The FACT complex interacts with the E3 ubiquitin ligase Psh1 to prevent ectopic localization of CENP-A

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Centromere identity and its epigenetic maintenance require the incorporation of a histone H3 variant called CENP-A at centromeres. CENP-A mislocalization to ectopic sites may disrupt chromatin-based processes and chromosome segregation, so it is important to uncover the mechanisms by which this variant is exclusively localized to centromeres. Here, we identify a role for the conserved chromatin-modifying complex FACT (facilitates chromatin transcription/transactions) in preventing budding yeast CENP-A\textsuperscript{Cse4} mislocalization to euchromatin by mediating its proteolysis. The Spt16 subunit of the FACT complex binds to Psh1 (Pob3/Spt16/histone), an E3 ubiquitin ligase that targets CENP-A\textsuperscript{Cse4} for degradation. The interaction between Psh1 and Spt16 is critical for both CENP-A\textsuperscript{Cse4} ubiquitylation and its exclusion from euchromatin. We found that Psh1 cannot efficiently ubiquitylate CENP-A\textsuperscript{Cse4} nucleosomes in vitro, suggesting that additional factors must facilitate CENP-A\textsuperscript{Cse4} removal from chromatin in vivo. Consistent with this, a Psh1 mutant that cannot associate with FACT has a reduced interaction with CENP-A\textsuperscript{Cse4} in vivo. Together, our data identify a previously unknown mechanism to maintain centromere identity and genomic stability through the FACT-mediated degradation of ectopically localized CENP-A\textsuperscript{Cse4}.

[Keywords: CENP-A; FACT; Psh1; proteolysis; ubiquitylation; centromere]

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et al. 2004; Hewawasam et al. 2010; Ranjitkar et al. 2010; Moreno-Moreno et al. 2011; Au et al. 2013). In budding yeast, the Psh1 E3 ligase is a critical regulator of CENP-A Cse4 stability (Hewawasam et al. 2010; Ranjitkar et al. 2010). CENP-A Cse4 degradation is impaired in psh1Δ cells, leading to the accumulation of CENP-A Cse4 in euchromatin. Overexpression of CENP-A Cse4 in psh1Δ cells is lethal, possibly due to perturbation of euchromatin-based processes. Psh1 recognizes the CATD (CENP-A targeting domain) of CENP-A, which includes loop 1 and helix 2 of the histone fold domain, to mediate CENP-A degradation, a property that is conserved with the Dro sophila Ppa1 F-box protein that mediates fly CENP-A Cid ubiquitylation (Ranjitkar et al. 2010; Moreno-Moreno et al. 2011). However, structural analysis of CENP-A nucleosomes has revealed that a substantial portion of the CATD is buried within the nucleosome, so it is unclear whether Psh1 can act on CENP-A Cse4 nucleosomes or instead targets a soluble pool (Tachiwana et al. 2011). Indeed, nucleosome structure hinders DNA-based processes, so cells use nucleosome remodeling, histone eviction, and histone tail post-translational modifications to alleviate the repressive nature of chromatin. It is not known whether Psh1 requires any of these processes to promote CENP-A Cse4 degradation.

One important factor that influences chromatin structure is the FACT (facilitates chromatin transcription/transactions) complex (Orphanides et al. 1998; Reinberg and Sims 2006). FACT facilitates many chromatin-based processes, including transcription initiation, transcription elongation, and DNA replication and repair (Belotserkovskaya et al. 2003; Biswas et al. 2005; VanDemark et al. 2006; Abe et al. 2011; Dinant et al. 2013). To promote these functions, FACT generates a more open chromatin configuration via multiple contacts with nucleosomes to allow polymerases to move through chromatin (Belotserkovskaya et al. 2003; Rhoades et al. 2004; Xin et al. 2009; Winkler et al. 2011). FACT also re-establishes chromatin structure in the wake of polymerase passage, thereby maintaining epigenetic states of chromatin (Kaplan et al. 2003; Jamai et al. 2009). Recently, FACT and other transcription elongation factors have been shown to have an indirect role in excluding fission yeast CENP-A Cnp1 from euchromatin by reassembling H3-containing nucleosomes during transcription elongation (Choi et al. 2012).

Here, we uncover a previously unknown role for the FACT complex in mediating proteolysis of the centromeric H3 variant. We found that the incorporation of CENP-A Cse4 into nucleosomes occludes access of CENP-A Cse4 to Psh1-mediated ubiquitylation in vitro, suggesting that additional factors must facilitate its degradation in vivo. Consistent with this, we identified a domain in the E3 ligase Psh1 that directly interacts with the Spt16 FACT subunit and mediates CENP-A Cse4 degradation to prevent its accumulation in euchromatin. Together, these data suggest that FACT destabilizes CENP-A Cse4 nucleosomes to facilitate Psh1-mediated degradation of CENP-A Cse4, revealing a critical role for the conserved FACT complex in maintaining genomic integrity by ensuring that CENP-A Cse4 localizes exclusively to centromeres.

**Results**

**FACT has a role in CENP-A Cse4 degradation**

The budding yeast FACT complex consists of a highly stable and abundant heterodimer of Spt16 and Pob3 (Brewster et al. 1998). Psh1 (Pob3/Spt16/histone) was identified in a coprecipitation experiment with the Pob3 and Spt16 proteins (Krogan et al. 2002), so we tested whether FACT affects CENP-A Cse4 stability. Because SPT16 and POB3 are essential genes, we generated a conditional allele of SPT16 by fusing the C terminus of the endogenous copy to an auxin-inducible degradation signal (AID) (Nishimura et al. 2009). spt16-aid cells grew normally, but their growth was inhibited in the presence of the synthetic auxin hormone 1-naphthaleneacetic acid (NAA) (data not shown). We analyzed the levels of the Spt16-AID protein in the presence and absence of NAA and found that the bulk of Spt16-AID was degraded 60 min after NAA addition (Fig. 1A).

To determine whether FACT has a role in CENP-A Cse4 stability, we transiently overexpressed CENP-A Cse4 after depleting Spt16, inhibited translation with cycloheximide, and monitored CENP-A Cse4 levels. Although CENP-A Cse4 was quickly degraded in control cells, it was stabilized twofold in Spt16-depleted cells (Figs. 1B; Supplemental Fig. S1A). We were unable to generate a viable pob3-aid strain, so the contribution of Pob3 to CENP-A Cse4 degradation could not be analyzed. The FACT complex requires Nhp6, a high-mobility group (HMG) domain-containing protein that binds DNA in a sequence-independent manner, to localize to chromatin (Formosa et al. 2001). We therefore tested whether it also affects CENP-A Cse4 stability. Nhp6 is encoded by a gene duplication, and nhp6Δ nhp6Δ cells (hereafter called nhp6Δ) are viable but exhibit a slow growth phenotype. Surprisingly, CENP-A Cse4 levels are not stabilized in nhp6Δ mutants (Supplemental Fig. S1B), indicating that the canonical method for FACT recruitment to nucleosomes is not involved in CENP-A Cse4 degradation.

We previously found that CENP-A Cse4 overexpression in psh1Δ cells is lethal (Ranjitkar et al. 2010), so we asked whether CENP-A Cse4 overproduction affects the viability of FACT mutants. We analyzed the spt16-22 and pob3- L78R point mutants that are thermosensitive, hydroxyurea-sensitive, and have an spt− phenotype (Schlesinger and Formosa 2000; Formosa et al. 2002). Indeed, spt16-22 and pob3- L78R cells exhibited partial sensitivity to CENP-A Cse4 overexpression (Fig. 1C,D), similar to findings in fission yeast (Choi et al. 2012). However, we were unable to detect a significant change in CENP-A Cse4 stability in these mutants (data not shown), so additional mechanisms must contribute to the underlying synthetic sickness. In contrast, nhp6Δ cells were insensitive to CENP-A Cse4 overexpression (Supplemental Fig. S1C), consistent with the canonical FACT recruitment mechanism not being required for CENP-A Cse4 degradation.

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Psh1-mediated degradation of CENP-A\textsuperscript{Cse4} prevents its accumulation in euchromatin (Hewawasam et al. 2010; Ranjitkar et al. 2010). To determine whether this is also true in cells depleted of Spt16, we overexpressed CENP-A\textsuperscript{Cse4} in spt16-aid cells and analyzed its levels in the soluble and chromatin fractions after inhibiting translation with cycloheximide. CENP-A\textsuperscript{Cse4} incorporation into the chromatin fraction was increased in cells depleted of Spt16, similar to the levels that occur in psh1Δ cells (Figs. 1E; Supplemental Fig. S1D). Because budding yeast CENP-A\textsuperscript{Cse4} is constitutively localized to the 125-base-pair [bp] point centromere, all additional CENP-A\textsuperscript{Cse4} in the chromatin pool must be mislocalized to euchromatin (Biggins 2013). Soluble CENP-A\textsuperscript{Cse4} levels also showed a modest increase in psh1Δ cells (Ranjitkar et al. 2010), and darker exposures of the immunoblots revealed a similar increase in Spt16-depleted cells (data not shown). Together, these data reveal the existence of a novel FACT mechanism independent of Nhp6 that regulates CENP-A\textsuperscript{Cse4} stability and inhibits its euchromatic localization.

The Spt16 subunit of the FACT complex interacts with Psh1

Although FACT could exclude CENP-A\textsuperscript{Cse4} from euchromatin by promoting H3 incorporation (Choi et al. 2012), the association of FACT with Psh1 in immunoprecipitation experiments suggested a more direct role in CENP-A\textsuperscript{Cse4} degradation (Krogan et al. 2002, Ranjitkar et al. 2010). We therefore asked whether Psh1 directly interacts with FACT in vitro. We expressed and purified recombinant FACT subunits (Nhp6, Pob3, and Spt16) that were fused to the maltose-binding protein (MBP) and assayed for binding to immobilized glutathione-S-transferase [GST] or GST-Psh1. We detected an interaction between recombinant GST-Psh1 and MBP-Spt16 [Fig. 2A]. To narrow down the domain in Spt16 that interacts with Psh1, we tested Spt16 fragments in the binding assays. Prior work defined four structured domains in Spt16: an N-terminal domain that binds weakly to H3/H4, a Pob3 dimerization domain, an M domain that also contributes to H3/H4 binding, and a C-terminal domain that binds histone H2A/H2B and can actively displace nucleosomal DNA (Supplemental Fig. S2A; O’Donnell et al. 2004; VanDemark et al. 2006; Stuwe et al. 2008; Kemble et al. 2013). An N-terminal Pob3 fragment has been reported to bind to the dimerization domain (VanDemark et al. 2006), while another study showed that full-length Pob3 binds with highest affinity to a Spt16 construct that contained the dimerization domain and an N-terminal region of the M domain (O’Donnell et al. 2004). In our assays, we found that Pob3 and Psh1 both interact with a large fragment containing the dimerization and M domain [residues 485–804] (Supplemental Fig. S2B, data not shown). Because this fragment may contain an N-terminal unstructured region that could mediate nonspecific interactions (O’Donnell et al. 2004), we tested a smaller fragment lacking this region [residues 643–804] and found that both Psh1 and Pob3 associate with this smaller region in the M domain [Supplemental Fig. S2B].
We were not able to identify distinct binding domains for Pob3 and Psh1 within the M domain of Spt16 [data not shown], so we tested whether Pob3 and Psh1 binding to Spt16 might be mutually exclusive. To do this, we performed tandem affinity purification (TAP) tag isolations of Spt16 and Pob3 and tested whether Psh1 copurifies. Spt16 is not known to form homodimers, so the presence of Psh1 in Pob3-TAP purifications could not be a consequence of dimerization of Spt16/Pob3 with Spt16/Psh1. We found that Psh1 is present in both Pob3 and Spt16 TAP tag purifications, suggesting that it likely associates with the FACT complex [Fig. 2B]. However, because quantitative immunoblotting revealed that Spt16 is at least 30-fold more abundant than Psh1 [Supplemental Fig. S2C; data not shown], Psh1 is bound to only a subset of FACT complexes.

We next identified a region of Psh1 required for Spt16 association. Psh1 contains a catalytic RING domain that mediates ubiquitylation of CENP-A$^{Cse4}$ in the N terminus [residues 29–71], a zinc finger motif [residues 150–171], and a C-terminal region lacking obvious motifs [residues 171–406] [Fig. 2C]. The only region of Psh1 that directly interacted with recombinant Spt16 was the C-terminal region [data not shown]. Consistent with this, a truncation of the C-terminal 106 amino acids of GST-Psh1 (residues 301–406) eliminated the interaction between Psh1 and MBP-Spt16 in vitro [Fig. 2D]. Together, these data indicate that the C terminus of Psh1 is required for the Psh1 interaction with Spt16.

The identification of a region in Psh1 required for Spt16 binding allowed us to ask whether this domain is required for Psh1 activity in vitro. First, we tested whether a Psh1 mutant lacking this domain (hereafter called Psh1$^{ΔC}$) affects CENP-A$^{Cse4}$ binding. Bacterially expressed and purified CENP-A$^{Cse4}$ octamers were used in these assays, but we cannot distinguish Cse4 ubiquitylation from Psh1 autoubiquitylation. Furthermore, Psh1 autoubiquitylation makes the comparison of Psh1 levels in the reactions difficult, so parallel reactions were performed in the absence of the ubiquitin cocktail and 2V5-Cse4 octamers [shown in the three right lanes].

RING domain mutant [C45S, C50S], followed by washing and elution from the resin. The levels of the GST proteins were assessed with α-GST antibodies, and the presence of Cse4 was assessed with α-Cse4 antibodies. [F] The indicated GST-Psh1 proteins and 2V5-Cse4 octamers were added to reactions in the presence (+) or absence (−) of a ubiquitin (Ub) cocktail. The top immunoblot shows Cse4 ubiquitylated species, while Psh1 levels are shown in the bottom immunoblot. The blots were not probed with α-ubiquitin antibodies, since we cannot distinguish Cse4 ubiquitylation from Psh1 autoubiquitylation. Furthermore, Psh1 autoubiquitylation makes the comparison of Psh1 levels in the reactions difficult, so parallel reactions were performed in the absence of the ubiquitin cocktail and 2V5-Cse4 octamers [shown in the three right lanes].
since CENP-A<sup>Cse4</sup> is an unstable protein in bacteria when produced in the absence of the other histones (data not shown). In contrast to Spt16, CENP-A<sup>Cse4</sup> associated normally with GST-Psh1ΔC in vitro (Fig. 2E). Consistent with this, we did not detect an interaction between the C terminus (CT) of Psh1 and CENP-A<sup>Cse4</sup>. However, mutations in the catalytic domain (CD) affected CENP-A<sup>Cse4</sup> binding, as previously shown (Hewawasam et al. 2010). We next tested the catalytic activity of GST-Psh1ΔC toward CENP-A<sup>Cse4</sup> in ubiquitylation assays. CENP-A<sup>Cse4</sup> octamers were incubated in the presence of the requisite E1 and E2 proteins, ubiquitin, ATP, and either GST-Psh1, GST-Psh1ΔC, or a catalytically dead RING mutant, GST-Psh1(CD). When analyzed by immunoblotting, the unmodified CENP-A<sup>Cse4</sup> band predominates in the absence of Psh1, as previously described (Ranjitkar et al. 2010). However, the addition of either GST-Psh1 or GST-Psh1ΔC produced similar levels of ubiquitin-conjugated CENP-A<sup>Cse4</sup> species and a corresponding decrease in unmodified CENP-A<sup>Cse4</sup> (Fig. 2F). Therefore, the GST-Psh1ΔC mutant protein binds to and ubiquitylates CENP-A<sup>Cse4</sup> in vitro as efficiently as full-length GST-Psh1. Together, these data identify a specific region of Psh1 required for association with the Spt16 subunit of FACT that is separable from the domains required for CENP-A<sup>Cse4</sup> binding and ubiquitylation in vitro.

The Psh1 mutant that cannot bind FACT is defective in CENP-A<sup>Cse4</sup> degradation in vivo

We next analyzed the function of the interaction between Spt16 and Psh1 in vivo. We incorporated 13Myc epitope tags after residue 300 of Psh1 (hereafter psh1ΔC-13Myc), to simultaneously truncate the domain required for Spt16 binding and to epitope-tag Psh1. Consistent with our in vitro binding results, Spt16-3Flag coimmunoprecipitated full-length Psh1-13Myc from yeast extracts but failed to associate with Psh1ΔC-13Myc (Fig. 3A). To determine whether FACT association with Psh1 influences CENP-A<sup>Cse4</sup> ubiquitylation in vivo, we analyzed the ubiquitin conjugates of CENP-A<sup>Cse4</sup> isolated from wild-type, psh1Δ, and psh1ΔC-13myc cells. Multiple slower-migrating CENP-A<sup>Cse4</sup> species that correspond to ubiquitin conjugates were detected when CENP-A<sup>Cse4</sup> was overexpressed and isolated from wild-type cells (Figs. 3B, Supplemental Fig. S3; Ranjitkar et al. 2010). As expected, these species were substantially decreased but not eliminated in psh1Δ cells, consistent with additional Psh1-independent pathways contributing to CENP-A<sup>Cse4</sup> ubiquitylation (Collins et al. 2004; Au et al. 2013). Strikingly, CENP-A<sup>Cse4</sup> purified from psh1ΔC-13Myc cells also displayed a significant decrease in CENP-A<sup>Cse4</sup> ubiquitylation (Figs. 3B, Supplemental Fig. S3). To confirm that the decreased CENP-A<sup>Cse4</sup> ubiquitylation in psh1ΔC-13Myc cells affects CENP-A<sup>Cse4</sup> degradation, CENP-A<sup>Cse4</sup> protein levels were monitored after transient overexpression followed by translational repression with cycloheximide. CENP-A<sup>Cse4</sup> was stabilized in psh1ΔC-13Myc cells to an extent similar to psh1Δ cells (Fig. 3C), indicating that the Psh1 C terminus that is required for Spt16 association is also required for CENP-A<sup>Cse4</sup> degradation.

Because CENP-A<sup>Cse4</sup> overexpression is lethal in a psh1Δ mutant, we tested how overexpression affects the...
viability of psh1ΔC cells. PSH1 is a nonessential gene, and psh1ΔC cells grew similarly to both wild-type and psh1Δ cells [Fig. 3D]. However, CENP-A Cse4 overexpression strongly inhibited the growth of psh1ΔC-13Myc cells. Because the Psh1ΔC mutant has normal activity toward CENP-A Cse4 in vitro [Fig. 2], these data reveal a critical role for the C terminus of Psh1 to mediate CENP-A Cse4 degradation in vivo. Although the Psh1ΔC mutant may affect interactions with other factors, the similar phenotypes between this mutant that is defective in Spt16 association with cells that are depleted of Spt16 strongly suggest that FACT binding to Psh1 plays a crucial role in CENP-A Cse4 degradation.

**Figure 4.** CENP-A Cse4 localizes to the euchromatin in psh1ΔC cells. (A) Whole-cell extracts [WCE] from wild-type [WT] (SBY8851), psh1Δ [SBY8903], and psh1ΔC-13Myc [SBY10921] cells expressing pGAL-3Flag-CSE4 were fractionated into soluble and chromatin fractions. Cse4 levels were monitored in each fraction with α-Flag antibodies. Pgk1 and dimethylation of histone H3 at Lys4 [H3K4Me2] are markers of the soluble and chromatin fractions, respectively. (B) 3Flag-Cse4 was transiently overexpressed for 1 h in wild-type [SBY8851], psh1Δ [SBY8903], and psh1ΔC-13Myc [SBY10921] cells, and its localization to chromatin was analyzed by immunofluorescence on chromosome spreads. DAPI staining was used to visualize DNA, while α-Flag staining revealed Cse4 localization. (C) Quantification of the percentage of chromatin masses in which Cse4 staining was coincident with total DNA staining instead of with kinetochores that contain discrete Cse4 foci. Wild type [SBY8851], n = 132, psh1Δ [SBY8903], n = 137, psh1ΔC-13Myc [SBY10921], n = 130.

**Nucleosome structure is a barrier to CENP-A Cse4 ubiquitylation by Psh1**

Because our data indicated a role for FACT in mediating CENP-A Cse4 degradation, we considered the possibility that its role in nucleosome disassembly might facilitate Psh1 degradation by destabilizing CENP-A Cse4 from chromatin. Consistent with this, Psh1 interacts with CENP-A Cse4 via CATD residues that exist in the histone fold domain, and much of the CATD is occluded within the nucleosome [Tachiwana et al. 2011]. Our in vitro ubiquitylation assays were performed with CENP-A Cse4 octamers, which presumably behave like H3 octamers and dissociate into a CENP-A Cse4/H4 tetramer and two H2A/H2B dimers in the low-salt buffers used for the ubiquitylation reaction [Eickbush and Moudrianakis 1978]. We therefore tested whether CENP-A Cse4 nucleosomes, which have a more tightly associated histone core that is stabilized by nucleosome-wrapped DNA, can serve as a substrate for Psh1.

We generated two species of CENP-A Cse4 nucleosomes in vitro [Fig. 5A]. Mononucleosomes were assembled by the traditional step-salt gradient dialysis of recombinant histones and a 146-bp DNA sequence containing a strong nucleosome-positioning sequence as described [Kingston et al. 2011]. In addition, polynucleosomes were made by incubation of histones and a 146-bp DNA sequence containing a strong nucleosome-positioning sequence as described [Kingston et al. 2011]. In addition, polynucleosomes were made by incubation of histones and a 146-bp DNA sequence containing a strong nucleosome-positioning sequence as described [Kingston et al. 2011]. In addition, polynucleosomes were made by incubation of histones and a 146-bp DNA sequence containing a strong nucleosome-positioning sequence as described [Kingston et al. 2011].
ubiquitylation of lysines in the N-terminal tail of CENP-A^Cse4 (Au et al. 2013). To ensure that the decrease in activity was not due to direct inhibition of Psh1 by DNA, we added the DNA used to assemble the mononucleosomes to the ubiquitylation assays containing CENP-A^Cse4 octamers. The ubiquitylation of CENP-A^Cse4 was unaltered, indicating that Psh1 ubiquitin ligase activity is unaffected by the presence of DNA (Fig. 5C). Together, these results reveal that nucleosome structure is a barrier to CENP-A^Cse4 ubiquitylation by Psh1 in vitro.

FACT facilitates the interaction between Psh1 and CENP-A^Cse4

Because FACT is known to facilitate chromatin-based processes by destabilizing nucleosomes, we speculated that it might help expose CENP-A^Cse4 nucleosomes to Psh1 to promote ubiquitylation. We therefore tested whether the addition of FACT enhanced the ubiquitylation of CENP-A^Cse4 in vitro. However, FACT inhibited the in vitro ubiquitylation reactions containing either Psh1 or Psh1ΔC [Supplemental Fig. S5A]. Because we do not know the underlying reason for the inhibition, we could not further test whether FACT facilitates the ubiquitylation of CENP-A^Cse4 incorporated into nucleosomes.

If FACT releases CENP-A^Cse4 from nucleosomes to promote an interaction with Psh1, we reasoned that FACT might enhance the association of Psh1 with CENP-A^Cse4 in vivo. To test this, 3Flag-CENP-A^Cse4 was immunoprecipitated from whole-cell extracts, and the amount of coprecipitating full-length Psh1-13Myc or Psh1ΔC-13Myc was analyzed. Indeed, the interaction of CENP-A^Cse4 with Psh1ΔC-13Myc was strongly reduced compared with full-length Psh1 (Fig. 6A). To determine whether a specific pool of CENP-A^Cse4 was defective in binding to Psh1ΔC-13Myc, we immunoprecipitated overexpressed 3Flag-CENP-A^Cse4 from the soluble and chromatin fractions in cells expressing full-length Psh1-13Myc or Psh1ΔC-13Myc. Although the Psh1ΔC-13Myc protein localized to chromatin normally, it had impaired binding to CENP-A^Cse4 in both the soluble and chromatin fractions [Fig. 6B]. These data are consistent with the idea that FACT promotes an interaction between Psh1 and CENP-A^Cse4 that facilitates CENP-A^Cse4 degradation.

Discussion

Here we identify a direct physical interaction between Psh1, an E3 ubiquitin ligase that targets CENP-A^Cse4 for degradation, and the Spt16 subunit of the FACT complex. Cells depleted of Spt16 stabilize CENP-A^Cse4, which leads to CENP-A^Cse4 accumulation in the euchromatin. Similarly, a psh1 mutant that has perturbed Spt16 association cannot efficiently degrade CENP-A^Cse4 or prevent its localization to euchromatin in vivo despite its efficient activity against CENP-A^Cse4 in vitro. We found that chromatin structure inhibits CENP-A^Cse4 ubiquitylation by Psh1 in vitro, and FACT potentiates the interaction between Psh1 and CENP-A^Cse4 in vivo. We therefore propose that the nucleosome-reorganizing activity of FACT is a key factor that promotes the Psh1-mediated ubiquitylation of CENP-A^Cse4 in vivo at least in part by increasing the association of Psh1 with CENP-A^Cse4.

FACT may have multiple functions in regulating CENP-A chromatin. Although our data identify a role for FACT in preventing ectopic localization of CENP-A^Cse4 via an association with the CENP-A^Cse4 degradation machinery, FACT has also been implicated in CENP-A deposition at centromeres. Human FACT copurifies with inner kinetochore proteins that associate with centromeres and has been postulated to be involved in CENP-A deposition [Walfridsson et al. 2005; Foltz et al. 2006; Okada et al. 2009]. However, the conserved histone chaperone HJURP/Scm3 appears to be the key deposition factor both in vivo and in vitro [Camahort et al. 2007; Foltz et al. 2009]. FACT might facilitate CENP-A de-
position and kinetochore assembly indirectly through its role in forming pericentromeric heterochromatin in organisms with regional centromeres (Lejeune et al. 2007). FACT has also been implicated in recruiting CHD1, an ATP-dependent chromatin remodeler that influences histone H4 deacetylation, to centromeres to promote CENP-A deposition (Walfridsson et al. 2005; Okada et al. 2009). However, Chd1 is absent from budding yeast centromeres (Zentner et al. 2013), and it is unknown whether FACT influences CENP-ACse4 deposition in this organism.

Our work identifies a previously unknown role for FACT in facilitating the degradation of the centromeric H3 variant. Although most of our experiments focused on the Spt16 protein, a pob3 mutant was also sensitive to increased CENP-A^Cse4 dosage and Psh1 coprecipitated with Pob3. While it is formally possible that Psh1 interacts with a pool of Spt16 that does not include Pob3, the simplest interpretation is that the FACT complex mediates CENP-A^Cse4 degradation. FACT defects were previously associated with CENP-A^Cnp1 misincorporation into euchromatin (Choi et al. 2012), consistent with evidence that maintaining the integrity of H3 chromatin via FACT and other elongation factors provides a crucial barrier to CENP-A misincorporation. Here, we found that FACT also has a more direct role in controlling CENP-A chromatin association by promoting its proteolysis to prevent ectopic localization.

The role of FACT in CENP-A^Cse4 degradation may be dependent on events that occur during transcription. Transcription elongation factors, including Spt16 and Spt6, have been shown to inhibit CENP-A^Cnp1 incorporation into euchromatin (Choi et al. 2012), and CENP-A^Cse4 stability is partially dependent on RNA polymerase II [Pol II] transcription [Lopes da Rosa et al. 2011]. An attractive possibility is that FACT’s role in re-establishing H3-containing chromatin during transcription is coupled to its role in CENP-A^Cse4 degradation to ensure the exclusion of CENP-A^Cse4 from euchromatin. However, FACT may also have a role in mediating CENP-A^Cse4 degradation independent of transcription, since FACT can regulate chromatin disassembly at promoter

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**Figure 6.** The Psh1 mutant that is defective in binding to FACT has reduced association with CENP-A^Cse4 in vivo. (A) α-Flag-conjugated beads were used to immunoprecipitate 3Flag-Cse4 from cells expressing 13Myc-tagged full-length [FL] (SBY11102) or the C-terminal mutant [ΔC] Psh1-13Myc (SBY11104). Cells expressing full-length Psh1-13Myc but not 3Flag-Cse4 (SBY6423) served as a control. The immunoblots were probed with α-Flag and α-Myc antibodies to detect the levels of Cse4 and Psh1, respectively. (B) pGAL-3Flag-CSE4 was overexpressed with galactose in cells expressing 13Myc-tagged full-length [FL] or the C-terminal mutant [ΔC] Psh1-13Myc (SBY11090 and SBY10920). Cells expressing full-length Psh1-13Myc but not 3Flag-Cse4 (SBY6423) served as a control. Whole-cell extracts [WCE] were fractionated into soluble and chromatin fractions. α-Flag-conjugated beads were used to immunoprecipitate 3Flag-Cse4 from the soluble and chromatin fractions. The immunoblots were probed with α-Flag and α-Myc antibodies to detect the levels of Cse4 and Psh1, respectively. Pgk1 and H3K4me2 are markers of the soluble and chromatin fractions, respectively. (C) In this model, Cse4 mis-localizes to the euchromatin, where it is protected from Psh1-dependent ubiquitylation due to the repressive nature of chromatin structure on trans-acting factors. In order to override this accessibility barrier, Cse4 nucleosomes must be restructured so that Psh1 can bind and ubiquitylate Cse4. The FACT complex plays a crucial role in this process by increasing the association of Psh1 with Cse4. In part a, FACT reorganizes nucleosome structure (denoted by the black and white gradient) to facilitate the interaction and subsequent ubiquitylation of Cse4 by Psh1. In part b, FACT activity might release Cse4 from nucleosomes to create a soluble pool of Cse4 that is competent in interacting with Psh1. In either scenario, FACT exposes critical Cse4 residues to which Psh1 binds, ultimately leading to Cse4 ubiquitylation, degradation, and elimination from euchromatin.
regions outside of transcriptional activation of the downstream gene [Takahata et al. 2009]. Moreover, CENP-A mislocalizes to promoter regions that have high histone turnover [Krassovsky et al. 2012; Lacoste et al. 2014], and FACT can evict histones at promoters [Ransom et al. 2009; Takahata et al. 2009]. In the future, it will be important to determine whether polymerases or other factors exist in the Psh1-associated FACT complex to couple CENP-A<sup>Cse4</sup> degradation to chromatin-based processes.

We found that the mechanism by which FACT facilitates CENP-A<sup>Cse4</sup> degradation is independent of Nhp6, the protein known to mediate FACT association with canonical nucleosomes [Formosa et al. 2001]. Nhp6 constitutes an initial step in FACT-dependent nucleosome reorganization that involves DNA bending and altered histone–DNA contacts to allow FACT to substantively change nucleosome structure [Xi et al. 2009]. Recent data suggest that DNA topology at the nucleosomal entry/exit site might influence FACT activity on nucleosomes [Hondele et al. 2013]. Reconstituted CENP-A<sup>Cse4</sup> nucleosomes have weakened DNA–histone contacts at the DNA entry/exit site compared with H3 nucleosomes [Dechassa et al. 2011; Tachiwana et al. 2011], which may generate an open chromatin configuration that allows FACT to function on CENP-A<sup>Cse4</sup> nucleosomes independent of Nhp6. Psh1 has a pair of C4-type zinc finger motifs that are found in many DNA-binding proteins, so it may bind to CENP-A<sup>Cse4</sup> nucleosomes to recruit FACT.

FACT is required for the association of Psh1 with both the soluble and chromatin pools of CENP-A<sup>Cse4</sup> in vivo, although it is not required for Psh1 and CENP-A<sup>Cse4</sup> to interact in vitro. We speculate that the altered interaction in the chromatin reflects a requirement for FACT to expose CENP-A<sup>Cse4</sup> when it is incorporated into nucleosomes to the Psh1 ligase in vivo. The decreased interaction of Psh1 with CENP-A<sup>Cse4</sup> in the soluble pool may also be a result of the decreased chromatin interaction in the absence of FACT. Alternatively, Psh1 may prevent the soluble FACT complex from binding to CENP-A<sup>Cse4</sup> to prevent FACT from misincorporating CENP-A<sup>Cse4</sup> into euchromatin.

Taken together, our data lead to the following model (Fig. 6C). CENP-A<sup>Cse4</sup> overexpression leads to its misincorporation into euchromatin [Camahort et al. 2009; Lefrancois et al. 2009; Krassovsky et al. 2012; Lacoste et al. 2014]. Psh1 must recognize and target the mislocalized CENP-A<sup>Cse4</sup> for degradation, but we found that CENP-A<sup>Cse4</sup> nucleosomes are refractory to its activity. The FACT complex destabilizes nucleosomes and interacts with Psh1, so we propose that FACT exposes CENP-A<sup>Cse4</sup> for Psh1 association and subsequent degradation in vivo. Psh1 can still associate with chromatin when it does not interact with FACT, so Psh1 may help FACT associate with chromatin. CENP-A<sup>Cse4</sup> localized to the centromere is protected from proteolysis [Collins et al. 2004], and this may be because the histone chaperone Scm3 binds to the CATD of CENP-A<sup>Cse4</sup> at the centromere [Zhou et al. 2011]. However, we also found that FACT inhibited Psh1 activity in vitro, so it may also have a role in protecting CENP-A<sup>Cse4</sup> from degradation at the centromere. Additional mechanisms might also inhibit Psh1 at the centromere. For example, CENP-A<sup>Cse4</sup> proline isomerization is required for its interaction with Psh1, so this activity may be inactive at centromeres [Ohkuni et al. 2014]. Together, our work identifies a previously unknown function for the conserved FACT complex in maintaining euchromatin integrity through degradation of the CENP-A<sup>Cse4</sup> histone variant. In the future, it will be critical to further understand the mechanisms that promote FACT activity on CENP-A<sup>Cse4</sup> nucleosomes to maintain genomic stability.

Materials and methods

Strain construction and microbial techniques

Microbial techniques and media were as described [Sherman et al. 1974; Rose et al. 1990]. Yeast strains were constructed using standard genetic techniques. PCR integration was used to truncate the C terminus of PSH1 with 13myc [Longtine et al. 1998]. All oligonucleotide sequences are available on request. Specific plasmid construction and yeast strains used in this study are described in the Supplemental Material and Supplemental Tables S1 and S2. For Spt16-AID degradation experiments, NAA [dissolved in 95% ethanol] was added to a final concentration of 500 μM. For galactose induction experiments, cells were grown in YEP + lactic acid medium to mid-log phase, and galactose was added to a final concentration of 2%. For nocodazole arrest, nocodazole was added to a final concentration of 75 μg/mL.

Stability assays

Cells were grown in YEP + lactic acid until mid-log phase, followed by a 1-h 3Flag-Cse4 induction with 2% galactose. Glucose was added to a final concentration of 2% to inhibit further 3Flag-Cse4 transcription, and cycloheximide was added to a final concentration of 50 μg/mL. Time point 0 was taken immediately, followed by sample collection at the indicated time points. Extracts were prepared and analyzed as described in the Supplemental Material.

Ubiquitylation assays

Ubiquitylation assays were performed as described [Ranjitkar et al. 2010] with the following slight modifications. Twenty-microliter reactions containing 150 ng of E1 enzyme [human Uba1; catalog no. E-305, Boston Biochemicals], 115 ng of E2 enzyme [human UbcH5a; catalog no. E-616, Boston Biochemicals], 100 ng of purified GST-Psh1 (or C45S, C50S RING mutant), ~250 ng of Cse4 octamers, 2.5 μg of ubiquitin, 2 mM Mg-ATP, 50 mM Tris-HCl (pH 7.5), 2.5 mM MgCl2, and 0.5 mM DTT were incubated for 15–30 min at 23°C. Reactions were stopped by adding sample buffer and boiling for 4 min. Ubiquitin and Mg-ATP were obtained from Boston Biochemicals. Cse4 ubiquitin conjugates were detected by immunoblotting with anti-V5 or anti-Cse4 antibodies (gift from Carl Wu, National Institutes of Health) as indicated.

For ubiquitylation assays with Cse4 nucleosomes, ~250 ng of nucleosomal template (mononucleosomes or polynucleosomes) conjugated to streptavidin–Dynabeads was washed twice with TE (10 mM Tris-HCl at pH 8.0, 1 mM EDTA) resuspended in TE, and added to the ubiquitylation reactions. For ubiquitylation
assays with octamers and exogenously added DNA, an equal amount of biotinylated 146-bp DNA (purified as described in the Supplemental Material) to Cse4 octamer was added to the reactions.

### Immunofluorescence

Cells were grown in YEP + lactic acid until mid-log phase, followed by nocodazole arrest for 3 h. For the last hour of arrest, 3Flag-Cse4 was induced with 2% galactose. Chromosome spreads were performed as described previously (Collins et al. 2004). DAPI (4',6-diamidino-2-phenylindole; Molecular Probes) was used at a 1 μg/mL final concentration, and LipsoL was obtained from Fisher. Anti-Flag M2 antibodies were used at 1:1000 dilution, and Cy3 secondary antibodies (Jackson Immunoresearch) were used at a 1:1000 dilution.

### Chromatin fractionation assays

Chromatin fractionations were performed similar to Keogh et al. (2006) with modifications described in the Supplemental Material.

### Protein techniques and Cse4 nucleosome assembly

All protein techniques (purifications, immunoprecipitations, and immunoblotting) as well as Cse4 nucleosome generation are described in the Supplemental Material.

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