Title

Cdc48Ufd1/Npl4 segregase removes mislocalized centromeric histone H3 variant CENP-A from non-centromeric chromatin.

Journal

Nucleic Acids Research, 50(6)

ISSN

0305-1048

Authors

Ohkuni, Kentaro
Gliford, Loran
Au, Wei-Chun
et al.

Publication Date

2022-04-08

DOI

10.1093/nar/gkac135

Peer reviewed

3276–3291 *Nucleic Acids Research, 2022, Vol. 50, No. 6* *Published online 2 March 2022*
https://doi.org/10.1093/nar/gkac135

Cdc48$^{Ufd1/Npl4}$ segregase removes mislocalized centromeric histone H3 variant CENP-A from non-centromeric chromatin

Kentaro Ohkuni[1], Loran Gliford[1], Wei-Chun Au[1], Evelyn Suva[1], Peter Kaiser[2] and Munira A. Basrai[1,*]

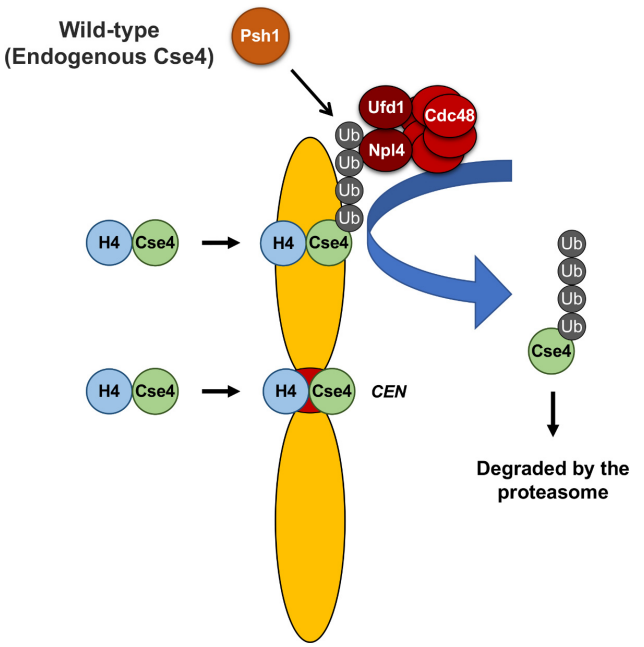
[1]Genetics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA and [2]Department of Biological Chemistry, School of Medicine, University of California, Irvine, CA 92697, USA

Received September 17, 2021; Revised January 10, 2022; Editorial Decision February 06, 2022; Accepted February 14, 2022

ABSTRACT

Restricting the localization of CENP-A (Cse4 in *Saccharomyces cerevisiae*) to centromeres prevents chromosomal instability (CIN). Mislocalization of overexpressed CENP-A to non-centromeric chromatin contributes to CIN in budding and fission yeasts, flies, and humans. Overexpression and mislocalization of CENP-A is observed in cancers and is associated with increased invasiveness. Mechanisms that remove mislocalized CENP-A and target it for degradation have not been defined. Here, we report that Cdc48 and its cofactors Ufd1 and Npl4 facilitate the removal of mislocalized Cse4 from non-centromeric chromatin. Defects in removal of mislocalized Cse4 contribute to lethality of overexpressed Cse4 in *cdc48*, *ufd1* and *npl4* mutants. High levels of polyubiquitinated Cse4 and mislocalization of Cse4 are observed in *cdc48-3, ufd1-2* and *npl4-1* mutants even under normal physiological conditions, thereby defining polyubiquitinated Cse4 as the substrate of the ubiquitin directed segregase Cdc48$^{Ufd1/Npl4}$. Accordingly, Npl4, the ubiquitin binding receptor, associates with mislocalized Cse4, and this interaction is dependent on Psh1-mediated polyubiquitination of Cse4. In summary, we provide the first evidence for a mechanism that facilitates the removal of polyubiquitinated and mislocalized Cse4 from non-centromeric chromatin. Given the conservation of Cdc48$^{Ufd1/Npl4}$ in humans, it is likely that defects in such pathways may contribute to CIN in human cancers.

GRAPHICAL ABSTRACT



INTRODUCTION

Centromeres are specialized chromatin structures 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. Centromeric localization of the evolutionarily conserved histone H3 variant CENP-A (Cse4 in *Saccharomyces cerevisiae*, Cnp1 in *Schizosaccharomyces pombe*, CID in *Drosophila melanogaste* and CENP-A in humans) serves as a platform for kinetochore assembly (1–3). Recruitment of Cse4/Cnp1/CID/CENP-A at the centromeres is regulated by the evolutionarily conserved

*To whom correspondence should be addressed. Tel: +1 240 760 6746; Fax: +1 240 541 4529; Email: basraim@mail.nih.gov

CENP-A specific histone chaperone Scm3/CAL1/HJURP (Holliday Junction Recognition Protein) in budding and fission yeasts, flies, and humans, respectively (4–11).

Overexpression of CENP-A and its homologs lead to mislocalization to non-centromeric chromatin and contributes to chromosomal instability (CIN) in budding and fission yeasts, flies, and humans (12–18). Overexpression and mislocalization of CENP-A is observed in many cancers, which correlates with poor patient survival and increased risk of disease progression (19–26). Hence, characterization of pathways that prevent mislocalization of CENP-A will provide better diagnosis and treatment of CENP-A overexpressing cancers.

Studies with *S. cerevisiae* have identified pathways that prevent or promote mislocalization of Cse4. For example, post-translational modifications (PTMs) of Cse4 such as ubiquitination, sumoylation, and isomerization regulate steady-state levels of Cse4 and prevent its mislocalization to non-centromeric regions, thereby maintaining chromosomal stability (12,27–33). Ubiquitin-mediated proteolysis of Cse4 initiated by E3 ubiquitin ligases such as Psh1 (27,28), Slx5 (30,32,34), Ubr1 (32), SCF-Rcy1 (31) and SCF-Met30/Cdc4 (33) and by proline isomerase Fpr3 (29) regulate the cellular levels of Cse4. Psh1-mediated proteolysis of overexpressed Cse4 is regulated by several factors such as FACT (Facilitates Chromatin Transcription/Transactions) complex (35), CK2 (casein kinase 2) (36), HIR histone chaperone complex (37), and DDK (Dbf1-dependent kinase) complex (38). Mutants for genes that prevent mislocalization of Cse4 exhibit synthetic dosage lethality (SDL) when Cse4 is overexpressed from a *GAL* promoter (*GAL-CSE4*).

In addition to factors that prevent mislocalization of Cse4, defining pathways that promote mislocalization of Cse4 is also an area of active research. For example, evolutionarily conserved replication dependent Chromatin Assembly Factor 1 (CAF-1) promotes mislocalization of Cse4 to non-centromeric chromatin (39). We have recently shown that the interaction of overexpressed Cse4 with histone H4 facilitates the C-terminal sumoylation of Cse4, which promotes its interaction with CAF-1 and the deposition of Cse4 into non-centromeric chromatin (40,41). Mutants for genes that promote mislocalization of Cse4 suppress the SDL phenotype of *psh1*Δ *GAL-CSE4* strain.

Despite advances in the characterization of factors that prevent or promote mislocalization of Cse4, the mechanism by which mislocalized Cse4 is removed from non-centromeric chromatin and targeted for degradation has not been clearly defined. We reasoned that mutants defective in removal of mislocalized Cse4 from non-centromeric chromatin may exhibit SDL with *GAL-CSE4.* Our previous genome-wide screen identified mutants that exhibit SDL with *GAL-CSE4*; among these, Psh1, HIR complex, DDK, SCF-Met30 and SCF-Cdc4 were top hits, which we have pursued in previous studies (33,37,38). In this study, we characterized the role of Cdc48, also identified from the screen. Cdc48 is referred to as p97/VCP (valosin-containing protein) in metazoans and is a highly conserved chaperone of the AAA (ATPase associated with diverse cellular activities)-ATPase family (42,43). Cdc48 plays an essential role in cellular processes such as endoplasmic reticulum-associated degradation (ERAD), spindle disassembly, membrane fusion, autophagy, and transcriptional control (42–46). Mechanistically, Cdc48/p97 acts as a ubiquitin-dependent segregase that extracts polyubiquitinated clients from macromolecular complexes and delivers them to the 26S proteasome for degradation (47,48). The cellular function and localization of the Cdc48/p97 ATPase is controlled by many cofactors (44,49,50); the best-characterized of which is the Ufd1–Npl4 heterodimer. Polyubiquitinated proteins can be directly targeted to the proteasome, whereas polyubiquitinated proteins that are embedded into cell membranes, chromatin-associated, or protein multi-subunit complexes often need to be segregated by the $Cdc48^{Ufd1/Npl4}$ complex (43,45,46). Npl4 is responsible for the recognition of the substrate-attached ubiquitin chain in the initial step (51,52).

Here, we show that $Cdc48^{Ufd1/Npl4}$ segregase is necessary for the removal of mislocalized Cse4 from non-centromeric regions. Loss-of-function mutants of *CDC48*, *UFD1* and *NPL4* show mislocalization of Cse4 to non-centromeric regions, accumulation of polyubiquitinated Cse4 in chromatin, and an enrichment of chromatin-bound Cse4, expressed from its endogenous promoter. Furthermore, Npl4, which is also enriched in chromatin, interacts with chromatin-bound Cse4 facilitated by the attached polyubiquitin chain. Taken together, we define a role for $Cdc48^{Ufd1/Npl4}$ segregase in removing mislocalized Cse4 from non-centromeric regions and targeting Cse4 for degradation under normal physiological conditions.

MATERIALS AND METHODS

Yeast strains, plasmids and methods

Supplementary Table S1 and S2 describe the genotype of yeast strains and plasmids used for this study, respectively. Gene deletions and epitope-tagged alleles were constructed at the endogenous loci using standard PCR-based integration (53). All epitope tagging was confirmed by Western blot analysis.

Yeast cells were grown in rich media (YPD: 1% yeast extract, 2% bacto-peptone, 2% glucose) or synthetic complete (SC) media containing 2% glucose, 2% galactose or 2% sucrose + 2% galactose. For cell cycle assays, logarithmically growing cells in YPD at 25°C were treated with α-factor (3 μM) for G1 phase, hydroxyurea (0.2 M) for S phase, and nocodazole (20 μg/ml) for M phase arrests. Cell cycle arrest was confirmed by fluorescence-activated cell sorting (FACS) and microscopic analyses as described previously (54,55). To induce degradation of Cdc48 fused to the auxin-inducible degron, we added 3-indoleacetic acid (Sigma-Aldrich, I3750) to the medium at a final concentration of 5 mM. Chromosome spreads, protein stability assay, and plasmid retention assay were performed as described previously (33).

Ubiquitin (Ub) pull-down assay using whole cell extracts

Levels of ubiquitinated endogenous Cse4 were determined with ubiquitin pull-down assay as described previously (56) with some modifications. Cell lysates were prepared from

50 ml culture of logarithmically growing cells in YPD at 25°C. Cells were pelleted, rinsed with sterile water, and suspended in 0.5 ml of 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, 5 mM *N*-ethylmaleimide, 1 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich, P8215)). Cells were homogenized with Matrix C (MP Biomedicals) using a bead beater (MP Biomedicals, FastPrep-24 5G). Cell lysates were clarified by centrifugation at 6000 rpm for 5 min and protein concentration 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 lysis buffer (50 μl of aliquot was saved as input). The equal concentration of lysates was incubated with tandem ubiquitin binding entities (Agarose-TUBE1, Life Sensors, Inc., UM401) overnight at 4°C. Proteins bound to the beads were washed three times with TBS-T at room temperature and eluted in 2× Laemmli buffer at 100°C for 10 min. The eluted protein was resolved on a 4–12% Bis–Tris gel (Novex, NP0322BOX) and ubiquitinated Cse4 was detected by western blot using anti-HA (12CA5) antibody (Roche, 11583816001). Input samples were analyzed using anti-HA (12CA5) and anti-Tub2 (Basrai laboratory) antibodies.

Co-immunoprecipitation (Co-IP) and Ub pull-down assay using solubilized chromatin lysates

Cells were grown to logarithmic phase of growth in YPD at 25°C. Subcellular fractionation was performed using 50 OD_{600} cells as described previously (15) with minor modifications. Whole cell extracts (WCEs) were prepared from lysates before the sucrose gradient centrifugation. Chromatin pellets were resuspended in IP lysis buffer (50 mM Tris–HCl at pH 8.0, 5 mM EDTA, 1% Triton X-100, 150 mM NaCl, 50 mM NaF, 10 mM beta-glycerophosphate, 1 mM PMSF, 1× protease inhibitor cocktail) for Co-IP or in Ub lysis buffer for Ub pull-down assay, and bead beat with Matrix C (MP Biomedicals) using a bead beater (MP Biomedicals, FastPrep-24 5G) for 40 s. Chromatin lysates were clarified by centrifugation at 6000 rpm for 5 min. For Co-IP, equal amounts of chromatin lysates were incubated with anti-HA agarose (Sigma-Aldrich, A2095) overnight at 4°C. After washing with IP lysis buffer three times, beads were incubated with 2× Laemmli buffer at 100°C for 5 min. For Ub pull-down assay, equal amounts of chromatin lysates were incubated with tandem ubiquitin binding entities (Agarose-TUBE1, Life Sensors, Inc., UM401) overnight at 4°C. After washing with TBS-T three times, beads were incubated with 2× Laemmli buffer at 100°C for 10 min. Protein samples were analyzed by western blot. Primary antibodies were as follows: anti-HA (12CA5) mouse (Roche, 11583816001), anti-HA rabbit (Sigma-Aldrich, H6908), anti-Pgk1 mouse (Invitrogen, 459250), anti-H2B rabbit (Abcam, ab1790), anti-c-Myc (9E10) mouse (Sigma-Aldrich, M4439; Covance, MMS-150P), anti-Cdc48 rabbit (AS ONE International, Inc., 62-303) and anti-Cse4 rabbit (Strahl laboratory). Protein levels were quantified using Image Lab software (version 6.0.0) from Bio-Rad Laboratories, Inc (Hercules, CA).

CHIP-QPCR

Chromatin immunoprecipitations were performed as previously described (41) with modifications. Briefly, logarithmic phase cultures (120–150 OD_{600}) were grown in sucrose/galactose (2% final concentration each) media for 3–3.5 h and were treated with formaldehyde (1%) for 20 min at 30°C followed by the addition of 2.5 M glycine for 5 min. Cell pellets were washed twice in 1× PBS then resuspended in 1.5 ml FA Lysis Buffer (1 mM EDTA pH8.0, 50 mM HEPES–KOH pH7.5, 140 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100) with protease inhibitor cocktail (Sigma) and PMSF (1 mM). The cell suspension was split into three Lysing Matrix C tubes (MP Biomedicals) and lysed in a FastPrep-24 5G (MP Biomedicals) for 40 s ten times with rest on ice between every three consecutive beads-beating. The crude chromatin pellet was washed in FA Lysis Buffer twice. Each pellet was resuspended in 700 μl of FA Lysis Buffer and combined into one 5 ml tube. The chromatin suspension was sonicated on ice with a Branson digital sonifer 24 times at 20% amplitude with a repeated 15 s on/off cycle. After 10 min of centrifugation (12 000 rpm, 4°C), the supernatant was transferred to a new tube. 50 μl of the resulting solubilized chromatin was taken as input. The remaining solubilized chromatin (600 μl each) was incubated with anti-HA-agarose beads (Sigma, A2095), anti-Myc (Sigma M4439) bound or anti-GST (Invitrogen MA4-004) bound protein G magnetic beads (Invitrogen 10004D) overnight at 4–8°C. The beads were washed in 1 ml FA, FA-HS (500 mM NaCl), RIPA, and TE buffers for 5 min on a rotor two times each. The beads were suspended in ChIP Elution Buffer (25 mM Tris–HCl pH7.6, 100 mM NaCl, 0.5% SDS) and incubated at 65°C overnight. The beads were treated with proteinase K (0.5 mg/ml) and incubated at 50°C for 2 h followed by phenol/chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in a total of 100 μl sterile water. Samples were analyzed by quantitative PCR (qPCR) performed with the 7500 Fast Real Time PCR System with Fast SYBR Green Master Mix (Applied Biosystems). The occupancy of the respective protein was calculated as % input. As indicated, two to three biological replicates were performed for each strain tested. Primers used for this study are listed in Supplementary Table S3.

RESULTS

$Cdc48^{Ufd1/Npl4}$ segregase complex mutants exhibit synthetic dosage lethality (SDL) with *GAL-CSE4* and show mislocalization of endogenously expressed Cse4

Mutations or deletions of genes that prevent mislocalization of Cse4 manifest synthetic dosage lethality (SDL) (27,28,30,33,35–38). We have previously used synthetic genetic arrays (SGA) in a genome-wide effort to identify pathways that prevent mislocalization of Cse4 (33,37). The SGA screen for SDL with *GAL-CSE4* with essential gene mutants identified *cdc48-3* mutant, which has two missense substitutions (P257L and R387K) in the D1 ATPase domain (Supplementary Figure S1A, (47)), as a strong genetic interactor (33). To confirm the SDL phenotype, we examined the growth of *cdc48-3* strain with vector or *GAL-*

CSE4 plasmid on plates containing glucose or galactose. The *cdc48-3* strain exhibits SDL with *GAL-CSE4* on galactose plates at the permissive temperature of 25°C (Supplementary Figure S1B). To test the allele specificity of the SDL phenotype, we examined growth of other *cdc48* mutants (*cdc48-1* and *cdc48-4601*). Sequence analysis of *cdc48* alleles identified the mutations for each allele (Supplementary Figure S1A). Surprisingly, the two *cdc48-1* strains from independent sources showed additional six mutations in YMB8734 when compared to YMB8735. Growth assays showed that all *cdc48* mutants we examined exhibit SDL with *GAL-CSE4* (Supplementary Figure S1B), suggesting that the *GAL-CSE4* SDL phenotype is not specific to a particular allele of *cdc48*. Previous studies have shown that defects of Cse4 proteolysis contribute to *GAL-CSE4*-mediated SDL (27,28,30–33,38,56). To define a physiological role for Cdc48 in preventing mislocalization of Cse4, we examined the stability of endogenously expressed HA-Cse4 from its own promoter at the permissive temperature of 25°C. Protein stability assays showed that endogenous HA-Cse4 was rapidly degraded in the wild-type strain ($t_{1/2}$ = 78 min) and was stabilized in the *cdc48-3* strain ($t_{1/2}$ = 127 min) (Supplementary Figures S1C and D). Thus, we conclude that defects in Cdc48 leads to *GAL-CSE4* SDL and Cdc48 regulates proteolysis of Cse4 under normal physiological conditions *in vivo*.

Cdc48 cofactors Ufd1-Npl4 were previously shown to be required for degradation of Cdc48 substrates in the endoplasmic reticulum and for CMG helicase disassembly during DNA replication termination (42,57,58). We sought to examine whether *UFD1* and *NPL4* mutants exhibit SDL with Cse4 overexpression. Since *npl4-1* mutant was not present on the array of temperature sensitive mutants used for the SGA screen, we decided to use an isogenic set of strains with wild-type, *cdc48-3*, *ufd1-2* and *npl4-1* for our analysis. Growth assays showed that *ufd1-2* and *npl4-1* strains also exhibit SDL with *GAL-CSE4* on galactose plates at 25°C similar to that observed for the *cdc48-3* strain (Figure 1A); albeit the growth defect was moderate in *ufd1-2* strain, likely due to the weaker defect associated with the *ufd1-2* allele at the permissive temperature. The SDL phenotypes are specific to *GAL-CSE4*, since we did not observe growth defects with overexpressed histone H3 or H4 (*GAL-H3* or *GAL-H4*) in *cdc48-3*, *ufd1-2*, or *npl4-1* strain (Supplementary Figure S2). The *GAL-CSE4* dosage lethality is linked to mutations in the *cdc48-3*, *ufd1-2* or *npl4-1* strain as lethality and temperature sensitivity were suppressed by expressing the cognate wild-type gene in these mutants (Figure 1B).

Previous studies have shown that the dosage lethality with *GAL-CSE4* correlates with mislocalization of Cse4 to non-centromeric regions (39–41). In order to define the physiological role of $Cdc48^{Ufd1/Npl4}$ segregase, we pursued all further studies with endogenous HA-Cse4 expressed from its own promoter grown at the permissive temperature of 25°C. Chromosome spreads were used to examine the localization of Cse4 as this technique removes soluble material and allows visualization of chromatin bound HA-Cse4. Wild-type, *cdc48-3*, *ufd1-2* or *npl4-1* cells were arrested in M phase with nocodazole to compare Cse4 localization in all strains in the same cell cycle stage (Figure 1C). Cell cycle arrest was confirmed by flow cytometry (Supplementary Figure S3). Consistent with clustering of kinetochores, immunofluorescence staining of HA-Cse4 showed one or two Cse4 foci coincident with DAPI (DNA) signal in the majority of wild-type cells (Figure 1C). Mislocalization of Cse4 was defined as cells showing more than two Cse4 foci or diffused Cse4 signals within DAPI stained region (Figure 1C). The percentage of *cdc48-3*, *ufd1-2* or *npl4-1* cells exhibiting Cse4 mislocalization was significantly higher than that observed in wild-type cells, confirming that $Cdc48^{Ufd1/Npl4}$ activity is required to prevent Cse4 mislocalization (Figure 1D). Taken together, we conclude that $Cdc48^{Ufd1/Npl4}$ complex mutants are hypersensitive to overexpression of Cse4 and show mislocalization of Cse4 under normal physiological conditions.

Chromatin-associated Cse4 contributes to SDL in the *cdc48-3* strain

The ubiquitin ligase Psh1 constitutes one of the several Cse4 degradation pathways. Previous studies have shown that *GAL-CSE4* dosage lethality in *psh1Δ* mutants is linked to enrichment of Cse4 in chromatin (27,28). If a similar mechanism applies to *cdc48* mutants, we expected that preventing Cse4 mislocalization would suppress dosage lethality. To address this, we took advantage of our recent studies in which either reduced gene dosage of histone H4 or expression of *cse4 K215/216R* mutant (defective in C-terminal sumoylation) prevent mislocalization of overexpressed Cse4 and suppress dosage lethality of a *psh1Δ GAL-CSE4* strain (40,41). We examined dosage lethality phenotypes of *cdc48-3* mutants with and without deletion of one copy of H4 alleles (*hhf1Δ* or *hhf2Δ*) and observed suppression of synthetic dosage lethality (Figure 2A and Supplementary Figure S4). Moreover, overexpression of the sumoylation deficient Cse4 K215/216R also partially suppressed dosage lethality in the *cdc48-3* mutants (Figure 2B and Supplementary Figure S5), similar to that observed in the *psh1Δ GAL-cse4 K215/216R* strain (40).

Next, we examined if the severity of *GAL-CSE4* SDL correlates with enrichment of Cse4 in chromatin. Subcellular fractionation was done using wild-type, *cdc48-3*, *ufd1-2*, and *npl4-1* strains expressing endogenous HA-Cse4 grown at 25°C. Pgk1 and histone H2B served as controls for the soluble and the chromatin fractions, respectively. Levels of Cse4 in the soluble fraction were similar in all strains (Figure 2C and Supplementary Figure S6). In contrast, levels of chromatin-associated Cse4 in *cdc48-3* and *npl4-1* are significantly higher when compared to the wild-type strain. The level of chromatin-associated Cse4 is less pronounced in the *ufd1-2* strain (Figure 2C and Supplementary Figure S6), consistent with the milder dosage lethality phenotype of the *ufd1-2 GAL-CSE4* strain (Figure 1A). Taken together, these data suggest that increased chromatin-associated Cse4 causes dosage lethality in the *cdc48-3 GAL-CSE4* strain.

Accumulation of polyubiquitinated Cse4 in *cdc48-3, ufd1-2* and *npl4-1* mutants

Previous studies have shown that defects in ubiquitin-mediated Cse4 proteolysis contribute to *GAL-CSE4* dosage

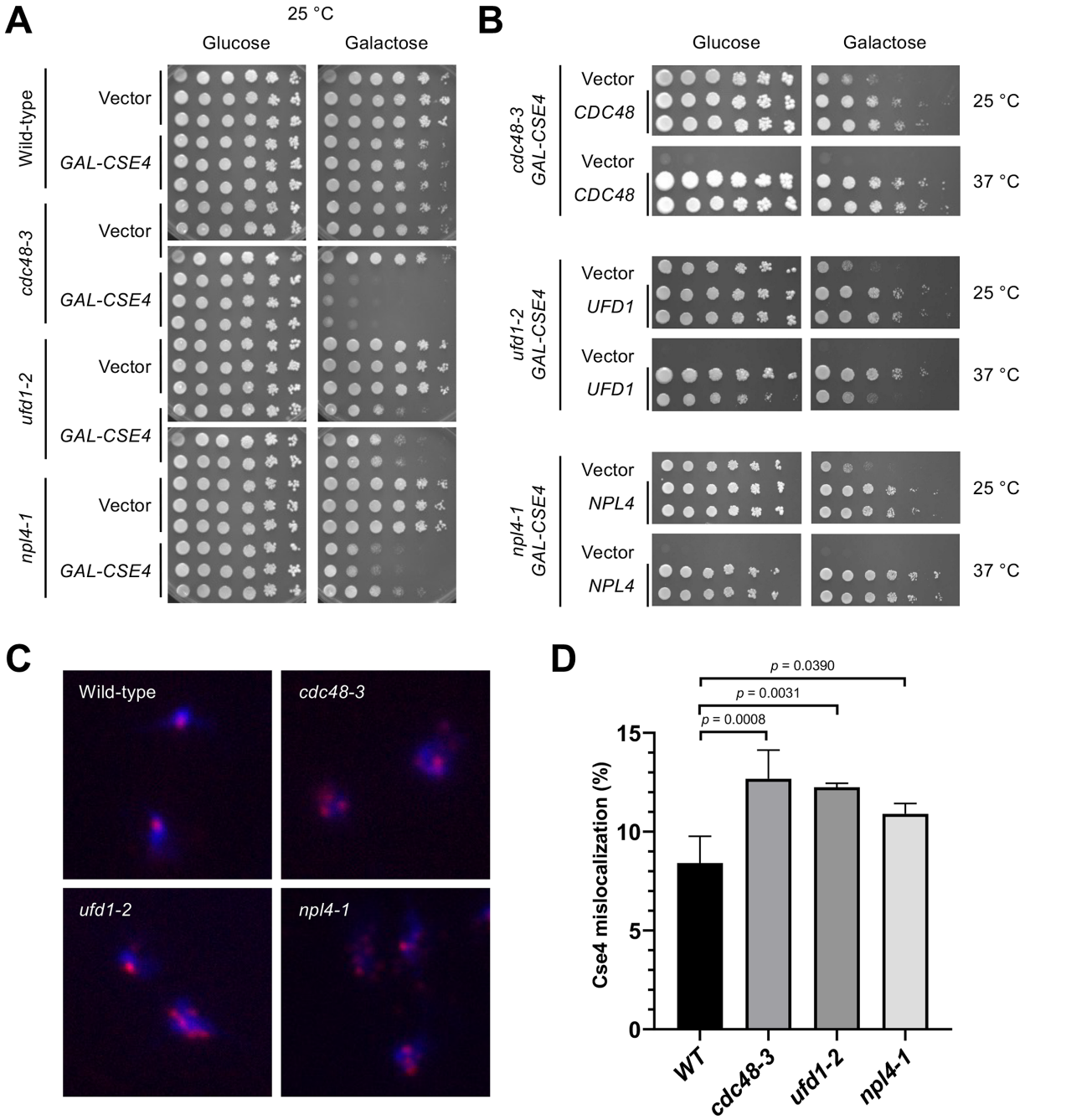


Figure 1. Cdc48$^{Ufd1/Npl4}$ segregase complex mutants exhibit SDL with *GAL-CSE4* and show mislocalization of endogenous Cse4. (**A**) Overexpression of Cse4 results in SDL in *cdc48-3*, *ufd1-2*, and *npl4-1* mutants. Wild-type, *cdc48-3*, *ufd1-2* and *npl4-1* cells containing either vector or *GAL-CSE4* were spotted in five-fold serial dilutions on glucose (2%)- or galactose (2%)-containing synthetic medium selective for the plasmid. The plates were incubated at the permissive temperature of 25°C for 4 days. Three independent transformants for each strain are shown. (**B**) Plasmid (2μ)-borne *CDC48*, *UFD1*, and *NPL4* can complement the *GAL-CSE4* SDL phenotypes of *cdc48-3*, *ufd1-2*, and *npl4-1* strains, respectively. Serial dilutions were conducted as described in A. The plates were incubated at 25°C and 37°C for 4–7 days. (**C**) Endogenous Cse4 is mislocalized in *cdc48-3*, *ufd1-2* and *npl4-1* strains. Localization of Cse4 was examined by chromosome spreads prepared from nocodazole (M) arrested wild-type, *cdc48-3*, *ufd1-2* and *npl4-1* cells. The cell cycle arrest was verified by FACS analysis (Supplementary Figure S3). DAPI (blue) and α-HA (red) staining were used to visualize DNA and Cse4 localization, respectively. Representative images of cells showing normal localization (counted as nuclei with one or two Cse4 foci) and mislocalization (counted as nuclei with more than two foci or a diffuse signal in the nucleus). (**D**) Quantification of Cse4 mislocalization as a percentage over total cell count. Error bars represent the standard deviation of multiple experiments. The increased mislocalization observed in the *cdc48-3*, *ufd1-2* and *npl4-1* strains was highly significant (one-way ANOVA, numbers of cells scored (n) = 627, 409, 418, 507, n = 503, 429, 340, 406, n = 349, 326, 555 and n = 330, 347, 448 for wild-type, *cdc48-3*, *ufd1-2* and *npl4-1*, respectively in three or four independent experiments).

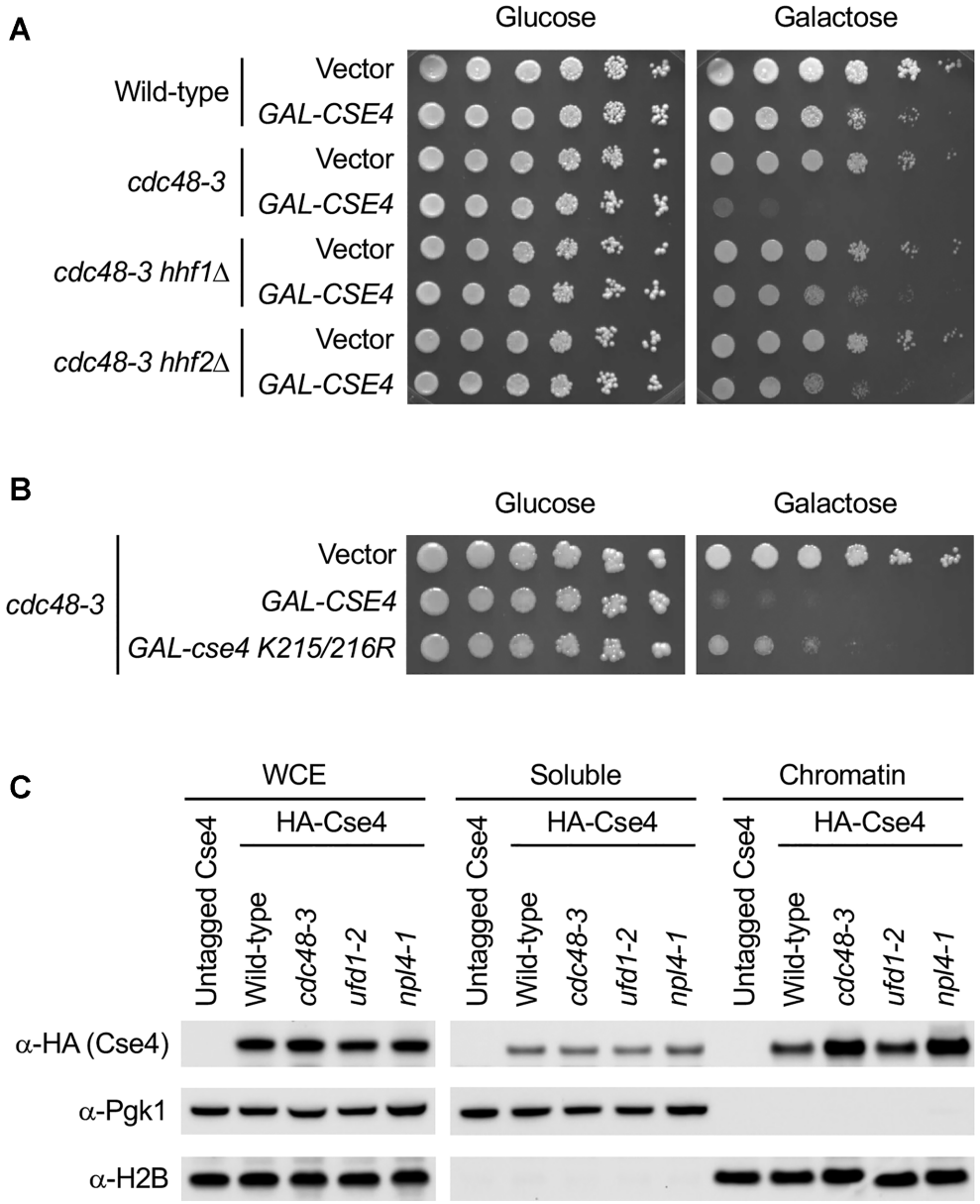


Figure 2. Chromatin-bound Cse4 contributes to SDL. (**A**) Reduced dosage of histone H4 (*hhf1*Δ or *hhf2*Δ) suppresses the *cdc48-3 GAL-CSE4* SDL. The indicated strains containing either vector or *GAL-CSE4* were spotted in five-fold serial dilutions on glucose (2%)- or galactose (2%)-containing synthetic medium selective for the plasmid. The plates were incubated at 25°C for 4 days. (**B**) Overexpression of *cse4 K215/216R* mutant exhibits reduced SDL compared to *GAL-CSE4* in *cdc48-3* strain. Growth assay was conducted as described in A. The plates were incubated at 25°C for 5 days. (**C**) Chromatin-bound endogenous Cse4 is enriched in *cdc48-3* and *npl4-1* strains. Whole cell extracts (WCEs) prepared from equal numbers of logarithmically growing cells in YPD were fractionated into soluble and chromatin fractions. Cse4 levels in each fraction were monitored by Western blot analysis with anti-HA antibody. Pgk1 and histone H2B were used as markers for soluble and chromatin fractions, respectively.

lethality in *psh1*Δ, *doa1*Δ and *cdc7-7* strains and this phenotype can be suppressed by overexpression of *UBI4* (encodes ubiquitin) presumably by driving Cse4 ubiquitination (38,56). To determine if defects in ubiquitination of Cse4 contribute to SDL in the *cdc48-3 GAL-CSE4* strain, we examined the effect of *UBI4* overexpression in the *cdc48-3 GAL-CSE4* strain. In contrast to mutations that reduce Cse4 ubiquitination, the dosage lethality of *cdc48-3 GAL-CSE4* was not suppressed by *UBI4* overexpression (Supplementary Figure S7). This result suggests that $Cdc48^{Ufd1/Npl4}$ is required for steps after the ubiquitination of Cse4.

The $Cdc48^{Ufd1/Npl4}$ complex is an important factor in recognizing lysine 48-linked polyubiquitinated substrates for processing and delivery to the proteasomal degradation pathway (51). Tetraubiquitin is the minimum signal for efficient proteasomal targeting of the ubiquitinated substrate for degradation (59,60). Accordingly, a post-ubiquitination role for $Cdc48^{Ufd1/Npl4}$ predicts an accumulation of polyubiquitinated Cse4 with more than four ubiquitin moieties in *cdc48-3*, *ufd1-2* and *npl4-1* mutants. To determine the status of Cse4 ubiquitination *in vivo*, we performed an affinity pull-down assay using agarose with tandem ubiquitin-

binding entities (Ub^+) from wild-type, *cdc48-3*, *ufd1-2* and *npl4-1* strains expressing endogenous HA-Cse4. Polyubiquitinated Cse4 corresponding to higher molecular weight species (>53 kDa) accumulated in *cdc48-3* and *npl4-1* mutants, and to a lesser extent in the *ufd1-2* mutant, confirming that $Cdc48^{Ufd1/Npl4}$ is dispensable for Cse4 polyubiquitination, but required for the processing of polyubiquitinated Cse4 (Figure 3A and Supplementary Figure S8A). The more modest effect observed in the *ufd1-2* mutant is consistent with the lower phenotypic penetrance of this mutant at 25°C, as compared to *cdc48-3* and *npl4-1* (Figure 1A). A faster migrating species was also observed in all strains expressing HA-Cse4 (Figure 3A and Supplementary Figure S8A, asterisk), as reported previously (30,34,56). Since these faster migrating Cse4 species were similar in size to that in the input lanes, these represent unmodified HA-Cse4, which likely interacts with ubiquitinated proteins such as canonical histones. These results support a role of $Cdc48^{Ufd1/Npl4}$ in the processing of polyubiquitinated Cse4; thus polyubiquitination of endogenous Cse4 is barely detectable in wild-type cells.

All our studies so far were done at the permissive temperature of 25°C to avoid cell cycle position effects, because complete inactivation of *cdc48-3* at the non-permissive temperature of 37°C shows cell cycle arrest in metaphase due to defects in bipolar attachment of kinetochores to microtubules (61). Nevertheless, to rule out a possible indirect effect of cell cycle arrest on polyubiquitination of Cse4, we performed ubiquitin pull-down assays in wild-type and *cdc48-3* strains that were arrested in G1 (α-factor treatment), S (hydroxyurea treatment), or M phase (nocodazole treatment). Flow cytometry and nuclear morphology confirmed the cell cycle arrest of the strain (Supplementary Figure S9). The levels of polyubiquitinated Cse4 in wild-type strain (>53 kDa) are barely detectable in any stage of cell cycle (Figure 3A and Supplementary Figure S8B). In contrast, levels of polyubiquitinated Cse4 (>53 kDa) were much higher in the *cdc48-3* strain in G1, S and M phases. Hence, accumulation of polyubiquitinated Cse4 in *cdc48-3* strain is not due to defects in cell cycle.

We next examined if the accumulation of polyubiquitinated Cse4 in *cdc48-3* mutant is allele specific using an auxin-inducible degron for Cdc48, as described previously (57). Treatment with auxin for 2 h led to efficient depletion of Cdc48 in the *cdc48-aid* strain (Supplementary Figures S10A and B). Ubiquitin pull-down assays were done with logarithmically grown and M phase arrested cells with and without auxin treatment. Flow cytometry confirmed the cell cycle arrest of the cells (Supplementary Figure S11). The *cdc48-aid* cells show higher levels of Cse4 when compared to the control wild type *CDC48* cells, suggesting a defect in Cse4 degradation (Supplementary Figures S10A, C andD). More importantly, we observed higher levels of polyubiquitinated Cse4 (>53 kDa) in logarithmically grown (–Noc) and M phase arrested (+Noc) *cdc48-aid* cells when compared to the control *CDC48* cells and non-auxin treated *cdc48-aid* cells (Figure 3B and Supplementary Figure S12). Taken together, we conclude that accumulation of polyubiquitinated Cse4 in *cdc48-3* strain is cell cycle independent and due to the defect in Cdc48 activity rather than an allele specific effect.

Chromatin-associated Cse4 accumulates in a polyubiquitinated form in *cdc48-3* strain

Our results so far have shown that defects in the $Cdc48^{Ufd1/Npl4}$ segregase cause dosage lethality with *GAL-CSE4* and leads to enrichment of Cse4 in chromatin as well as accumulation of polyubiquitinated Cse4. Based on these results, we hypothesized that chromatin-associated Cse4 is polyubiquitinated in a *cdc48-3* strain. We used Cse4 mutants where Cse4 Y193 was changed to alanine (Cse4 Y193A) or phenylalanine (Cse4 Y193F) to examine a correlation between dosage lethality, chromatin enrichment, and accumulation of polyubiquitinated Cse4 in the *cdc48-3* strain. Cse4 Y193 is located at the center of alpha helix 2 and interacts with the alpha helix 2 of H4 (62). Phenylalanine (F) is identical to tyrosine (Y) except for the hydroxyl group present on Y. Interestingly, human CENP-A has a phenylalanine (F101), instead of tyrosine (Y193) (Figure 4A). We previously showed that the *cse4 Y193A* mutant exhibits a severe defect in Cse4 sumoylation and suppresses the Cse4 synthetic dosage lethality with *psh1*Δ mutants (41). Similarly, the structural mimic mutant *cse4 Y193F*, which shows low levels of Cse4 sumoylation, partially suppresses dosage lethality of *psh1*Δ (41). These results show that sumoylation of Cse4 promotes mislocalization to non-centromeric regions and mislocalized Cse4 contributes to the dosage lethality. Analogous to the effects of Cse4 Y193A/F in the *psh1*Δ strain (41), *GAL-cse4 Y193A* does not exhibit dosage lethality, whereas *GAL-cse4 Y193F* shows moderate lethality in the *cdc48-3* strain (Figure 4B and Supplementary Figure S5).

We next examined the levels of chromatin-associated endogenous Cse4 Y193A/F expressed from its own promoter in *cdc48-3* mutants. Consistent with previous experiment (Figure 2C), wild-type Cse4 was enriched in chromatin in *cdc48-3* mutant. Lower levels of Cse4 Y193A were present in chromatin (Figure 4C and D). On the other hand, no significant difference in chromatin enrichment was observed for the Cse4 Y193F mutant. Both *cse4 Y193A/F* alleles complement *cse4*Δ mutants and do not show temperature sensitivity (Supplementary Figure S13), suggesting that Cse4 Y193A/F is not defective for centromere function. These results show that dosage lethality correlates with enrichment in chromatin; lack of dosage lethality of *GAL-Cse4 Y193A* correlates with lower levels of this protein in the chromatin in *cdc48-3* strain.

We next asked if the *cdc48-3 GAL-cse4 Y193A/F* dosage lethality phenotype and enrichment in chromatin correlate with levels of polyubiquitinated Cse4 in these strains. As described earlier (Figure 3A), we observed an accumulation of polyubiquitinated Cse4 in the *cdc48-3* mutant; this was greatly reduced in *cse4 Y193A* but not affected in the *cse4 Y193F* strain (Figure 4E and Supplementary Figure S14). Taken together, our results suggest that Cse4 associated with non-centromeric chromatin is polyubiquitinated and Cdc48 activity is necessary to remove mislocalized, chromatin-associated, polyubiquitinated Cse4.

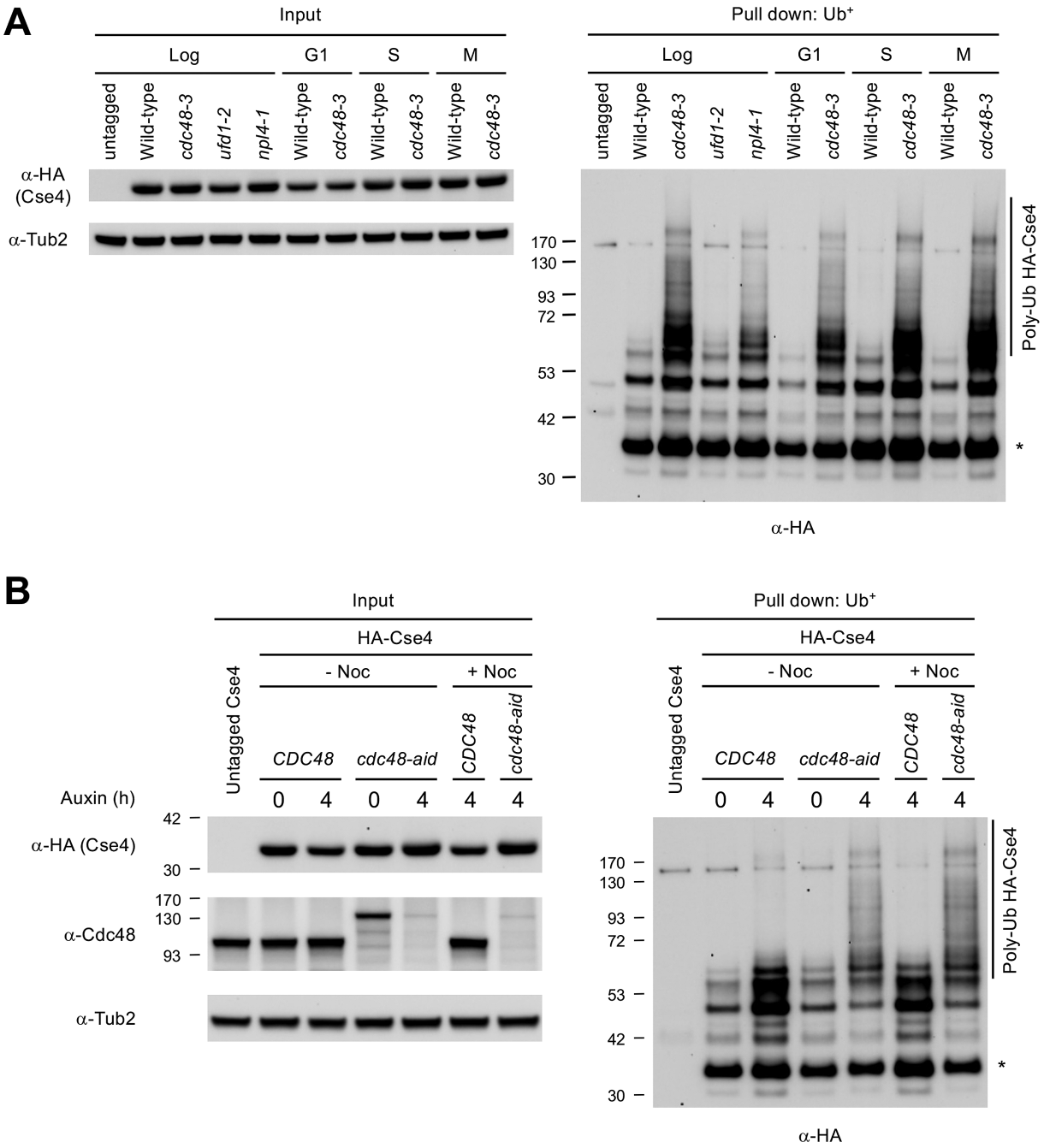


Figure 3. Polyubiquitinated Cse4 is enriched in *cdc48-3, ufd1-2,* and *npl4-1* mutants under normal physiological conditions. (**A**) Levels of Cse4 polyubiquitination, which are not specific to any cell cycle stage, are enhanced in *cdc48-3*, *ufd1-2* and *npl4-1* strains. Ub pull-down assay was performed using protein extracts from logarithmically growing cells, G1 phase cells synchronized with α-factor, S phase cells synchronized with hydroxyurea (HU), and M phase cells synchronized with nocodazole (Noc), in YPD at 25°C. The cell cycle arrest was verified by FACS analysis and cell morphology using propidium iodide-stained cells (Supplementary Figure S9). Input and ubiquitin pull down samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Untagged Cse4 was used as a negative control. Asterisk shows nonmodified Cse4. (**B**) Levels of Cse4 polyubiquitination are enhanced by inactivation of Cdc48. Control (*CDC48*) and *cdc48-aid* strains were grown in YPD to logarithmic phase at 25°C, then treated with auxin at the indicated time point. For M phase arrested cells, nocodazole (Noc) was added to the media after 2 h auxin treatment. Ub pull-down assay was conducted as described in (A).

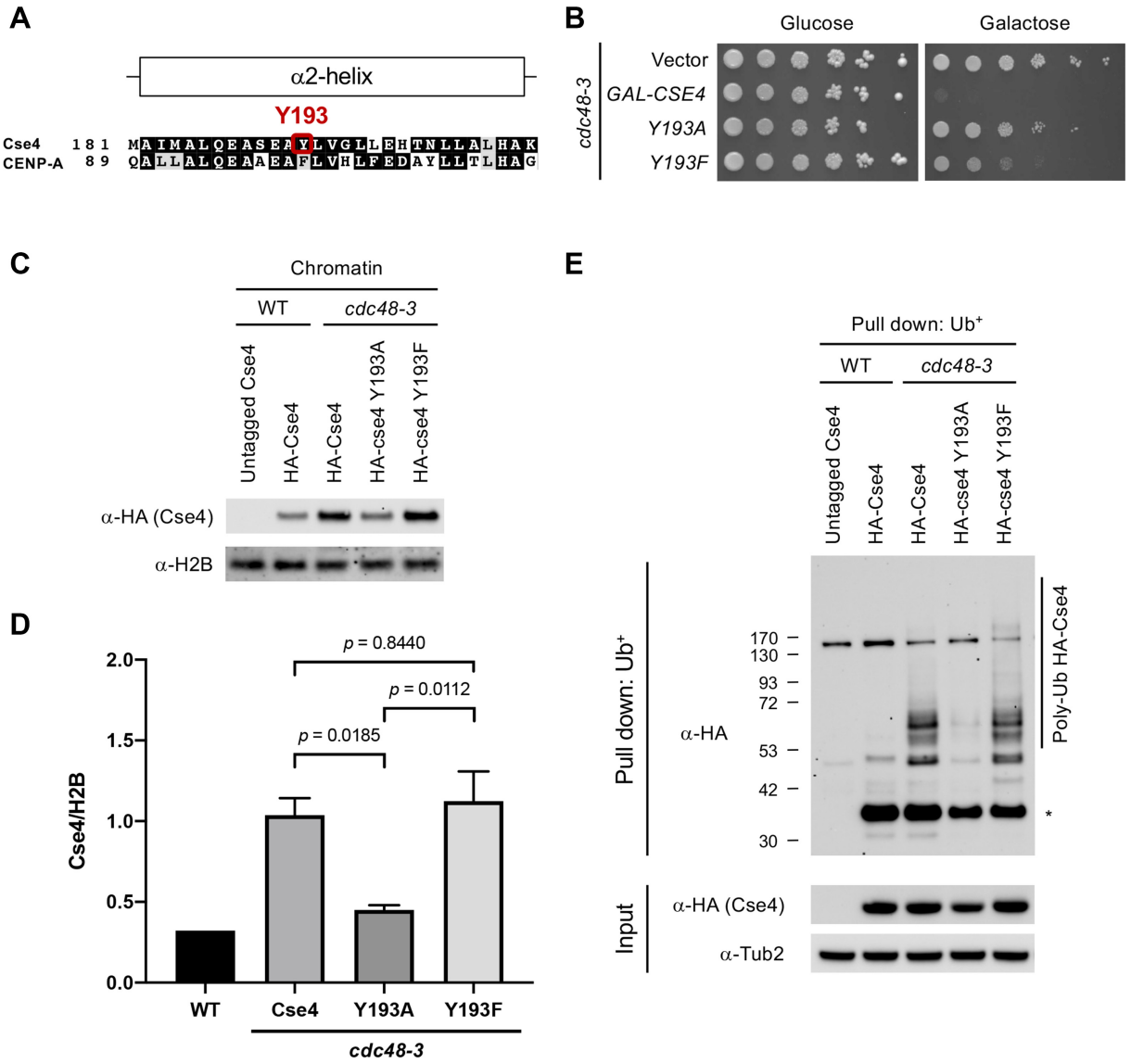


Figure 4. Chromatin-bound Cse4 is polyubiquitinated under normal physiological condition. (**A**) Sequence comparison of α2-helix domain of Cse4 and CENP-A. Human CENP-A has F101, instead of Y. (**B**) The Y193A mutation in Cse4 does not cause SDL in a *cdc48-3 GAL-CSE4* strain. Growth assay was conducted using a *cdc48-3* strain containing vector, *GAL-CSE4*, or *GAL-cse4 Y193A/F*. Five-fold serial dilutions of the indicated strain were plated on glucose (2%)- or galactose (2%)-containing synthetic medium selective for the plasmid. The plates were incubated at 25°C for 4 days. (**C**) Endogenous Cse4 Y193A exhibits reduced enrichment in chromatin. Whole cell extracts (WCEs) prepared from equal numbers of logarithmically growing cells in YPD were fractionated into soluble and chromatin fractions. Cse4 levels in chromatin fraction were monitored by Western blot analysis with anti-HA antibody. Histone H2B was used as marker for chromatin fraction. (**D**) Levels of chromatin-bound Cse4 from C were quantified in arbitrary density units after normalization to H2B. Error bars represent average deviation of two biological repeats. Statistical significance was assessed by one-way ANOVA (P = 0.0036) followed by Tukey's multiple comparisons test. (**E**) Endogenous Cse4 Y193A exhibits reduced Cse4 polyubiquitination. Ub pull-down assay was performed using protein extracts from logarithmically growing cells in YPD at 25°C. Input and ubiquitin pull down samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Untagged Cse4 was used as a negative control. Asterisk shows nonmodified Cse4.

Npl4 interacts with chromatin-associated Cse4 in a *cdc48-3* strain

Our model predicts that the Cdc48$^{Ufd1/Npl4}$ segregase recognizes polyubiquitinated Cse4 to facilitate its removal from non-centromeric regions. Npl4 binds specifically to lysine 48-linked polyubiquitinated substrate and functions as a co-factor for the Cdc48 ATPase (51,52). We therefore used co-immunoprecipitation (Co-IP) experiments to test whether Npl4 binds chromatin-associated Cse4 *in vivo*. We reasoned that Npl4-Cse4 interaction may be transient in a wild-type strain due to removal of chromatin-associated Cse4. Hence, in addition to wild-type, we performed Co-IP experiments in *cdc48-3* mutant, which we expected to enrich the recognition state because removal of mislocalized Cse4 is prevented by defects in segregase activity. Cse4 was immunopurified from chromatin fractions of cells expressing Npl4-Myc and HA-Cse4 and lysates were analyzed by anti-myc immunoblotting. An enrichment of not only Cse4, but also Npl4 and Cdc48 was observed in the chromatin when Cdc48

activity was reduced in *cdc48-3* strains (Figure 5A and B). Importantly, Npl4 co-purified with Cse4 specifically in *cdc48-3* mutant where the substrate recognition state is enriched (Figure 5C and Supplementary Figure S15).

Psh1 contributes to polyubiquitination of endogenous Cse4

E3 ubiquitin ligases such as Psh1 and Slx5 regulate ubiquitin-mediated proteolysis of overexpressed Cse4 (27,28,30–33). Our results for accumulation of polyubiquitinated endogenous Cse4 in *cdc48-3* strain prompted us to examine the role of Psh1 and Slx5 in the polyubiquitination of endogenous Cse4, which is then targeted by $Cdc48^{Ufd1/Npl4}$. Our results showed that deletion of *PSH1* almost completely eliminated accumulation of polyubiquitinated endogenous Cse4 in *cdc48*-3 strain (Figure 6 and Supplementary Figure S16). In contrast, deletion of *SLX5* did not show an obvious reduction of polyubiquitinated endogenous Cse4 in *cdc48*-3 strain (Supplementary Figure S17). We conclude that Psh1-mediated polyubiquitination of Cse4 is subsequently recognized by $Cdc48^{Ufd1/Npl4}$ segregase for removal from chromatin and targeted for degradation.

Psh1 facilitates the association of Npl4 with mislocalized Cse4

Based on our results thus far, we hypothesize that Psh1 recognizes mislocalized Cse4, ubiquitinates these species at non-centromeric regions, and thereby generates a substrate for $Cdc48^{Ufd1/Npl4}$ segregase for further processing. To test this hypothesis, we asked if Npl4 localizes to sites of mislocalized Cse4 in a Psh1-dependent manner. Previous studies have identified that promoters of *SAP4* and *RDS1* and pericentromeric R1 region of *CEN3* are enriched for mislocalized Cse4 when it is overexpressed in a *psh1*Δ mutants (41,63). We examined the ubiquitination status of chromatin-associated Cse4 and the association of Cse4 and Npl4 at the non-centromeric regions in *cdc48-3* and *cdc48-3 psh1*Δ strains overexpressing Cse4. We showed that polyubiquitinated chromatin-associated Cse4 is detected in a *cdc48-3* strain and that the polyubiquitinated Cse4 is greatly reduced although not completely abolished in a *cdc48-3 psh1*Δ strain (Figure 7A and B).

We performed chromatin immunoprecipitation (ChIP)-qPCR experiments to examine if Cse4 is mislocalized to the non-centromeric regions in *cdc48-3* strain. An enrichment of Cse4 was observed at the non-centromeric regions *SAP4, RSD1* and *R1* in a *cdc48-3* mutant (Figure 7C). We next examined if association of Npl4 with Cse4 is dependent on Psh1 by comparing the non-centromeric occupancy of Npl4 in *cdc48-3* and *cdc48-3 psh1Δ* strains. Steady state levels of Npl4 were not affected in a *cdc48-3 psh1*Δ strain (Supplementary Figure S18). Since chromatin-bound Cse4 was increased in a *cdc48-3 psh1Δ* strain, compared to a *cdc48-3* strain (Supplementary Figure S19), we normalized the enrichment of Npl4 to Cse4 occupancy at each region. The ChIP-qPCR results showed that enrichment of Npl4 in the *cdc48-3 psh1Δ* strain was significantly reduced at *SAP4* and *RDS1*, but not *CEN3* and R1 region of *CEN3* (Figure 7D). These results support our hypothesis that association of Npl4 to sites of Cse4 mislocalization is facilitated by Psh1-mediated polyubiquitination of Cse4.

We have previously shown that mislocalization of Cse4/CENP-A contributes to CIN in budding yeast and human cells (15,17,18). Hence, we examined if *cdc48-3* strain exhibits CIN by measuring the ability of cells to retain a centromere-containing plasmid (pRS416 *URA3*) after growth in nonselective medium at 25°C (33,41). Plasmid retention was measured as the ratio of the number of colonies grown on SD-Ura versus SD+Ura medium. In wild-type strain, plasmid retention after 10 generations (10G) was about 80% (Supplementary Figure S20). In contrast, we observed reduced plasmid retention after 10 generations in nonselective medium (10G) in *cdc48-3* strain. We conclude that Cdc48 contributes to the chromosomal stability. Taken together, our results show that Psh1 facilitates the association of Npl4 with mislocalized Cse4.

DISCUSSION

The incorporation of CENP-A into centromeric chromatin is essential for establishing centromere identity. Overexpressed CENP-A leads to its mislocalization to non-centromeric chromatin and contributes to aneuploidy in budding and fission yeasts, flies, and humans. Mechanisms that remove mislocalized CENP-A and target it for degradation have not been defined. Here, we report that $Cdc48^{Ufd1/Npl4}$ segregase removes mislocalized Cse4 for degradation under normal physiological conditions in *S. cerevisiae*. We propose a model (Figure 8) in which Psh1-mediated polyubiquitination of mislocalized Cse4 facilitates the interaction with $Cdc48^{Ufd1/Npl4}$ segregase via Npl4 thereby promoting the removal of mislocalized Cse4 and targeting it for proteasomal degradation.

Cdc48 is an abundant protein and is a member of the ATPase associated with diverse cellular activities (AAA) family that functions to maintain protein homeostasis. The *cdc48-3* mutant shows synthetic dosage lethality with overexpressed Cse4. There are many substrates regulated by Cdc48 and hence we examined if the phenotypes of *cdc48-3* strain are linked to mislocalization of Cse4. We recently reported that reduced mislocalization of Cse4 contributes to the suppression of lethality of *psh1Δ GAL-CSE4* strain with either reduced gene dosage of histone H4 or *cse4 K215/216R* mutant (40,41). Consistent with these results, we observed that reduced gene dosage of histone H4 or expression of *GAL-cse4 K215/216R* suppresses the dosage lethality in the *cdc48-3* strain. Hence, the dosage lethality of overexpressed Cse4 is due to mislocalization of Cse4 in the *cdc48-3* strain. More importantly, our studies with endogenous Cse4 showed an enrichment in chromatin and mislocalization in *cdc48-3*, *ufd1-2*, and *npl4-1* mutants at the permissive temperature of 25°C. Cdc48 associates with a large number of cofactors in diverse cellular processes, among which Ufd1 and Npl4 together with Cdc48 functions as a segregase. The enrichment in chromatin and mislocalization of endogenous Cse4 in all *cdc48-3*, *ufd1-2* and *npl4-1* mutants suggest that the Cse4-related phenotypes are specifically linked to defective segregase activity of $Cdc48^{Ufd1/Npl4}$. We determined that *cdc48-3* strain exhibits a CIN phenotype, however, this may not be solely due to mislocalization

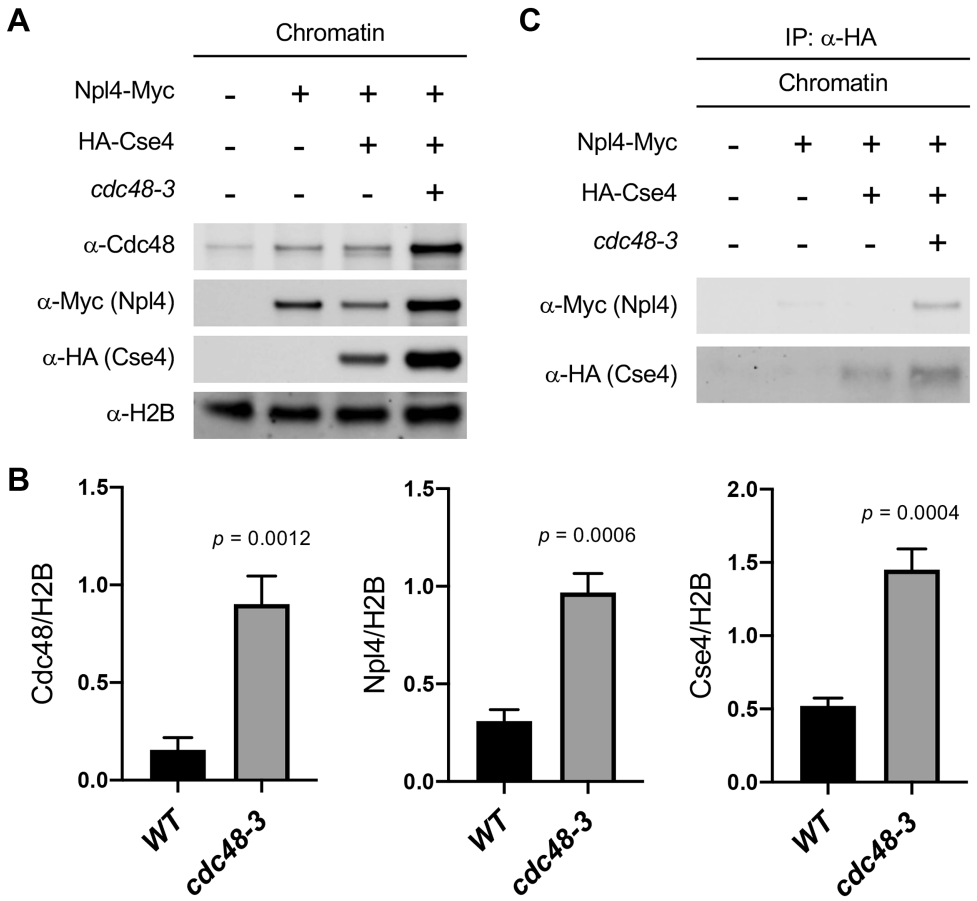


Figure 5. Npl4 interacts with chromatin-bound Cse4 in a *cdc48-3* mutant. (**A**) Endogenous Cse4, Npl4, and Cdc48 are enriched in chromatin of *cdc48-3* strain. Chromatin was prepared from equal numbers of logarithmically growing cells. Levels of Cdc48, Npl4, and Cse4 in chromatin were monitored by Western blot analysis with anti-Cdc48, anti-Myc, and anti-HA antibodies, respectively. Histone H2B was used as marker for chromatin fraction. (**B**) Levels of chromatin-bound Cdc48, Npl4, and Cse4 from A were quantified in arbitrary density units after normalization to H2B. Error bars represent the standard deviation of three biological repeats. Statistical significance was assessed by unpaired *t*-test. (**C**) Npl4 interacts with chromatin-bound Cse4. *In vivo* interaction of Cse4 with Npl4 in chromatin was determined by Co-IP using chromatin lysates. α-Myc and α-HA antibodies were used to detect Npl4 and Cse4 on the Western blot, respectively.

of Cse4 as Cdc48 has many other cell cycle regulated substrates (64,65).

Cdc48/p97 along with its cofactors acts as a ubiquitin-dependent segregase that extracts polyubiquitinated substrates from macromolecular complexes and targets them for degradation (44,49,50). Polyubiquitinated endogenous Cse4 is barely detectable in wild-type cells, probably due to its rapid removal by $Cdc48^{Ufd1/Npl4}$ segregase and subsequent degradation. Studies with *S. cerevisiae* have identified multiple E3 ubiquitin ligases such as Psh1, Slx5, Ubr1, SCF-Rcy1 and SCF-Met30/Cdc4 (27,28,30–33). Each single mutant shows partial reduction of polyubiquitinated Cse4 when Cse4 is overexpressed. Our results are consistent with these reports as we observed reduced levels of polyubiquitinated chromatin-associated Cse4 in a *cdc48-3 psh1*Δ *GAL-CSE4* strain. We propose that Psh1 is a major E3 ligase that contributes to polyubiquitination of chromatin-associated Cse4 and/or other E3 ligases may be functionally dependent on priming by Psh1 in a *cdc48-3* strain. Alternatively, other E3 ligases may contribute to proteolysis of soluble Cse4 when Cse4 is overexpressed. Future studies will provide insights into how multiple E3 ligases and other factors target soluble versus chromatin bound Cse4 to prevent its mislocalization under normal physiological conditions.

Using *cse4 Y193A/F* mutants as a tool for Cse4 mislocalization, we uncovered a strong correlation between dosage lethality, chromatin enrichment, and accumulation of polyubiquitinated Cse4 in *cdc48-3* strain. Both *cse4 Y193A* and *cse4 Y193F* alleles complement the *cse4*Δ strain, suggesting that sumoylation status of Cse4 does not affect haploid growth. These results also provide evidence that the role of Cdc48 segregase is specifically towards chromatin-bound mislocalized Cse4, but not centromeric Cse4. Consistent with the correlation between chromatin enrichment and accumulation of polyubiquitinated Cse4 in *cdc48-3 cse4 Y193A/F* mutants, we also showed that polyubiquitinated chromatin-associated Cse4 is detected in a *cdc48-3* strain. We observed the enrichment of Cse4, Cdc48, and Npl4 in chromatin, the interaction of Npl4 with chromatin-associated Cse4, and the enrichment of Npl4 as well as overexpressed Cse4 at non-centromeric

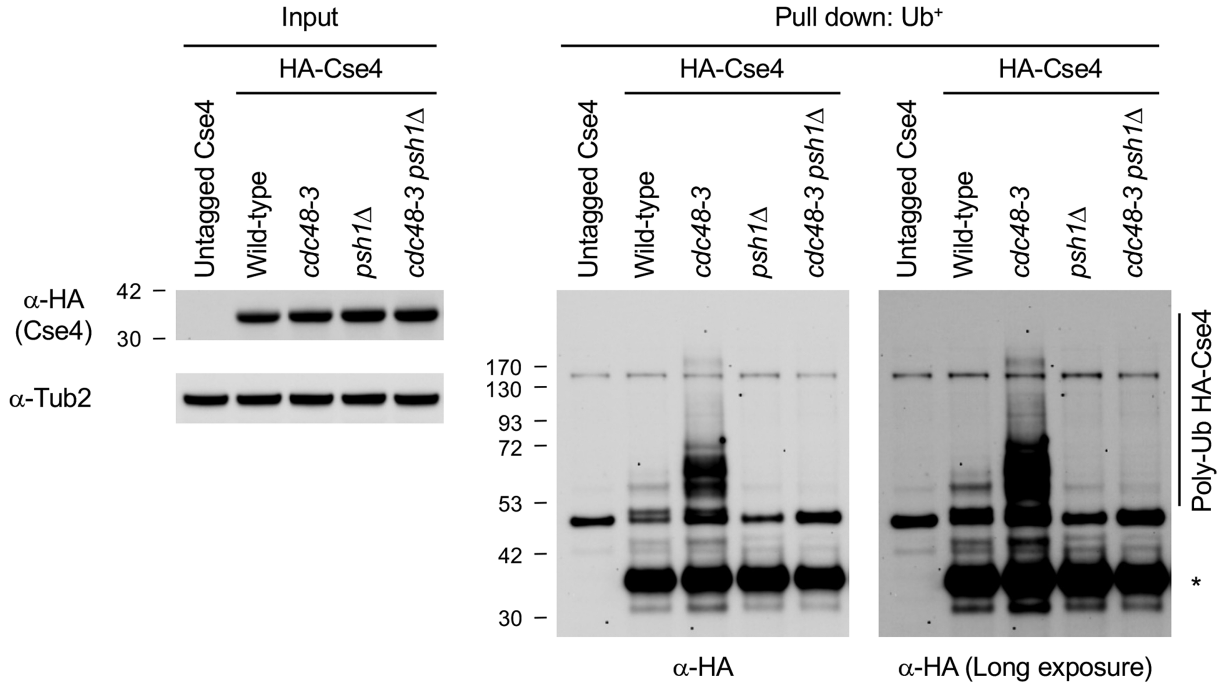


Figure 6. Psh1 contributes to polyubiquitination of endogenous Cse4 in *cdc48-3* strain. Ub pull-down assay was performed using protein extracts from logarithmically growing cells in YPD at 25°C. Input and ubiquitin pull down samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Untagged Cse4 was used as a negative control. Asterisk shows nonmodified Cse4.

regions in the *cdc48-3* mutant. Furthermore, the reduced enrichment of Npl4 at non-centromeric regions in *cdc48-3* strain with deletion of *PSH1*, suggests that Cse4 that is polyubiquitinated by Psh1 is a substrate for Cdc48$^{Ufd1/Npl4}$ segregase. These data support our model that mislocalized Cse4 is polyubiquitinated in a *cdc48-3* strain and define a role for Cdc48$^{Ufd1/Npl4}$ segregase in the removal of polyubiquitinated mislocalized Cse4 at non-centromeric regions even under normal physiological conditions (Figure 8).

The Cdc48 ATPase often acts upstream of the 26S proteasome. A recent study shows that cofactors Ufd1-Npl4 bind one unfolded ubiquitin and three to four folded ubiquitin molecules for stable binding (66). Cdc48 ultimately releases the unfolded, oligo-ubiquitinated polypeptides. In fact, a minimum of four ubiquitin moieties are required for recognition by the 26S proteasome for degradation (59,60). Our results are consistent with this conclusion as mono- or di-ubiquitination of Cse4 (around 53 kDa) were more abundant in wild-type cells due to rapid removal of polyubiquitinated Cse4 and higher molecular weight polyubiquitinated Cse4 signals (> 53 kDa) are enhanced in *cdc48-3* and *cdc48-aid* strains.

The *cdc48-3* mutant protein has two missense substitutions (P257L and R387K) in the D1 ATPase domain (47). It is of interest to examine if the ATPase activity of Cdc48 is important for the removal of mislocalized Cse4. The complementation of *cse4Δ* by *cse4 Y193A/F* suggests that polyubiquitination of Cse4 is not essential for centromeric roles of Cse4, and Cdc48 does not target centromeric Cse4 at the point centromeres of *S. cerevisiae*, which lacks heterochromatin. It is also possible that centromeric Cse4 is protected from Cdc48 activity due to its association with Scm3, similar to the protection of Scm3 associated Cse4 at centromeres from Psh1-mediated ubiquitination (55). It has been proposed that mislocalized Cse4 at non-centromeric regions is not associated with Scm3 and hence better targeted by E3 ligases for Ub proteasome-mediated degradation (67).

Non-proteolytic functions for the Cdc48 segregase have been demonstrated in various systems such as membrane extraction of transcription factors and dissociation of the SCF complex (68–72). Our data suggest that Cdc48$^{Ufd1/Npl4}$ regulates the removal of Cse4 from non-centromeric chromatin with subsequent delivery to the proteasome for degradation. However, we do not have direct evidence for the involvement of Cdc48 in escorting Cse4 to the proteasome. It is possible that other proteasome shuttling factors such as Rad23, Ddi1, and Dsk2 (42) bind chromatin extracted ubiquitinated Cse4 for delivery.

In summary, we have defined a molecular role for Cdc48$^{Ufd1/Npl4}$ segregase in the removal of mislocalized Cse4 from non-centromeric chromatin (Figure 8). Our studies expand the role of VCP/p97/CDC48 in the recognition of ubiquitinated substrates and extraction of misfolded proteins from multisubunit complexes or membranes such as ERAD to the regulation of spatial nucleosome composition. VCP/p97/CDC48 is a key regulator and provides the main quality control for soluble, as well as membrane- associated and chromatin-associated proteins. Mutations in p97 have been linked to a number of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases. Overex-

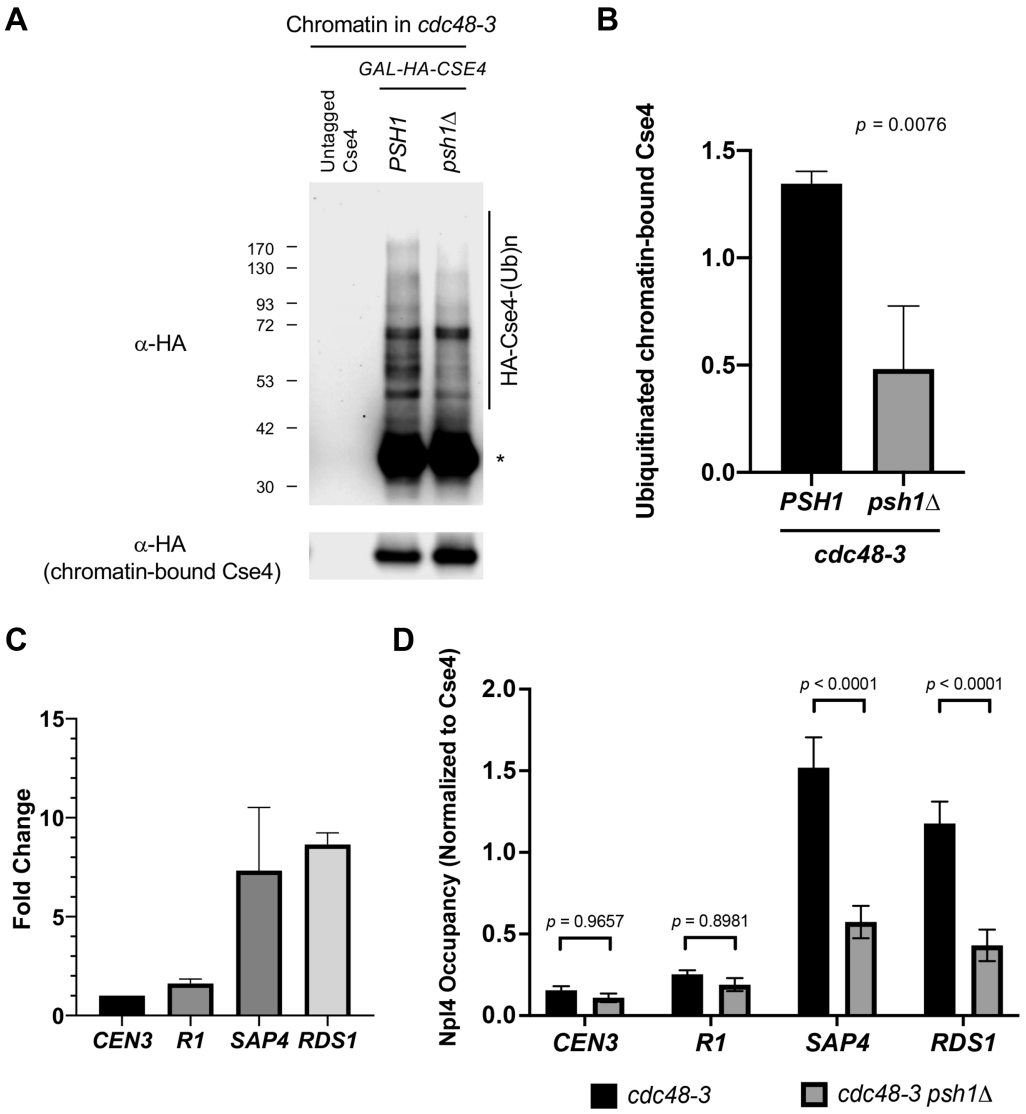


Figure 7. Psh1 facilitates the association of Npl4 with mislocalized Cse4. (**A**) Ubiquitinated chromatin-bound Cse4 is reduced in a *cdc48-3 psh1*Δ strain. Solubilized chromatin lysate was prepared from *cdc48-3 GAL-CSE4* or *cdc48-3 psh1*Δ *GAL-CSE4* strains. Cultures were grown in sucrose/galactose (2%) for 4hrs at 25°C to induce expression of Cse4 and Ub pull-down assay was performed using chromatin lysates. Ub pull down samples and chromatin-bound Cse4 were analyzed using anti-HA (Cse4) antibody. Untagged Cse4 was used as a negative control. Asterisk shows nonmodified Cse4. (**B**) Levels of chromatin-bound Cse4 ubiquitination from A were quantified in arbitrary density units after normalization to chromatin-bound Cse4. Error bars represent the standard deviation of three biological repeats. Statistical significance was assessed by unpaired *t*-test. (**C**) Cse4 is mislocalized upon overexpression of Cse4 in a *cdc48-3* mutant. ChIP-qPCR was performed on chromatin lysate from *cdc48-3* strain transformed with vector (endogenous Cse4) or *GAL-CSE4* (overexpressed Cse4). Equal volume of solubilized, crosslinked chromatin from each strain was used for ChIP with anti-HA (Cse4), anti-Myc (Npl4) and anti-GST. qPCR was performed for association with *CEN3*, pericentromeric R1 region of *CEN3*, and *SAP4* and *RDS1* promoter regions. Enrichment of Cse4 is shown as a fold change over endogenous Cse4. Error bars represent standard deviation of the mean of two independent experiments. (D) Chromatin-bound Npl4 was significantly reduced at the promoters of *SAP4* and *RDS1* in a *cdc48-3 psh1Δ* strain. ChIP-qPCR was performed on chromatin lysate from *cdc48-3* or *cdc48-3 psh1*Δ strain transformed with *GAL-CSE4*. Equal volume of solubilized, crosslinked chromatin from each strain was used for ChIP with anti-HA (Cse4) and anti-Myc (Npl4). Occupancy of Npl4 normalized to that of Cse4 was measured. Error bars represent standard deviation of the mean of three independent experiments.

pression of wild-type p97 is observed in numerous cancers including human melanomas and breast carcinoma, most likely due to the activation of protein degradation pathways in cancer cells (73,74). Inhibitors of p97 have been explored for treatment of various cancers (73,75), however the precise mechanism for their action has not been fully elucidated. We recently reported that mislocalization of overexpressed CENP-A leads to aneuploidy with karyotypic heterogeneity in human cells and xenograft mouse model (18). Our studies are important from a clinical standpoint given the poor prognosis of CENP-A overexpressing cancers. It is of interest to examine the molecular role of p97 as well as co-factors Ufd1 and Npl4 in preventing stable association of mislocalized CENP-A to non-centromeric regions and how defects in this pathway may contribute to aneuploidy in human cancers.

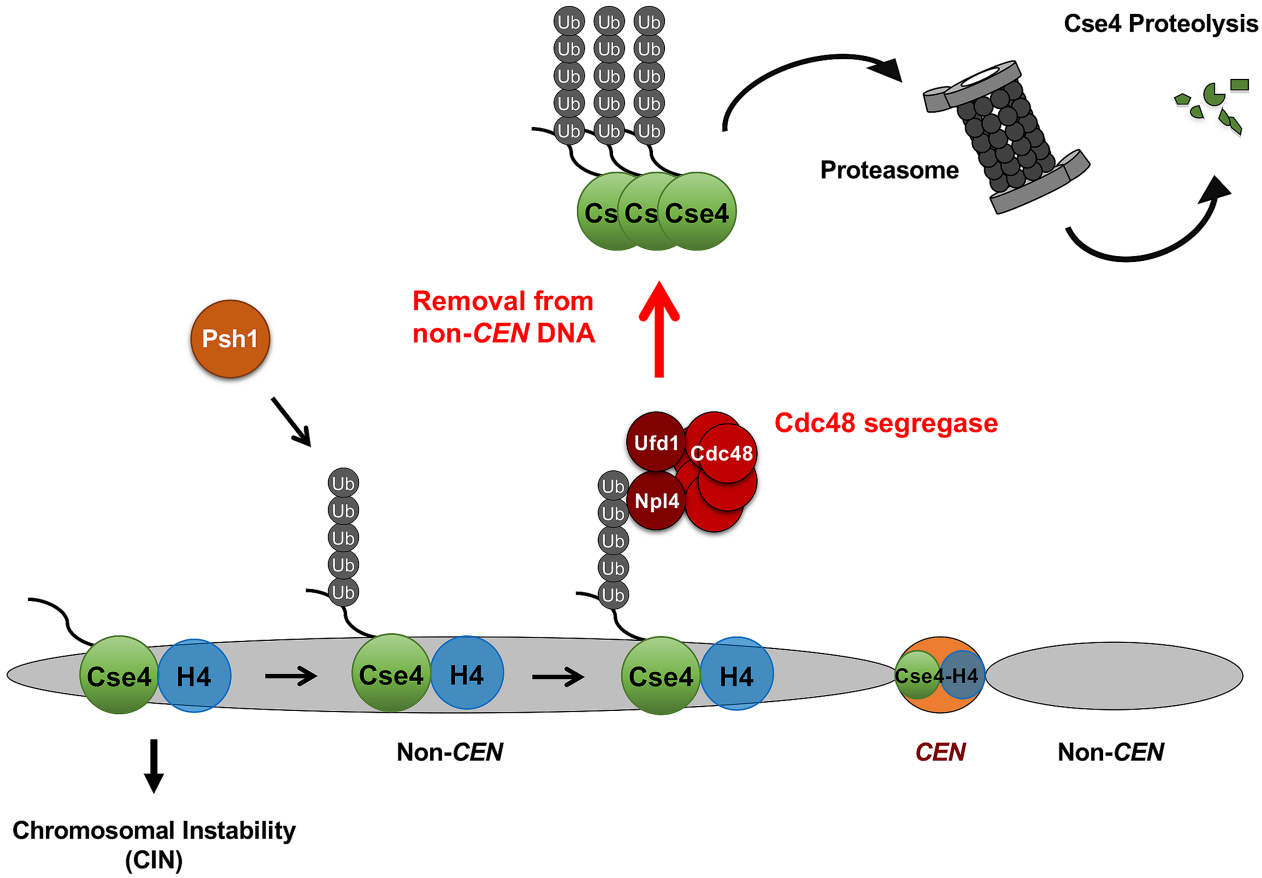


Figure 8. Molecular role of $Cdc48^{Ufd1/Npl4}$ segregase in removal of mislocalized Cse4 from non-centromeric regions. Defects in several pathways contribute to mislocalization of Cse4 to non-centromeric regions and CIN. We propose that Psh1 contributes to polyubiquitination of mislocalized Cse4 and this facilitates the interaction between polyubiquitinated Cse4 and $Cdc48^{Ufd1/Npl4}$ segregase complex via Npl4, resulting in the removal of Cse4 from non-centromeric regions for proteasomal degradation.

DATA AVAILABILITY

FACS data were deposited with Flow Repository under ID: FR-FCM-Z4RS.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We gratefully acknowledge Charles Boone, Michael Costanzo, Anastasia Baryshnikova, and Chad Myers for the SGA screen, Karim Labib for *cdc48-aid* strain, Brian D. Strahl for rabbit anti-Cse4 antibody, Kathy McKinnon of the National Cancer Institute Vaccine Branch FACS Core, Prashant K. Mishra of the Basrai laboratory for assistance with FACS analysis, and the members of the Basrai laboratory for helpful discussions and comments on the manuscript.

FUNDING

National Institutes of Health Intramural Research Program at the National Cancer Institute to M.A.B. and National Institues of Health [R01GM066164 to P.K.]. Funding for open access charge: NIH Intramural Research Program at the National Cancer Institute.
Conflict of interest statement. None declared.

REFERENCES

1. Torras-Llort,M., Moreno-Moreno,O. and Azorin,F. (2009) Focus on the centre: the role of chromatin on the regulation of centromere identity and function. *EMBO J.*, **28**, 2337–2348.
2. Biggins,S. (2013) The Composition, Functions, and Regulation of the Budding Yeast Kinetochore. *Genetics*, **194**, 817–846.
3. McKinley,K.L. and Cheeseman,I.M. (2016) The molecular basis for centromere identity and function. *Nat. Rev. Mol. Cell. Biol.*, **17**, 16–29.
4. Camahort,R., Li,B., Florens,L., Swanson,S.K., Washburn,M.P. and Gerton,J.L. (2007) Scm3 is essential to recruit the histone H3 variant Cse4 to centromeres and to maintain a functional kinetochore. *Mol. Cell*, **26**, 853–865.
5. Mizuguchi,G., Xiao,H., Wisniewski,J., Smith,M.M. and Wu,C. (2007) Nonhistone Scm3 and histones CenH3-H4 assemble the core of centromere-specific nucleosomes. *Cell*, **129**, 1153–1164.
6. Stoler,S., Rogers,K., Weitze,S., Morey,L., Fitzgerald-Hayes,M. and Baker,R.E. (2007) Scm3, an essential Saccharomyces cerevisiae centromere protein required for G2/M progression and Cse4 localization. *Proc. Natl. Acad. Sci. U.S.A.*, **104**, 10571–10576.
7. Williams,J.S., Hayashi,T., Yanagida,M. and Russell,P. (2009) Fission yeast Scm3 mediates stable assembly of Cnp1/CENP-A into centromeric chromatin. *Mol. Cell*, **33**, 287–298.

8. Pidoux,A.L., Choi,E.S., Abbott,J.K., Liu,X., Kagansky,A., Castillo,A.G., Hamilton,G.L., Richardson,W., Rappsilber,J., He,X. *et al.* (2009) Fission yeast Scm3: a CENP-A receptor required for integrity of subkinetochore chromatin. *Mol. Cell*, **33**, 299–311.
9. Foltz,D.R., Jansen,L.E., Bailey,A.O., Yates,J.R. 3rd, Bassett,E.A., Wood,S., Black,B.E. and Cleveland,D.W. (2009) Centromere-specific assembly of CENP-A nucleosomes is mediated by HJURP. *Cell*, **137**, 472–484.
10. Shuaib,M., Ouararhni,K., Dimitrov,S. and Hamiche,A. (2010) HJURP binds CENP-A via a highly conserved N-terminal domain and mediates its deposition at centromeres. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 1349–1354.
11. Chen,C.C., Dechassa,M.L., Bettini,E., Ledoux,M.B., Belisario,C., Huen,P., Luger,K. and Mellone,B.G. (2014) CAL1 is the Drosophila CENP-A assembly factor. *J. Cell. Biol.*, **204**, 313–329.
12. Collins,K.A., Furuyama,S. and Biggins,S. (2004) Proteolysis contributes to the exclusive centromere localization of the yeast Cse4/CENP-A histone H3 variant. *Curr. Biol.*, **14**, 1968–1972.
13. Heun,P., Erhardt,S., Blower,M.D., Weiss,S., Skora,A.D. and Karpen,G.H. (2006) Mislocalization of the Drosophila centromere-specific histone CID promotes formation of functional ectopic kinetochores. *Dev. Cell*, **10**, 303–315.
14. Moreno-Moreno,O., Torras-Llort,M. and Azorin,F. (2006) Proteolysis restricts localization of CID, the centromere-specific histone H3 variant of Drosophila, to centromeres. *Nucleic Acids Res.*, **34**, 6247–6255.
15. Au,W.C., Crisp,M.J., DeLuca,S.Z., Rando,O.J. and Basrai,M.A. (2008) Altered dosage and mislocalization of histone H3 and Cse4p lead to chromosome loss in Saccharomyces cerevisiae. *Genetics*, **179**, 263–275.
16. Gonzalez,M., He,H., Dong,Q., Sun,S. and Li,F. (2014) Ectopic centromere nucleation by CENP-A in fission yeast. *Genetics*, **198**, 1433–1446.
17. Shrestha,R.L., Ahn,G.S., Staples,M.I., Sathyan,K.M., Karpova,T.S., Foltz,D.R. and Basrai,M.A. (2017) Mislocalization of centromeric histone H3 variant CENP-A contributes to chromosomal instability (CIN) in human cells. *Oncotarget*, **8**, 46781–46800.
18. Shrestha,R.L., Rossi,A., Wangsa,D., Hogan,A.K., Zaldana,.S., Suva,E., Chung,Y.J., Sanders,C.L., Difilippantonio,S., Karpova,T.S. *et al.* (2021) CENP-A overexpression promotes aneuploidy with karyotypic heterogeneity. *J. Cell. Biol.*, **220**, e202007195.
19. Tomonaga,T., Matsushita,K., Yamaguchi,S., Oohashi,T., Shimada,H., Ochiai,T., Yoda,K. and Nomura,F. (2003) Overexpression and mistargeting of centromere protein-A in human primary colorectal cancer. *Cancer Res.*, **63**, 3511–3516.
20. Amato,A., Schillaci,T., Lentini,L. and Di Leonardo,A. (2009) CENPA overexpression promotes genome instability in pRb-depleted human cells. *Mol. Cancer*, **8**, 119.
21. Hu,Z., Huang,G., Sadanandam,A., Gu,S., Lenburg,M.E., Pai,M., Bayani,N., Blakely,E.A., Gray,J.W. and Mao,J.H. (2010) The expression level of HJURP has an independent prognostic impact and predicts the sensitivity to radiotherapy in breast cancer. *Breast Cancer Res.*, **12**, R18.
22. Li,Y., Zhu,Z., Zhang,S., Yu,D., Yu,H., Liu,L., Cao,X., Wang,L., Gao,H. and Zhu,M. (2011) ShRNA-targeted centromere protein A inhibits hepatocellular carcinoma growth. *PLoS One*, **6**, e17794.
23. Wu,Q., Qian,Y.M., Zhao,X.L., Wang,S.M., Feng,X.J., Chen,X.F. and Zhang,S.H. (2012) Expression and prognostic significance of centromere protein A in human lung adenocarcinoma. *Lung Cancer*, **77**, 407–414.
24. Lacoste,N., Woolfe,A., Tachiwana,H., Garea,A.V., Barth,T., Cantaloube,S., Kurumizaka,H., Imhof,A. and Almouzni,G. (2014) Mislocalization of the centromeric histone variant CenH3/CENP-A in human cells depends on the chaperone DAXX. *Mol. Cell*, **53**, 631–644.
25. Athwal,R.K., Walkiewicz,M.P., Baek,S., Fu,S., Bui,M., Camps,J., Ried,T., Sung,M.H. and Dalal,Y. (2015) CENP-A nucleosomes localize to transcription factor hotspots and subtelomeric sites in human cancer cells. *Epigenetics Chromatin*, **8**, 2.
26. Sun,X., Clermont,P.L., Jiao,W., Helgason,C.D., Gout,P.W., Wang,Y. and Qu,S. (2016) Elevated expression of the centromere protein-A(CENP-A)-encoding gene as a prognostic and predictive biomarker in human cancers. *Int. J. Cancer*, **139**, 899–907.
27. Hewawasam,G., Shivaraju,M., Mattingly,M., Venkatesh,S., Martin-Brown,S., Florens,L., Workman,J.L. and Gerton,J.L. (2010) Psh1 is an E3 ubiquitin ligase that targets the centromeric histone variant Cse4. *Mol. Cell*, **40**, 444–454.
28. Ranjitkar,P., Press,M.O., Yi,X., Baker,R., MacCoss,M.J. and Biggins,S. (2010) An E3 ubiquitin ligase prevents ectopic localization of the centromeric histone H3 variant via the centromere targeting domain. *Mol. Cell*, **40**, 455–464.
29. Ohkuni,K., Abdulle,R. and Kitagawa,K. (2014) Degradation of centromeric histone H3 variant Cse4 requires the Fpr3 peptidyl-prolyl cis-trans isomerase. *Genetics*, **196**, 1041–1045.
30. Ohkuni,K., Takahashi,Y., Fulp,A., Lawrimore,J., Au,W.C., Pasupala,N., Levy-Myers,R., Warren,J., Strunnikov,A., Baker,R.E. *et al.* (2016) SUMO-Targeted Ubiquitin Ligase (STUbL) Slx5 regulates proteolysis of centromeric histone H3 variant Cse4 and prevents its mislocalization to euchromatin. *Mol. Biol. Cell*, **27**, 1500–1510.
31. Cheng,H., Bao,X. and Rao,H. (2016) The F-box protein Rcy1 is involved in the degradation of histone H3 variant Cse4 and genome maintenance. *J. Biol. Chem.*, **291**, 10372–10377.
32. Cheng,H., Bao,X., Gan,X., Luo,S. and Rao,H. (2017) Multiple E3s promote the degradation of histone H3 variant Cse4. *Sci. Rep.*, **7**, 8565.
33. Au,W.C., Zhang,T., Mishra,P.K., Eisenstatt,J.R., Walker,R.L., Ocampo,J., Dawson,A., Warren,J., Costanzo,M., Baryshnikova,A. *et al.* (2020) Skp, cullin, F-box (SCF)-Met30 and SCF-Cdc4-mediated proteolysis of CENP-A prevents mislocalization of CENP-A for chromosomal stability in budding yeast. *PLoS Genet.*, **16**, e1008597.
34. Ohkuni,K., Levy-Myers,R., Warren,J., Au,W.C., Takahashi,Y., Baker,R.E. and Basrai,M.A. (2018) N-terminal sumoylation of centromeric histone H3 variant Cse4 regulates its proteolysis to prevent mislocalization to non-centromeric chromatin. *G3 (Bethesda)*, **8**, 1215–1223.
35. Deyter,G.M. and Biggins,S. (2014) The FACT complex interacts with the E3 ubiquitin ligase Psh1 to prevent ectopic localization of CENP-A. *Genes Dev.*, **28**, 1815–1826.
36. Hewawasam,G.S., Mattingly,M., Venkatesh,S., Zhang,Y., Florens,L., Workman,J.L. and Gerton,J.L. (2014) Phosphorylation by casein kinase 2 facilitates Psh1 protein-assisted degradation of Cse4 protein. *J. Biol. Chem.*, **289**, 29297–29309.
37. Ciftci-Yilmaz,S., Au,W.C., Mishra,P.K., Eisenstatt,J.R., Chang,J., Dawson,A.R., Zhu,I., Rahman,M., Bilke,S., Costanzo,M. *et al.* (2018) A genome-wide screen reveals a role for the HIR histone chaperone complex in preventing mislocalization of budding yeast CENP-A. *Genetics*, **210**, 203–218.
38. Eisenstatt,J.R., Boeckmann,L., Au,W.C., Garcia,V., Bursch,L., Ocampo,J., Costanzo,M., Weinreich,M., Sclafani,R.A., Baryshnikova,A. *et al.* (2020) Dbf4-dependent kinase (DDK)-mediated proteolysis of CENP-A prevents mislocalization of CENP-A in *Saccharomyces cerevisiae*. *G3 (Bethesda)*, **10**, 2057–2068.
39. Hewawasam,G.S., Dhatchinamoorthy,K., Mattingly,M., Seidel,C. and Gerton,J.L. (2018) Chromatin assembly factor-1 (CAF-1) chaperone regulates Cse4 deposition into chromatin in budding yeast. *Nucleic Acids Res.*, **46**, 4440–4455.
40. Ohkuni,K., Suva,E., Au,W.C., Walker,R.L., Levy-Myers,R., Meltzer,P.S., Baker,R.E. and Basrai,M.A. (2020) Deposition of centromeric histone H3 variant CENP-A/Cse4 into chromatin is facilitated by its C-terminal sumoylation. *Genetics*, **214**, 839–854.
41. Eisenstatt,J.R., Ohkuni,K., Au,W.C., Preston,O., Gliford,L., Suva,E., Costanzo,M., Boone,C. and Basrai,M.A. (2021) Reduced gene dosage of histone H4 prevents CENP-A mislocalization and chromosomal instability in *Saccharomyces cerevisiae*. *Genetics*, **218**, iyab033.
42. Finley,D., Ulrich,H.D., Sommer,T. and Kaiser,P. (2012) The ubiquitin-proteasome system of *Saccharomyces cerevisiae*. *Genetics*, **192**, 319–360.
43. Meyer,H., Bug,M. and Bremer,S. (2012) Emerging functions of the VCP/p97 AAA-ATPase in the ubiquitin system. *Nat. Cell. Biol.*, **14**, 117–123.
44. Franz,A., Ackermann,L. and Hoppe,T. (2016) Ring of change: CDC48/p97 drives protein dynamics at chromatin. *Front. Genet.*, **7**, 73.
45. Bodnar,N. and Rapoport,T. (2017) Toward an understanding of the Cdc48/p97 ATPase. *F1000Res*, **6**, 1318.

46. van den Boom,J. and Meyer,H. (2018) VCP/p97-mediated unfolding as a principle in protein homeostasis and signaling. *Mol. Cell*, **69**, 182–194.
47. Verma,R., Oania,R., Fang,R., Smith,G.T. and Deshaies,R.J. (2011) Cdc48/p97 mediates UV-dependent turnover of RNA Pol II. *Mol. Cell*, **41**, 82–92.
48. Raman,M., Havens,C.G., Walter,J.C. and Harper,J.W. (2011) A genome-wide screen identifies p97 as an essential regulator of DNA damage-dependent CDT1 destruction. *Mol. Cell*, **44**, 72–84.
49. Dargemont,C. and Ossareh-Nazari,B. (2012) Cdc48/p97, a key actor in the interplay between autophagy and ubiquitin/proteasome catabolic pathways. *Biochim. Biophys. Acta*, **1823**, 138–144.
50. Hanzelmann,P. and Schindelin,H. (2017) The interplay of cofactor interactions and post-translational modifications in the regulation of the AAA+ ATPase p97. *Front. Mol. Biosci.*, **4**, 21.
51. Tsuchiya,H., Ohtake,F., Arai,N., Kaiho,A., Yasuda,S., Tanaka,K. and Saeki,Y. (2017) In vivo ubiquitin linkage-type analysis reveals that the Cdc48-Rad23/Dsk2 axis contributes to K48-linked chain specificity of the proteasome. *Mol. Cell*, **66**, 488–502.
52. Sato,Y., Tsuchiya,H., Yamagata,A., Okatsu,K., Tanaka,K., Saeki,Y. and Fukai,S. (2019) Structural insights into ubiquitin recognition and Ufd1 interaction of Npl4. *Nat. Commun.*, **10**, 5708.
53. Longtine,M.S., McKenzie,A. 3rd, Demarini,D.J., Shah,N.G., Wach,A., Brachat,A., Philippsen,P. and Pringle,J.R. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in *Saccharomyces cerevisiae*. *Yeast*, **14**, 953–961.
54. Mishra,P.K., Au,W.C., Choy,J.S., Kuich,P.H., Baker,R.E., Foltz,D.R. and Basrai,M.A. (2011) Misregulation of Scm3p/HJURP causes chromosome instability in Saccharomyces cerevisiae and human cells. *PLoS Genet.*, **7**, e1002303.
55. Mishra,P.K., Guo,J., Dittman,L.E., Haase,J., Yeh,E., Bloom,K. and Basrai,M.A. (2015) Pat1 protects centromere-specific histone H3 variant Cse4 from Psh1-mediated ubiquitination. *Mol. Biol. Cell*, **26**, 2067–2079.
56. Au,W.C., Dawson,A.R., Rawson,D.W., Taylor,S.B., Baker,R.E. and Basrai,M.A. (2013) A novel role of the N-terminus of budding yeast histone H3 variant Cse4 in ubiquitin-mediated proteolysis. *Genetics*, **194**, 513–518.
57. Maric,M., Maculins,T., De Piccoli,G. and Labib,K. (2014) Cdc48 and a ubiquitin ligase drive disassembly of the CMG helicase at the end of DNA replication. *Science*, **346**, 1253596.
58. Maric,M., Mukherjee,P., Tatham,M.H., Hay,R. and Labib,K. (2017) Ufd1-Npl4 recruit Cdc48 for disassembly of ubiquitylated CMG helicase at the end of chromosome replication. *Cell. Rep.*, **18**, 3033–3042.
59. Chau,V., Tobias,J.W., Bachmair,A., Marriott,D., Ecker,D.J., Gonda,D.K. and Varshavsky,A. (1989) A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science*, **243**, 1576–1583.
60. Thrower,J.S., Hoffman,L., Rechsteiner,M. and Pickart,C.M. (2000) Recognition of the polyubiquitin proteolytic signal. *EMBO J.*, **19**, 94–102.
61. Cheng,Y.L. and Chen,R.H. (2010) The AAA-ATPase Cdc48 and cofactor Shp1 promote chromosome bi-orientation by balancing Aurora B activity. *J. Cell. Sci.*, **123**, 2025–2034.
62. Zhou,Z., Feng,H., Zhuou,B.-R., Ghirlando,R., Hu,K., Zwolak,A., Miller Jenkins,L.M., Xiao,H., Tjandra,N., Wu,C. *et al.* (2011) Structural basis for recognition of centromere histone variant CenH3 by the chaperone Scm3. *Nature*, **472**, 234–237.
63. Hildebrand,E.M. and Biggins,S. (2016) Regulation of budding yeast CENP-A levels prevents misincorporation at promoter nucleosomes and transcriptional defects. *PLoS Genet.*, **12**, e1005930.
64. Meyer,H. and Popp,O. (2008) Role(s) of Cdc48/p97 in mitosis. *Biochem. Soc. Trans.*, **36**, 126–130.
65. Buchberger,A. (2013) Roles of Cdc48 in regulated protein degradation in yeast. *Subcell. Biochem.*, **66**, 195–222.
66. Twomey,E.C., Ji,Z., Wales,T.E., Bodnar,N.O., Ficarro,S.B., Marto,J.A., Engen,J.R. and Rapoport,T.A. (2019) Substrate processing by the Cdc48 ATPase complex is initiated by ubiquitin unfolding. *Science*, **365**, eaax1033.
67. Folco,H.D. and Desai,A. (2010) A PSHaver for centromeric histones. *Mol. Cell.*, **40**, 351–352.
68. Hoppe,T., Matuschewski,K., Rape,M., Schlenker,S., Ulrich,H.D. and Jentsch,S. (2000) Activation of a membrane-bound transcription factor by regulated ubiquitin/proteasome-dependent processing. *Cell*, **102**, 577–586.
69. Rape,M., Hoppe,T., Gorr,I., Kalocay,M., Richly,H. and Jentsch,S. (2001) Mobilization of processed, membrane-tethered SPT23 transcription factor by CDC48(UFD1/NPL4), a ubiquitin-selective chaperone. *Cell*, **107**, 667–677.
70. Shcherbik,N. and Haines,D.S. (2007) Cdc48p(Npl4p/Ufd1p) binds and segregates membrane-anchored/tethered complexes via a polyubiquitin signal present on the anchors. *Mol. Cell.*, **25**, 385–397.
71. Yen,J.L., Flick,K., Papagiannis,C.V., Mathur,R., Tyrrell,A., Ouni,I., Kaake,R.M., Huang,L. and Kaiser,P. (2012) Signal-induced disassembly of the SCF ubiquitin ligase complex by Cdc48/p97. *Mol. Cell.*, **48**, 288–297.
72. Lauinger,L., Flick,K., Yen,J.L., Mathur,R. and Kaiser,P. (2020) Cdc48 cofactor Shp1 regulates signal-induced SCF(Met30) disassembly. *Proc. Natl. Acad. Sci. U.S.A.*, **117**, 21319–21327.
73. Huryn,D.M., Kornfilt,D.J.P. and Wipf,P. (2020) p97: an emerging target for cancer, neurodegenerative diseases, and viral infections. *J. Med. Chem.*, **63**, 1892–1907.
74. Tang,W.K. and Xia,D. (2016) Mutations in the human AAA(+) chaperone p97 and related diseases. *Front. Mol. Biosci.*, **3**, 79.
75. Vekaria,P.H., Home,T., Weir,S., Schoenen,F.J. and Rao,R. (2016) Targeting p97 to disrupt protein homeostasis in cancer. *Front. Oncol.*, **6**, 181.