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Dbf4-Dependent Kinase (DDK)-Mediated Proteolysis of CENP-A Prevents Mislocalization of CENP-A in *Saccharomyces cerevisiae*

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ABSTRACT

The evolutionarily conserved centromeric histone H3 variant (Cse4 in budding yeast, CENP-A in humans) is essential for faithful chromosome segregation. Mislocalization of CENP-A to non-centromeric chromatin contributes to chromosomal instability (CIN) in yeast, fly, and human cells and CENP-A is highly expressed and mislocalized in cancers. Defining mechanisms that prevent mislocalization of CENP-A is an area of active investigation. Ubiquitin-mediated proteolysis of overexpressed Cse4 (GALCSE4) by E3 ubiquitin ligases such as Psh1 prevents mislocalization of Cse4, and psh1Δ strains display synthetic dosage lethality (SDL) with GALCSE4. We previously performed a genome-wide screen and identified five alleles of CDC7 and DBF4 that encode the Dbf4-dependent kinase (DDK) complex, which regulates DNA replication initiation, among the top twelve hits that displayed SDL with GALCSE4. We determined that cdc7-7 strains exhibit defects in ubiquitin-mediated proteolysis of Cse4 and show mislocalization of Cse4. Mutation of MCM5 (mcm5-bob1) bypasses the requirement of Cdc7 for replication initiation and rescues replication defects in a cdc7-7 strain. We determined that mcm5-bob1 does not rescue the SDL and defects in proteolysis of GALCSE4 in a cdc7-7 strain, suggesting a DNA replication-independent role for Cdc7 in Cse4 proteolysis. The SDL phenotype, defects in ubiquitin-mediated proteolysis, and the mislocalization pattern of Cse4 in a cdc7-7 psh1Δ strain were similar to that of cdc7-7 and psh1Δ strains, suggesting that Cdc7 regulates Cse4 in a pathway that overlaps with Psh1. Our results define a DNA replication initiation-independent role of DDK as a regulator of Psh1-mediated proteolysis of Cse4 to prevent mislocalization of Cse4.
INTRODUCTION

The centromere, a specialized region of the chromosome that is essential for faithful chromosome segregation, and associated proteins make up the kinetochore, which serves as an attachment site for microtubules to promote segregation of sister chromatids during mitosis (Allshire and Karpen 2008; Verdaasdonk and Bloom 2011; Burrack and Berman 2012; Choy et al. 2012; Maddox et al. 2012; McKinley and Cheeseman 2016). Budding yeast “point centromeres” consist of approximately 125 base pairs (bp) of unique DNA sequences, whereas other eukaryotic organisms have “regional centromeres” consisting of several mega-bp of repeated DNA sequences, satellite DNA arrays, or retrotransposon-derived sequences. Despite the difference in the size of centromeres, the centromeric histone H3 variant (Cse4 in Saccharomyces cerevisiae, Cnp1 in Schizosaccharomyces pombe, CID in Drosophila melanogaster, and CENP-A in mammals) is evolutionarily conserved from yeast to human cells and is essential for faithful chromosome segregation (Przewloka and Glover 2009; Choy et al. 2012; Henikoff and Furuyama 2012; Biggins 2013; Wong et al. 2020). Mislocalization of overexpressed CENP-A and its homologs to non-centromeric regions contributes to chromosomal instability (CIN) in yeast, fly, and human cells (Heun et al. 2006; Au et al. 2008; Mishra et al. 2011; Lacoste et al. 2014; Athwal et al. 2015; Shrestha et al. 2017). CIN and high expression of CENP-A have been observed in cancer cells and this correlates with poor prognosis and increased invasiveness (Tomonaga et al. 2003; Amato et al. 2009; Li et al. 2011; McGovern et al. 2012; Sun et al. 2016; Zhang et al. 2016). The mechanisms that prevent the mislocalization of CENP-A and its homologs are not fully understood. Defining these mechanisms will provide insight into how mislocalization of CENP-A contributes to aneuploidy in human cancers.
Stringent regulation of cellular levels of Cse4 by post-translational modifications such as ubiquitination prevents its mislocalization to non-centromeric regions in budding yeast, fission yeast, and flies (Collins et al. 2004; Moreno-Moreno et al. 2006; Moreno-Moreno et al. 2011; Au et al. 2013; Gonzalez et al. 2014). In addition to ubiquitination of Cse4, we have recently defined a role for sumoylation in proteolysis of Cse4 (Ohkuni et al. 2016). Multiple ubiquitin ligases, such as Psh1, Ubr1, the Sumo-targeted ubiquitin ligase Slx5, and the F-box protein Rcy1 regulate proteolysis of overexpressed Cse4 (Hewawasam et al. 2010; Ranjitkar et al. 2010; Cheng et al. 2016; Ohkuni et al. 2016; Cheng et al. 2017; Ohkuni et al. 2018). Psh1 is one of the best characterized E3 ligases for proteolysis of overexpressed Cse4 and prevents mislocalization of Cse4 to non-centromeric regions (Hewawasam et al. 2010; Ranjitkar et al. 2010). Psh1 interacts with the CENP-A targeting domain (CATD) in the C-terminus of Cse4 (Hewawasam et al. 2010; Ranjitkar et al. 2010) and mediates Cse4 degradation through the interaction of Psh1 with Spt16, a component of the FACT (facilitates chromatin transcription) complex (Deyter and Biggins 2014). It has also been shown that phosphorylation of Psh1 by casein kinase 2 (CK2) promotes degradation of Cse4 (Hewawasam et al. 2014). In addition to targeting the C-terminus of Cse4, we have shown that the N-terminus of Cse4 regulates Cse4 proteolysis (Au et al. 2013).

Mutant strains that show defects in Cse4 proteolysis display synthetic dosage lethality (SDL) when Cse4 is overexpressed. However, Cse4 is not completely stabilized in psh1Δ, ubr1Δ, doa1Δ, slx5Δ, or rcy1Δ strains (Cheng et al. 2017), suggesting the existence of additional genes/pathways that regulate Cse4 proteolysis. We previously performed a Synthetic Genetic Array (SGA) using conditional mutant alleles of essential genes to identify additional factors that regulate Cse4 proteolysis (Au et al. 2020). The screen identified mutants encoding the F-box
proteins Met30 and Cdc4 of the Skp1, Cullin, F-box (SCF) complex. We defined a cooperative role for Met30 and Cdc4 in the proteolysis of endogenous Cse4 to prevent its mislocalization and promote chromosome stability (Au et al. 2020). Here, we pursued studies of the evolutionarily conserved Dbf4-dependent kinase (DDK) complex as we identified five mutant dbf4 and cdc7 alleles among the top twelve significant SDL hits. The DDK complex, which is essential for the initiation of DNA replication, consists of the Cdc7 kinase and the regulatory subunit Dbf4 (Jackson et al. 1993; Stillman 1996). DDK promotes the initiation of DNA replication by phosphorylating Cdc45 and subunits of the mini-chromosome maintenance complex (Mcm2-7) at origins of replication (Lei et al. 1997; Owens et al. 1997; Zou and Stillman 2000; Bruck and Kaplan 2009). DDK also phosphorylates histone H3 at threonine 45 (H3T45) during S-phase, which occurs in response to replication stress (Baker et al. 2010), suggesting that H3T45 phosphorylation is linked with DNA replication. Previous studies have shown that centromeric association of Cdc7 is important for early replication of centromeres (Raghuraman et al. 2001; Rossbach et al. 2017), which are among the earliest firing origins.

The identification of five cdc7 and dbf4 alleles that display SDL with overexpressed Cse4 led us to investigate the role of DDK in regulating Cse4 proteolysis. We determined that Cdc7 regulates Cse4 proteolysis in a pathway that overlaps with Psh1, and this prevents mislocalization of Cse4. The role of Cdc7 in Cse4 proteolysis is independent of its role in the initiation of DNA replication.
MATERIAL AND METHODS

Strains and Plasmids
Yeast strains were grown in YPD (1% yeast extract, 2% bacto-peptone, 2% glucose) or synthetic medium with glucose or raffinose/galactose (2% final concentration each) and supplements to allow for selection of the indicated plasmids. Yeast strains and plasmids used in this study are described in Table S1 and Table S2, respectively. To integrate the cdc7-7 allele marked with the G418 resistance marker (KanMX), the cdc7-7 sequence amplified from RSY302 and the KanMX sequence were cloned into pGEM-T-Easy. cdc7-7:KanMX from the vector was transformed into yeast strains as per standard lithium acetate procedure. Transformants were screened for temperature sensitivity at 37°C and sequenced (CCR Genomics Core) to confirm the G1137A mutation. Wild type CDC7 marked with G418 resistance strains were selected from the non-temperature sensitive transformants and sequenced to verify the wild type CDC7 sequence. To replace endogenous CSE4 with HA-tagged CSE4, a PCR-based method was used as described previously (BOECKMANN et al. 2013). Replacement of the CSE4 gene with HA-tagged CSE4 was verified by sequencing and Western blots confirmed the expression of the HA-tagged protein. At least two independent strains were analyzed for each experiment.

Growth assays
Wild type and mutant strains were transformed with the indicated plasmids or the empty vector. Transformants grown on synthetic medium, selective for the plasmid, were suspended in water to a concentration with an optical density of 1 measured at a wavelength of 600 nm (OD\textsubscript{600}, approximately 1.0 X 10\textsuperscript{7} cells per ml). Five-fold serial dilutions starting with 1 OD\textsubscript{600} were generated and 3 μl of each dilution spotted on synthetic growth medium selecting for the plasmid and containing either glucose (2% final concentration) or galactose and raffinose (2%
Strains were grown at the indicated temperatures for 3-5 days. Three independent transformants were assayed for growth unless otherwise stated.

**Protein stability assays**

Protein stability assays were performed as previously described (Au et al. 2008). Briefly, strains were grown to logarithmic phase overnight in selective media, re-suspended in fresh media containing galactose/raffinose (2% final concentration each) and grown for 1.75 or four hours as indicated in figure legends at 23°C. 10 µg/ml cycloheximide (CHX) and glucose (2% final concentration) were added to cultures and aliquots were collected 0, 30, 60, 90, and 120 minutes after CHX addition. Proteins were isolated using the TCA method as described previously (Kastenmayer et al. 2006). Protein levels were standardized using the Bio-Rad DCTM Protein Assay. Samples were diluted 1:1 with Laemmli buffer containing BME and stored at -20°C for Western blot analysis. Proteins were separated by SDS-PAGE on 4-12% Bis-TRIS SDS-polyacrylamide gels (Novex, NP0322BOX). Western blot analysis was done using primary antibodies α-HA (1:1000, Roche, 12CA5), α-Flag (1:5000, Sigma, F3165), or α-Tub2 (1:4500, custom made for Basrai Laboratory) in TBST containing 5% (w/v) dried skim milk. HRP-conjugated sheep α-mouse IgG (Amersham Biosciences, NA931V) and HRP-conjugated donkey α-rabbit IgG (Amersham Biosciences, NA934V) were used as secondary antibodies. Blots were washed after primary and secondary antibodies with TBST (Tris-buffered saline plus 0.1% Tween 20) three times for 10 minutes. Western blots were quantified with the SynGene program (SynGene, Cambridge, UK) or the Image Lab Software (BioRad). Protein stability of Cse4 was measured as the percent remaining after normalization to Tub2 signal.
Ubiquitination (Ub) Pull-down Assay

Ub pull-down assays for determining the levels of ubiquitinated Cse4 were performed as described previously (Au et al. 2013) with minor modifications. Strains were grown to logarithmic phase overnight in selective media, re-suspended in fresh media containing galactose/raffinose (2% final concentration each) and grown for four hours at 23°C. Cells were resuspended in Cell Lysis Buffer with freshly added protease inhibitor cocktail, PMSF, and NEM (inhibitor for de-ubiquitination) and lysed by vortexing for 1 hour at 4°C in the presence of glass beads. The concentration of proteins in each resulting lysate was measured and normalized. 50 µl lysate was saved for input and the remaining lysate was added to Tandem Ubiquitin Binding Entity (TUBE) beads (LifeSensors) and incubated overnight at 4°C. Beads were centrifuged and washed three times with TBST on a rocking platform; unbound lysate was collected. Beads were resuspended in Laemmli buffer and incubated for 10 minutes at 100°C. Input and unbound fraction containing Laemmli buffer were processed in parallel. Samples were analyzed using Western Blot. Western blots were quantified with the SynGene program (SynGene, Cambridge, UK). p-value was determined using a paired t-test (GraphPad Prism).

Chromosome spreads

Chromosome spreads were performed as previously described (Collins et al. 2004; Crotti and Basrai 2004; Collins et al. 2007) with minor modifications. Cultures were grown to logarithmic phase in selective medium containing 2% raffinose and treated with Nocodazole (20 µg/mL final) for three hours to arrest cells in the G2/M phase of the cell cycle. FACS analysis confirmed the cell cycle arrest. For the last hour of the Nocodazole arrest, galactose was added to 2% final concentration. Cells were lysed gently by treatment with zymolase-100T and BME. Spheroplasts were then spread onto glass slides and fixed with paraformaldehyde and 1%
lipsol and allowed to air dry. Slides were washed with 1 X PBS for 10 minutes and incubated in
16B12 Mouse anti-HA primary antibody (1:2500). Slides were washed three times with 1 X PBS
for 10 min and incubated with Cy3 conjugated Goat anti-mouse secondary antibody (1:5000).
Slides were washed with 1 X PBS and mounted with antifade containing DAPI and visualized
using DeltaVision Microscopy Imaging Systems. Nuclei with a single or two HA- or Flag-Cse4
foci were counted as normal Cse4 localization and nuclei with multiple foci or a diffused signal
in the nucleus were counted as mislocalized Cse4. At least 360 cells were counted for each
experiment. p-values were determined using Ordinary one-way ANOVA (GraphPad Prism)

ChIP-Seq
Chromatin immunoprecipitations were performed as previously described (COLE et al.
2014; CHEREJI et al. 2017) with modifications. Cultures grown to logarithmic phase in glucose or
raffinose/galactose media for 1.75 hours were treated with formaldehyde (final 1%) for 20
minutes at 30°C followed by the addition of 2.5 M glycine for 10 minutes. Cells were washed
twice with 1 X PBS and resuspended in 2 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 1 x
protease inhibitors (Sigma) and 1 mM PMSF final concentration. The cell suspension was split
into four screw top tubes with glass beads (0.4-0.65 mm diameter) and lysed three times for 40
seconds each, followed by a five-minute rest on ice, and lysed two times for 40 seconds each in
an MP Bio FastPrep-24 5G. The cell lysate was collected, and the chromatin pellet was washed
twice in FA Lysis Buffer. Each pellet was resuspended in 600 µl of FA Lysis Buffer and
combined into one 5 ml tube. The chromatin suspension was sonicated 24 times with repeated 15
seconds on/off at 20% amplitude using a Branson digital sonifer. After 3 minutes of
centrifugation (13000 rpm, 4°C), the supernatant was transferred to another tube. About 5% was
used for input and checking the size of sheared DNA. The remaining was incubated with 150 µl anti-FLAG® M2 Affinity Gel (Sigma, A2220-5ML) overnight. The beads were washed for five minutes on a rotator in 1 ml FA, FA-HS (500 mM NaCl), RIPA, and TE buffers twice each. The beads were resuspended in ChIP Elution Buffer (25 mM Tris-HCl pH7.6, 100 mMNaCl, 0.5% SDS) and incubated at 65°C overnight. The beads were treated with proteinase K (0.5 mg/ml) at 55°C for four hours followed by Phenol/Chloroform extraction and ethanol precipitation. The DNA pellet was resuspended in a total of 50 µl sterile water.

Input and IP samples were repaired using the NEB Pre-PCR Repair Mix (New England Biolabs, M0309). Paired-end libraries for input and IP samples were prepared using the NEBNext® End Prep (New England Biolabs, E7370) and NEBNext® Multiplex Oligos for Illumina (New England Biolabs, E7335). Agencourt AMPure XP beads (Beckman-Coulter, A63880) were used to purify adaptor-ligated DNA samples and PCR products (input adapters diluted 1/3 and IP 1/250). The 50-base paired-end Illumina reads were aligned to the S. cerevisiae S288C reference (R64-2-1) using Bowtie version 1.0.0 with command line options -n2 -m1 -X 500. Duplicate reads (20-89%) were removed using Samtools rmdup (version 0.1.19). Between 1.4M and 5.3M unique alignments remained for the ChIP libraries and 14M-24M for the input libraries. The input alignments were randomly down sampled to 10M alignments each. Peaks were called using MACS (Zhang et al. 2008) version 2.1.1.20160226 in paired-end mode with default parameters and no additional down sampling.

The annotatePeaks tool of the Hypergeometric Optimization of Motif EnRichment suite (HOMER v5.10; http://homer.ucsd.edu/homer/) was used to assign peaks of Cse4 enrichment to genomic features. Customized annotations were utilized. Similar to the approach of Hildebrand and Biggins (Hildebrand and Biggins 2016), 5’- and 3’-UTR’s were annotated using the data
of Nagalakshmi et al. (NAGALAKSHMI et al. 2008) downloaded from the yeast genome browser (https://browse.yeastgenome.org). 5’- and 3’-prime UTR data was available for 4605 and 5175 genes, respectively. For genes lacking UTR data, UTR’s were assigned a median length (53 and 105 nucleotides, respectively). Promoters were defined as the region lying 500 bp upstream of the transcription start site (i.e., position 1 of the 5’-UTR). Transcription termination sites were defined as ± 50 bp from the end of the 3’-UTR.

Intersections between peak sets were computed using the IntersectRegions function of the USeq suite (http://useq.sourceforge.net) which also provides an estimate of statistical significance by randomization of one of the target peak sets across the genome. Coverage tracks were computed by MACS and normalized to 1M reads and displayed using the Integrative Genomics Viewer (ROBINSON et al. 2011).

Data availability
Strains and plasmids are available upon request. Supplemental file S1 contains Table S1, which describes the strains used in this study, and Table S2, which lists the plasmids used. Figures S1, S2, and S3 are available as supplemental files. Supplemental files are available at FigShare. ChIP-seq data for wild type and cdc7-7 strains with Flag-Cse4 expressed from its own promoter and GAL-Flag-Cse4 integrated into the genome are available at GEO with accession number GSE148068.
RESULTS

Mutants of the Cdc7-Dbf4 kinase complex exhibit SDL with GALCSE4

To identify mutants of essential genes that display synthetic dosage lethality (SDL) when Cse4 is overexpressed (GALCSE4), we performed a Synthetic Genetic Array (SGA) (AU et al. 2020). A strain in which GAL-HA-CSE4 was integrated in the genome was mated to an array of 786 conditional temperature sensitive strains. Growth at 26°C of the haploid meiotic progeny was scored on galactose plates and the p-value was determined as previously described (BARYSHNIKOVA et al. 2010; COSTANZO et al. 2010; COSTANZO et al. 2016). Among the top twelve hits that show SDL are five alleles of genes encoding the Dbf4-dependent kinase (DDK) complex, the gene encoding calmodulin, and regulators of proteasome assembly, mRNA polyadenylation, and cell cycle progression (Table 1). The identification of multiple alleles encoding components of the DDK complex led us to further investigate a possible role of DDK in regulating cellular levels of Cse4 to prevent mislocalization of Cse4 to non-centromeric regions. We confirmed the SDL phenotype using growth assays in which yeast strains transformed with a plasmid containing GALCSE4 or empty vector (vector) were plated on media with glucose or galactose to induce expression of GALCSE4. Strains with mutations in either CDC7 (Figure 1A; cdc7-4) or DBF4 (Figure 1A; dbf4-1, dbf4-2) exhibited GALCSE4 SDL at the permissive temperature of 23°C on galactose media. A cdc7-7 mutant, which was not included in the SGA screen, also exhibited GALCSE4 SDL (Figure 1A). We pursued in-depth studies with the cdc7-7 mutant because the cdc7-7 allele displays a stronger SDL phenotype at 23°C, has a low frequency of induced mutagenesis, does not have defects in the cell cycle at 23°C, and exhibits DNA replication defects only at the non-permissive temperature of 37°C (HOLLINGSWORTH Jr et al. 1992). To establish that the SDL phenotype of a cdc7-7 GALCSE4 strain is linked to the CDC7 gene, we performed growth assays with cdc7-7 GALCSE4 strains
with plasmid-borne \textit{CDC7} or empty vector. The plasmid-borne \textit{CDC7} rescued the temperature sensitivity of the \textit{cdc7-7} strain at 37°C and the SDL phenotype of \textit{cdc7-7 GALCSE4} at 23°C (Figure 1B).

\textbf{Cdc7 regulates ubiquitin-mediated proteolysis of Cse4}

Previous studies have shown that defects in ubiquitin-mediated proteolysis of overexpressed Cse4 contribute to \textit{GALCSE4} SDL in \textit{psh1Δ}, \textit{slx5Δ}, and \textit{hir2Δ} strains (Hewawasam \textit{et al.} 2010; Ranjitkar \textit{et al.} 2010; Ohkuni \textit{et al.} 2016; Ciftci-Yilmaz \textit{et al.} 2018). The SDL phenotype of DDK mutants led us to hypothesize that proteolysis of Cse4 is regulated by the DDK complex. Therefore, we examined the stability of overexpressed HA-Cse4 in wild type, \textit{cdc7-7}, and \textit{dbf4-1} strains after treatment with cycloheximide at 23°C. Increased stability of HA-Cse4 was observed in \textit{cdc7-7} (Figure 2A) and \textit{dbf4-1} (Figure 2B) strains when compared to that in a wild type strain.

It has been shown that defects in ubiquitination of Cse4 contribute to increased protein stability and mislocalization of Cse4 in a \textit{psh1Δ} strain (Hewawasam \textit{et al.} 2010; Ranjitkar \textit{et al.} 2010). The increased stability of Cse4 led us to examine if a \textit{cdc7-7} strain exhibits defects in poly-ubiquitination of overexpressed HA-Cse4 (Ub\textsubscript{n}-Cse4). We performed an affinity pull-down of ubiquitinated proteins and consistent with previous studies (Au \textit{et al.} 2013), we detected ubiquitinated Cse4 as a laddering pattern in wild type cells (Figure 2C). Quantification of signal intensities of Ub\textsubscript{n}-Cse4 normalized to signal intensities of Cse4 in input samples showed a significant reduction in the levels of ubiquitinated Cse4 in a \textit{cdc7-7} strain compared to a wild type strain (Figure 2C, \textit{p}-value < 0.05). The defects in Cse4 proteolysis and Cse4 ubiquitination in the \textit{cdc7-7} strains suggest that Cdc7 regulates ubiquitin-mediated proteolysis of Cse4.
Cdc7 regulates proteolysis of Cse4 independently of its role in DNA replication initiation

Previous studies have shown that DDK activates the initiation of DNA replication through phosphorylation of the MCM2-7 complex (Lei et al. 1997; Oshiro et al. 1999; Weinreich and Stillman 1999; Zou and Stillman 2000; Bruck and Kaplan 2009). DNA replication defects are observed in cdc7 strains at the non-permissive temperature of 37°C (Sclafani 2000), but DNA replication is unperturbed in cdc7-7 strains grown at 23°C (Jackson et al. 1993). Mutation of proline 83 of MCM5 to leucine (mcm5-bob1) bypasses specifically the requirement of Cdc7 for replication initiation and rescues the temperature sensitivity and replication defects of cdc7-1 and cdc7-7 strains at 37°C (Hardy et al. 1997; Sclafani et al. 2002; Hoang et al. 2007). We observed the GALCSE4 SDL phenotype and stability of HA-Cse4 in cdc7-7 strains at 23°C. To further confirm that Cdc7-mediated proteolysis of Cse4 is independent of its role in initiating DNA replication, we performed growth assays for the SDL phenotype with the cdc7-7 mcm5-bob1 double mutant with GALCSE4. Our results showed that the cdc7-7 mcm5-bob1 GALCSE4 strain exhibited SDL similar to that observed in the cdc7-7 GALCSE4 strain at 23°C (Figure 3A). Next, we determined if the mcm5-bob1 mutation affects the proteolysis of overexpressed HA-CSE4 in a cdc7-7 strain. Protein stability assays were done with extracts from wild type, cdc7-7, mcm5-bob1, and cdc7-7 mcm5-bob1 strains expressing GAL-HA-CSE4. The stability of HA-Cse4 in the mcm5-bob1 strain was similar to that of the wild type strain (Figure 3B). Furthermore, the defects in proteolysis of HA-Cse4 observed in the cdc7-7 strain were not suppressed in the cdc-7 mcm5-bob1 strain (Figure 3B). The inability of the mcm5-bob1 mutation to rescue the SDL phenotype and proteolysis defect in a cdc7-7 GALCSE4 strain suggests that the role of Cdc7 in regulating Cse4 proteolysis is independent of Cdc7’s role in initiating DNA replication.
Cse4 is mislocalized to non-centromeric regions with an enrichment at promoters in a cdc7-7 strain

We next examined the localization pattern of Cse4 using chromosome spreads, a method that eliminates soluble material to visualize chromatin-bound HA-Cse4 in WT and cdc7-7 strains. Previous studies have shown that Cse4 is localized to kinetochores that are clustered in one or two discrete nuclear foci in wild type cells, whereas mislocalization of Cse4 shows more than two foci or diffuse signal through the nuclear mass in psh1Δ, slx5Δ, and hir2Δ strains (Hewawasam et al. 2010; Ranjikar et al. 2010; Ohkuni et al. 2016; Ciftci-Yilmaz et al. 2018). In the cdc7-7 strain, we found that, in contrast to wild type cells, HA-Cse4 was mislocalized with signal at more than two foci or diffused across the nuclear mass (Figure 4A and 4B, cdc7-7, p-value = 0.0028). To determine if the mislocalization of HA-Cse4 in a cdc7-7 strain is due to a kinetochore clustering defect, we examined the localization of the kinetochore protein Mtw1-GFP (Pinsky et al. 2003; Westermann et al. 2003). Our results showed a similar localization pattern of Mtw1-GFP to one or two foci in both the wild type (97.5%) and cdc7-7 (94.6%) cells (Figure 4C and 4D). This suggests that the mislocalization of HA-Cse4 in a cdc7-7 mutant is not due to kinetochore de-clustering. Based on these results, we conclude that DDK regulates ubiquitin-mediated proteolysis of Cse4 and prevents mislocalization of Cse4 to non-centromeric regions.

We next performed ChIP-seq experiments to define the genome-wide localization pattern of endogenous and overexpressed Cse4 in a cdc7-7 strain. ChIP-seq was performed using chromatin from wild type and cdc7-7 strains with endogenous Flag-Cse4 expressed from its own promoter grown at 23°C in glucose or with galactose-inducible Flag-Cse4 integrated in the genome and grown at 23°C in galactose media for 1.75 hours to overexpress Flag-Cse4. Consistent with previous reports (Hildebrand and Biggins 2016), endogenous Flag-Cse4
showed peaks of enrichment primarily at centromeric (CEN) regions in the wild type strain (Figure S1, WT). Endogenous Flag-Cse4 also showed enrichment primarily at CEN regions in the cdc7-7 strain (Figure S1, cdc7-7), indicating that Flag-Cse4 expressed from its own promoter is not mislocalized to distinct non-centromeric genomic loci in a cdc7-7 strain. For overexpressed Flag-Cse4, at the sequencing depth of our experiments (1.5-5.3 million non-duplicates, uniquely-mapped reads), Flag-Cse4 was found enriched at only 30 non-CEN sites in a wild type strain. In contrast, 2,187 non-CEN peaks of Flag-Cse4 were detected in a cdc7-7 GAL-FLAG-CSE4 strain. In addition, a higher generalized background of Flag-Cse4 was observed across the genome as evidenced by a lower signal to noise ratio of the CEN peaks (Figure 5A, S2). Our results show that overexpressed Flag-Cse4 is highly enriched at promoters (60.4% of total peaks and 2.75-fold enriched relative to feature target size), but not at 3'-UTR’s, transcription termination sites (TTS), exons, introns, and intergenic regions in the cdc7-7 strain (Figure 5B). Significant enrichment (3.1-fold) was also found at 5'-UTR’s, although only 9.8% of the total peaks were found at these locations. This result is likely attributable to peaks overlapping the boundary between promoter and 5'-UTR.

The phenotypes of SDL with GALCSE4, defects in Cse4 proteolysis, and mislocalization of Cse4 to non-centromeric regions in a cdc7-7 strain are similar to that observed in a psh1Δ strain (Hewawasam et al. 2010; Ranjitkar et al. 2010). Since the ChIP-seq experiments were performed with an isogenic set of strains with an integrated copy of GAL-FLAG-CSE4 in the same genetic background used previously to examine localization of overexpressed Flag-Cse4 in a psh1Δ background (Hildebrand and Biggins 2016), we compared our results to the ChIP-seq results from Hildebrand and Biggins. The raw sequencing data was downloaded from the Sequence Read Archive (GEO Series GSE69696) and subjected to the same alignment and peak
calling procedures used for our ChIP-seq analyses with the \textit{cdc7-7} strain. Of the 2,129 regions of Flag-Cse4 enrichment identified in the \textit{cdc7-7} strain 2,059 (97\%) overlapped with one or more peaks of Cse4 enrichment identified in the \textit{psh1\Delta} strain (\textit{p}-value $< 10^{-4}$) (Figure 5C). As observed for the \textit{psh1\Delta} strain, a high proportion (1,994/2,129, 94\%) of Cse4 mislocalization in the \textit{cdc7-7} strain occurs in promoter regions (\textit{p}-value $< 10^{-4}$); virtually all were common to promoter-localized Cse4 found in the \textit{psh1\Delta} strain (Figure 5C). We note that in making the 3-way comparison, closely-spaced peaks are merged to eliminate inconsistency in counts when a single peak in one set overlaps multiple peaks in another set; thus, the total number of intervals shown in Figure 5C differs from the actual \textit{cdc7-7} peak count indicated in Figure 5B. Overall, these results show that the mislocalization pattern of Cse4 in the \textit{cdc7-7} strain is similar to that observed in a \textit{psh1\Delta} strain.

\textbf{Cdc7 regulates Psh1-mediated proteolysis of overexpressed Cse4}

We have previously shown that overexpression of the ubiquitin-encoding gene \textit{UBI4} suppresses the SDL of a \textit{psh1\Delta GALCSE4} strain (Au \textit{et al.} 2013). The overlapping pattern of Cse4 mislocalization in \textit{cdc7-7} and \textit{psh1\Delta} strains prompted us to examine if overexpression of \textit{UBI4} would suppress the SDL of a \textit{cdc7-7 GALCSE4} strain. Growth assays showed that \textit{UBI4} suppresses the \textit{cdc7-7 GALCSE4} SDL phenotype at the permissive temperature of 23°C (Figure 6A). \textit{UBI4} did not suppress the TS growth defect of \textit{cdc7-7} strains at 37°C.

We took multiple approaches to evaluate if Cdc7 functions in an overlapping pathway with Psh1 to regulate Cse4 proteolysis. We generated \textit{cdc7-7} and \textit{psh1\Delta} single and \textit{cdc7-7 psh1\Delta} double mutant strains with \textit{GAL-FLAG-CSE4} integrated in the genome. Growth assays confirmed the SDL phenotype of \textit{GAL-FLAG-CSE4} for \textit{cdc7-7} (Figure 1A and 6B), and that reported previously for \textit{psh1\Delta} strains on galactose media (Hewawasam \textit{et al.} 2010; Ranjitkar...
et al. 2010). The *psh1Δ* strain showed a more severe growth defect than the *cdc7-7* strain with

*GAL-FLAG-CSE4*. The *cdc7-7 psh1Δ* double mutant displays SDL similar to that observed for the *psh1Δ* strain (Figure 6B). We also examined the stability of overexpressed Flag-Cse4 in the *cdc7-7, psh1Δ*, and *cdc7-7 psh1Δ* strains. Consistent with previous results (Figure 2A and (HEWAWASAM et al. 2010; RANJITKAR et al. 2010)), Flag-Cse4 was more stable in *cdc7-7* and *psh1Δ* strains when compared to the wild type strain (Figure 6C). The stability of Flag-Cse4 in the *psh1Δ* strain is higher than that in the *cdc7-7* strain, however the stability of Flag-Cse4 in the *cdc7-7 psh1Δ* strain was similar to that observed in the *psh1Δ* strain (Figure 6C). Lastly, we examined the mislocalization of Flag-Cse4 in wild type, *cdc7-7, psh1Δ*, and *cdc7-7 psh1Δ* strains using chromosome spreads. We observed significantly higher levels of Flag-Cse4 mislocalization in *cdc7-7* as described earlier (Figures 4A and 4B) and as reported previously for *psh1Δ* strains (HEWAWASAM et al. 2010; RANJITKAR et al. 2010) when compared to the wild type strain. Consistent with results for the SDL phenotype and protein stability, the mislocalization of Flag-Cse4 was not further enhanced in *cdc7-7 psh1Δ* strains when compared to the single *cdc7-7* and *psh1Δ* strains (Figure 6D, *p*-value > 0.999). We propose that Cdc7 and Psh1 are epistatic for proteolysis of Cse4 to prevent Cse4 mislocalization to non-centromeric regions.
DISCUSSION

In this study, we investigated the role of the Dbf4-dependent kinase (DDK) complex in proteolysis of Cse4. Five alleles of genes encoding DDK were among the top twelve hits in a screen to identify mutant strains displaying SDL with GALCSE4. Our results show that cdc7-7 strains exhibit an SDL phenotype with GALCSE4, defects in ubiquitin-mediated proteolysis of Cse4, and mislocalization of Cse4 to non-centromeric regions, particularly to gene promoters. The lack of a rescue of the GALCSE4 SDL or Cse4 proteolysis defect in the cdc7-7 strain by mcm5-bob1 indicates a DNA replication-independent role of Cdc7 in Cse4 proteolysis. Additionally, several experimental approaches showed that Cdc7 functions in a pathway overlapping with Psh1 to promote proteolysis of Cse4 and prevent Cse4 mislocalization to non-centromeric regions. Our studies define the first essential kinase, DDK, to regulate proteolysis of overexpressed Cse4 and prevent mislocalization of Cse4.

DDK is most well-studied for its role in initiating DNA replication through phosphorylation of the MCM2-7 DNA helicase complex at origins of replication, allowing cells to proceed through the G1/S phase of the cell cycle (Lei et al. 1997; Oshiro et al. 1999; Weinreich and Stillman 1999; Zou and Stillman 2000; Bruck and Kaplan 2009). Temperature sensitive cdc7 mutants exhibit defects in the cell cycle and are unable to complete DNA replication at the restrictive temperature of 37°C; replication and cell cycle defects are not observed at the permissive temperature of 23°C (reviewed in Sclafani 2000). All the assays in our current study, including growth, protein stability, and chromosome localization, were performed at 23°C. Based on these results, we conclude that the GALCSE4 SDL phenotype, defect in Cse4 proteolysis, and decrease in Ub{n}-Cse4 levels in cdc7-7 strains observed at 23°C are independent of defects in cell cycle progression.
Phosphorylation of MCM2-7 by DDK causes a conformational change in the MCM2-7 complex and this regulates replication initiation (Hoang et al. 2007). A mutation in MCM5, P83L (mcm5-bob1), is thought to mimic the conformational change that results from DDK-mediated phosphorylation of MCM2-7. The mcm5-bob1 mutation rescues the temperature sensitivity, bypasses the cell cycle defects of cdc7 strains (Jackson et al. 1993), and the DNA distribution by FACS of a cdc7 mcm5-bob1 strain is normal (Hardy et al. 1997). We used genetic and biochemical approaches to examine if the role of Cdc7 in proteolysis of Cse4 is independent of its role in replication initiation. We reasoned that if the regulation of Cse4 proteolysis by Cdc7 was dependent on replication initiation, the mcm5-bob1 mutation should rescue the SDL phenotype and Cse4 proteolysis defect in cdc7-7 strains. However, we did not observe suppression of the GALCSE4 SDL or defects in Cse4 proteolysis in the cdc7-7 mcm5-bob1 strain at 23°C. Furthermore, ChIP-seq using a cdc7-7 strain did not reveal a significant enrichment of Cse4 to origins of DNA replication which are normally occupied by Cdc7 (Rössbach et al. 2017). Similar to our observations, a previous study has shown that mcm5-bob1 cannot suppress the defect of cdc7-induced mutagenesis (Pessoa-Brandao and Sclafani 2004), indicating a different Cdc7 substrate in mutagenesis than the MCM2-7 complex (Rössbach and Sclafani 2016). Together, our results support a DNA replication-independent role of Cdc7 in regulating Cse4 proteolysis.

Cse4 expressed from its own promoter is not detectably mislocalized to specific genomic regions in a cdc7-7 strain (Figure S1). Additionally, degradation of endogenous Flag-Cse4 in a cdc7-7 strain is similar to that in a wild type strain (Figure S3). Genome-wide studies have shown that mislocalization of Cse4 is barely detectable in wild type (Camahort et al. 2009; Lefrancois et al. 2009; Hildebrand and Biggins 2016) or psh1Δ strains (Hildebrand and
suggesting that cellular levels of endogenous Cse4 are stringently regulated to ensure that it is not mislocalized to non-centromeric regions in a wild type cell. In the context of overexpressed Cse4, wild type cells do not show growth inhibition with GAL-CSE4, in part because overexpressed Cse4 is proteolyzed by Psh1, Rey1, Slx5, Ubr1, and other regulators. Mutants of these regulators display defects in proteolysis of Cse4, which contributes to mislocalization of overexpressed Cse4 and lethality with GAL-CSE4 (HEAWASAM et al. 2010; RANJITKAR et al. 2010; OHKUNI et al. 2016; CIFTCI-YILMAZ et al. 2018).

Our studies here provide evidence that Cdc7 plays a role in regulating levels of overexpressed Cse4. Chromosome spreads showed mislocalization of overexpressed Cse4 in a cdc7-7 strain and ChIP-seq confirmed these results. We observed a significant amount of Cse4 mislocalization throughout the genome, and analysis of the localization pattern showed a preferential enrichment of Flag-Cse4 at promoter regions with a high degree of overlap to that observed in the psh1Δ strain. We propose that Cdc7 functions in a pathway that overlaps with Psh1 in Cse4 proteolysis. We provide several lines of evidence to support our hypothesis. The GALCSE4 SDL phenotype, increased stability of Cse4, and levels of Cse4 mislocalization observed in the cdc7-7 psh1Δ strain were not significantly different than that observed in the cdc7-7 or psh1Δ strains. Additionally, the preferential localization of Cse4 to promoters is observed in both cdc7-7 and psh1Δ strains and the GALCSE4 SDL phenotype is suppressed by overexpression of UBI4 in both cdc7-7 and psh1Δ strains. Future studies will allow us to investigate the mechanism by which Cdc7 affects the Psh1 pathway and if Cdc7 regulates pathways other than Psh1-mediated proteolysis for Cse4.

Previous studies have shown that Cdc7 and Dbf4 associate with replication origins, including the early-firing replication origins at the centromere (NATSUME et al. 2013; ROSSBACH
et al. 2017) and that low levels of DDK at centromeres contributes to delay in the replication of
centromeres (NATSUME et al. 2013). DDK associates with kinetochores through the COMA
complex, consisting of Ctf19, Mcm21, Okp1, and Ame1, and this regulates sister chromatid cohesion independently of the role of DDK in initiating DNA replication (NATSUME et al. 2013).
DDK phosphorylates the N-terminal tail of Ctf19 and this recruits the cohesin loader Scc2/4, for proper sister chromatid cohesion (HINSHAW et al. 2017). Recent studies have shown that the N-terminal tail of Cse4 interacts with Okp1, which directs kinetochore loading distinct from Mif2-directed loading (FISCHBÖCK-HALWACHS et al. 2019). Phosphorylation of the N-terminal tail of Cse4 promotes the interaction of Cse4 with Ame1/Okp1 and this likely regulates recruitment of kinetochore components (HINSHAW AND HARRISON 2019). It is of great interest to examine if Cse4 is a substrate of DDK and define the role of DDK-mediated phosphorylation of Ctf19 for the association of the COMA complex with Cse4. Future studies will allow us to examine if DDK-mediated phosphorylation of kinetochore substrates such as Cse4, Psh1, and Ctf19 contribute to the proteolysis of overexpressed Cse4 and prevent its mislocalization to non-centromeric regions.

In this study, we have described a new role for the essential kinase Cdc7 in regulating Psh1-mediated proteolysis of Cse4 independently of Cdc7’s role in initiating DNA replication. Based on our results for SDL of GALCSE4 in cdc7-7 strains, we propose that inhibition of Cdc7 in cancers with high levels of CENP-A would lead to cancer cell-specific cell death. These studies are relevant from a clinical standpoint because high levels of Cdc7 and Dbf4 expression have been reported in several types of cancers (BONTE et al. 2008) and this correlates with accelerated progression through the cell cycle, mutation of p53, resistance to DNA damaging agents and chemotherapy, and poor survival rates (MONTAGNOLI et al. 2004; BONTE et al. 2008;
Targeting Cdc7 through siRNA knockdown in cancer cells has been shown to result in cancer cell-specific apoptotic cell death (Bonte et al. 2008; Kulkarni et al. 2009; Hou et al. 2012), whereas non-cancerous cells arrest in G1 and resume proliferation after Cdc7 activity is restored (Rodriguez-Acebes et al. 2010). Currently, Cdc7 inhibitors are in clinical trials to downregulate Cdc7 activity in cancer cells (clinicaltrials.gov #’s NCT02699749, NCT03096054). The evolutionary conservation of CENP-A and DDK makes budding yeast an excellent model to investigate the molecular role of DDK in preventing mislocalization of CENP-A and CIN.
Acknowledgements

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Table 1. Twelve mutant alleles with the lowest score from a Synthetic Genetic Array (SGA) with temperature sensitive gene mutants overexpressing *CSE4*. Listed are the top twelve conditional alleles of essential genes that displayed SDL when *CSE4* is expressed from a galactose-inducible promoter (Au *et al.* 2020). Shown are the mutant allele, SGA score as the epsilon value calculated as previously in (Costanzo *et al.* 2010; Costanzo *et al.* 2016) with a negative value indicating a defect in growth, human orthologue (https://yeastmine.yeastgenome.org/yeastmine), and gene ontology (GO) annotation (https://www.yeastgenome.org/).

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<td>CDC23</td>
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Figure 1. DDK mutants exhibit synthetic dosage lethality (SDL) to GALCSE4. A. Validation of GALCSE4 SDL in cdc7 and dbf4 strains. Growth assays were done with wild type [BY4741 (for cdc7-4, dbf4-1, and dbf4-2) and RSY299 (for cdc7-7)], cdc7-4 (tsa131), dbf4-1 (tsa161), dbf4-2 (tsa162), and cdc7-7 (RSY302) strains transformed with vector (pMB433, vector) or GAL-HA-CSE4 (SB878, GALCSE4). Cells were spotted in five-fold serial dilutions on medium selective for the plasmid containing either glucose (2%, Cse4 expression off) or raffinose/galactose (2% each, Cse4 expression is on) and incubated at 23°C for 3-5 days. Two independent transformants of dbf4-1, dbf4-2, and cdc7-4 strains and three independent transformants of cdc7-7 strains were assayed and a representative image is shown. B. The GALCSE4 SDL phenotype of a cdc7-7 strain is linked to the cdc7 mutant allele. Growth assays were done with cdc7-7 strains (RSY302 with pMB433 and RSY302 with pMB1597) transformed with empty vector (pRS425) or plasmid-born CDC7 (pMB1898). Cells were spotted in five-fold serial dilutions on medium selective for the plasmids with glucose (2%) or raffinose/galactose (2% each). Plates were incubated at the indicated temperature for 5-7 days. Three independent transformants for each strain were assayed and a representative image is shown.

Figure 2. Cdc7 regulates ubiquitin-mediated proteolysis of Cse4. A. Cse4 is stabilized in a cdc7 strain and B. Cse4 is stabilized in a dbf4 strain. Western blot analysis of protein extracts prepared from wild type (BY471 for dbf4-1 and RSY299 for cdc7-7), (A) cdc7-7 (RSY302), and (B) dbf4-1 (TSA161) strains transformed with GAL-HA-CSE4 (pMB1597). Strains were grown to logarithmic phase of growth in raffinose-containing media (2%), and expression of GAL-HA-
CSE4 was induced with galactose (2%) for four hours. Cells were then treated with cycloheximide (CHX, 10 µg/ml) and glucose (2%). Aliquots were taken at the indicated timepoints. Protein extracts were analyzed using Western blot analysis and blots were probed with anti-HA (Cse4) and anti-Tub2. (loading control). Quantification of the levels of HA-Cse4 remaining after treatment with CHX relative to Tub2 from two independent experiments is shown in the graphs. Error bars represent SEM. **C. Ubiquitination of Cse4 is decreased in a cdc7 strain.** Ub-pull down assays were performed using protein extracts from wild type and cdc7-7 strains as described above and lysates were incubated with Tandem Ubiquitin Binding Entity beads (LifeSensors). Input and ubiquitin-enriched (Pull down: Ub⁺) samples were analyzed via Western blot against HA (left). Arrow indicates the unmodified Cse4 band. Quantification of levels of poly-ubiquitinated Cse4 (Ubⁿ-Cse4) normalized to the levels in the input from three independent experiments is shown in the graph, p-value < 0.05.

**Figure 3. Cdc7 regulates stability of Cse4 independently of its role in initiation of DNA replication.** A. A cdc7-7 mcm5-bob1 strain shows SDL with GALCSE4. Growth assays with wild type (RSY299), mcm5-bob1 (RSY867), cdc7-7 (RSY302), or cdc7-7 mcm5-bob1 (RSY847) strains transformed with vector (pMB433, vector) or GAL-HA-CSE4 (SB878, GALCSE4). Cells were spotted in five-fold serial dilutions on media selective for the plasmid containing either glucose (2%) or raffinose/galactose (2% each) and incubated at 23°C for 3-5 days. Three independent transformants for each strain were assayed and the representative image is shown. B. A cdc7-7 mcm5-bob1 strain exhibits defects in Cse4 proteolysis. Western blot analysis of protein extracts from wild type (RSY299), mcm5-bob1 (RSY867), cdc7-7 (RSY302), or cdc7-7 mcm5-bob1 (RSY847) strains transformed with GAL-HA-CSE4 (pMB1597). Strains were grown
to logarithmic phase of growth in raffinose-containing media (2%) and expression of \( \text{GAL-HA-CSE4} \) was induced with galactose (2%) for four hours. Cells were then treated with cycloheximide (CHX, 10 \( \mu \)g/ml) and glucose (2%). Aliquots were taken at the indicated timepoints. Protein extracts were analyzed using Western blot analysis and blots were probed with anti-HA (Cse4) and anti-Tub2. (loading control). The graph shows the quantification of levels of HA-Cse4 remaining after treatment with CHX relative to Tub2 from two independent experiments. Error bars represent SEM.

**Figure 4. Cdc7 prevents mislocalization of Cse4 to non-centromeric regions.**

A. **Cse4 is mislocalized in a cdc7 strain.** Localization of Cse4 was examined using chromosome spreads prepared from nocodazole arrested wild type (RSY299) and \( \text{cdc7-7} \) (RSY302) strains transformed with \( \text{GAL-HA-CSE4} \) (pMB1597). HA-Cse4 was labeled with Cy3 (red) and DNA with DAPI (blue). Representative images of cells showing normal localization counted as nuclei with one or two Cse4 foci (WT) and mislocalization counted as nuclei with more than two foci or a diffuse signal in the nucleus (\( \text{cdc7-7} \)). Arrow indicates HA-Cse4 foci.

B. **Quantification of Cse4 localization from A.** The graph displays the quantification of Cse4 localization as a percentage over total cell count. The SEM of two independent experiments is shown, WT 1 or 2 foci vs \( \text{cdc7-7} \) 1 or 2 Foci \( p \)-value = 0.0028; WT 3+ foci vs \( \text{cdc7-7} \) 3+ Foci \( p \)-value = 0.0028.

C. **The kinetochore protein Mtw1 is not mislocalized in a cdc7-7 strain.** Wild type (YMB9337) and \( \text{cdc7-7} \) (YMB9338) cells were transformed with Mtw1-GFP on a plasmid (pMB1058), grown to logarithmic phase of growth, and analyzed for Mtw1-GFP (green) foci with live cell imaging. Representative images of cells showing single Mtw1-GFP foci are shown. Arrow indicates Mtw1-GFP foci.

D. **Quantification of Mtw1-GFP localization from C.** The graph
displays the quantification of cells with one or two GFP foci (normal) or with greater than three foci (mislocalized) with the SEM of two independent experiments; WT 1 or 2 foci vs cdc7-7 1 or 2 Foci p-value = 0.1683; WT 3+ foci vs cdc7-7 3+ Foci p-value = 0.1683.

Figure 5. Cse4 is mislocalized to non-centromeric regions in a cdc7-7 strain. ChIP-seq was performed using chromatin lysates from wild type (YMB10044) and cdc7-7 (YMB10041) strains. A. Flag-Cse4 is mislocalized in a cdc7-7 strain. Genome browser of input and ChIP samples for Chromosome I and Chromosome V in wild type (top) and cdc7-7 (bottom) strains overexpressing Flag-Cse4. Regions of CEN1 and CEN5 are shown. B. Flag-Cse4 is enriched at promoters in a cdc7-7 strain. The annotatePeaks tool of HOMER v5.10 (http://homer.ucsd.edu/homer/) was used to define genomic locations of Flag-Cse4 enrichment in the cdc7-7 strain. The genomic feature, peak number, percent of total peaks, region size, fold-enrichment (relative to sequence content), and LogP enrichment are indicated. C. FLAG-Cse4 is preferentially enriched at promoters in cdc7-7 and psh1Δ strains. Overlap between Flag-Cse4 enrichment in cdc7-7 and psh1Δ strains and at promoters.

Figure 6. Cdc7 regulates Psh1-mediated proteolysis of Cse4. A. Overexpression of UBI4 suppresses the SDL of a cdc7-7 GALCSE4 strain. Growth assays of wild type (RSY299) and cdc7-7 (RSY302) cells transformed with empty vector (pMB433, GALCSE4-) or GAL-HA-CSE4 (pMB1597, GALCSE4+) and subsequently transformed with empty (pRS425, 2μ UBI4-) or UBI4 (pMB1604, UBI4+). Cells were spotted in five-fold serial dilutions on media selective for the plasmids containing either glucose (2%) or raffinose/galactose (2% each) and incubated at 23°C or 37°C as indicated for 3-5 days. Three independent transformants for each strain were
assayed and the representative image is shown. **B. The GALCSE4 SDL phenotype of the cdc7-7 psh1Δ strain is similar to that observed for psh1Δ and cdc7-7 strains.** Growth assays of wild type, psh1Δ, cdc7-7, and cdc7-7 psh1Δ strains with endogenously expressed Flag-Cse4 (vector; YMB10043, YMB10126, YMB10040, and YMB10124, respectively) or Flag-Cse4 expressed from a galactose-inducible promoter integrated into the genome (GALCSE4; YMB10044, YMB10127, YMB10041, and YMB10125, respectively) spotted in five-fold serial dilutions on to rich media containing either glucose (2%) or raffinose/galactose (2% each) and incubated at 23°C for 5 days. Three independent transformants for each strain were assayed and the representative image is shown. **C. The Cse4 proteolysis defect in a cdc7-7 psh1Δ double mutant is similar to that observed for a psh1Δ strain.** Western blot analysis of protein extracts from wild type (YMB10044), cdc7-7 (YMB10041), psh1Δ (YMB10127), and cdc7-7 psh1Δ (YMB10125) strains grown to logarithmic phase of growth in raffinose-containing media (2%). Expression of GAL-FLAG-CSE4 was induced with galactose (2%) for 1.75 hours. Cells were then treated with cycloheximide (CHX, 10 µg/ml) and glucose (2%). Aliquots were taken at the indicated timepoints. Protein extracts were analyzed using Western blot analysis and blots were probed with anti-FLAG (Cse4) and anti-Tub2. (loading control). The graph shows the quantification of the levels of FLAG-Cse4 remaining after treatment with CHX relative to Tub2 from two independent experiments. Error bars represent SEM. **D. Mislocalization of Cse4 is not further enhanced in the cdc7-7 psh1Δ strain.** Localization of Cse4 was examined using chromosome spreads prepared from nocodazole arrested wild type (YMB10044), cdc7-7 (YMB10041), psh1Δ (YMB10127), and cdc7-7 psh1Δ (YMB10125). FLAG-Cse4 was labeled with Cy3 and DNA with DAPI. The graph displays quantification of Cse4 localization as a percentage over total cell count. The graph displays the SEM of two independent experiments,
$psh1\Delta$ 3+ foci vs $cdc7^{-7}$ 3+ Foci, $cdc7^{-7}$ 3+ foci vs $cdc7^{-7} \ psh1\Delta$ 3+ Foci, and $psh1\Delta$ 3+ foci vs $cdc7^{-7} \ psh1\Delta$ 3+ Foci $p$-value > 0.999.
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Figure 1

A

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image1" alt="vector GALCSE4" /></td>
<td><img src="image2" alt="vector GALCSE4" /></td>
</tr>
<tr>
<td>cdc7-4</td>
<td><img src="image3" alt="vector GALCSE4" /></td>
<td><img src="image4" alt="vector GALCSE4" /></td>
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<tr>
<td>dbf4-1</td>
<td><img src="image5" alt="vector GALCSE4" /></td>
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<tr>
<td>dbf4-2</td>
<td><img src="image7" alt="vector GALCSE4" /></td>
<td><img src="image8" alt="vector GALCSE4" /></td>
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</tbody>
</table>

23°C

B

<table>
<thead>
<tr>
<th>GAL CSE4</th>
<th>CDC7</th>
<th>Glucose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>cdc7-7</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cdc7-7</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

23°C

37°C
Figure 2

A. WT vs. cdc7-7

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
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<tr>
<td>cdc7-7</td>
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HA (Cse4) Tub2

B. WT vs. dbf4-1

<table>
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<tr>
<th>Time (min)</th>
<th>0</th>
<th>30</th>
<th>60</th>
<th>90</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dbf4-1</td>
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</tbody>
</table>

HA (Cse4) Tub2

C. Input vs. Ub

<table>
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<tr>
<th>WT</th>
<th>dbf4-1</th>
<th>cdc7-7</th>
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</thead>
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<tr>
<td>Input</td>
<td>Ub+</td>
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</tr>
<tr>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>Ub</td>
<td>Ub</td>
<td>Ub</td>
</tr>
</tbody>
</table>

HA (Cse4) Ub-Cse4

Figure legend:
- kDa: molecular weight of the protein
- WT: wild type
- cdc7-7: cdc7-7 mutant
- dbf4-1: dbf4-1 mutant
- Relative Cse4-Ubiquitination
- p < 0.05: statistical significance
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td><img src="image1.png" alt="Image" /></td>
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<tr>
<td>cdc7-7</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
<tr>
<td>mcm5-bob1</td>
<td><img src="image5.png" alt="Image" /></td>
<td><img src="image6.png" alt="Image" /></td>
</tr>
<tr>
<td>cdc7-7 mcm5-bob1</td>
<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
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</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>cdc7-7</th>
<th>mcm5-bob1</th>
<th>cdc7-7 mcm5-bob1</th>
</tr>
</thead>
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<td><img src="image11.png" alt="Image" /></td>
<td><img src="image12.png" alt="Image" /></td>
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<tr>
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<td><img src="image14.png" alt="Image" /></td>
<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
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<tr>
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<td><img src="image18.png" alt="Image" /></td>
<td><img src="image19.png" alt="Image" /></td>
<td><img src="image20.png" alt="Image" /></td>
</tr>
<tr>
<td>90</td>
<td><img src="image21.png" alt="Image" /></td>
<td><img src="image22.png" alt="Image" /></td>
<td><img src="image23.png" alt="Image" /></td>
<td><img src="image24.png" alt="Image" /></td>
</tr>
</tbody>
</table>

HA (Cse4) and Tub2 expression in different conditions over time.
Figure 4

A

DAPI  HA-Cse4  Merge

WT

cdc7-7

B

% Cells with Cse4 foci

wt  cdc7-7

n=105  n=106

p = 0.0028

C

GFP

WT

cdc7-7

D

% Cells with Mtw1-GFP foci

wt  cdc7-7

n=123  n=126

p = 0.1683
Figure 5

### B

<table>
<thead>
<tr>
<th>Peak Annotations in cdc7-7 GALCSE4</th>
<th>Number of Peaks</th>
<th>% of Total</th>
<th>Total Size (bp)</th>
<th>Enrichment (Obs/Exp)</th>
<th>LogP Enrichment (+values depleted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3' UTR</td>
<td>28</td>
<td>1.3%</td>
<td>868412</td>
<td>0.2222</td>
<td>60.544</td>
</tr>
<tr>
<td>Transcription Termination Site</td>
<td>24</td>
<td>1.1%</td>
<td>666400</td>
<td>0.2481</td>
<td>42.749</td>
</tr>
<tr>
<td>Exons</td>
<td>457</td>
<td>20.7%</td>
<td>9031012</td>
<td>0.3487</td>
<td>694.356</td>
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<tr>
<td>Introns</td>
<td>12</td>
<td>0.5%</td>
<td>66236</td>
<td>1.2483</td>
<td>-1.347</td>
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<td>Intergenic</td>
<td>136</td>
<td>6.2%</td>
<td>739256</td>
<td>1.2675</td>
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<tr>
<td>Promoters</td>
<td>1330</td>
<td>60.4%</td>
<td>3330000</td>
<td>2.7511</td>
<td>-758.277</td>
</tr>
<tr>
<td>5' UTR</td>
<td>216</td>
<td>9.8%</td>
<td>476819</td>
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<tr>
<td><strong>Total</strong></td>
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</tbody>
</table>

**Promoters (4604)**

**cdd7-7 (2129)**

**psh1Δ (4798)**
Figure 6

A

<table>
<thead>
<tr>
<th>GAL</th>
<th>CSE4</th>
<th>UBI4</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>Glucose</td>
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<td>Glucose</td>
</tr>
<tr>
<td>WT</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>psh1Δ</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cdc7-7</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>cdc7-7 psh1Δ</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Glucose</th>
<th>23°C</th>
<th>Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>vector</td>
<td>vector</td>
</tr>
<tr>
<td>psh1Δ</td>
<td>vector</td>
<td>GALCSE4</td>
</tr>
<tr>
<td>cdc7-7</td>
<td>vector</td>
<td>GALCSE4</td>
</tr>
<tr>
<td>cdc7-7 psh1Δ</td>
<td>vector</td>
<td>GALCSE4</td>
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</tbody>
</table>

C

<table>
<thead>
<tr>
<th>WT</th>
<th>cdc7-7</th>
<th>psh1Δ</th>
<th>cdc7-7 psh1Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>0</td>
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</tr>
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<td>30</td>
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<tr>
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</tr>
<tr>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>

D

% Cells with CSE4 foci

WT: n=447, p > 0.999
cdc7-7: n=360, p > 0.999
psh1Δ: n=497, p > 0.999
cdc7-7 psh1Δ: n=448, p > 0.999