors in an otherwise white colony. Colonies that are at least half red indicate loss of the reporter chromosome in the first cell division.

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We 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 advice, Tatiana Karpova (Fluorescent Imaging Facility, National Cancer Institute) for assistance with cell biology experiments, Kathy McKinnon (Vaccine Branch FACS Core, National Cancer Institute) for assistance with FACS, and Anita Corbett, Ian Cheseman, Michael Lichtten, Tom Mistel, and Peter Kaiser for comments on the manuscript. This work was supported by the National Institutes of Health Intramural Research Program to M.B., National Science Foundation Grant MBC 1051970 to O.K., and National Institutes of Health R37 Grant GM32238 to K.B.

REFERENCES


SUPPLEMENTAL INFORMATION

**Supplemental Figure S1.** Purified Cse4 used for *in vitro* reaction. Coomassie blue staining of purified Cse4 for *in vitro* reaction is shown.

**Supplemental Figure S2.** Deletion of *SIZ1* and *SIZ2* stabilizes Cse4 protein level *in vivo.* Cse4 expression from *pGAL-6His-3HA-CSE4* (pMB1458) in wild-type (BY4741) and *siz1Δ siz2Δ* (YMB7277) 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.

**Supplemental Figure S3.** Synthetic dosage lethality of *GAL-CSE4* in *psh1Δ, slx5Δ,* or *siz1Δ siz2Δ* mutants. Wild-type (BY4741), *psh1Δ* (YMB9034), *slx5Δ* (YMB9035), and *siz1Δ siz2Δ* (YMB7277) strains were transformed with either vector (pMB433) or *pGAL-6His-3HA-CSE4* (pMB1458). Yeast cells were spotted in 5-fold dilutions on glucose (2%)- or galactose (2%)- containing synthetic medium selective for vector or *GAL-CSE4.* The plates were incubated at 25°C for 4 days and photographed.

**Supplemental Figure S4.** (A) *psh1Δ slx5Δ* strains exhibit temperature and benomyl sensitivities. Cells were spotted in 5-fold dilutions on YPD and YPD supplemented with benomyl. The plates were incubated at indicated temperatures for 2 days and photographed. Isogenic yeast strains used are wild-type (BY4741), *psh1Δ* (YMB9034),
slx5Δ (YMB9035), psh1Δ::natR slx5Δ::kanMX (YMB9037 and 9038), and
psh1Δ::kanMX slx5Δ::natR (YMB9039 and 9040). (B) psh1Δ slx5Δ strains exhibit
defects in chromosome transmission fidelity. Chromosome loss was determined by
counting the number of colonies that show loss of the reporter chromosome in the first
division and exhibit a half sector phenotype. Statistical significance was determined by
chi-square test. NS, not significant. Isogenic yeast strains used are wild-type (YMB9378
and 9379), psh1Δ (YMB9425 and 9426), slx5Δ (YMB9375 and 9376), and psh1Δ slx5Δ
(YMB9427 and 9428).

**Supplemental Figure S5.** Cse4 is highly enriched in the chromatin fraction in psh1Δ
slx5Δ cells. Wild-type (BY4741), psh1Δ (YMB9034), slx5Δ (YMB9035), and psh1Δ
slx5Δ (YMB9040) strains transformed with pGAL-3HA-CSE4 (pMB1597) were used for
subcellular fractionation. Cse4 expression was induced by the addition of galactose (2%)
for 4 hrs. Whole cell extracts (WCE) were fractionated into soluble and chromatin
fractions. Cse4 levels in each fraction were monitored by western blot analysis with anti-
HA antibody. Tub2 and histone H3 were used as markers for soluble and chromatin
fractions, respectively.
## Supplemental Table S1. *S. cerevisiae* stains used in this study

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Supplemental Table S2. Plasmids used in this study.

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**Figure S2**
Glucose

Galactose

Wild-type

Vector

GAL-CSE4

psh1Δ

Vector

GAL-CSE4

slx5Δ

Vector

GAL-CSE4

siz1Δ siz2Δ

Vector

GAL-CSE4

Figure S3
A

Wild-type
psh1Δ::kanMX
slx5Δ::kanMX

psh1Δ::natR slx5Δ::kanMX

psh1Δ::kanMX slx5Δ::natR

22°C  25°C  30°C  37°C

30°C

Benomyl:
10 µg/ml  15 µg/ml  20 µg/ml

Wild-type
psh1Δ::kanMX
slx5Δ::kanMX

psh1Δ::natR slx5Δ::kanMX

psh1Δ::kanMX slx5Δ::natR

B

Chromosome Transmission Fidelity (CTF)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Half sector/Total colonies</th>
<th>Loss per 1000 divisions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>2/2804</td>
<td>0.71</td>
</tr>
<tr>
<td>psh1Δ</td>
<td>2/3145</td>
<td>0.64 (NS)</td>
</tr>
<tr>
<td>slx5Δ</td>
<td>1/1422</td>
<td>0.70 (NS)</td>
</tr>
<tr>
<td>psh1Δ slx5Δ</td>
<td>4/1619</td>
<td>2.47 (P &lt; 0.01)</td>
</tr>
</tbody>
</table>

Figure S4
<table>
<thead>
<tr>
<th></th>
<th>WCE</th>
<th>Soluble</th>
<th>Chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
<td><img src="image3.png" alt="Image" /></td>
</tr>
<tr>
<td>psh1Δ</td>
<td><img src="image4.png" alt="Image" /></td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>slx5Δ</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
<tr>
<td>psh1Δ slx5Δ</td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
</tr>
</tbody>
</table>

- **α-HA (Cse4)**
- **α-Tub2**
- **α-H3**

*Figure S5*