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Zhang, Tianyi

Au, Wei-Chun

Ohkuni, Kentaro

et al.

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Brief Investigation

Mck1-mediated proteolysis of CENP-A prevents mislocalization of CENP-A for chromosomal stability in *Saccharomyces cerevisiae*

Tianyi Zhang,[1,3,†] Wei-Chun Au,[1,†] Kentaro Ohkuni,[1,†] Roshan L. Shrestha,[1] Peter Kaiser,[2] Munira A. Basrai[1,*]

[1]Genetics Branch, Center for Cancer Research, National Cancer Institute. National Institute of Health, Bethesda, MD 20892, USA
[2]Department of Biological Chemistry, School of Medicine, University of California, Irvine, Irvine, CA 92697, USA
[3]Present address: Laboratory of Genetics and Genomics, National Institute on Aging, Intramural Research Program, National Institutes of Health, Baltimore, MD 21224, USA

*Corresponding author: Genetics Branch, Center for Cancer Research, National Cancer Institute. National Institute of Health, Bethesda, MD 20892, USA. Email: basraim@nih.gov
[†]These authors contributed equally to this work.

Centromeric localization of evolutionarily conserved CENP-A (Cse4 in *Saccharomyces cerevisiae*) is essential for chromosomal stability. Mislocalization of overexpressed CENP-A to noncentromeric regions contributes to chromosomal instability in yeasts, flies, and humans. Overexpression and mislocalization of CENP-A observed in many cancers are associated with poor prognosis. Previous studies have shown that F-box proteins, Cdc4 and Met30 of the Skp, Cullin, F-box ubiquitin ligase cooperatively regulate proteolysis of Cse4 to prevent Cse4 mislocalization and chromosomal instability under normal physiological conditions. Mck1-mediated phosphorylation of Skp, Cullin, F-box-Cdc4 substrates such as Cdc6 and Rcn1 enhances the interaction of the substrates with Cdc4. Here, we report that Mck1 interacts with Cse4, and Mck1-mediated proteolysis of Cse4 prevents Cse4 mislocalization for chromosomal stability. Our results showed that *mck1Δ* strain overexpressing *CSE4* (*GAL-CSE4*) exhibits lethality, defects in ubiquitin-mediated proteolysis of Cse4, mislocalization of Cse4, and reduced Cse4–Cdc4 interaction. Strain expressing *GAL-cse4-3A* with mutations in three potential Mck1 phosphorylation consensus sites (S10, S16, and T166) also exhibits growth defects, increased stability with mislocalization of Cse4-3A, chromosomal instability, and reduced interaction with Cdc4. Constitutive expression of histone H3 (*Δ16H3*) suppresses the chromosomal instability phenotype of *GAL-cse4-3A* strain, suggesting that the chromosomal instability phenotype is linked to Cse4-3A mislocalization. We conclude that Mck1 and its three potential phosphorylation sites on Cse4 promote Cse4–Cdc4 interaction and this contributes to ubiquitin-mediated proteolysis of Cse4 preventing its mislocalization and chromosomal instability. These studies advance our understanding of pathways that regulate cellular levels of CENP-A to prevent mislocalization of CENP-A in human cancers.

Keywords: CENP-A; Cse4; Mck1; Cdc4; centromere

Introduction

The centromere (*CEN*) is a specialized region of the chromosome for the formation of kinetochore (*CEN* and associated proteins) which is essential for faithful chromosome segregation. Despite the divergence in DNA sequences in different species, centromeres contain an evolutionarily conserved histone H3 variant CENP-A (Cse4 in *Saccharomyces cerevisiae*, Cnp1 in *Schizosaccharomyces pombe*, and centromere identifier in *Drosophila melanogaster*) (Choy *et al.* 2012; Henikoff and Furuyama 2012; Biggins 2013). Mislocalization of overexpressed CENP-A or its homologs to noncentromeric regions contributes to chromosomal instability (CIN) in budding and fission yeasts, flies, 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). Overexpression and mislocalization of CENP-A have been observed in many cancers 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). Hence, defining pathways that prevent mislocalization of CENP-A is an area of active research.

Ubiquitin-mediated protein degradation plays a critical role in regulating the cellular levels of CENP-A homologs in budding, fission yeasts, and flies, and this prevents mislocalization to noncentromeric regions (Collins, Furuyama and Biggins 2004; Moreno-Moreno *et al.* 2006; Moreno-Moreno *et al.* 2011; Au *et al.* 2013; Gonzalez *et al.* 2014). Studies with budding yeast have defined multiple E3 ubiquitin ligases such as Psh1, Sumo-targeted ubiquitin ligases (STUbLs) Slx5/Slx8, Skp-Cullin-F-box (SCF)-Rcy1, and SCF-Cdc4/Met30 for ubiquitin-mediated proteolysis of Cse4 (Hewawasam *et al.* 2010; Ranjitkar *et al.* 2010; Cheng *et al.* 2016, 2017; Ohkuni *et al.* 2016, 2018; Au *et al.* 2020). The latter describes an unusual heterodimeric SCF complex with the two different substrate acceptors, Cdc4 and Met30, a specific configuration required for Cse4 degradation (Au *et al.* 2020). Psh1-mediated proteolysis of overexpressed Cse4 has been well characterized and shown to be regulated by the facilitates chromatin transcription/transactions complex (Deyter and Biggins 2014), Casein kinase 2 (CK2; Hewawasam *et al.* 2014), Fpr3 (proline isomerase; Ohkuni *et al.* 2014), histone regulation histone chaperone complex (Ciftci-Yilmaz *et al.* 2018), and

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Dbf4-dependent kinase complex (Eisenstatt *et al.* 2020). Slx5-mediated Cse4 proteolysis is regulated by sumoylation of Cse4 K65 (Ohkuni *et al.* 2018). Removal of Psh1-mediated polyubiquitinated Cse4 from noncentromeric regions is facilitated by $Cdc48^{Ufd1/Npl4}$ segregase (Ohkuni *et al.* 2022). We have also pursued studies to define pathways that promote mislocalization of Cse4, showing that sumoylation of Cse4 and an in vivo conformational change of Cse4 upon interaction with histone H4 contribute to mislocalization of Cse4 to noncentromeric regions (Ohkuni *et al.* 2020, 2024; Eisenstatt *et al.* 2021).

We previously defined a role for the essential E3 ligase SCF-Cdc4/Met30 in proteolysis of endogenous Cse4 to prevent mislocalization of Cse4 for chromosomal stability (Au *et al.* 2020). F-box subunits of SCF, such as Cdc4 or Met30, recruit substrates by interacting with degrons with specific amino acid sequence motifs. Phosphorylation of substrates for some F-box proteins such as Cdc4 is essential for the interaction between the F-box protein and the substrate. For example, phosphorylation of cell-cycle regulators, such as Sic1, Cdc6, Ash1, and Eco1, by Cdk1 is necessary for their recognition by Cdc4 (Perkins *et al.* 2001; Hao *et al.* 2007; Liu *et al.* 2011; Lyons *et al.* 2013). Subsequent studies indicated that Cdc4 prefers to bind peptides with two phosphorylated sites (Hao *et al.* 2007). Mck1 kinase or its metazoan homolog glycogen synthase kinase (GSK)-3β phosphorylates amino acid four residues upstream of a previously phosphorylated residue (consensus motif: S/T*-x-x-x-pS/pT; Fiol *et al.* 1987; Doble and Woodgett 2003; Mok *et al.* 2010). Phosphorylation by Mck1 increases affinity of the target substrates with Cdc4. Indeed, Mck1 phosphorylated sites have been demonstrated to be important in the degrons of several Cdc4 substrates in yeast, including Cdc6, Hsl1, Hst3, Rcn1, and Eco1 (Mizunuma *et al.* 2001; Kishi *et al.* 2007; Ikui *et al.* 2012; Lyons *et al.* 2013; Edenberg *et al.* 2014). Furthermore, the degrons of many substrates of vertebrate Fbw7, the Cdc4 homolog, are phosphorylated by GSK-3β (Welcker and Clurman 2008; Crusio *et al.* 2010; Yeh *et al.* 2018). Mck1 was reported to be a dosage suppressor of CDEIII *CEN* mutations and proposed to phosphorylate Ndc10 (Shero and Hieter 1991; Jiang *et al.* 1995). However, the molecular role for Mck1 in chromosome segregation has not been investigated.

In this study, we have defined a role for Mck1 in proteolysis of Cse4 to prevent its mislocalization for chromosomal stability. Our results showed that Mck1 interacts with Cse4 and the *GAL-CSE4 mck1Δ* strain exhibits synthetic dosage lethality (SDL), defects in ubiquitin-mediated proteolysis of Cse4, mislocalization of Cse4, and reduced interaction of Cse4 with Cdc4. Studies with *GAL-CSE4* strain with mutations corresponding to three potential Mck1 phosphorylation sites in Cse4 (*cse4*-3A: *cse4* S10A S16A T166A) exhibits phenotypes similar to that for *GAL-CSE4 mck1Δ* strain. Our results show that Mck1 and consensus Mck1 phosphorylation sties in Cse4 S10, S16, and T166 promote Cse4–Cdc4 interaction, and this contributes to ubiquitin-mediated proteolysis of Cse4 preventing its mislocalization and CIN.

Materials and methods

Strains, plasmids, and growth conditions

The yeast strains and plasmids used in this study are described in Supplementary Tables 1 and 2, respectively. Unless otherwise noted, the yeast strains used are derived from *S. cerevisiae* BY4741. Yeast strain of an indicated gene integration or deletion was created by homologous recombination (Longtine *et al.* 1998). Triple-hemagglutinin (HA)-Cse4 was tagged within the N-terminus at amino acids 80 (Baker and Rogers 2006; Ohkuni *et al.* 2016; Au *et al.* 2020). Triple-Myc-Cse4 was tagged to the N-terminus end.

Yeast strains were grown in rich media (YPD: 1% yeast extract, 2% bacto-peptone, and 2% glucose) or synthetic medium with glucose or raffinose and galactose (2% final concentration each) allowing selection of the indicated plasmids.

Protein stability assays

Protein stability assays were done as previously described (Au *et al.* 2020). For strains expressing *GAL*-induced genes, cultures were grown to logarithmic phase in glucose media, washed and resuspended in raffinose (2%)- and galactose (2%)-containing medium for 1.5–4 h so that initial steady-state levels of Cse4 in wild-type (WT) and mutants were similar. Cycloheximide (CHX) and glucose were then added to final concentrations of 10 μg/mL and 2%, respectively. Samples were taken at the indicated time points after CHX treatment. The protein samples were analyzed by SDS-PAGE and Western blot using anti-HA (12CA5) mouse (Roche, 11583816001) and anti-Tub2 rabbit (Basrai Laboratory) antibodies. Protein levels were quantified using Gene Tools software (version 3.8.8.0) from SynGene (Frederick, MD).

Chromosome spreads

Chromosome spreads were performed as described previously (Collins *et al.* 2004; Crotti and Basrai 2004). Three independent biological repeats were performed and scored in blind experiments. A more detailed description is provided in Supplementary material.

Co-immunoprecipitation experiments

For experiments with genes expressed from their native promoter, cultures were grown in selective medium with 2% glucose. For experiments with genes expressed from *GAL* promoter, cultures were overnight in selective medium containing 2% raffinose to logarithmic phase, diluted in the same selective medium containing 2% galactose and incubated at 25°C for 3 h. Cell lysis, beads incubation, and Western blot detection were done as described previously (Au *et al.* 2020). The protein samples were analyzed by SDS-PAGE and Western blot using anti-HA (12CA5) mouse (Roche, 11583816001), anti-Myc rabbit (Santa Cruz Inc, sc789), and anti-FLAG mouse (Sigma, F3165) antibodies.

Chromosome transmission fidelity (*ctf*) assays

Assays for the loss of the reporter chromosome (RC) were done as previously reported (Spencer *et al.* 1990; Basrai *et al.* 1996). Chromosome loss was quantified by measuring % of colonies with ≥ 50% red sectors and visible white sectors. Colonies that were completely red were not counted.

Ub pull-down assays

The levels of ubiquitinated protein were determined by Ub pull-down assay as described previously (Au *et al.* 2020; Ohkuni *et al.* 2022). A more detailed description is provided in Supplementary material.

Results

Mck1 interacts with Cse4 and regulates ubiquitin-mediated proteolysis of Cse4 to prevent its mislocalization

Mutants of SCF-Cdc4/Met30 components exhibit SDL when Cse4 is overexpressed from a galactose-inducible promoter

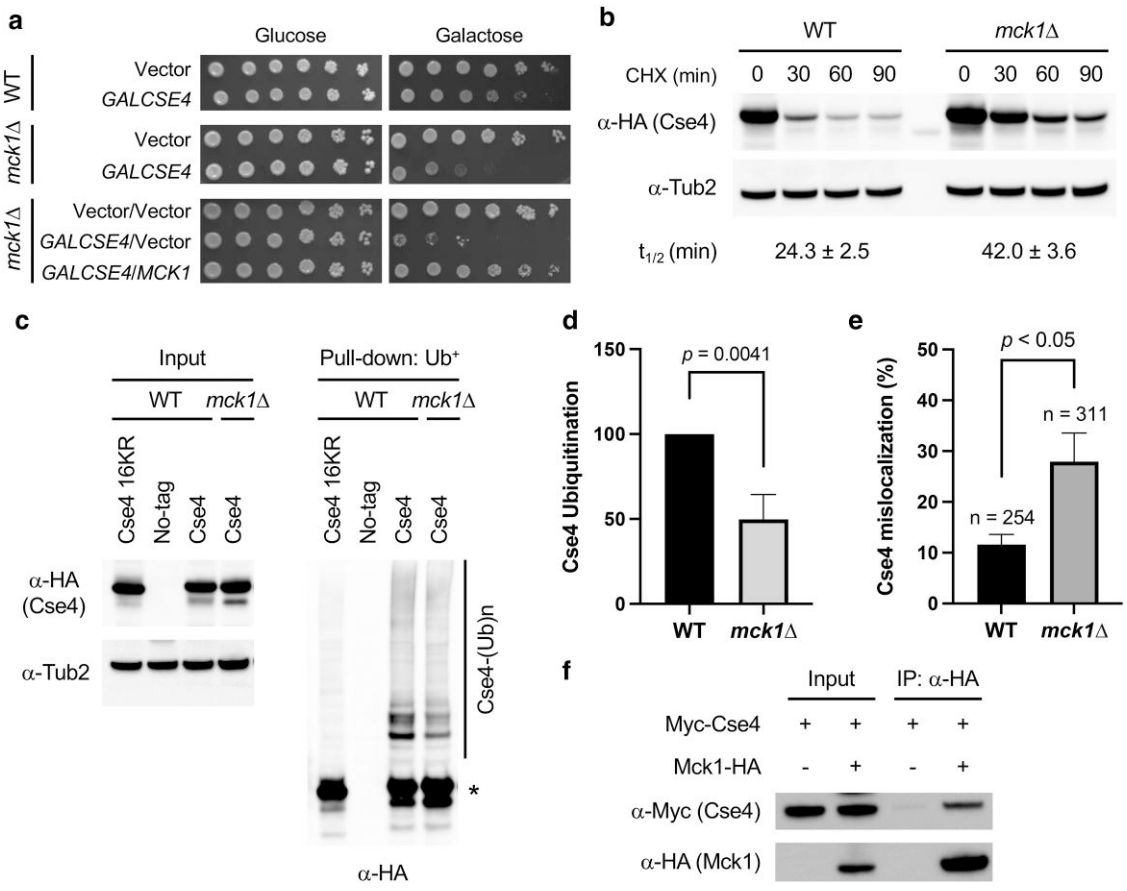

Fig. 1. Mck1 interacts with Cse4 and regulates ubiquitin-mediated proteolysis of Cse4 to prevent its mislocalization. a) SDL phenotype in *mck1Δ GAL-CSE4* strain. Growth assays were performed with WT (BY4741) and *mck1Δ* (YMB10154) strains transformed with vector (pMB433), or *GAL-HA-CSE4* (pMB1458) plated on either 2% glucose or 2% galactose medium. Complementation of *mck1Δ GAL-CSE4* SDL was performed with *mck1Δ* strain with or without *GAL-CSE4* transformed with vector (pRS425) or plasmid expressing *MCK1* (pMB1872). The plates were incubated at 25 °C for 6 days and photographed. b) Mck1 regulates Cse4 proteolysis. Protein stability assays were done with extracts from WT (YMB4741) and *mck1Δ* (YMB10154) strains transformed with *GAL-HA-CSE4* (pMB1807) grown in galactose (2%) medium for 1.5 and 4 h at 25 °C and later shift to glucose (2%) containing medium, followed by addition of CHX (10 µg/mL). Protein extracts were prepared from cells collected at the indicated time points. Western blots were probed with anti-HA (HA-Cse4) or anti-Tub2 (loading control) antibody. Cse4 protein half-lives ($t_{1/2}$) are reported as mean ± SD of three independent experiments. The difference in $t_{1/2}$ is statistically significant ($P = 0.0022$). c) Cse4 ubiquitination is reduced in *mck1Δ* strain. Protein extracts were prepared from WT and *mck1Δ* strains used in (b). WT strain expressing either vector (pMB430) or *cse4* 16KR (pMB1892) were used as negative controls. These strains were grown in galactose medium for 2–3.5 h to express comparable levels of Cse4. Agarose-TUBE1 (tandem ubiquitin binding entities) was used to pull-down and ubiquitination levels of Cse4 were detected by Western blot analysis with anti-HA antibody. Input samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Asterisk shows nonmodified Cse4. d) Levels of polyubiquitinated Cse4 from (c) were quantified after normalization to input Cse4. Statistical significance from three biological repeats was assessed by unpaired *t*-test. Error bars indicate standard deviation from the mean. e) Cse4 is mislocalized to noncentromeric regions in *mck1Δ* strain. Chromosome spreads were prepared from WT (BY4741) and *mck1Δ* (YMB10154) strains transformed with *GAL-HA-CSE4* (pMB1597) after transient induction in galactose (2%) medium at 25 °C for 1.5 h. Cse4 localization was determined using Cy3-conjugated secondary antibody and DNA was stained with DAPI. Centromeric Cse4 localization is restricted to 1–2 foci. Mislocalization of Cse4 results in more than three foci or increased area of Cse4 localization within the nucleus. Images were acquired with 63× objective with the same exposure time. Error bars represent the standard deviation of three independent experiments. The total number of cells used is indicated (*n*) and the numbers (mislocalized/total) of three replicates are 10/83, 10/75, and 9/96 in WT and 32/104, 30/95, and 24/112 in *mck1Δ*. f) Mck1 interacts with Cse4 in vivo. Protein extracts from WT strain (BY4741) expressing *GAL-Myc-CSE4* (pMB1878) with either vector (pMB433) or *GAL-MCK1-HA-3C-ZZ* (MCK1 MORF, GE Healthcare) were prepared after transient induction in 2% galactose medium for 3 h at 25 °C. Input or IP (IgG beads, 3C protease treatment, and anti-HA beads) samples were analyzed by Western blot and probed with anti-Myc and anti-HA antibodies.

(*GAL-CSE4*; Au *et al.* 2020). Since Mck1 kinase phosphorylates substrates for SCF-Cdc4, we examined whether *mck1Δ* strain also exhibits SDL phenotype with *GAL-CSE4*. Growth assays showed that *mck1Δ* strain exhibits SDL with *GAL-CSE4* on galactose plates at the permissive temperature of 25 °C (Fig. 1a). The observed SDL is linked to deletion of *MCK1* as the lethality was suppressed by expressing the cognate wild-type gene in the *mck1Δ* strain (Fig. 1a).

GAL-CSE4-mediated SDL correlates with defects in Cse4 proteolysis which contributes to its mislocalization (Ohkuni *et al.* 2016; Ciftci-Yilmaz *et al.* 2018; Au *et al.* 2020; Eisenstatt *et al.* 2020). Hence, we examined the stability, ubiquitination, and chromosomal localization of Cse4 in a *mck1Δ* strain. Protein stability assays showed that Cse4, which was rapidly degraded in wild-type strain ($t_{1/2} = 24.3 \pm 2.5$ min), was stabilized in the *mck1Δ* strain ($t_{1/2} = 42.0 \pm 3.6$ min; Fig. 1b). Our results showed that consistent with defects in proteolysis of Cse4, the levels of ubiquitinated Cse4 were reduced in the *mck1Δ* strain (Fig. 1, c and d). We performed chromosome spreads to examine if defects in Cse4 proteolysis contribute to mislocalization of Cse4 to noncentromeric regions in *mck1Δ* strain. In wild-type cells, Cse4 localization is

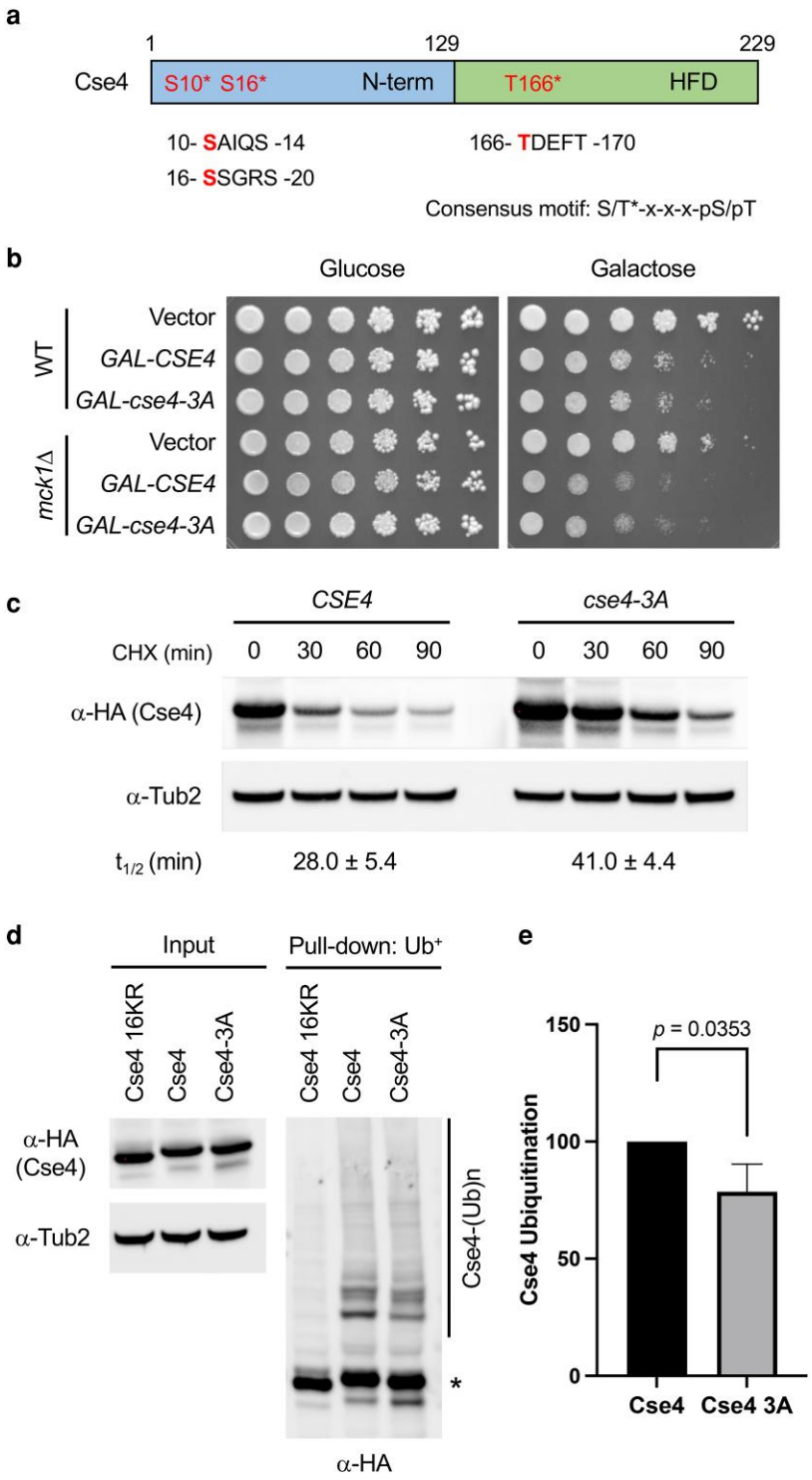

Fig. 2. *cse4*-3A mutant for Mck1 consensus phosphorylation sites exhibits defects in ubiquitin-mediated proteolysis. a) Cartoon showing the three potential Mck1 phosphorylation sites (S/T*-X-X-X-pS/pT): Cse4 S10, S16, and T166. *cse4*-3A mutant was generated by mutating S10, S16, and T166 sites to alanine. HFD, histone-fold domain. b) SDL phenotype of *GAL-cse4*-3A *mck1*Δ is similar to that of *GAL-CSE4 mck1*Δ. Growth assays were performed with WT (BY4741) and *mck1Δ* (YMB10154) strains transformed with vector (pMB433), *GAL-HA-CSE4* (pMB1597), or *GAL-HA-cse4*-3A (pMB1909) plated on either 2% glucose or 2% galactose medium. The plates were incubated at 25 °C for 4 days and photographed. c) Cse4 S10, S16, and T166 regulate its proteolysis. Protein stability assays were done using extracts from WT (BY4741) strain transformed with *GAL-HA-CSE4* (pMB1807) or *GAL-HA-cse4*-3A (pMB1833) grown in galactose (2%) medium for 1.5 h at 25 °C and later shift to glucose (2%) containing medium, followed by addition of CHX (10 µg/mL). Protein extracts were prepared from cells collected at the indicated time points. Western blots were probed with anti-HA (HA-Cse4) or anti-Tub2 (loading control) antibody. Cse4 protein half-lives ($t_{1/2}$) are reported as mean ± SD of four independent experiments. The difference in $t_{1/2}$ is statistically significant ($P = 0.0095$). d) Cse4-3A ubiquitination is reduced. Protein extracts were prepared from same strains used in (c). WT strain expressing *cse4 16KR* was used as a negative control. These strains were grown in galactose medium for 2 h. Agarose-TUBE1 was used to pull-down and ubiquitination levels of Cse4 were detected by Western blot analysis with anti-HA antibody. Input samples were analyzed using anti-HA (Cse4) and anti-Tub2 antibodies. Asterisk shows nonmodified Cse4. e) Levels of polyubiquitinated Cse4 from (d) were quantified after normalization to input Cse4. Statistical significance from three biological repeats was assessed by unpaired *t*-test. Error bars indicate standard deviation from the mean.

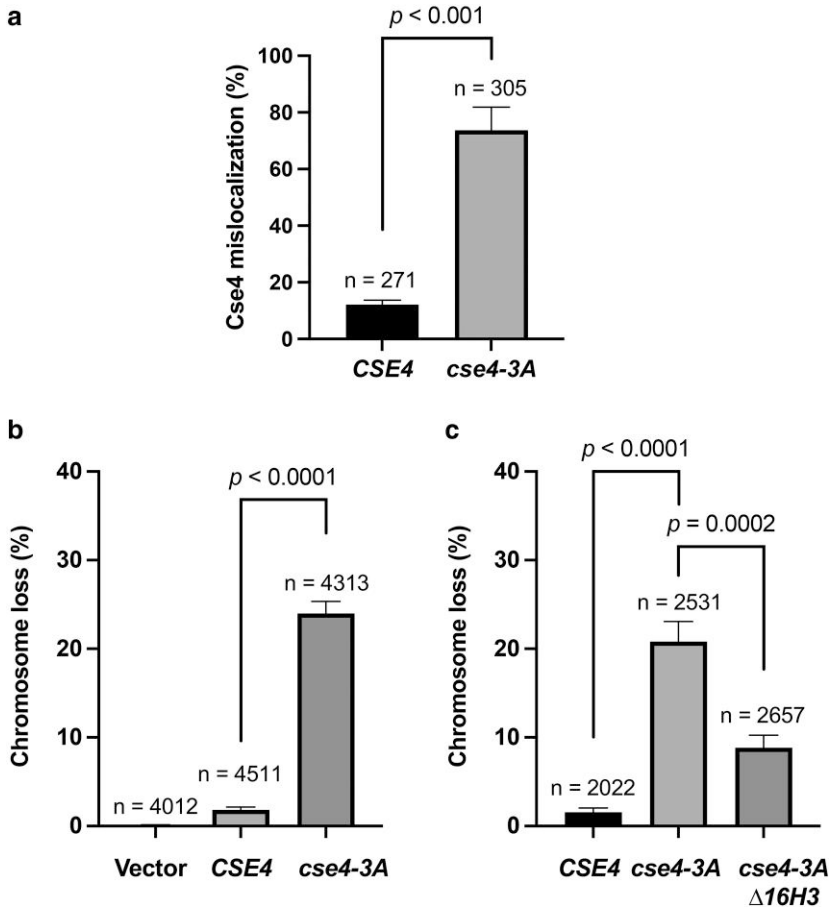

Fig. 3. Cse4-3A is mislocalized and this contributes to CIN. a) Cse4-3A is mislocalized to noncentromeric regions. Localization of Cse4 was examined by chromosome spreads as described in Fig. 1e in WT (BY4741) strain transformed with *GAL-HA-CSE4* (pMB1807) or *GAL-HA-cse4-3A* (pMB1833). Error bars represent the standard deviation of three independent experiments. The total number of cells used is indicated (*n*) and the numbers (mislocalized/total) of three replicates are 13/95, 11/91, and 9/85 in *CSE4* and 88/107, 67/92, and 70/106 in *cse4-3A*. b) *GAL-cse4-3A* strain exhibits CIN. Loss of RC was measured in wild-type strain (YPH1015) transformed with vector (pMB430), *GAL-CSE4* (pMB1807), or *GAL-cse4-3A* (pMB1833). Strains with reporter RC were spread on 2% galactose plates and incubated at 25 °C. Loss of the RC was determined by counting the number of colonies that show loss of the RC in the first division with greater than or equal to≥ half sector phenotype. At least 4,000 colonies from three independent transformants were assayed and the total number of colonies counted is indicated as (*n*). c) CIN phenotype of *cse4-3A* strain is suppressed by constitutive expression of histone H3 (*Δ16H3*). Loss of RC was measured as described in (b) using wild-type (YPH1015) or *Δ16H3* (YMB6331) strain transformed with *GAL-CSE4* (pMB1597) or *GAL-cse4-3A* (pMB1909).

usually restricted to one or two foci coincident with DAPI signal, which correspond to kinetochore clusters (Supplementary Fig. 1a). In contrast, a higher incidence of multiple or diffused Cse4 foci within DAPI stained region were observed in *mck1*Δ cells (Fig. 1e and Supplementary Fig. 1a). To rule out the possibility that the mislocalization of Cse4 is due to declustering of centromeres, we examined the localization of kinetochore protein Mtw1-GFP. We previously showed that Mtw1-GFP is not declustered in mutants that show mislocalization of Cse4 (Ciftci-Yilmaz *et al.* 2018; Au *et al.* 2020; Eisenstatt *et al.* 2020). We determined that the localization pattern of Mtw1-GFP was restricted to one or two foci in both wild-type and *mck1*Δ strains (Supplementary Fig. 2). These results support a role for Mck1 in Cse4 proteolysis to prevent Cse4 mislocalization.

We next examined whether Mck1 interacts with Cse4 in vivo. Co-immunoprecipitation (Co-IP) experiments were done with strains co-expressing *Myc-CSE4* with *MCK1-HA*. Immunoprecipitation with anti-HA antibody revealed an interaction between Mck1 and Cse4, which was not observed in control strain (Fig. 1f). Taken together, we conclude that Mck1 interacts with Cse4 in vivo and regulates ubiquitin-mediated proteolysis of Cse4 to prevent Cse4 mislocalization.

Consensus Mck1 phosphorylation sites in Cse4 (Cse4 S10, S16, and T166) regulate ubiquitin-mediated proteolysis of Cse4

Our results for Mck1-mediated Cse4 proteolysis led us to investigate the possibility that Cse4 is a potential substrate for Mck1 kinase. The consensus motif (S/T*-x-x-x-pS/pT) is frequently found in Mck1 substrates (Ikui *et al.* 2012; Lyons *et al.* 2013; Edenberg *et al.* 2014). We identified S10, S16, and T166 as potential phosphorylation sites in Cse4 (Fig. 2a). To test the role of these sites for Mck1-mediated proteolysis of Cse4, we generated a strain expressing *GAL-cse4-3A* (S10A, S16A, and T166A) and examined this strain for growth, protein stability, ubiquitination, and mislocalization phenotypes that we have shown in the *mck1Δ* strain (Fig. 1). Growth assays showed that *GAL-cse4-3A* mutant exhibits increased lethality when compared to that observed for *GAL-CSE4* in a wild-type strain (Fig. 2b and Supplementary Fig. 3). Furthermore, the growth defect of *GAL-cse4-3A mck1*Δ strain was similar to that of *GAL-CSE4 mck1*Δ strain, suggesting that mutation of S10, S16, and T166 phenocopies loss of Mck1 in the context of Cse4 biology (Fig. 2b and Supplementary Fig. 3). These results suggest that Cse4 S10, S16, and T166 contribute to the growth defects of the *GAL-CSE4 mck1*Δ strain.

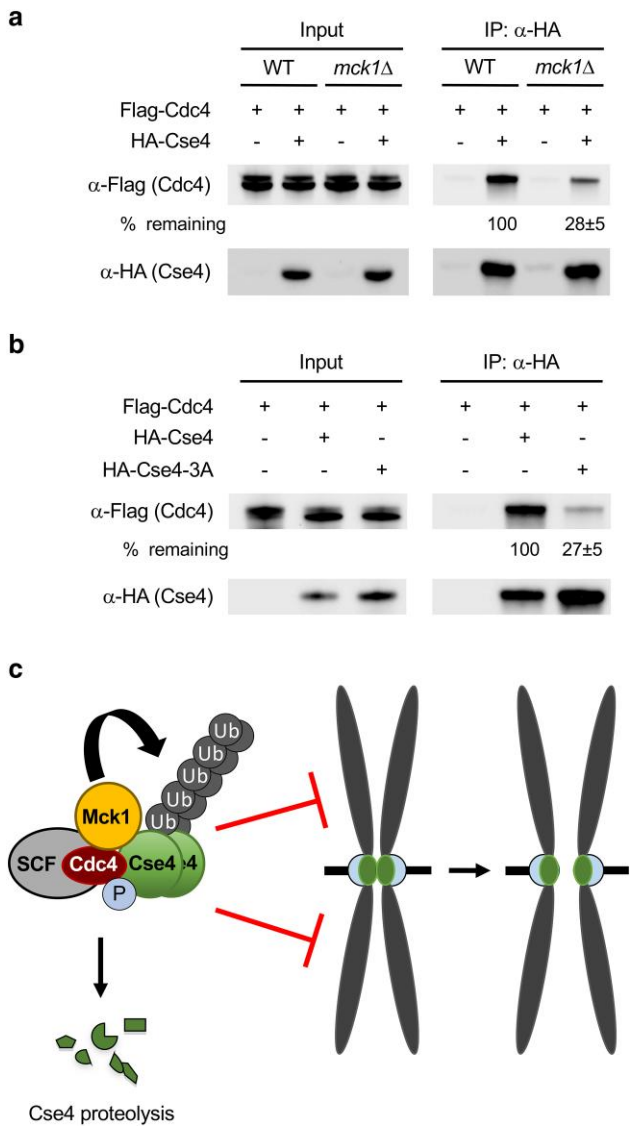

Fig. 4. Mck1 and Cse4 S10, S16, and T166 facilitate the interaction of Cse4 with Cdc4 in vivo. a) The interaction of Cse4 with Cdc4 is reduced in *mck1Δ* strain. Co-IP experiments were performed using whole cell extracts (WCEs) from WT (BY4741) and *mck1Δ* (YMB10154) strains expressing *Flag-CDC4* (pMB1840) with vector (pRS426) or *HA-CSE4* (pMB1831). Input and IP (anti-HA) samples were analyzed by Western blot analysis and probed with anti-Flag and anti-HA antibodies. All tagged proteins are expressed from their native promoter. Percentages of remaining interaction of Flag-Cdc4 and HA-Cse4 in *mck1Δ* strain from two independent experiments are measured as mean ± average deviation. b) Recued interaction of Cse4-3A with Cdc4. Co-IP experiments were done using WCE from WT strain (BY4741) expressing Flag-Cdc4 (pMB1860) with vector (pMB433), *GAL-HA-CSE4* (pMB1807), or *GAL-HA-cse4*-3A (pMB1833) prepared after transient induction in 2% galactose medium for 3 h at 25 °C. Input or IP (anti-HA beads) samples were analyzed by Western blot and probed with anti-Myc and anti-HA antibodies. Percentage of remaining interaction of Flag-Cdc4 and HA-Cse4-3A from two independent experiments is shown as mean ± average deviation. c) Model proposing a role for Mck1-mediated proteolysis of Cse4 by the SCF-Cdc4 pathway in preventing Cse4 mislocalization and CIN.

We next examined the protein stability and levels of ubiquitinated Cse4-3A. Cse4 was rapidly degraded in a wild-type strain ($t_{1/2}$ = 28.0 ± 5.4 min), whereas Cse4-3A protein was stabilized ($t_{1/2}$ = 41.0 ± 4.4 min; Fig. 2c). Consistent with these results, Cse4-3A showed reduced ubiquitination (Fig. 2, d and e), similar to what was observed in *mck1Δ* strain (Fig. 1c) and described in previous reports (Ciftci-Yilmaz *et al.* 2018; Eisenstatt *et al.* 2020). Based on these results, we conclude that consensus Mck1 phosphorylation sites in Cse4 (Cse4 S10, S16, and T166) regulate ubiquitin-mediated proteolysis of Cse4.

Strain expressing *GAL-cse4-3a* (Cse4 S10A, S16A, and T166A) exhibits mislocalization of Cse4-3a and elevated CIN

To determine whether increased stability of Cse4-3A contributes to its mislocalization, we performed chromosome spreads. Similar to results observed in *mck1*Δ strain (Fig. 1e and Supplementary Fig. 1a), higher incidence of multiple or diffused Cse4 foci within DAPI stained region were observed in *cse4*-3A expressing cells (Fig. 3a and Supplementary Fig. 1b). To determine the physiological consequence of these mutations on chromosomal stability, we performed a colony color assay to measure the rate of loss of a nonessential RC in wild-type strain transformed with vector, *GAL-CSE4*, and *GAL-cse4*-3A. Consistent with previous studies (Au *et al.* 2008, 2020), *GAL-CSE4* strain exhibits higher chromosome loss compared to strain with vector alone (Fig. 3b). In contrast, *GAL-cse4*-3A showed more than 10-fold increase in loss of RC when compared to that observed in wild-type *GAL-CSE4* strain.

Constitutive expression of histone H3 (*Δ16H3*) has been shown to suppress loss of RC in *cdc4-1* mutant or *GAL-cse4*^{16KR} strain due to reduced mislocalization of Cse4 (Au *et al.* 2008, 2020). Hence, we examined whether loss of RC of *GAL-cse4*-3A would be suppressed by *Δ16H3*. Our results showed that RC loss was significantly reduced in *GAL-cse4-3A Δ16H3* strain (Fig. 3c). Taken together, these results show that Cse4 S10, S16, and T166 prevent mislocalization of Cse4 and CIN.

Mck1 and its three potential phosphorylation sites in (Cse4 S10, S16, and T166) facilitate the interaction of Cse4 with Cdc4 in vivo

Based on our results and the role of Mck1 in promoting the interaction of Cdc4 with its substrates, we hypothesized that Mck1 facilitates the interaction of Cse4 with Cdc4. We examined whether reduced interaction of Cdc4 with Cse4 contributes to defects in ubiquitin-mediated proteolysis of Cse4 in a *mck1Δ* strain. Co-IP experiments were done with wild-type and *mck1*Δ strain co-expressing *Flag-CDC4* with *HA-CSE4*. Immunoprecipitation with anti-HA antibody confirmed our previous results for a positive interaction between Cse4 and Cdc4 (Fig. 4a; Au *et al.* 2020). Consistent with our hypothesis, we observed greatly reduced interaction between Cse4 and Cdc4 in the *mck1Δ* strain when compared to that observed for wild-type strain (Fig. 4a).

The similar phenotypes observed for *GAL-CSE4 mck1*Δ with *GAL-cse4*-3A led us to examine whether Cse4-3A will also show reduced interaction with Cdc4. Co-IP experiments were done with strains co-expressing *Flag-CDC4* with either *HA-CSE4* or *HA-cse4*-3A. Reduced interaction between Cse4 and Cdc4 was observed in *GAL-cse4*-3A strain when compared to wild-type Cse4 strain (Fig. 4b). Taken together, these results show that Mck1 and Cse4 S10, S16, and T166 facilitate the interaction of Cse4 with Cdc4 in vivo.

Discussion

We have identified and defined a role for Mck1 kinase in proteolysis of Cse4 and propose a model (Fig. 4c) in which Mck1 kinase facilitates the interaction of Cse4 with SCF-Cdc4 for ubiquitin-mediated Cse4

proteolysis that prevents mislocalization of Cse4 and CIN. Our results also define a role for Cse4 S10, S16, and T166 as the potential phosphorylation sites of Mck1 in ubiquitin-dependent proteolysis of Cse4 to prevent mislocalization of Cse4. We were unable to provide direct evidence that Cse4 S10, S16, and T166 are phosphorylated by Mck1 in vivo and/or in vitro due to functional redundancy of multiple kinases capable of regulating Cse4 proteolysis. Hence, we cannot rule out the possibility that specific mutation(s) in the Cse4 S10A S16A T166A mutant or defective phosphorylation of Cse4 by other kinases such as Cdc5 may contribute to the phenotypes we have reported. For example, we previously showed that Cse4 S10 and S16 are phosphorylated by Cdc5 using an in vitro kinase assay (Mishra *et al.* 2019). In addition, structural defects of Cse4 S10A S16A T166A mutant cannot be excluded. However, our genetic, biochemical, and cell biology approaches showed that the phenotypes of *GAL-cse4-3A* in a wild-type strain are similar to those observed in a *GAL-CSE4 mck1Δ* strain. Taken together, we have shown that Mck1 and Cse4 S10, S16, and T166 residues promote Cse4–Cdc4 interaction and regulate ubiquitin-mediated proteolysis of Cse4 to prevent its mislocalization and CIN. Our studies are clinically relevant given the poor prognosis of CENP-A overexpressing cancers. Hence, defining pathways that prevent mislocalization of CENP-A is an area of active research. Our studies show that mislocalization of overexpressed CENP-A and defects in histone chaperone lead to aneuploidy with karyotypic heterogeneity in human cells and xenograft mouse model (Shrestha *et al.* 2021). Since degrons of many substrates of vertebrate Fbw7 (Cdc4 homolog) are phosphorylated by GSK-3β (Mck1 homolog), it is of interest to examine if GSK-3β prevents mislocalization of CENP-A to noncentromeric regions and how defects in this pathway may contribute to aneuploidy in human cancers.

Data availability

All experimental data sets are available at Figshare (https://doi.org/10.6084/m9.figshare.26049877).

Supplemental material available at GENETICS online.

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Conflicts of interest

The authors declare no conflict of interest.

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