N-terminal sumoylation of centromeric histone H3 variant Cse4 regulates its proteolysis to prevent mislocalization to non-centromeric chromatin

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Abstract

Stringent regulation of cellular levels of evolutionarily conserved centromeric histone H3 variant (CENP-A in humans, CID in flies, Cse4 in yeast) prevents its mislocalization to non-centromeric chromatin. Overexpression and mislocalization of CENP-A has been observed in cancers and leads to aneuploidy in yeast, flies and human cells. Ubiquitin-mediated proteolysis of Cse4 by E3 ligases Psh1 and Sumo-Targeted Ubiquitin Ligase (STUbL) Slx5 prevent mislocalization of Cse4. Previously, we found that sumoylation of Cse4 by E3 ligases Siz1 and Siz2 and ubiquitin mediated proteolysis of Cse4 by Slx5 prevents mislocalization to non-centromeric chromatin. In this study, we identify lysine 65 (K65) in Cse4 as a SUMO site and show that sumoylation of Cse4 K65 regulates its ubiquitin-mediated proteolysis by Slx5. Strains expressing *cse4 K65R* exhibit reduced levels of sumoylated and ubiquitinated Cse4 *in vivo*. Furthermore, co-immunoprecipitation experiments reveal reduced interaction of cse4 K65R with Slx5. Defects in sumoylation of cse4 K65R contribute to increased stability and mislocalization under normal physiological conditions. Based on the increased stability of cse4 K65R in a *psh1*Δ strain but not in an *slx5*Δ strain, we conclude that sumoylation of Cse4 K65 regulates Slx5-mediated proteolysis independent of Psh1. In summary, we have identified and characterized the physiological role of Cse4 sumoylation and determined that sumoylation of Cse4 K65 regulates ubiquitin-mediated proteolysis and prevents mislocalization of Cse4 which is required for genome stability.

Introduction

Evolutionarily conserved centromeric histone H3, CENP-A (Cse4 in *Saccharomyces cerevisiae*, Cnp1 in *Schizosaccharomyces pombe*, CID in *Drosophila*, and CENP-A in humans), is essential for faithful chromosome segregation. Overexpression of CID, Cse4 and CENP-A leads to its mislocalization to non-centromeric regions and contributes to aneuploidy in flies, yeast and human cells respectively (1-5). Overexpression and mislocalization of CENP-A are observed in many cancers and is associated with poor prognosis and increased invasiveness (6-13). Hence, characterization of pathways that regulate cellular levels of CENP-A and its homologs will help us to understand how overexpression and mislocalization of CENP-A contributes to tumorigenesis in human cells.

In budding yeast, ubiquitin-mediated proteolysis of Cse4 has been shown to regulate cellular levels of Cse4 and this prevents its mislocalization to non-centromeric regions (3). Proteolysis of Cse4 is regulated by several proteins: Psh1 (E3 ubiquitin ligase), Doa1 (WD-repeat protein), Fpr3 (proline isomerase), Ubp8 (ubiquitin protease), and Rcy1 (F-box protein) (14-19). Psh1-mediated proteolysis of Cse4 is regulated by its interaction with Spt16, a component of the FACT (facilitates chromatin transcription/transactions) complex, and casein kinase 2 (CK2) (20,21). Even though Psh1 interacts with the CENP-A targeting domain (CATD) and ubiquitinates lysine (K) residues (K131, K155, K163, and K172) in the C-terminus of Cse4 (14,15), several observations implicate the N-terminus of Cse4 in ubiquitin-mediated proteolysis. For example, ubiquitination of cse4 Δ129 (a deletion of the entire N-terminus of Cse4) is barely detectable (16). The stability of cse4 KC, where all K in the N-terminus of Cse4

are mutated to arginine, is at least three-fold higher than that of wild-type Cse4 (16). Furthermore, the stability of cse4 KC is at least four-fold higher than that of cse4 CK, where all K in the C-terminus of Cse4 are mutated to arginine (16). Proteolysis defects in N-terminus Cse4 mutants contribute to chromosome loss, as strains expressing *cse4 KC* exhibit a four-fold increase in chromosome segregation defects when compared to *cse4 CK* strains (16). These results indicate that ubiquitination and interaction of Psh1 with the C-terminus of Cse4 is not sufficient for Cse4 ubiquitin-mediated proteolysis. Studies with fission yeast have also shown that the N-terminus of Cnp1 mediates ubiquitin-mediated proteolysis of Cnp1 to prevent its mislocalization to non centromeric chromatin (22).

In addition to ubiquitination, we have recently reported that Cse4 is sumoylated by the small ubiquitin-related modifier (SUMO) E3 ligases Siz1 and Siz2 and that SUMO modification of Cse4 regulates its proteolysis (23). We showed that *slx5*Δ strains exhibit defects in Cse4 proteolysis and localization that are independent of Psh1. Slx5 is a SUMO-targeted ubiquitin ligase which regulates many substrates with diverse biological functions such as transcription and genome stability. Slx5 associates with centromeric chromatin and *slx5*Δ strains exhibit aneuploidy, spindle mispositioning, and aberrant spindle kinetics (24). Given the multiplicity of Slx5 substrates, it is possible that the defect in Cse4 proteolysis in *slx5*Δ strains may be indirect due to modification of another substrate. Hence, we sought to identify sumoylation sites in the N-terminus of Cse4 and investigate the role of sumoylation of Cse4 in proteolysis.

Here we report the identification of K65 in Cse4 as a SUMO site that is regulated by Slx5 in a Psh1-independent manner. We found that reduced levels of sumoylation and

ubiquitination of cse4K65R contribute to its increased stability and mislocalization under normal physiological conditions.

Results

Cse4 K65 is a target of sumoylation.

The consensus motif Ψ-K-x-D/E (Ψ is a hydrophobic residue, K is the lysine to be conjugated to SUMO, x is any amino acid, D or E is an acidic residue) is frequently observed in sumoylated substrates (25-27). We identified lysine (K) 65 (64-S**K**SD-67) in the N-terminus of Cse4 and K215/K216 (214-M**KK**D-217) in the C-terminus of Cse4 as potential sumoylation sites. Given the importance of the Cse4 N-terminus in ubiquitination and proteolysis (16), we focused on identification and characterization of the role of Cse4 sumoylation on K65. We created strains expressing either vector alone, *8His-Hemagglutinin (HA)-tagged CSE4*, *8His-HA-cse4 16KR* (all K mutated to R), or *8His-HA-cse4 K65R* (K65 mutated to R65), performed a pull down of 8His-HA-Cse4 using Ni-NTA agarose beads, and detected SUMO-modified Cse4 by western blot analysis with an anti-Smt3 antibody (Fig. 1). At least three distinct molecular weight bands were observed in wild-type cells (Fig. 1A, denoted by arrows), as previously described (23). These SUMO-modified Cse4 species were not detected in strains expressing vector alone or *8His-HA-cse4 16KR*. Strains expressing *8His-HA-cse4 K65R* showed greatly reduced levels of Cse4 sumoylation even though similar levels of Cse4 and cse4 K65R were pulled down (Fig. 1, A and B). We conclude that Cse4 K65 is sumoylated in wild-type cells.

Cse4 ubiquitination is reduced in a *cse4 K65R* mutant.

The reduced sumoylation of cse4 K65R prompted us to investigate the role of K65 in the ubiquitination of Cse4. An affinity pull down assay was done using agarose with tandem ubiquitin-binding entities (Ub^+) from a strain expressing *3HA-tagged CSE4* (Fig. 2). A faster migrating species (Fig. 2A, asterisk) was observed in all strains expressing Cse4. These faster migrating Cse4 species were similar in size to that in the input lanes. These species were also observed in experiments with *cse4 16KR* mutant (all K mutated to R) (16). Since cse4 16KR cannot be ubiquitinated, this faster migrating species represents unmodified Cse4, which likely interacts with ubiquitinated proteins such as canonical histones. Ubiquitinated Cse4 was detected as a laddering pattern in wild-type cells but was absent in strains with vector alone (Fig. 2A). However, reduced levels of ubiquitinated cse4 K65R were observed (Fig. 2, A and B). Lack of a drastic reduction in ubiquitination of cse4 K65R is not surprising given that the other fifteen lysines can still be ubiquitinated. Our results show that Cse4 K65 contributes to Cse4 ubiquitination.

Cse4 K65 is regulated by STUbL Slx5.

We have previously shown that STUbL Slx5 interacts with Cse4 and promotes its ubiquitin-mediated proteolysis (23). Slx5 contains SUMO-interacting motifs (SIMs), which interact with sumoylated substrates (28,29). To determine if the observed defect in sumoylation of cse4 K65 would result in a reduced interaction with Slx5, co-immunoprecipitation (Co-IP) experiments were carried out using strains co-expressing

either *3HA-CSE4* or *3HA-cse4 K65R* with *SLX5-Myc* (Fig. 3). As expected, immunoprecipitation with anti-HA showed an interaction between wild-type Cse4 and Slx5 (Fig. 3A). In contrast, reduced interaction was observed between cse4 K65R and Slx5 in two indepedent strains (Fig. 3, A and B). Hence, sumoylation of Cse4 K65 contributes to its interaction with Slx5.

Sumoylation of Cse4 K65 regulates its proteolysis and localization under normal physiological conditions.

To determine if cse4 K65R was defective in proteolysis under normal physiological conditions, protein stability assays were carried out with cells expressing either *CSE4* or *cse4 K65R* from its endogenous promoter. Cells were grown logarithmically and then treated with cycloheximide (CHX) to inhibit protein translation. Cell extracts were assayed for Cse4 levels at different times after CHX treatment. Cse4 is rapidly degraded ($t_{1/2}$ = 33.5 ± 0.8 min), whereas cse4 K65R is modestly stabilized ($t_{1/2}$ = 50.8 ± 6.1 min) (Fig. 4, A and B). These observations show that Cse4 K65 regulates its proteolysis.

Previous studies have shown that defects in ubiquitin-mediated proteolysis of Cse4 in *slx5*Δ and *psh1*Δ strains lead to mislocalization of Cse4 to non-centromeric regions under normal physiological conditions (23). Therefore, we examined whether the increased stability observed for endogenously expressed *cse4 K65R* were sufficient to cause mislocalization. Chromosome spreads were used to visualize localization of chromatin-bound Cse4. In wild-type cells, majority of the cells showed localization of Cse4 restricted to one or two dots, corresponding to kinetochore clusters (Fig. 4, C and

D). In contrast to wild-type cells, higher incidence of multiple or diffused Cse4 foci were observed in *cse4 K65R* strains (Fig. 4, C and D). Hence, ubiquitin-mediated proteolysis of Cse4 via sumoylation of K65 prevents its mislocalization to non-centromeric chromatin.

SUMO modification of Cse4 K65 is required for Slx5-mediated proteolysis independent of Psh1.

Based on the reduced interaction of cse4 K65R with Slx5 (Fig. 3), we predicted that the stability of cse4 K65R would not differ significantly from that of Cse4 in *slx5*Δ strains. In agreement with previous results (23), protein stability assays showed that Cse4 was more stable in the *slx5*Δ strain ($t_{1/2}$= 43.0 ± 6.5 min) when compared to that in the wild-type strain ($t_{1/2}$= 33.5 ± 0.8 min) (Fig. 5A). In contrast, the stability of cse4 K65R ($t_{1/2}$= 43.5 ± 2.7 min) was not significantly different than that of Cse4 ($t_{1/2}$ = 43.0 ± 6.5 min) in the *slx5*Δ strain (Fig. 5, A and B).

We showed previously that Slx5 regulates proteolysis of Cse4 in a Psh1-independent manner even under normal physiological conditions (23). Hence, we reasoned that in a *psh1*Δ strain, cse4 K65R would be more stable compared to Cse4. Protein stabililty assays showed that Cse4 was more stable in a *psh1*Δ strain ($t_{1/2}$= 42.9 ± 3.4 min) compared to that observed in a wild type strain ($t_{1/2}$= 33.5 ± 0.8 min) (Fig. 5C). Consistent with our prediction, the stability of cse4 K65R ($t_{1/2}$ = 58.0 ± 2.8 min) was much higher than that of Cse4 in the *psh1*Δ strain ($t_{1/2}$ = 42.9 ± 3.4 min) (Fig. 5, C and D). Therefore, we conclude that SUMO modification of Cse4 K65 is required for Slx5-mediated proteolysis that is independent of Psh1.

Discussion

We have identified Cse4 K65 as a sumoylation site and showed that ubiquitin-mediated proteolysis due to sumoylation of Cse4 K65 is regulated by Slx5 in a Psh1-independent manner. Defects in Cse4 sumoylation, proteolysis, localization and its interaction with Slx5 were observed in strains expressing *cse4 K65R*. The increased stability of cse4 K65R in a *psh1*Δ strain but not in an *slx5*Δ strain indicates that sumoylation of Cse4 K65 regulates Slx5-mediated ubiquitination of Cse4 and its proteolysis, independently of Psh1. We propose that in wild-type cells sumoylation of Cse4 K65 regulates its interaction with Slx5, and this is required for Slx5-mediated proteolysis of Cse4 to prevent mislocalization of Cse4.

Reduced Cse4 sumoylation observed for the *cse4 K65R* mutant supports the role of Cse4 K65 as a sumoylation site in the N-terminus of Cse4. A four-fold reduction in sumoylation of cse4 K65R was observed compared to wild-type Cse4. Sumoylation of Cse4 K65 regulates its proteolysis and localization. This is supported by our observations of reduced ubiquitination levels in *cse4 K65R*, defects in proteolysis of cse4 K65R, and mislocalization of cse4 K65R to non-centromeric chromatin under normal physiological conditions. Hence, we conclude that Cse4 K65 sumoylation regulates ubiquitin-mediated proteolysis of Cse4 to prevent its mislocalization to non-centromeric chromatin. Defects in sumoylation and ubiquitination of cse4 K65R are not as severe as that observed for cse4 16KR. Furthermore, ubiquitination defects of Cse4 are more pronounced in *slx5*Δ strains (23), compared to that observed for cse4 K65R. These results show that

sumoylation and ubiquitination of other lysine residues also contribute to proteolysis of Cse4 by E3 ligases such as Psh1, Slx5, and F-box protein, Rcy1.

We previously showed that Slx5-mediated proteolysis is a downstream consequence of Cse4 sumoylation, resulting in accumulation of high molecular weight sumoylated Cse4 species in *slx5*Δ strains but not in the wild type strain (23). Based on the phenotypes of strains expressing *cse4 K65R*, we propose that Cse4 K65 sumoylation regulates its proteolysis by Slx5 but not by Psh1 as we observed: 1) a reduced interaction of mutant cse4 K65R with Slx5, 2) no additional proteolysis defects of cse4 K65R in *slx5*Δ strains and 3) increased proteolysis defects of cse4 K65R in *psh1*Δ strains. Moreover, reduced levels of sumoylated cse4 K65R were also detected in a *slx5*Δ strain (data not shown). This result show that sumoylation of cse4 K65 contributes to the high molecular weight sumoylated species observed for wild type Cse4 in *slx5*Δ strains. Taken together, we conclude that SUMO modification of Cse4 K65 is required for Slx5-mediated proteolysis.

In summary, our studies have identified and characterized the physiological role of Cse4 sumoylation and shown that sumoylation of Cse4 K65 regulates ubiquitin-mediated proteolysis to prevent mislocalization of Cse4. Several studies have shown that deregulation of the sumoylation machinery causes severe defects in cell proliferation and genome stability and promote tumorigenesis (30,31). Sumoylation of kinetochore proteins or proteins required for sister chromatid cohesion and spindle assembly checkpoint regulate faithful chromosome segregation (30,32). Since ubiquitination and sumoylation prevents mislocalization of Cse4 for genome stability, it will be of interest to

determine if these post-translational modifications of CENP-A also prevent its mislocalization to preserve genome stability in human cells.

Experimental Procedures

Yeast strains and plasmids

Table 1 and 2 describe the genotype of yeast strains and plasmids used for this study, respectively.

Cell lysate preparation for pull down assays and coimmunoprecipitation (co-IP)

For pull down assays and immunoprecipitation, cell lysates were prepared as follows. 50 OD_{600} of logarithmically growing cells were pelleted, rinsed with H_2O, and lysed with 0.5 ml of guanidine buffer (0.1 M Tris-HCl at pH 8.0, 6.0 M guanidine chloride, 0.5 M NaCl) for sumoylation assay, or Ub lysis buffer (20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 50 mM NaF, 5 mM tetra-sodium pyrophosphate, 10 mM beta-glycerophosphate, 2 mM EDTA, 1 mM DTT, 1 % NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM N-ethylmaleimide (NEM), 1x protease inhibitor cocktail (Sigma, P8215)) for ubiquitin pull down assay, or IP lysis buffer (50 mM Tris-HCl at pH 8.0, 5 mM EDTA, 1 % TritonX-100, 150 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM PMSF, 1x protease inhibitor cocktail) for coimmunoprecipitation. Then cells were disrupted with 0.5 mm diameter glass beads or Matrix C (MP Biomedicals) using a bead beater (MP Biomedicals, FastPrep-24 5G). Cell lysates were clarified by centrifugation at 6,000 rpm for 5 min and the concentration of the extracted proteins was determined using a DC

protein assay kit (Bio-Rad). Samples containing equal amounts of protein were brought to a total volume of 1 ml with appropriate buffer.

Sumoylation assay *in vivo*

In vivo sumoylation was assayed in crude yeast extracts using Ni-NTA agarose beads to pull down His-HA-tagged Cse4 as described previously (33). Cell lysates were incubated with 100 μl of Ni-NTA superflow beads (Qiagen, 30430) overnight at 4 °C. After being washed with guanidine buffer one time and with breaking buffer (0.1 M Tris-HCl at pH 8.0, 20 % glycerol, 1 mM PMSF) four times, beads were incubated with 2x Laemmli buffer including imidazole at 100°C for 10 min. The protein samples were subjected to SDS-PAGE and Western Blotting.

Ubiquitin pull down assay

Ubiquitin-pull down assay was performed as described previously (16). Cell lysates were incubated with 25 μl of tandem ubiquitin-binding entities Agarose-TUBE1 (LifeSensors, Inc. UM401) overnight at 4 °C. After being washed with TBS-T (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.1 % Tween20) three times, beads were incubated with 2x Laemmli buffer at 100°C for 5 min. The protein samples were subjected to SDS-PAGE and Western Blotting.

Coimmunoprecipitation (Co-IP)

Cell lysates were incubated with 25 μl of Anti-HA-Agarose antibody (Sigma, A2095) overnight at 4 °C. After being washed with IP lysis buffer three times, beads were

incubated with 2x Laemmli buffer at 100°C for 5 min. The protein samples were subjected to SDS-PAGE and Western Blotting.

Protein stability assay

Protein stability assays were performed as described previously (23). Logarithmically growing cells in YPD were treated with cycloheximide (CHX) (20 μg/ml) for various time points. Multiple independent measurements were made, and means are given on the applicable figures ± the standard error of the mean with the number of replicates given in parentheses. Statistical significance was assessed by one-way ANOVA ($P = 0.0075$) with selected pairwise post hoc tests. Bonferroni correction was applied to adjust P values for multiple comparisons.

SDS-PAGE and Western Blotting

SDS-PAGE was performed using 4-12 % Bis-Tris gels (Novex). After running the gels, proteins were transferred onto nitrocellulose membrane (VitaScientific, DBOC80014). Western blotting was performed according to a standard protocol. SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, 34580) or One Component Chemiluminescent Substrate (Rockland, UniGlow-0100) was used for the detection. Antibodies were as follows: anti-HA (12CA5) mouse (Roche, 11583816001), anti-HA rabbit (Sigma, H6908), anti-c-Myc (A-14) (Santa Cruz Biotechnology, sc-789), anti-Smt3 (y-84) (Santa Cruz Biotechnology, sc-28649), anti-Tub2 antibodies (Basrai laboratory), and anti-FLAG (Sigma, F3165). Anti-HA was used at a dilution from 1:1000 to 1:5000, and anti-c-Myc, anti-Smt3, anti-FLAG, and anti-Tub2 were used at a dilution

from 1:3000 to 1:5000. Second antibodies were ECL Mouse IgG, HRP-Linked Whole Ab (GE Healthcare Life Sciences, NA931V) or ECL Rabbit IgG, HRP-linked whole Ab (GE Healthcare Life Sciences, NA934V) at 1:5000 dilution.

Chromosome spreads

Chromosome spreads were performed as described previously (3,34) with some modifications. Cells were grown in YPD logarithmically. 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.).

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Conflict of interest

The authors declare that they have no conflict of interest with the contents of this article.

Author contributions

K.O. designed the study, conducted all the experiments, analyzed the data, and wrote the manuscript. R.L.-M. contributed to sumoylation assays, J. W. performed some of the chromosome spreads experiments. W.C.A. generated plasmids and provided technical advice for biochemical assays. Y.T. made comments on the manuscript. R.E.B. performed statistical analyses and assisted with the writing of the manuscript. M.A.B. guided the project and contributed to the writing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Table 1. *S. cerevisiae* stains used in this study.

Strains	Parent	Genotype	Reference
BY4741		*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4742		*MAT*α *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
YMB9470	BY4741/BY4742	*MAT*α *cse4Δ::kanMX6* [pRS415-*6His-3HA-CSE4*]	This study
YMB9471	BY4741/BY4742	*MAT*α *cse4Δ::kanMX6 psh1Δ::kanMX6* [pRS415-*6His-3HA-CSE4*]	This study
YMB9472	BY4741/BY4742	*MAT*α *cse4Δ::kanMX6 slx5Δ::kanMX6* [pRS415-*6His-3HA-CSE4*]	This study
YMB9547	BY4741/BY4742	*MAT*α *cse4Δ::kanMX6* [pRS415-*6His-3HA-cse4 K65R*]	This study
YMB9548	BY4741/BY4742	*MAT*α *cse4Δ::kanMX6 psh1Δ::kanMX6* [pRS415-*6His-3HA-cse4 K65R*]	This study
YMB9549	BY4741/BY4742	*MAT*α *cse4Δ::kanMX6 slx5Δ::kanMX6* [pRS415-*6His-3HA-cse4 K65R*]	This study
YMB9694	BY4741	*MAT***a** *SLX5-Myc:kanMX6*	This study

Table 2. Plasmids used in this study.

Plasmid	Relevant characteristics	Reference
pYES2	Vector (*pGAL*, *2μ*, *URA3*)	(35)
pMB433	Vector (p426-*pGAL1*, *2μ*, *URA3*)	(36)
pMB1344	pYES2-*pGAL-8His-HA-cse4 16KR* (*2μ*, *URA3*)	(23)
pMB1345	pYES2-*pGAL-8His-HA-CSE4* (*2μ*, *URA3*)	(23)
pMB1458	pMB433-*pGAL-6His-3HA-CSE4* (*2μ*, *URA3*)	(16)
pMB1597	pMB433-*pGAL-3HA-CSE4* (*2μ*, *URA3*)	(23)
pMB1725	pRS415-*6His-3HA-CSE4* (*CEN, LEU2*)	This study
pMB1749	pRS415-*6His-3HA-cse4 K65R* (*CEN, LEU2*)	This study
pMB1762	pYES2-*pGAL-8His-HA-cse4 K65R* (*2μ*, *URA3*)	This study
pMB1791	pMB433-*pGAL-6His-3HA-cse4 K65R* (*2μ*, *URA3*)	This study
pMB1814	pMB433-*pGAL-3HA-cse4 K65R* (*2μ*, *URA3*)	This study

Figure Legends

Figure 1. Cse4 K65 is sumoylated *in vitro*. (A) Wild-type strain (BY4741) transformed with vector (pYES2), *pGAL-8His-HA-CSE4* (pMB1345), *pGAL-8His-HA-cse4 16KR* (pMB1344), or *pGAL-8His-HA-cse4 K65R* (pMB1762) was grown in raffinose/galactose (2%) for 4 hours to induce expression of Cse4. Sumoylation of 8His-HA-Cse4 and nonmodified 8His-HA-Cse4 was assayed using protein extracts after pull down on Ni-NTA beads followed by western blot analysis with anti-Smt3 and anti-HA (Cse4) antibodies, respectively. At least three high molecular weight bands of 8His-HA-Cse4 (arrows) were detected. Input samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies served as loading control for input. Asterisk shows nonspecific sumoylated proteins that bind to beads. (B) Quantitation of sumoylation in arbitrary density units (normalized to non-modified Cse4 probed by anti-HA in pull down sample) determined in multiple experiments (N = 3, 3, 2 for wild-type, Cse4 16KR, and Cse4 K65R, respectively). Statistical significance was assessed by one-way ANOVA (P = 0.007) followed by all possible pairwise comparisons of the means with Bonferroni correction. **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

Figure 2. Cse4 ubiquitination is reduced in a *cse4 K65R* mutant. (A) Protein extracts were prepared from wild-type strain (BY4741) transformed with vector (pMB433), *pGAL-3HA-CSE4* (pMB1597), or *pGAL-3HA-cse4 K65R* (pMB1814) grown in raffinose/galactose (2%) for 4 hours to induce expression of Cse4. Agarose-TUBE1 was used to pull down tandem ubiquitin binding entities and ubiquitination levels of Cse4 were detected by western blot analysis with anti-HA antibody. Input samples were

analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Asterisk shows non-modified Cse4. (B) Quantitation of ubiquitination in arbitrary density units (normalized to levels of Cse4 in input) in replicate determinations (N = 3). Unpaired t test of the means is shown.

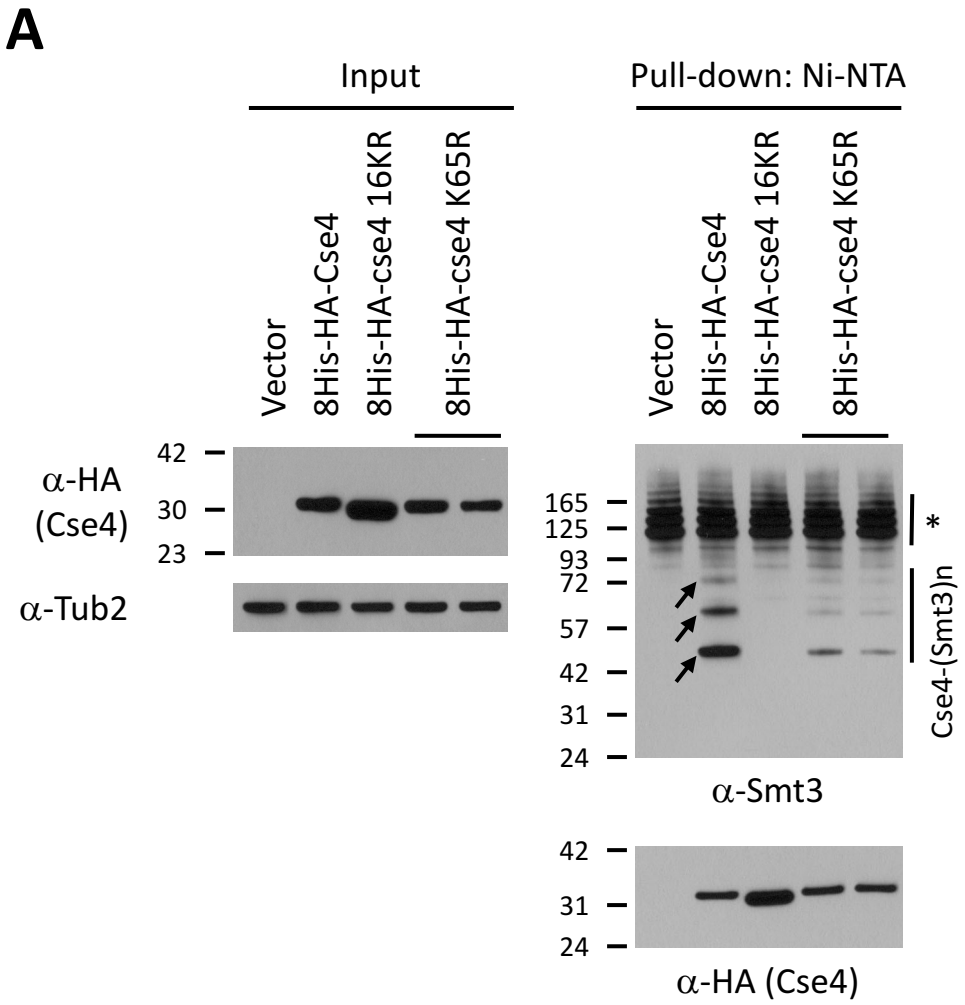
Figure 3. K65R mutation in Cse4 contributes to reduced interaction with Slx5. (A) Protein extracts were prepared from the indicated strains grown in raffinose/galactose (2%) for 4 hours to induce expression of Cse4 and incubated with anti-HA agarose antibody. Samples were resolved by SDS-PAGE and levels of Slx5 and Cse4 were detected by western blot analysis with anti-Myc and anti-HA antibodies, respectively. Isogenic yeast strains were: untagged (BY4741), or Slx5-Myc (YMB9694) strain transformed with vector (pMB433), *pGAL-3HA-CSE4* (pMB1458), or *pGAL-3HA-cse4 K65R* (pMB1791). (B) Levels of Slx5 (α-Myc, IP) after immunoprecipitation of 3HA-Cse4 were quantified in arbitrary density units after normalization to Cse4 levels in the IP (α-HA, IP) using replicate determinations (N = 2, 3 for HA-Cse4 and HA-Cse4 K65R, respectively). Unpaired t test of the means is shown.

Figure 4. Sumoylation of Cse4 K65 regulates its proteolysis and localization under normal physiological conditions. (A) Protein extracts from *CSE4* (YMB9470) or *cse4 K65R* (YMB9547) strain were prepared using logarithmically growing cells, treated with CHX (20 μg/ml) for various time points. Blots were probed with anti-HA (Cse4) or anti-Tub2 (loading control) antibody. A representative blot is shown. Cse4 protein half-life ($t_{1/2}$) is reported as the mean ± SEM of multiple biological repeats. The difference in $t_{1/2}$ is statistically significant (P = 0.013). See Experimental Procedures for details. (B)

Kinetics of turnover. The graph shows the percentage of Cse4 signals normalized to Tub2 at the indicated time points in wild-type and *cse4 K65R* mutant. Standard deviations of the means are shown. (C) Chromosome spreads of logarithmically-grown wild-type (YMB9470) or *cse4 K65R* (YMB9547) cells. 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. (D) Quantification of Cse4 mislocalization in two independent experiments. The increased mislocalization observed for the *cse4 K65R* strain was highly significant ($P < 0.001$) in both biological replicates (Chi-square test, N = 237, 237 and N = 251, 281 for wild-type and *cse4 K65R*, respectively in replicate 1 and replicate 2).

Figure 5. Cse4 K65 sumoylation regulates its proteolysis by Slx5, but not by Psh1. (A and C) Protein extracts were prepared using logarithmically growing cells, treated with CHX (20 μg/ml) for various time points. Blots were probed with anti-HA (Cse4) or anti-Tub2 (loading control) antibody. A representative blot is shown. Cse4 protein half-lives ($t_{1/2}$) are reported as the mean ± SEM of multiple biological repeats. The difference in $t_{1/2}$ between wild-type Cse4 and Cse4 K65R is statistically significant in the *psh1*Δ strain ($P = 0.043$) but not in the *slx5*Δ strain ($P > 0.99$). See Experimental Procedures for details. (B and D) Kinetics of turnover. The graph shows the percentage of Cse4 signals normalized to Tub2 at the indicated time points in the indicated strains. Average or standard deviations of the means are shown. (A) Strains analyzed were YMB9470 (*CSE4*), YMB9472 (*CSE4 slx5*Δ), and YMB9549 (*cse4 K65R slx5*Δ). (C) Strains

analyzed were YMB9470 (*CSE4*), YMB9471 (*CSE4 psh1*Δ), and YMB9548 (*cse4 K65R psh1*Δ).


A
Input
Pull-down: Ni-NTA
Vector
8His-HA-Cse4
8His-HA-cse4 16KR
8His-HA-cse4 K65R
α-HA (Cse4)
α-Tub2
42
30
23
165
125
93
72
57
42
31
24
*
Cse4-(Smt3)n
α-Smt3
α-HA (Cse4)

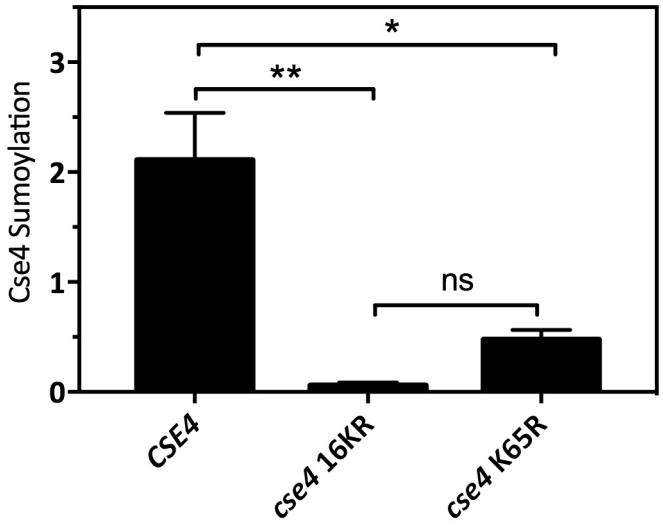
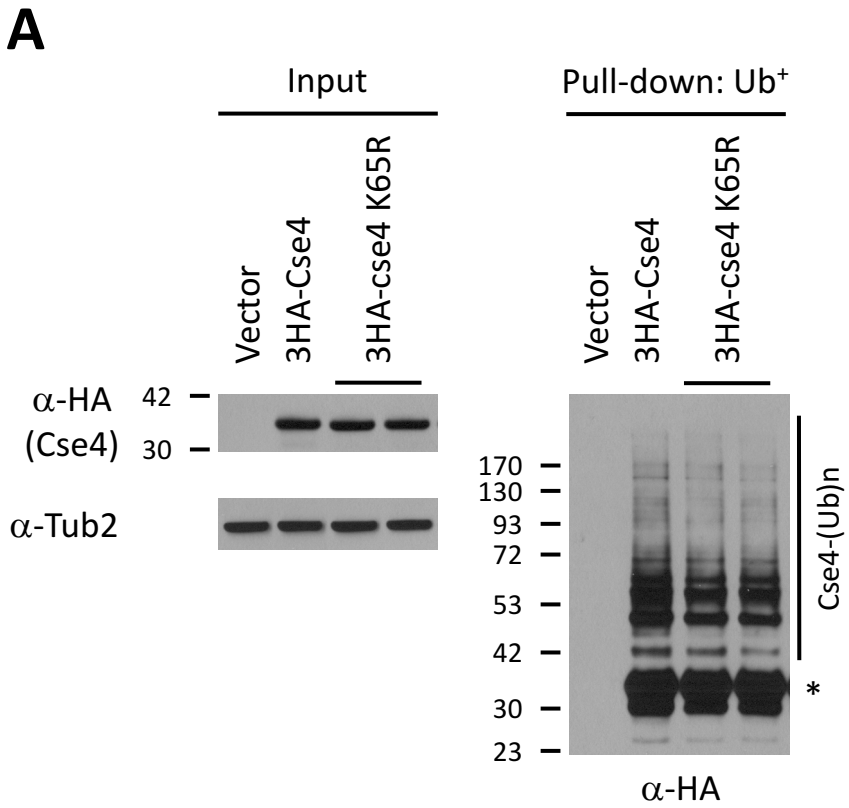

B
Cse4 Sumoylation
0
1
2
3
*
**
ns
CSE4
cse4 16KR
cse4 K65R

Figure 1


A
Input
Pull-down: Ub+
Vector
3HA-Cse4
3HA-cse4 K65R
Vector
3HA-Cse4
3HA-cse4 K65R
α-HA
(Cse4)
42
30
α-Tub2
170
130
93
72
53
42
30
23
Cse4-(Ub)n
*
α-HA

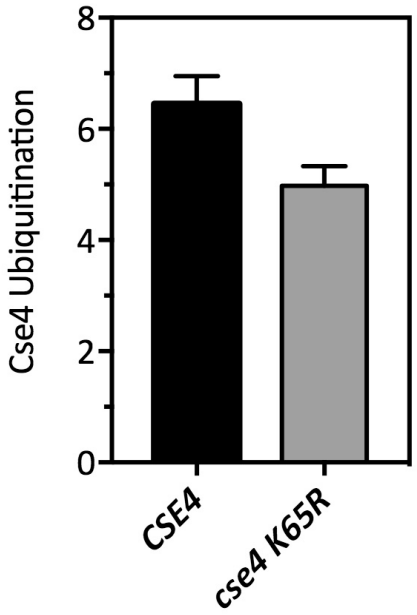
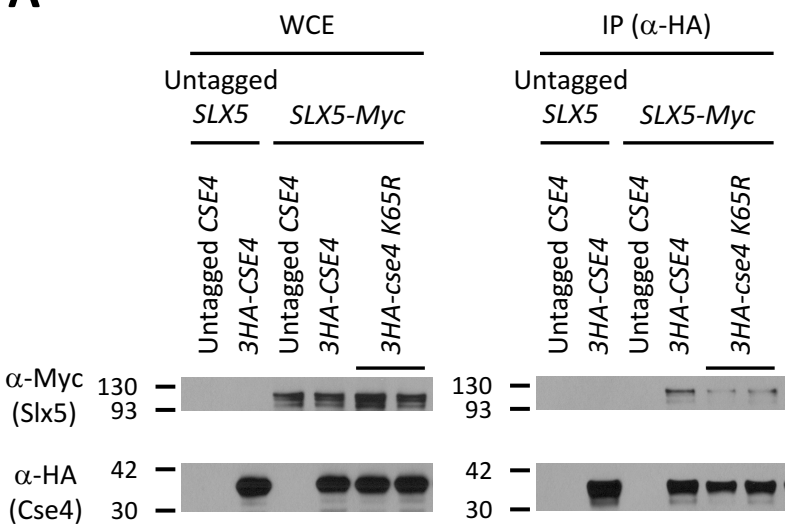

B
Cse4 Ubiquitination
8
6
4
2
0
CSE4
cse4 K65R

Figure 2

A


WCE
IP (α-HA)
Untagged
SLX5
SLX5-Myc
Untagged CSE4
3HA-CSE4
3HA-cse4 K65R
α-Myc
(Slx5)
α-HA
(Cse4)
130
93
42
30

B

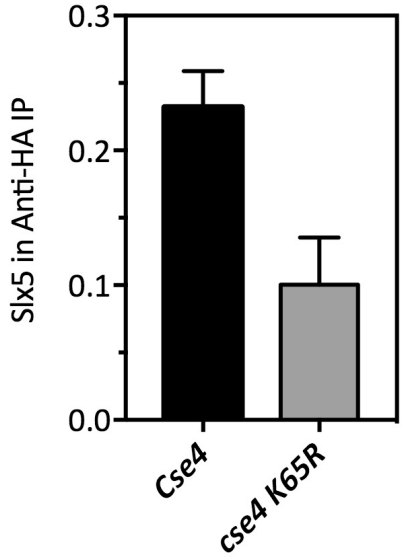

Slx5 in Anti-HA IP
0.3
0.2
0.1
0.0
Cse4
cse4 K65R

Figure 3

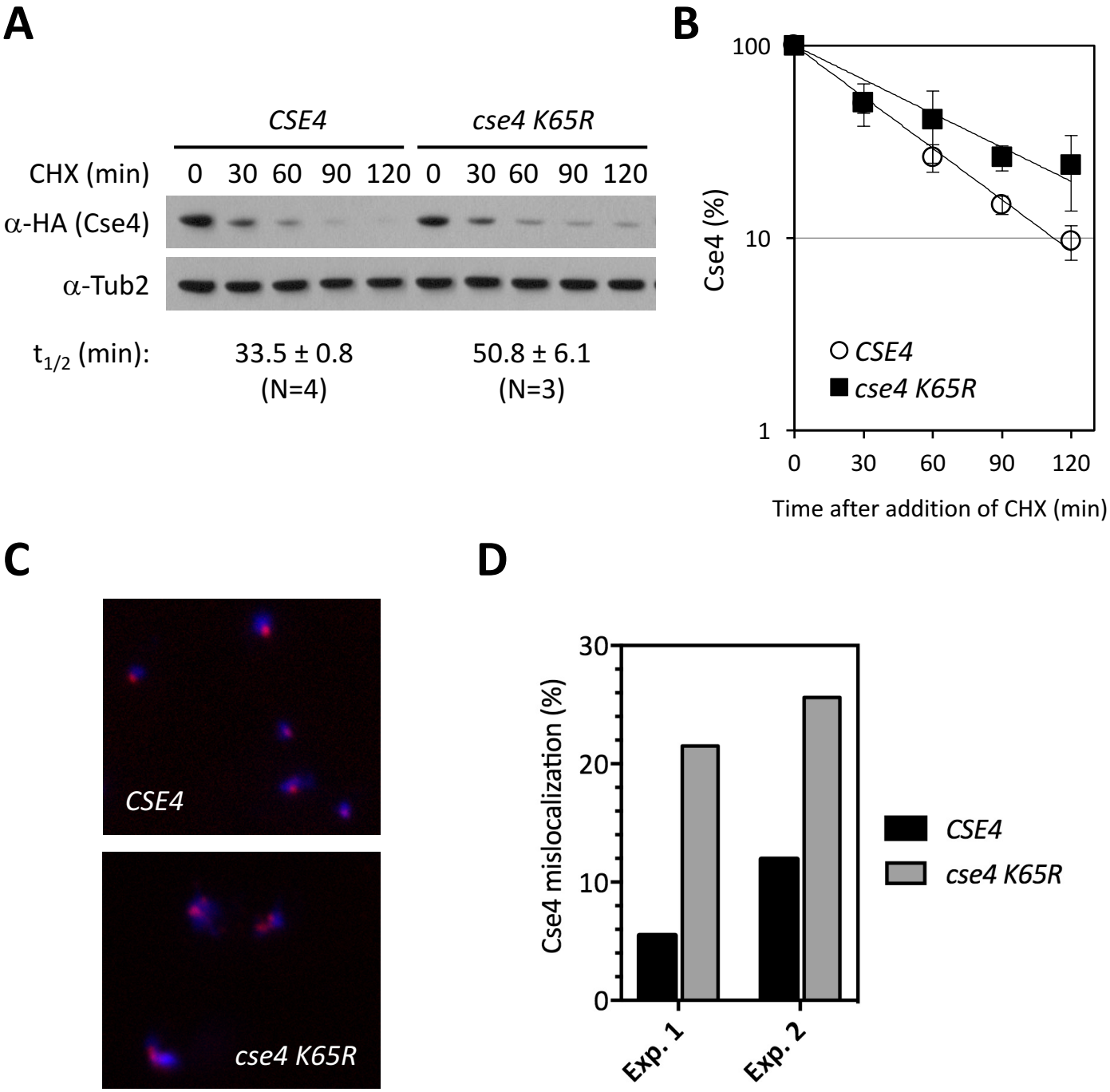
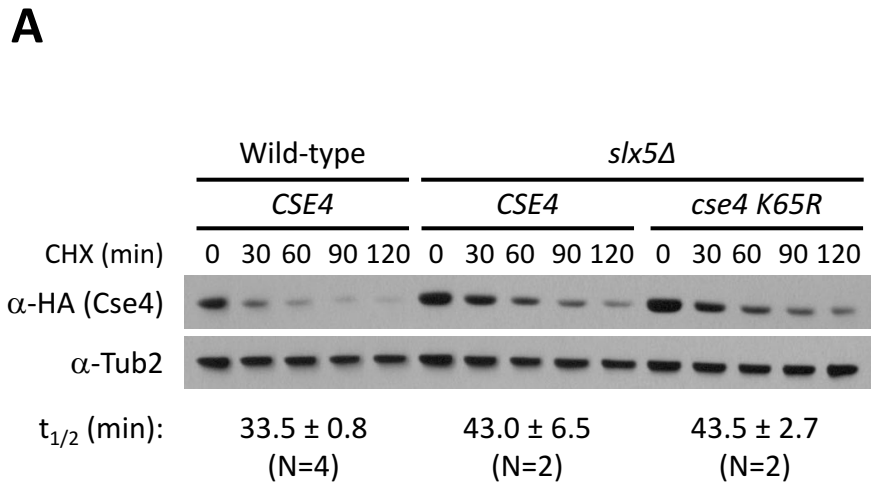
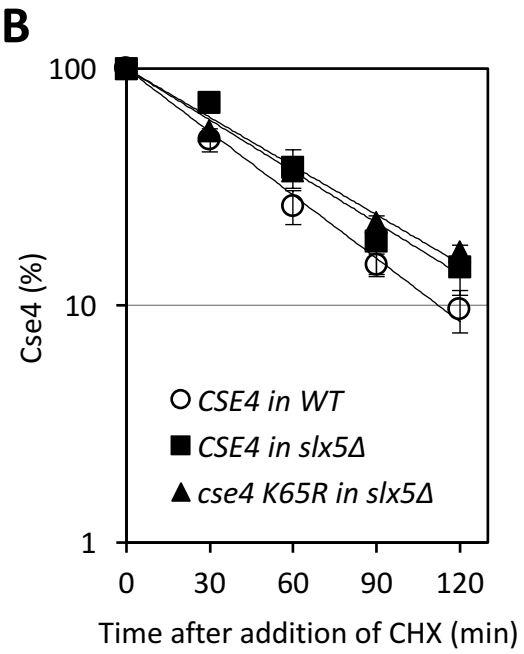
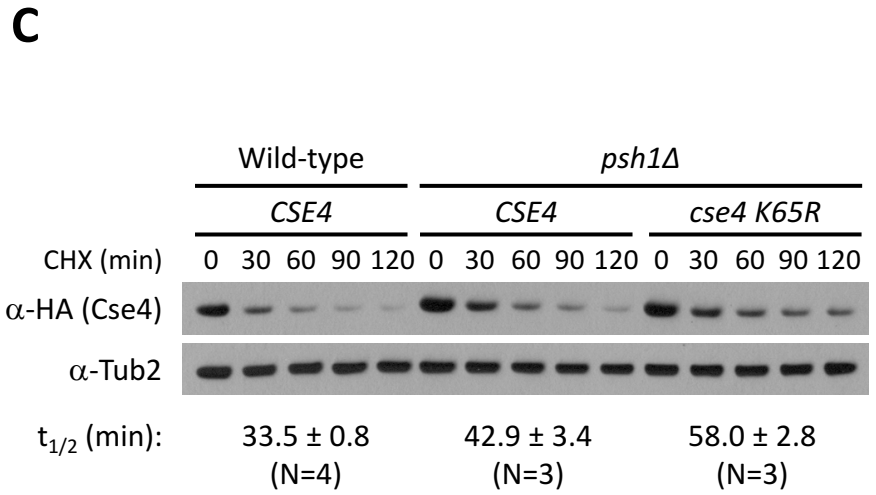

A
CSE4
cse4 K65R
CHX (min)
0 30 60 90 120 0 30 60 90 120
α-HA (Cse4)
α-Tub2
t1/2 (min):
33.5 ± 0.8
(N=4)
50.8 ± 6.1
(N=3)
B
100
10
1
Cse4 (%)
CSE4
cse4 K65R
0 30 60 90 120
Time after addition of CHX (min)
C
CSE4
cse4 K65R
D
30
20
10
0
Cse4 mislocalization (%)
CSE4
cse4 K65R
Exp. 1
Exp. 2

Figure 4


A
Wild-type
slx5Δ
CSE4
CSE4
cse4 K65R
CHX (min)
0 30 60 90 120 0 30 60 90 120 0 30 60 90 120
α-HA (Cse4)
α-Tub2
t1/2 (min):
33.5 ± 0.8
(N=4)
43.0 ± 6.5
(N=2)
43.5 ± 2.7
(N=2)


B
100
10
1
Cse4 (%)
CSE4 in WT
CSE4 in slx5Δ
cse4 K65R in slx5Δ
0 30 60 90 120
Time after addition of CHX (min)


C
Wild-type
psh1Δ
CSE4
CSE4
cse4 K65R
CHX (min)
0 30 60 90 120 0 30 60 90 120 0 30 60 90 120
α-HA (Cse4)
α-Tub2
t1/2 (min):
33.5 ± 0.8
(N=4)
42.9 ± 3.4
(N=3)
58.0 ± 2.8
(N=3)

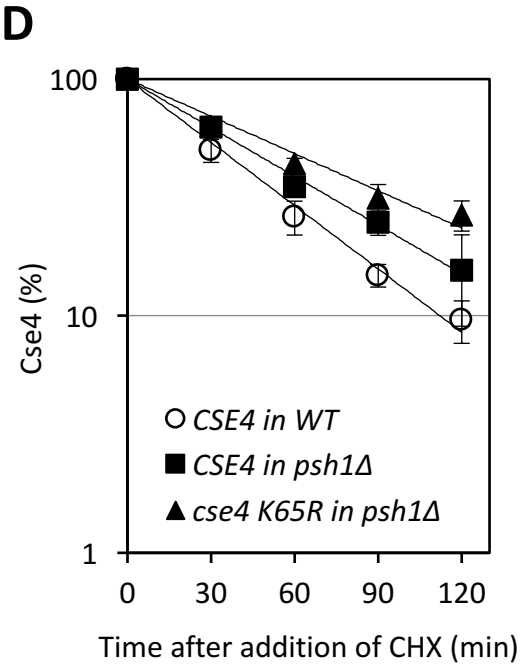

D
100
10
1
Cse4 (%)
CSE4 in WT
CSE4 in psh1Δ
cse4 K65R in psh1Δ
0 30 60 90 120
Time after addition of CHX (min)

Figure 5