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Chapter

E3 Ligase for CENP-A (Part 1)

Yohei Niikura and Katsumi Kitagawa

Abstract

CENP-A is a centromere-specific histone H3 variant that is required to ensure kinetochore assembly for proper chromosome segregation and its function is highly conserved among different species including budding yeast, *Saccharomyces cerevisiae*. The budding yeast *Saccharomyces cerevisiae* has genetically defined point centromeres, unlike other eukaryotes. Although, most eukaryotic centromeres are maintained epigenetically, currently only budding yeast *S. cerevisiae* centromeres are known to be genetically specified by DNA sequence, The small size and sequence specificity of the budding yeast centromere has made yeast a powerful organism for its study in many aspects. Many post-translational modifications (PTMs) of CENP-A and their functions have been recently reported, and studies with budding yeast are providing insights into the role of CENP-A/Cse4 PTMs in kinetochore structure and function. Multiple functions are controlled especially by ubiquitylation and sumoylation by E3 ligases that control CENP-A protein has initially emerged in the budding yeast as an important regulatory mechanism. Here we focus on what is known about the budding yeast E3 ligases for CENP-A/Cse4 ubiquitylation and sumoylation and their biological functions and significance.

Keywords: CENP-A, Cse4, Cnp1, E3 ligase, centromere, kinetochore, ubiquitylation, sumoylation, epigenetics, Psh1, Siz1 and Siz2, Slx5 and Slx8, CUL3/RDX, SCF, APC, CUL4A/RBX1/COPS8, DAXX (fruit fly DLP), Scm3, CAF-1 complex, CAL1, HJURP, Mis18 (human Mis18α and Mis18β) and Mis16 (human RbAp46 and RbAp48)

1. Introduction

The mechanistic process to establish centromeric chromatin of budding yeast and its structures have been actively studied [1–3]. In contrast to most eukaryotic centromeres that span megabases of DNA, in the budding yeast, *Saccharomyces cerevisiae* point centromeres are comprised of ~125 bp of DNA and are conserved among all 16 chromosomes [3]. There are three conserved centromere-determining elements (CDE) consisting of CDEI-III [1–3]. Although, most eukaryotic centromeres are maintained epigenetically, currently only budding yeast *S. cerevisiae* centromeres are known to be genetically specified by DNA sequence. The CDEIII consensus (TGTTT(T/A) TGNTTTCCGAAANNNAAAAA) binds to the CBF3 complex via a conserved CCG motif that is essential for centromere function, and the small size and sequence specificity of the budding yeast centromere has made yeast a powerful organism for its study in many aspects [1].

In *S. cerevisiae*, all pre-existing CENP-A[Cse4] is replaced by newly synthesized CENP-A[Cse4] during the S phase [4]. Centromeric assembly of CenH3 requires the adaptor protein, suppressor of chromosome mis-segregation (Scm3) in budding and fission yeasts [5–9], as well as the Holliday junction recognition protein (HJURP) in humans [10, 11]. Scm3/HJURP directly interacts with CenH3 and is essential for the assembly and maintenance of a functional kinetochore [5–12]. Scm3 recognizes CENP-A[Cse4] through the centromere-targeting domain (CATD) in the histone fold and mediates its incorporation into chromatin in vivo and in vitro [1].

Early studies showed that Scm3 is required for G2/M progression and Cse4 localization at centromeres. Scm3 contains 2 essential protein domains: a Leu-rich nuclear export signals and a heptad repeat domain that is widely conserved in fungi [5–11]. Localization of Cse4 to centromeres and the assembly activity is dependent on an evolutionarily conserved central core motif in Scm3 [13]. Camahort et al. showed that Scm3 is required throughout the whole cell cycle as well as the loading period for Cse4 [5, 14]. Consistent with these findings, Xiao et al. showed that Scm3 has an N-terminal nonspecific DNA binding domain for AT-rich DNA and a central histone chaperone domain (Cse4/H4 binding domain, CBD) that promotes specific loading of Cse4/H4 [15]. Moreover, Xiao et al. demonstrated that Scm3-GFP is enriched at centromeres in all cell cycle phases in live cells, and their results of ChIP analysis showed that Scm3 occupies centromere DNA throughout the cell cycle, even when Cse4 and H4 are temporarily dislodged in the S phase, suggesting Scm3 is a critical factor for recruitment of Cse4/H4 as well as maintenance of an H2A/H2B-deficient centromeric nucleosome [15]. Luconi et al. showed that Scm3 signals are present at centromeres when metaphase begins, and enriched in anaphase [14, 16] as observed for Scm3 in fission yeast *S. pombe* [7, 14]. However, HJURP is recruited to centromeres during early G1 [10, 11] (see also next chapter, section (4.1)).

Currently, the structure of budding yeast centromeric CENP-A[Cse4]-containing nucleosomes remains controversial among different research groups as in other species [17]. Dechassa et al. performed structural analysis and showed that the substitution of H3 with Cse4 results in octameric nucleosomes that organize DNA in a left-handed superhelix [18]. Cse4-nucleosomes exhibit an open conformation with weakly bound terminal DNA segments and do not preferentially form nucleosomes on its cognate centromeric DNA. The Cse4-specific octameric nucleosomes do not contain Scm3 as a stably bound component. Cho et al. reported the structure of a complex formed by an N-terminal fragment of Scm3 with the histone-fold domains of Cse4, and H4, which were all purified from the budding yeast *Kluyveromyces lactis* [19]. They described the structure of a (Cse4: H4)(2) heterotetramer; comparison with the structure of the Scm3:Cse4:H4 complex shows that tetramer formation and DNA-binding require displacement of Scm3 from the nucleosome core. Previously published structures of the Scm3 histone complex demonstrated that Scm3 binds only one copy of Cse4–H4 [20]. Dechassa et al. further showed that Scm3 deposits Cse4–H4 through a dimer intermediate onto DNA to form a (Cse4–H4)2–DNA complex (tetrasome) [20] (**Figure 1**, right). Recently, the budding yeast Yta7[ATAD2] (the homolog of AAA[+] ATPase and bromodomain factor ATAD2/ANCCA, which is overexpressed in many types of cancers) was shown to collaborate with Scm3 to deposit Cse4 at the centromere [25].

Recently, the importance of centromeric long non-coding RNA (cenRNA) for centromere integrity has been suggested in various species [35–37] including budding yeast [38–40]. Ling et al. reported that all the budding yeast centromere express long noncoding RNAs (cenRNAs), especially in S phase and induction of cenRNAs
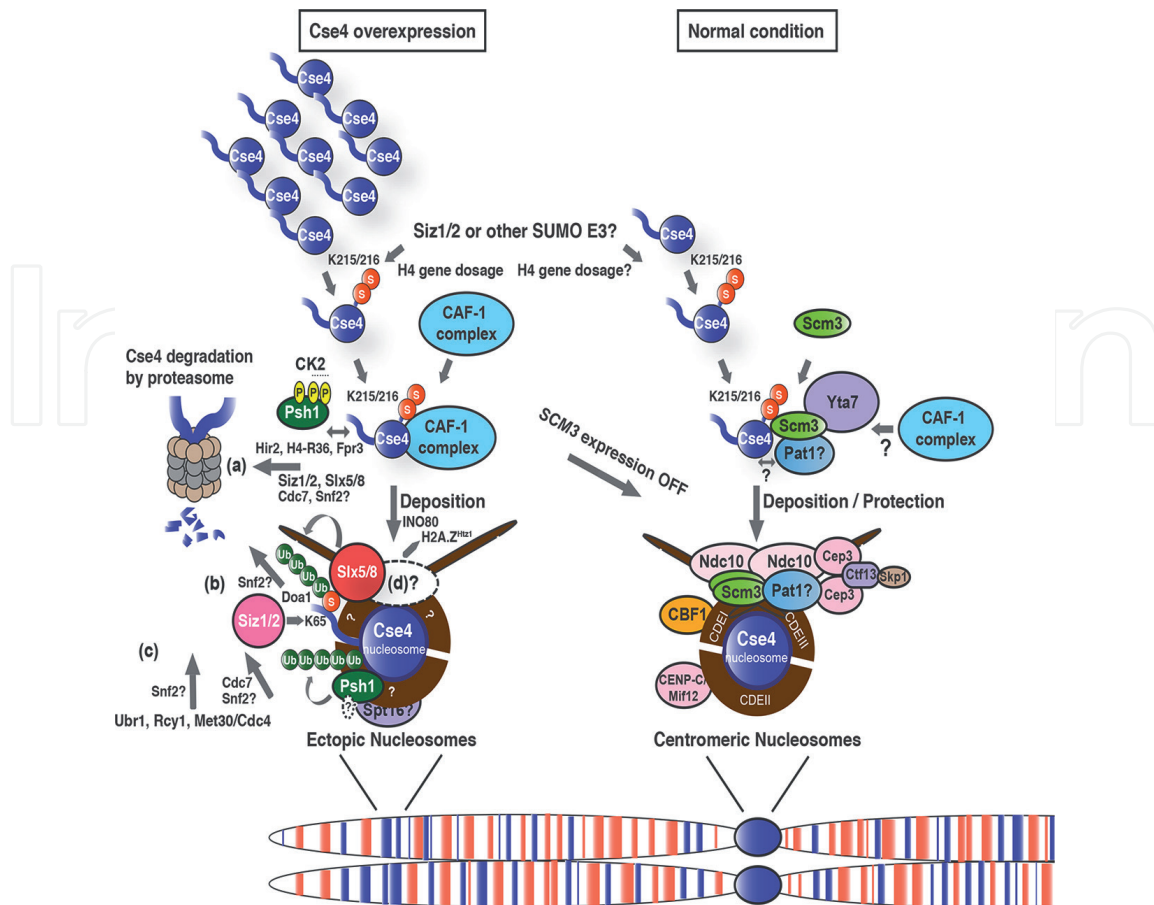
Figure 1.
*Mechanistic scheme for Saccharomyces Cerevisiae CENP-A^Cse4 pathways. (Right) In normal conditions, sumoylation of Cse4 K215/216 facilitates deposition into chromatin [21]. Centromeric Cse4 is protected by Scm3 from Psh1-mediated degradation [22, 23]. Interaction of Pat1 with Scm3 is required for its maintenance at kinetochores and Pat1 affects the structure of CEN chromatin and protects Cse4 from Psh1-mediated ubiquitylation [24]. Yta7 cooperates with Scm3 to deposit Cse4 at the centromere [25]. Note that histone H4 including one in Cse4–H4 that binds one copy of Scm3 [20] is omitted for simplicity. (Right-center) The functional role of Cse4 K215/216 sumoylation is distinct from that of Cse4 K65 sumoylation [21], although it is not yet clear if Siz1/Siz2 also target Cse4 C-terminal K215/216 as Cse4 N-terminal K65 (see also left) or a different unknown SUMO E3 target Cse4 K215/216. (Center) The interaction of sumoylated Cse4 K215/216 with CAF-1 promotes centromeric localization of overexpressed Cse4 only under conditions when Scm3 is depleted (SCM3 expression OFF) [26]. However, CAF-1 function in normal conditions is not clear, although CAF-1 promotes ubiquitylation of free Cse4, opposite to the effect of Scm3 (see also left and right). (Left-center) The histone H4 gene dosage promotes Cse4 sumoylation and mislocalization to noncentromeric regions [27], but its effect on Cse4 K215/216 sumoylation [21] to facilitate the deposition of overexpressed (or endogenous) Cse4 into CEN is not clear. (Left) When Cse4 is overexpressed, Psh1 promotes the degradation of free and ectopically incorporated Cse4. Ohkuni et al. suggested two independent pathways prevent the stable incorporation of Cse4 into non-centromeric chromatin [28]. (i) The first pathway depends on the interaction of Psh1 with Cse4. (ii) The second pathway requires Cse4 K65 sumoylation by Siz1/Siz2 and subsequent Cse4 K65-ubiquitination by Slx5. Both pathways contribute to (a) regulate soluble pools of Cse4 to prevent its mislocalization and/or (b) facilitate proteolysis of non-centromeric chromatin-bound Cse4. The Cse4 K65 sumoylation occurs downstream of Cse4 K215/ 216 sumoylation, i.e., after Cse4 is incorporated into chromatin [21]. Psh1 is phosphorylated by the Cka2 subunit of casein kinase 2 (CK2) to promote its E3 activity for Cse4, and Cse4 misincorporation is prevented by the intact Psh1-Cse4 association [29], but the status of Psh1's post-translational modifications, including the phosphorylation in ectopic Cse4-nucleosomes, is not yet elucidated. Hir2 [30], histone H4-R36 [31], and structural change in Cse4 by Fpr3 [32] might be important for the interaction between Cse4 and Psh1. (c) Ubr1, Rcy1, and Met30/Cdc4 E3 ligases could be in a Psh1-independent proteolysis pathway [33, 34], but the mechanistic details are unknown. (d) Factors/components that stabilize ectopically incorporated Cse4 are not yet clear. Preceding post-translational modifications before ubiquitylation or sumoylation of Cse4 and other proposed factors relevant to Psh1 function (e.g., Snf2, Doa1, Spt16, Pat1, Hir2, Cse4 MIMAS motif, Cdc7, Ubp8 [SAGA-DUB; not shown in this cartoon], etc.) and multiple E3s (Psh1, Ubr1, Rcy1, and Met30/Cdc4) are summarized in **Table 1**. While there are three conserved centromere-determining elements (CDE) consisting of CDEI-III [1–3], DNA sequence elements required for non-centromeric Cse4 nucleosome or its presence itself is unclear. Histone H4, including one in Cse4–H4 that binds one copy of Scm3 [20], is omitted for simplicity. This figure is partly adapted from Ohkuni et al. [21, 28].*

coincides with Cse4 loading time and is dependent on DNA replication [38]. The cenRNA is tightly regulated and repressed by the kinetochore protein Cbf1 and histone H2A variant H2A.Z^{Htz1}, and de-repressed during the S phase of the cell cycle, suggesting that an appropriate level of cenRNAs is essential for point centromere activity [38]. Interestingly, when they knocked down all cenRNAs from the endogenous chromosomes, but not the cenRNA from the circular minichromosome, they still observed an increase in minichromosome loss, suggesting that cenRNA functions in trans to regulate centromere activity. Chen et al. independently demonstrated that budding yeast cenRNA is negatively regulated by Cbf1 and binding of the Pif1 DNA helicase to the centromeres, which happens in mid–late S phase, occurred at about the same time as Cbf1 loss from the centromere [40]. These data suggest that Pif1 may facilitate this loss by its known ability to displace proteins from DNA. Ling et al. further showed that budding yeast cenRNAs are cryptic unstable transcripts (CUTs) that can be degraded by the nuclear RNA decay pathway suggesting that cenRNA can serve important cellular functions when it exists at the right time with the right level [39]. Together, these results in budding yeast indicate that the regulation of cenRNA is an essential factor for centromere structure and function.

1.1 Identification of Psh1 E3 ligase and its function

CENP-A (CenH3) proteolysis has also been reported in senescent human cells [41] or upon infection with herpes simplex virus 1 [42]. However, little had been known about the actual mechanisms that regulate CENP-A (CenH3) proteolysis. Collins et al. initially reported that the levels of the budding yeast CenH3, Cse4, are regulated by ubiquitin-proteasome-mediated proteolysis in 2004 [43]. They isolated a dominant lethal mutant, *CSE4-351*, and showed that the Cse4-351 mutant protein is stable and localized to euchromatin, suggesting that proteolysis prevents Cse4 euchromatic localization. They also constructed wild-type Cse4 fused to a degron signal, and showed that the soluble Cse4 protein was rapidly degraded, but the centromere-bound Cse4 was stable. These data indicate that centromere localization protects Cse4 from degradation. In 2010, two groups reported that budding yeast (*Saccharomyces cerevisiae*) CENP-A homolog, Cse4, is ubiquitylated by an E3 ubiquitin ligase called Psh1 (named for Pob3/Spt16/ histone associated [44]) [22, 45] (**Figure 1** and **Table 1**). Both groups identified independently that Psh1 leads to the degradation of Cse4 controlling the cellular level of Cse4 via ubiquitylation and proteolysis.

Hewawasam et al. performed TAP purification of Psh1 and identified Cse4 as well as several other kinetochore proteins by multidimensional protein identification technology analysis [22]. They described that Psh1 consists of three main domains: (i) a RING finger, (ii) a zinc finger, and (iii) a highly acidic domain [22, 23]. They performed co-immunoprecipitation using whole-cell extracts and showed that the RING finger of Psh1 is important to interact with Cse4. They also performed a pulse-chase assay and demonstrated that both RING and zinc fingers are critical for efficient control of Cse4 levels. They demonstrated the specificity of the ubiquitylation activity of Psh1 toward Cse4 in vitro and identified the sites of ubiquitylation. Mutation of these lysine sites prevents ubiquitylation of Cse4 by Psh1 in vitro and stabilizes Cse4 in vivo. Elimination of the Cse4-specific chaperone Scm3 destabilizes Cse4, and the addition of Scm3 to the Psh1-Cse4 ubiquitylation reaction prevents Cse4 ubiquitylation. Meanwhile, the deletion of Psh1 stabilizes Cse4. These data suggest that Scm3 and Psh1 might compete

lo	CENP-A homolog	E3 ligase (ubiquitylation or sumoylation)	Function	Preceding PTMs before ubiquitylation or sumoylation	Other proposed factor relevant to E3 function
Saccharomyces cerevisiae	Cse4	Psh1 (ubiquitylation)	Proteasomal degradation to remove non-centromeric CENP-A	P134 isomerization by Fpr3	Scm3, Snf2, Doa1, Fpr3, Spt16. Phosphorylation of Psh1 by Cka2, Pat1, Histone H4-R36, Gene dosage of histone H4 (HHF1 and HHF2), CAF-1, Hir2, Cdc7, Ubp8 (deubiquitylation, SAGA-DUB), Cse4 MIMAS motif
		Slx5/8 (vertebrate RNF4) (ubiquitylation)	Proteasomal degradation to remove non-centromeric CENP-A (Slx5-mediated Cse4 proteolysis could be independent of Psh1)	K65 sumoylation by Siz1/2	K65 sumoylation by Siz1/2
		Siz1/2 (sumoylation)	Proteasomal degradation to remove non-centromeric CENP-A	N.D.	N.D. (The effect of SUMO-proteases Ulp2/SENP6, on CenH3 was not confirmed.)
		Ubr1, Rcy1 (ubiquitylation)	Proteasomal degradation of Cse4	N.D.	N.D.
		Met30/Cdc4 (ubiquitylation)	Proteasomal degradation of Cse4 (Met30/Cdc4-mediated Cse4 proteolysis could be independent of Psh1)	N.D.	N.D.

Table 1.
E3 ligase for budding yeast (Saccharomyces Cerevisiae) CENP-A^Cse4.

for binding to Cse4. Cse4 that is not associated with Scm3 may be targeted by Psh1 for proteolysis, but Cse4 in a complex with Scm3 may be protected [23] (**Figure 1**, right) (see also next chapter, section 4.2). Cse4 overexpression is toxic without Psh1, and Cse4 is found at ectopic locations. Therefore, they suggested that the E3 activity of Psh1 prevents the mislocalization of Cse4 (**Figure 1**, left).

Ranjitkar et al. also identified Psh1 by mass spectrometry analysis after purification of 3xFLAG-Cse4^16R that is not ubiquitylated in vivo [45]. They demonstrated that Cse4 overexpression causes growth defects on *psh1*-deleted (*psh1Δ*) cells and

results in euchromatic localization of overexpressed Cse4. In immunoprecipitation analysis, they detected that full-length Cse4 and the histone fold domains (HFD)-Cse4 associate with Psh1, but the N-terminal domains (NTD)-Cse4 does not interact with Psh1. However, greater levels of full-length Cse4 associated with Psh1 compared to HFD-Cse4 were observed. These data suggest that the Cse4 N-terminus might contribute to the interaction of Cse4 with Psh1 in vivo. Because the CATD is critical for Psh1 binding to Cse4, they analyzed the stability of the chimeric proteins. Myc-Cse4-CATD levels in wild-type and *psh1* mutant cells after repressing transcription and translation were assessed. The degradation of H3^{CATD} was dependent on Psh1 in contrast to the Cse4 chimera lacking the CATD, suggesting that the Cse4 CATD is a key regulator of its stability and facilitates Psh1 to distinguish Cse4 from histone H3. Therefore, they proposed a new role of the CATD in maintaining the exclusive localization of Cse4 by preventing its mislocalization to euchromatin via Psh1-mediated degradation.

However, the new findings of E3 ligase, Psh1, by these two groups left these open questions and stimulated other researchers to study the Psh1-mediated ubiquitylation and degradation of Cse4 as well as CENP-A homologs of other species.

i. Why does deletion of *PSH1* not show a phenotype unless Cse4 is overexpressed [22, 45]? These data may suggest additional Cse4 regulatory mechanisms. In agreement with this concept, Cse4 is not completely stabilized when Psh1 is deleted and a lysine-free mutant of Cse4 is still degradable [22, 23, 43, 45]. Thus, it seems plausible that there are other destabilization mechanisms not yet discovered [22, 23, 43, 45].

ii. No Psh1 ortholog in other eukaryotes is yet identified. Because the RING and zinc fingers are highly conserved motifs in many proteins from yeast to human, it is difficult to verify such an ortholog. It is also unclear whether the ubiquitin–proteasome pathway that controls CENP-A proteolysis is conserved among different species.

iii. Can Psh1 be the unique E3 ligase in yeast? Is it possible to identify other E3 ligases that ubiquitylate Cse4 in the same or different function? Is the function of the Cse4 ubiquitylation restricted only to proteolysis?

iv. Are any other post-translational modifications of Cse4 involved in upstream or downstream functions of Cse4 ubiquitylation?

v. What is the genome-wide misincorporation pattern of Cse4? How does the pattern change in the presence and absence of Psh1? Does Cse4 misincorporation affect promotor function and transcriptional regulation?

vi. What is the molecular mechanism for the selective recognition and ubiquitylation of Cse4 by Psh1? Are other components required for such activities, or are other PTMs of Cse4 involved?

vii. What are the deubiquitylase and deubiquitylation mechanisms of Cse4?

In the following sections, answers to some of these questions are further described.

1.2 Additional Cse4 regulatory mechanisms and factors that are required for proper ubiquitylation of Cse4 in vivo

1.2.1 SWI/SNF complex

Gkikopoulos et al. had identified DNA sequences to which the *S. cerevisiae* ATP-dependent SWI/SNF chromatin remodeling complex is bound genome-wide to gain insight into that complex [46], and they observed that the complex is enriched at the centromeres of each chromosome. In their study, partial redistribution of the Cse4 to sites on chromosome arms was observed by deletion of the gene encoding the Snf2 subunit of the complex (**Figure 1** and **Table 1**). Cultures of *snf2Δ* yeast were found to progress through mitosis slowly, and this slow progress depends on the mitotic check-point protein Mad2; defects in chromosome segregation were observed in the absence of Mad2. Chromatin organization at centromeres is less distinct in the absence of Snf2, and especially hypersensitive sites flanking the Cse4-containing nucleosomes are less prominent. In addition, SWI/SNF complex was especially effective in the dissociation of Cse4 containing chromatin in their nucleosome reconstitution and remodeling assay in vitro. Taken together, these data suggest a role for Snf2 in the maintenance of point centromeres involving the removal of Cse4 from ectopic sites, rather than via directing incorporation of Cse4 at centromeres.

1.2.2 A novel role of the N-terminus of Cse4

The aforementioned groups had shown interactions of Psh1 with the C-terminus CATD of Cse4 and ubiquitylation of Cse4 at its C-terminus in vitro [22, 45]. Further, Au et al. demonstrated a role for ubiquitination of the N-terminus of Cse4 in regulating Cse4 proteolysis [47]. They initiated their studies with a mutant *cse4*[16KR] (16KR) and fusion mutants in which lysines (K) are mutated to arginines (R). Their results indicated that lack of ubiquitylation of the C-terminus due to KR mutations does not increase protein stability, while the mutations in the N-terminus do so significantly, suggesting that the N-terminus of Cse4 facilitates its proteolysis. They also investigated the role of Cse4 domains in directing Cse4 proteolysis using a genome-wide screen (a synthetic genetic array, SGA), and identified *DOA1* and *PSH1*. Their results using cse4KR mutants suggest that Psh1 is not the sole regulator of Cse4 proteolysis and that Doa1 facilitates Cse4 N-terminus-dependent proteolysis. We also note that N-terminal functions of CENP-A were described for some species [12, 28, 45, 47–55] (see also Sections 1.1, 1.4.1, 1.9 and next chapter, sections 2.1, 2.3, 2.4, 3.1, 4.1, 4.6, and 5.1).

1.2.3 Fpr3 peptidyl-prolyl cis-trans isomerase

Ohkuni et al. reported that the proline isomerase Fpr3 regulates Cse4 proteolysis [32] (**Figure 1** and **Table 1**). *FPR3* encodes a peptidylprolyl cis-trans isomerase (PPIase) which has a function in the meiotic recombination checkpoint pathway [56, 57]. In their study, the *fpr3Δ* or *fpr4Δ* strain displayed a significant chromosome missegregation phenotype. Cse4 protein levels were increased in *fprΔ* cells, and deletion of *FPR3* stabilized Cse4 protein levels in vivo. PPIase dead mutants (W363L and F402Y) stabilized Cse4 protein levels in vivo, suggesting that Fpr3 isomerization activity is necessary for Cse4 proteolysis. Interaction between Cse4 and Psh1 was diminished in *fpr3Δ* cells, and P134V mutation (a mutation of a putative target of Fpr3 isomerization) in Cse4 diminished the Psh1 interaction, suggesting that Fpr3 regulates the Cse4-Psh1 interaction. In summary, they

proposed that structural change in Cse4 by Fpr3 might be important for the interaction between Cse4 and the E3 ubiquitin ligase Psh1 (**Figure 1**, left). Prolyl isomerization of fission yeast CENP-A^{Cnp1} was discussed in this paper [32] (see also next chapter, section 2).

1.2.4 FACT complex

Deyter et al. identified a role for the conserved chromatin-modifying complex FACT (facilitates chromatin transcription/transactions) in preventing Cse4 mislocalization to euchromatin by mediating its proteolysis [58]. They initially found that Psh1 cannot efficiently ubiquitylate Cse4 nucleosomes in vitro, suggesting that additional factors must facilitate Cse4 removal from chromatin in vivo. The Spt16 subunit (**Figure 1b** and **Table 1**) of the FACT complex binds to Psh1, and this interaction between Psh1 and Spt16 is critical for both Cse4 ubiquitylation and its exclusion from euchromatin. Therefore, a Psh1 mutant that cannot associate with FACT has reduced interaction with Cse4 in vivo. Collectively, they proposed a previously unknown mechanism to maintain centromere identity and genomic stability through the FACT-mediated degradation of ectopically localized Cse4.

1.2.5 Phosphorylation by casein kinase 2 (CK2)

Hewawasam et al. reported that Psh1 is phosphorylated by the Cka2 subunit of casein kinase 2 (CK2) to promote its E3 activity for Cse4 [29] (**Figure 1a** and **Table 1**). They first showed that the deletion of *CKA2* significantly stabilized Cse4. Consistently, Cse4 has stabilized in a Psh1 phospho-depleted mutant strain in which all identified phosphorylation sites (total 10 sites) were changed to alanines. However, they showed that phosphorylation of Psh1 did not control Psh1-Cse4 or Psh1-Ubc3(E2) interactions. Mislocalization of Cse4 was mild, although Cse4 was highly stabilized in a *cka2Δ* strain. These data suggest that Cse4 misincorporation was prevented by the intact Psh1-Cse4 association. Supporting that idea, Psh1 was also stabilized in a *cka2Δ* strain. However, some questions remain if the phosphorylation of Psh1 by Cka2 is required for its E3 activity to degrade "already" mis-incorporated Cse4 on the non-centromeric chromatin and the status of Psh1's PTMs in such non-centromeric chromatin (**Figure 1b**). Collectively, these results suggest that phosphorylation is important for Psh1-assisted control of Cse4 levels and that the Psh1-Cse4 association itself functions to prevent Cse4 misincorporation.

1.2.6 Pat1, a protector of Cse4 against Psh1

Mishra et al. showed that a kinetochore protein, Pat1 (**Figure 1**, right and **Table 1**), protects *CEN*-associated Cse4 from ubiquitylation to maintain the proper structure and function of the kinetochore [24]. In their study, *PAT1*-deletion (*pat1Δ*) strains exhibit increased ubiquitylation of Cse4 and faster turnover of Cse4 at kinetochores. Psh1 interacts with Pat1 and contributes to the increased ubiquitylation of Cse4 in *pat1Δ* strains. Their results showed that transient induction of *PSH1* in a wild-type strain resulted in phenotypes similar to a *pat1Δ* strain (e.g., a reduction in *CEN*-associated Cse4, increased Cse4 ubiquitylation, defects in the spatial distribution of Cse4 at kinetochores, and altered structure of *CEN* chromatin). Pat1 interacts with Scm3 (a Cse4-specific chaperone) and *pat1Δ* strains showed reduced levels of centromeric Scm3, suggesting that the interaction of Pat1 with Scm3 is required for its maintenance at kinetochores. In summary, these results suggest a new mechanism

by which Pat1 affects the structure of *CEN* chromatin and protects Cse4 from Psh1-mediated ubiquitylation for faithful chromosome segregation.

1.3 Cse4 misincorporation affects promotor function and transcriptional regulation, and histone H4 facilitates the proteolysis of the Cse4

1.3.1 Regulation of Cse4 protein levels prevents misincorporation at promotor nucleosomes and transcriptional defects

One interesting question is if Cse4 misincorporation affects promotor function and transcriptional regulation. Hildebrand et al. addressed the genome-wide misincorporation pattern of Cse4 in the presence and absence of Psh1, performing chromatin immunoprecipitation analysis followed by high throughput sequencing [59]. They found that ectopic Cse4 mislocalized to intergenic regions of the genome. Mislocalized Cse4 is enriched at promoters that contain histone H2A. Z^{Htz1} nucleosomes flanking nucleosome-depleted regions (NDRs), however, Cse4 mislocalization does not depend on H2A.Z^{Htz1}. Instead, the chromatin remodeling inositol-requiring 80 (INO80) complex (INO80-C), which removes H2A.Z^{Htz1} from nucleosomes, contributes to the ectopic deposition of Cse4 [59] (**Figure 1**, left). However, the functional relationship of INO80-C with other factors (e.g., CAF-1 complex) for Cse4 ectopic deposition remains to be elucidated (**Figure 1**, left). Together, this transcriptional profiling revealed that mislocalized Cse4 significantly disturbs transcription in the absence of Psh1, suggesting that regulating centromeric nucleosome localization is important for ensuring accurate promoter function and transcriptional regulation.

1.3.2 Histone H4 facilitates the proteolysis of the Cse4

Because Cse4 proline residues though the Fpr3 regulation influence its degradation as reported by Ohkuni et al. [32] (see also Section 1.2.3), Deyter et al. hypothesized that additional features of the Cse4 nucleosome might be important for Cse4 proteolysis [31]. They initially asked whether histone H4 residues are important for Cse4 degradation, since Cse4 binds with high affinity to histone H4 before and after deposition on DNA, and they determined that Cse4 protein levels are stabilized in H4-R36A mutant cells and Cse4 is enriched in the euchromatin. Consistent with those data, they also demonstrated that H4-R36 is important for the interaction between Cse4 and Psh1 (**Figure 1** and **Table 1**). They also analyzed Psh1 localization in WT vs. H4- R36A cells at the 5′, 3′, and coding regions of two highly transcribed genes, *ADH1* and *PMA1*, because Psh1 interaction with FACT is important for Cse4 ubiquitylation and degradation, as previously reported [58] (see also Section 1.2.4). Their ChIP-qPCR revealed that Psh1 also shows a strong enrichment at the 3' UTRs of these genes in H4-R36A cells compared to wild-type cells, while the levels at the promoter and gene regions were similar to wild-type cells. These data suggest that altered Psh1 localization could contribute to the Cse4 stability phenotype in H4-R36A mutant cells.

This group previously had discovered that overexpressed Cse4 is mislocalized to nucleosomes in both tandem and divergent intergenic regions in the absence of Psh1, as shown earlier [59]. Therefore, they tested whether this is also true in the H4-R36A mutant cells by performing ChIP-qPCR. Cse4 mislocalization was negatively correlated with Psh1 enrichment in H4-R36A cells. Taken together, these data revealed H4-R36 is a key residue for efficient Cse4 degradation, likely by facilitating the interaction between Psh1 and Cse4.

1.3.3 Reduced gene dosage of histone H4 prevents Cse4 mislocalization and chromosomal instability

Eisenstatt et al. further utilized a genome-wide screen (SGA) to identify factors that facilitate the mislocalization of overexpressed Cse4 by characterizing suppressors of the *psh1Δ GAL-CSE4* SDL [27]. They found that deletions of histone H4 alleles (HHF1 or HHF2; **Table 1**), which were among the most major suppressors, also suppress *slx5Δ*, *cdc4-1*, *doa1Δ*, *hir2Δ*, and *cdc7-4 GAL-CSE4* SDL (**Table 1**). Defects in sumoylation and reduced mislocalization of overexpressed Cse4 are observed with a reduced dosage of H4, and these events lead to suppression of CIN when Cse4 is overexpressed (see about Cse4 sumoylation also in the following Section 1.4). *hhf1-20*, *cse4-102*, and *cse4-111* mutants, which have defective Cse4-H4 interactions, also show reduced sumoylation of Cse4 and do not cause *psh1Δ GAL-CSE4* SDL. Overall, these results identified a novel function of the histone H4 gene dosage in promoting Cse4 sumoylation and mislocalization to noncentromeric regions, which leads to CIN when Cse4 is overexpressed.

One question is how this H4 dosage balance affects the function of H4-R36 (see also Section 1.5.1). Deyter et al. reported that H4-R36 is a key residue for efficient Cse4 degradation, likely by facilitating the interaction between Psh1 and Cse4 [31]. This group also found that a basic residue at H4-R36, but not PTM (e.g., methylation) of the amino acid, is required to prevent sensitivity to Cse4 overexpression [31]. Then how is it possible that reduced dosage of H4 leads to sumoylation and reduced mislocalization of overexpressed Cse4? Eisenstatt et al. showed that deletion of either histone H4 allele resulted in reduced levels of sumoylated Cse4 and concluded that physiologic levels of histone H4 are required for Cse4 sumoylation [27] (see also Section 1.4). However, the level of sumoylation loss caused by the deletion of either histone H4 allele (in *hhf1Δ* or *hhf2Δ*) was similar to one caused by $cse4^{16KR}$ mutant that should be total loss of sumoylation of Cse4. Ohkuni et al. proposed a model in which Cse4 K215/216 sumoylation facilitates the deposition of overexpressed Cse4 into CEN and non-CEN regions, respectively [21] (**Figure 1**). If a total loss of sumoylation is achieved in the H4 loss (in *hhf1Δ* or *hhf2Δ*) as a $cse4^{16KR}$ mutant, and if the sumoylation of Cse4 K215/216 is required for centromeric deposition of Cse4 into chromatin as Ohkuni et al. suggest [21], how do the *hhf1Δ* or *hhf2Δ* strains keep centromeric Cse4 and survive? How H4 gene dosage on Cse4 K215/216 sumoylation facilitates deposition of overexpressed (or endogenous) Cse4 into CEN is not clear (**Figure 1**, left-center).

The mechanism by which other histones' PTMs and dosages are involved in the incorporation of CENP-A/CenH3 is highly interesting, but at the same time, it suggests many questions. A further question raised is whether H4 dosage affects heterotypic CENP-A-H3.3 nucleosomes (see also Section 1.6) or H3 dosage among species including humans? Results in both budding and fission yeast suggest that the balance among histones H3,H4 and CENP-A is important for centromeric chromatin assembly [60, 61]. In fission yeast, increasing cellular histone H3 levels relative to Cnp1 promotes accumulation of H3 and loss of Cnp1 from the central domain and leads to defects in kinetochore function, however, there does not appear to be an efficient mechanism for the active exclusion of histone H3 from the centromeric nucleosomes [60, 62]. If H4 dosage affects heterotypic CENP-A-H3.3 nucleosomes or H3 dosage, is there an indirect pathway through which H4 dosage affects CENP-A incorporation into chromatin through H3? The inter-regulation among different histones, including CENP-A/CenH3 for high(macro) and low(micro) order chromatin structures, must be intricate. However, this could make it difficult to elucidate the mechanisms of incorporation, maintenance, and inheritance of CENP-A/CenH3.

In fission yeast and human studies, Mis18 (human Mis18α and Mis18β homolog) and Mis16 (human RbAp46 and RbAp48 homolog) are required for loading of newly synthesized Cnp1/CENP-A into centromeric chromatin [63, 64] (see also next chapter, sections 2.1, 3.1, and 4.1). Mis16 and Mis18 are also required for the maintenance of the hypoacetylation of histone H4 specifically within the central domain of the centromere [64], and Mis16 homologs are components of several histone chaperone complexes [65]. Moreover, acetylation of histone H4 lysine 5 and 12 (H4K5ac and H4K12ac) within the pre-nucleosomal CENP-A-H4-HJURP complex mediated by the RbAp46/48-Hat1 complex is required for CENP-A deposition into centromeres in chicken and humans [66], consistent with the Hat1 role shown in *Drosophila melanogaster* [67] (see also next chapter, sections 3.1 and 4.1). In mouse studies, Mis18α interacts with DNMT3A/3B, and this interaction is required to maintain DNA methylation [68]. *Mis18α* deficiency leads to not only the reduction of DNA methylation, but altered histone H3 modifications, and uncontrolled non-coding transcripts in the centromere region (see also next chapter, section [4.1]). However, Mis16 and Mis18 proteins are absent from budding yeast *S. cerevisiae* with point centromeres [69]. In addition, how these proteins and H4 hypoacetylation facilitate the fission yeast Cnp1/CENP-A incorporation into chromatin is still not clear [62] (see also next chapter, section [2.1]).

1.4 Cse4 sumoylation

1.4.1 Slx5/RNF4 and Ulp2/SENP6

It is known that sumoylation is involved in multiple intercellular pathways, and a subset of polysumoylation-mediated polyubiquitylation processes lead to proteasome-mediated degradation [70, 71]. Such machineries of SUMO-dependent ubiquitylation and degradation of CENP-A are interesting and important issues. Recent research has revealed new insights about the sumoylation of Cse4.

Ohkuni et al. reported the first evidence that Cse4 is sumoylated by E3 ligases Siz1 and Siz2 in vivo and in vitro [28] (**Figure 1** and **Table 1**). Siz1 is the founding member of the Siz/PIAS (protein inhibitor of activated STAT) RING family of SUMO E3 ligases, and both Siz1 and Siz2 are normally bound to chromatin via their SAP domains [72]. The Siz/PIAS RING family is involved in the sumoylation of the septin protein group and several chromatin proteins including core histones and the replication clamp PCNA (proliferating cell nuclear antigen) [70, 72]. They showed that ubiquitylation of Cse4 by the small ubiquitin-related modifier (SUMO)-targeted ubiquitin ligase (STUbL), Slx5, is important for proteolysis of Cse4 and prevents mislocalization of Cse4 to euchromatin under normal physiologic conditions (**Figure 1b** and **Table 1**). Sumoylated Cse4 proteins are accumulated and protein stability of Cse4 is increased in *slx5Δ* strains, suggesting that sumoylation precedes ubiquitin-mediated proteolysis of Cse4 (**Figure 1b**). *slx5Δ psh1Δ* strains exhibit higher levels of Cse4 stability and mislocalization than either *slx5Δ* or *psh1Δ* strains, suggesting that Slx5-mediated Cse4 proteolysis is independent of Psh1 (**Figure 1b**). In addition to the Psh1-dependent ubiquitylation pathway, their results suggested a second pathway that requires sumoylation of Cse4 by Siz1/Siz2 and ubiquitination of sumoylated Cse4 by Slx5 to prevent its mislocalization and maintain genome stability.

Further, Ohkuni et al. identified lysine 65 (K65) in Cse4 as a site that regulates sumoylation and ubiquitin-mediated proteolysis of Cse4 through Slx5 [52] (**Figure 1b**). The abundance of sumoylated and ubiquitinated Cse4 in vivo is reduced

in budding yeast strains expressing cse4 K65R. They also showed that the interaction of cse4 K65R with Slx5 is significantly reduced, and stability and mislocalization of cse4 K65R are increased under normal physiologic conditions. The stability of cse4 K65R in *psh1Δ* strains is increased, but not in *slx5Δ* strains. Therefore, they concluded that Slx5 targets sumoylated Cse4 K65 for ubiquitination-mediated proteolysis, but Psh1 does not (**Figure 1b**). Overall, they clarified the function and biological significance of Cse4 K65 in sumoylation, ubiquitin-mediated proteolysis, and localization of Cse4 for genome stability.

In humans, depletion of the human Slx5 homolog ring finger protein 4 (RNF4) contributes to sumoylation-dependent degradation of the CCAN protein CENP-I, while SENP6 (a member of a large family of Sentrin-specific protease enzymes that belongs to the yeast Ulp2 group) stabilizes CENP-I by antagonizing RNF4 [73]. However, depletion of SENP6 in HeLa cells leads to the loss of the CENP-H/I/K complex from the centromeres, but not an apparent reduction in centromeric CENP-A/B/C levels recognized by CREST sera [73]. Recent analyses by some groups also indicated that CENP-A was not a direct substrate of SENP6 [74, 75]. Differences among species of roles of sumoylation in the regulation of CENP-A stability are described later (see also next chapter, section [4]).

1.4.2 Deposition of Cse4 into chromatin through its C-terminal sumoylation

C-terminal sumoylation of Cse4 also contributes to the deposition of Cse4 into chromatin. Ohkuni et al. identified sumoylation sites lysine (K) 215/216 in the C-terminus of Cse4 and showed that sumoylation of Cse4 K215/216 facilitates its genome-wide deposition into chromatin when overexpressed [21] (**Figure 1**). Their results showed reduced levels of sumoylation of mutant Cse4 K215/216R/A [K changed to arginine (R) or alanine (A)] and reduced interaction of mutant Cse4 K215/216R/A with Scm3 and CAF-1 (**Figure 1** and **Table 1**) (see also Section 1.5.3) when compared to wild-type Cse4. Consistently, levels of Cse4 K215/216R/A in the chromatin fraction and localization to centromeric and noncentromeric regions were reduced. In addition, GAL-cse4 K215/216R does not exhibit synthetic dosage lethality (SDL) in these strains—unlike *GAL-CSE4*, which exhibits SDL in *psh1Δ*, *slx5Δ*, and *hir2Δ* strains. Thus, they clearly demonstrated that the deposition of Cse4 into chromatin is facilitated by its C-terminal sumoylation. Based on their data, they also updated a model in which Cse4 K215/216 sumoylation promotes its interaction with the histone chaperones Scm3 and CAF-1, facilitating the deposition of overexpressed Cse4 into *CEN* and non-*CEN* regions, respectively (**Figure 1**). Their results suggest the importance of the SUMO-interaction motif in Slx5's targets and histone chaperone proteins (Scm3 and CAF-1), and it will be interesting to test if this sumoylation machinery is conserved in humans and if human CENP-A sumoylation regulates its interaction with HUJRP and/or DAXX.

Further questions remain about the SUMO E3 ligase of Cse4 C-terminal K215/216 sumoylation. Sumoylation of Cse4 is barely detectable in a *siz1Δ siz2Δ* strain [21]. Do Siz1/Siz2 also target the Cse4 C-terminal K215/216, as they do the Cse4 N-terminal K65? Or do different E3 entities target Cse4 K215/216 (**Figure 1**, right-center)? If Siz1/Siz2 are required for Cse4 C-terminal K215/216 sumoylation for proper Cse4 deposition at centromeres, how do Siz1/Siz2 distinguish between Cse4 for centromeric deposition and Cse4 for degradation? Functional comparisons among different species (esp. budding yeast and human) of sumoylation in the regulation of CENP-A stability are also described later (see also next chapter, section 4).

1.5 More intricate E3 network of Cse4 and other chaperones that function in proteolysis and mislocalization of Cse4

1.5.1 Cse4 R37 methylation and Ubr2/Mub1 E3 ligase

Samel et al. reported that the absence of the E3 ubiquitin ligase Ubr2, as well as its adaptor protein Mub1, suppresses the synthetic growth defects (or lethality) caused by the absence of Cse4-R37 methylation in *cbf1Δ* or deletion mutants of Ctf19/CCAN complex [76]. Previously Ubr2 had been shown to control the levels of the MIND complex protein Dsn1 via ubiquitination and proteasome-mediated degradation [77]. Consistent with these results, Samel et al. found that overexpression of *DSN1* also led to suppression of growth defects (or lethality) caused by the absence of Cse4-R37 methylation. Collectively, they proposed that the absence of Cse4 R37 methylation reduces the recruitment of kinetochore proteins to centromeric chromatin, and that this can be compensated for by stabilizing the outer kinetochore protein Dsn1.

However, the relationship between Ubr2 and Psh1, and E3 activity of Ubr2 on Cse4 is still not clear, although the absence of both E3s suppressed the synthetic growth defects (or lethality) shown in their study. The authors stated that most likely increased levels of kinetochore proteins other than Dsn1 in *ubr2Δ* cells can also compensate for the absence of R37 methylation, since *ubr2Δ* controls the levels of other proteins, possibly also kinetochore proteins. On the other hand, others had previously suggested that the role of Ubr2 at kinetochores seems to be partially redundant with the E3 ubiquitin ligase Psh1 [78]. Samel et al. also hypothesized that the ubiquitin ligase Psh1 restricts the loading of inner kinetochore proteins, whereas Ubr2 regulates the loading of outer kinetochore proteins, indicating that the mechanism of the suppression by *ubr2Δ* and *psh1Δ* differs.

1.5.2 Multiple E3 ligases promote the degradation of Cse4

In addition to the aforementioned report of Slx5 by Ohkuni et al. [28], Cheng et al. demonstrated that 4 ubiquitin ligases (i.e., Ubr1, Slx5, Psh1, and Rcy1) (**Figure 1c** and **Table 1**) contribute in parallel to the Cse4 proteolysis and turnover in budding yeast cells [33]. Cse4 overexpression generates cellular toxicity and cell cycle delay in budding yeast cells lacking *PSH1*, but not in cells lacking *UBR1*. These data suggest different roles of these two degradation pathways, and that various ubiquitin ligases collaborate to check and control Cse4 protein levels.

On the other hand, Cheng et al. also noted the lack of clarity about how this different E3s collaborate [33]. Their finding also generated these questions:

   i. How do these E3s specifically recognize Cse4?

   ii. How do they work with other cellular cues and pathways (e.g., casein kinase 2, Siz1- and Siz2-mediated sumoylation, SWI/SNF remodeling enzymes, the FACT complex, and the proline isomerase Fpr3)?

   iii. What is the functional role and mechanism of each degradation pathway?

Further study is required to address these intricate E3 networks of Cse4 as well as other kinetochore proteins. In addition, the ubiquitin ligase(s) involved in human CENP-A degradation still remains unclear, although the CUL4A complex was identified as an E3 ligase that is required for CENP-A deposition at the centromere [79]

(see also next chapter, section [4.2]). As Cheng et al. noted, while Psh1 does not seem to have a mammalian counterpart, Ubr1 (human UBR1), Slx5 (human RNF4) and Rcy1 (human EXOC5) are known to have human homologs. It is highly interesting to test CENP-A turnover in mammalian cells deficient for these homologs and also to determine if the human homologs of these E3s are altered in CENP-A-related cancer cells. Analogous questions are also raised in Section 1.6.2.

1.5.3 CAF-1 chaperone

Hewawasam et al. reported that chromatin assembly factor-1 (CAF-1) (**Figure 1** and **Table 1**) controls Cse4 deposition in budding yeast (see also Section 1.4.2). CAF-1 is an evolutionarily conserved histone H3/H4 chaperone; its subunits were shown to interact with CenH3 in flies and human cells. Previously, it had been reported that subunits of CAF-1 are required for building functional kinetochores [80], for recruitment of CenH3/Cnp1 and Scm3 to centromeres in fission yeast, *S. pombe* [7, 64], and for regulating Cse4/H3 exchange kinetics [81]. Hewawasam et al. showed that yCAF-1 interacts with Cse4 and can assemble Cse4 nucleosomes in vitro, using both *CEN* and non-*CEN* plasmids [26] (**Figure 1**). In their study, when Cse4 is overexpressed, loss of yCAF-1 markedly reduces Cse4 deposition into chromatin genome-wide. They suggest that incorporation of Cse4 genome-wide may induce multifactorial effects on growth and gene expression. Loss of yCAF-1 rescues growth defects and some changes in gene expression caused by Cse4 genome-wide misincorporation that occur in the absence of Psh1-mediated proteolysis. The incorporation of Cse4 into promoter nucleosomes at transcriptionally active genes is dependent on yCAF-1. In summary, these findings suggest CAF-1 can act as a Cse4 chaperone, controlling the amount and the incorporation of Cse4 in chromatin.

Some questions remain about the relationships among Cse4, Psh1, Scm3, and CAF-1. The first question is about the role of CAF-1 in Psh1-mediated proteolysis of Cse4: How does CAF-1 function in the process of Cse4 ubiquitylation by Psh1? Hewawasam et al. observed more ubiquitylation of Cse4 in the presence of CAF-1 compared with the absence of CAF-1 in vitro, suggesting CAF-1 can promote ubiquitylation of free Cse4, opposite to the effect of Scm3 that protects Cse4 from ubiquitylation by Psh1 in vitro [22]. They also tested CAF-1 interaction with Psh1, but their co-immunoprecipitation experiment in whole-cell extracts did not show any interactions. Thus, they speculated that soluble Cse4 bound to CAF-1 may expose ubiquitylation sites on Cse4, promoting ubiquitylation by Psh1.

The second question is whether CAF-1 could assemble Cse4 at centromere as Scm3. If so, do the roles of the two proteins simply overlap, or does each protein have a unique role in the process of Cse4 assembly at centromere? To test this question, Hewawasam et al. used the Scm3$^{on/off}$ strain, which can be toggled by galactose, along with copper-inducible Cse4 overexpression, so that Cse4 protein levels can be controlled by the concentration of copper [26]. Their results suggest that when Scm3 is absent and Cse4 levels are high, CAF1 may be a primary chaperone targeting Cse4 to the centromere (**Figure 1**, center). Meanwhile, in the fission yeast *S. pombe*, the CAF-1 subunit can recruit the Scm3 to centromeres [7]. Thus, Hewawasam et al. speculated that under normal conditions, Scm3 and CAF-1 both play important functions in the deposition of Cse4 at centromere; however, further study is required to reinforce this hypothesis (**Figure 1**, right).

The third question is how CAF-1 can be responsible for the mislocalization of Cse4/CENP-A in cancer development. The human CAF-1 subunit p60 was one of the overexpressed chaperones in CENP-A-overexpressing breast cancer cells [82],

and ectopic CENP-A nucleosomes from colorectal cancer cells keep a subpopulation of structurally distinct hybrid (chimeric) nucleosomes containing both CENP-A and H3.3 [82, 83]. Misregulation of Scm3/HJURP causes chromosome instability in both yeast and humans [84], and many previous reports suggested the functional relevance of Scm3/HJURP with the development of a wide spectrum of cancers (e.g., colon, lung, liver, breast, pancreatic, brain cancer) [85–94]. As aforementioned, CAF-1 may cooperate with Psh1 and Scm3 to regulate proteolysis of Cse4, in the way that CAF-1 association with free Cse4 may promote ubiquitylation and proteolysis. If so, how do these two chaperons (CAF-1 and Scm3/HJURP) cooperate together in genomic stability and anti-cancer development? Further in-depth study is required to elucidate the collaboration among Psh1, Scm3, and CAF-1 in genomic stability and anti-cancer development.

1.6 A genome-wide screen (a synthetic genetic array, SGA) revealed other proteins that are required for proteolysis and proper localization of Cse4

1.6.1 HIR histone chaperone complex

Deletion for genes encoding the replication-independent histone chaperone HIR complex (HIR1, HIR2, HIR3, HPC2) and a Cse4-specific E3 ubiquitin ligase, PSH1, showed the highest SDL using a genome-wide synthetic genetic array (SGA) to identify gene deletions that exhibit SDL when Cse4 is overexpressed [30]. Thus, Ciftci-Yilmaz et al. performed functional analysis for Hir2 (**Figure 1** and **Table 1**) in proteolysis of Cse4 that prevents mislocalization of Cse4 to noncentromeric regions for genome stability. They demonstrated the interaction of Hir2 with Cse4 in vivo, and defects in Cse4 proteolysis and stabilization of chromatin-bound Cse4 appear in *hir2Δ* strains. The *hir2Δ* strains also exhibit mislocalization of Cse4 to noncentromeric regions with a preferential enrichment at promoter regions. They also found that Hir2 facilitates the interaction of Cse4 with Psh1. In the *hir2Δ* strain, defects in Psh1-mediated proteolysis increase Cse4 stability and lead to mislocalization of Cse4 compared to wild-type cells. Collectively, they identified a novel role for the HIR complex to prevent mislocalization of Cse4 by facilitating proteolysis of Cse4, thereby promoting genomic stability.

Analogous questions can be raised regarding CAF-1, especially about the functional relationships among different Cse4 chaperone proteins (e.g., Scm3, CAF-1, and Hir2) and their roles in cancer development. In the Psh1-mediated proteolysis of free Cse4 using whole-cell lysates, CAF-1 and Hir2 promote proteolysis and Scm3 inhibits it [22, 26, 30]. CAF-1 and Hir2 could be involved in the proteolysis of noncentromeric Cse4, but Scm3 in the anti-proteolysis of the centromeric Cse4. However, CAF-1 may promote centromeric localization of overexpressed Cse4 only under conditions when Scm3 is depleted (*SCM3* expression OFF; see also Section 1.5.3) [26]. Furthermore, the centromeric Cse4 level is decreased in *hir2Δ* strains, suggesting that Hir2 might have anti-proteolytic activity in centromeric Cse4. If the functions of these chaperones change with the expression level and localization pattern of CENP-A that they target, how do these chaperones sense the dosage change of CENP-A (between overexpression and normal levels of CENP-A) and distinguish centromeric and ectopic CENP-A?

Studying the real-time 3D structure of free CENP-A/CenH3 after post-translational modification and before incorporation into chromatin could be a key future direction. In budding yeast, Ohkuni et al. proposed that structural change in Cse4 caused by the proline isomerase Fpr3 might be important for the interaction between Cse4 and the E3 ubiquitin ligase Psh1 [32] (see also Section 1.2.3).

1.6.2 Skp1, Cullin, F-box (SCF)-Met30 and SCF-Cdc4-mediated proteolysis of Cse4

Au et al. identified two Skp1, Cullin, F-box (SCF) ubiquitin ligases with the evolutionarily conserved F-box proteins Met30 and Cdc4 (**Figure 1c** and **Table 1**) as essential genes required for Cse4 homeostasis through a genome-wide SGA screen [34]. They showed that Met30 and Cdc4 interact through the Met30-WD40 domain, and these two proteins cooperatively regulate proteolysis of endogenous Cse4 and prevent its mislocalization for faithful chromosome segregation (**Figure 1**). The inter-action of Met30 with Cdc4 is independent of the Met30-D domain, which is essential for their homodimerization and ubiquitination of other substrates. Ubiquitin affinity pull-down assays showed that both Cdc4 and Met30 specifically target Cse4 for its ubiquitination. They suggest that Met30 is necessary for the interaction between Cdc4 and Cse4, and its defective interaction leads to stabilization and mislocaliza-tion of Cse4, which in turn promotes to CIN. They also provided the first direct link between Cse4 mislocalization and defects in kinetochore structure measured by the sensitivity against the restriction enzyme *DraI,* and collectively showed that proteoly-sis of Cse4 by SCF-Met30/Cdc4 prevents mislocalization and CIN.

Further studies are also required to address analogous questions as in Section 1.5.2: How does the Met30/Cdc4-pathway work with other cellular cues and multiple E3 path-ways, including Psh1-dependent and independent proteolysis? Are the human homologs of these E3s (e.g., human FBXO24, TRAF7, etc.) altered in CENP-A-related cancer cells?

1.6.3 Dbf4-dependent kinase (DDK)-mediated proteolysis of Cse4

Eisenstatt et al. identified five alleles of CDC7 and DBF4 that encode the Dbf4-dependent kinase complex, which regulates DNA replication initiation in their SGA [95]. They found that cdc7–7 strains show defects in ubiquitin-mediated proteolysis of Cse4 and mislocalization of Cse4 [95]. Mutation of MCM5 (mcm5-bob1) bypasses the requirement of Cdc7 for replication initiation and rescues replication defects in a cdc7-7 strain. They demonstrated that mcm5-bob1 does not rescue the SDL and defects in proteolysis of overexpressed Cse4 (*GALCSE4*) in a cdc7-7 strain. These data suggest a DNA replication-independent role for Cdc7 in Cse4 proteolysis. Their results of the SDL phenotype, defects in ubiquitin-mediated proteolysis, and the mislocalization pattern of Cse4 in a cdc7-7 *psh1Δ* strain were similar to that in the cdc7-7 and *psh1Δ* strains. These data suggest that Cdc7 regulates Cse4 in a pathway that overlaps with Psh1. They propose a role for the Dbf4-dependent kinase complex as a regulator of Psh1-mediated proteolysis of Cse4 to prevent mislocalization of Cse4, independent of DNA replication initiation.

1.6.4 Reduced gene dosage of histone H4

Recently, Eisenstatt et al. further utilized SGA to identify factors that facilitate the mislocalization of overexpressed Cse4 by characterizing suppressors of the *psh1Δ GALCSE4 SDL* [27]. See Section 1.3.3 for more details of this study.

1.7 Cse4 deubiquitylase

Compared to the ubiquitylation mechanism of Cse4, there are relatively few studies on the deubiquitylation mechanism of Cse4. We should also consider how the deubiquitylation affects the localization and the function of Cse4 at both centromere and ectopic sites along chromosomes.

Canzonetta et al. investigated the role of Ubp8-driven deubiquitylation of the Cse4 in budding yeast [96]. Ubp8 is a component of the SAGA (Spt-Ada-Gcn5-acetyltransferase) complex, a multicomponent regulator of acetylation. The SAGA complex is also involved in deubiquitylation through its deubiquitylation (DUB) module, and one example of its activity is upon histone H2B [97, 98]. Canzonetta et al. demonstrated that the deubiquitylation process was inhibited and a short ubiquitin oligomer on Cse4 was accumulated by the loss of Ubp8. Such defective deubiquitylation caused by Ubp8 loss leads to chromosome instability and Cse4 protein degradation, and induces ectopic localization of the Cse4 outside the centromere.

1.8 Psh1 is also involved in proper plasmid segregation, and two distinct cellular effects by Psh1 and Cse4

Interestingly, there was a report suggesting that Psh1 is also involved in proper plasmid segregation [99]. Metzger et al. initially sought to assess the involvement of the ubiquitin-proteasome system in the turnover of mitochondrial proteins in budding yeast [99]. Then, they found that deletion of a specific ubiquitin ligase (E3), Psh1p, increases the level of a temperature-sensitive mitochondrial protein, mia40-4pHA, when it is expressed from a centromere-containing (*CEN*) plasmid that remains, on average, at one copy per yeast cell. Unexpectedly, they also found deletion of Psh1p elevates the levels of other proteins (not only mitochondrial proteins) expressed from the *CEN* plasmids. The rate of turnover of mia40-4pHA, total protein synthesis, or the protein levels of chromosomal genes is not affected by the loss of Psh1p. On the other hand, *psh1Δ* appears to increase the occurrence of missegregation of centromeric plasmids compared to their normal 1:1 segregation. Their results showed that ongoing missegregation leads to elevated plasmid DNA, mRNA, and protein, all of which they observed in *psh1Δ* cells after generations of growth with selection for the plasmid. Elevation of Cse4p leads to an apparent increase in 1:0 plasmid segregation events, although Cse4p overexpression alone does not phenocopy *psh1Δ* in increasing plasmid DNA and protein levels. Moreover, 2 μm high-copy yeast plasmids also lead to missegregation in *psh1Δ*, but not when Cse4p alone is overexpressed. Their findings demonstrated that Psh1p is required for the faithful inheritance of both centromeric and 2 μm plasmids. In addition, the effects that loss of Psh1p has on plasmid segregation cannot be merely explained by increased levels of Cse4p, arguing two distinct cellular effects by Psh1p and Cse4p.

1.9 Molecular basis for the selective recognition and ubiquitination of Cse4 by Psh1 through Cse4 MIMAS motif

Zhou et al. first solved the structure of the Cse4-binding domain (CBD) of Scm3 in complex with Cse4 and H4 in a single chain model using nuclear magnetic spectroscopy [12]. They suggested that four Cse4-specific residues in the N-terminal region of helix 2 (MIMAS motif; **Table 1**) are sufficient for specific recognition by conserved and functionally important residues in the N-terminal helix of Scm3 through the formation of a hydrophobic cluster.

Scm3 (CBD) also induces major conformational changes and sterically occludes DNA-binding sites in the structure of Cse4 and H4. Furthermore, Zhou et al. showed that Psh1 uses a CBD (residues 1–211) to interact with Cse4-H4 instead of H3-H4, yielding a dissociation constant (K_d) of 27 nM in their isothermal titration calorimetric experiments [100]. They are in vitro pull-down assays revealed that Psh1 interacts with Cse4-specific residues in the L1 loop and α2 helix for Cse4 binding and ubiquitination.

They also mapped the Psh1-binding region of Cse4-H4 and identified a wide range of Cse4 specific residues required for the Psh1-mediated Cse4 recognition and ubiquitination. Consistent with the previous reports of the inhibitory effect of Scm3 on Cse4 ubiquitylation [22], their data showed that the histone chaperone Scm3 prevents Cse4 ubiquitination by abrogating Psh1-Cse4 binding. Their results suggest that Scm3 interacts with the Cse4 MMAS motif (a particular Cse4 region containing residues M181/M184/A189/S190 reported previously [12]) to prevent Psh1 from binding to Cse4. Elimination of the Psh1-binding residues outside of the Cse4 MMAS motif promotes the inhibitory effect of Scm3. Thus, the MMAS motif plays a central role in the activation or inhibition of Cse4 ubiquitination as well as yeast cell growth. Taken together, they elucidated a novel Cse4 binding mode distinct from those of known CenH3 chaperones and the mechanism by which Scm3 competes with Psh1 for Cse4 binding.

2. Conclusions

The budding yeast is a powerful organism for centromere-kinetochore research in many aspects. For example, the centromere sequence size of the budding yeast is small and the sequences can be easily mutated to identify the important functional regions [1]. Techniques such as ChIP are also possible, which cannot be easily performed on highly repetitive centromeres in other organisms. Moreover, the centromere can be shifted to other genomic regions, allowing the construction of artificial chromosomes and plasmids as well as tools such as conditional centromeres. As a result, the most common species studied and reported in the past for E3 ligase of CenH3 (Cse4) is budding yeast at present. However, many questions described in this chapter are unanswered even in the budding yeast model. Especially, little has been studied on how each of such multiple E3 ligases of budding yeast selectively recognizes Cse4 substrate and functions specifically. Currently, 4 types of E3 ligases for ubiquitylation and one type of E3 ligases for sumoylation (Slx5/8) have been reported (**Table 1**). In particular, the functions of the 4 types of E3 ligases for ubiquitylation including Psh1 are common, all of which are related to ectopic degradation and/or quality control of soluble or chromatin-bound Cse4, and the functional differences are not clear. It is neither clear why E3 ligases with overlapping functions exist in one species. The simple interpretation is that at least such a number (4–5) of E3 ligases of Cse4 is required as a backup system, so that it can be complemented if one of the E3 functions is defective. As we described the compensatory system of CENP-A PTM (see also the next chapter, Conclusion), compensatory systems and resilience of CENP-A^{Cse4} could be expected as future directions to study the spatiotemporal regulation of E3 ligase of CENP-A^{Cse4}.

No neocentromere has been found in budding yeast. As a simple reason, it seems that there is no or little possibility of ectopic centromere formation, because the kinetochore formation of budding yeast depends on centromeric DNA elements. However, in terms of considering centromeric evolution, it is interesting to question why budding yeast has maintained point centromere which relies on DNA elements, and other species have evolved to regional centromere which allows the system to generate neocentromere? There is also no clear answer as to whether simply introducing centromeric DNA elements into ectopic loci causes neocentromere or it is still eliminated by the specific E3 activity in budding yeast. The building and establishment of artificial chromosomes are facilitated by studying the mechanisms of formation and maintenance of neocentromeres, and these topics of other species are described in the next chapter.

The regulation of budding yeast cenRNA is an essential factor for centromere structure and function as other eukaryotes, but we have little understanding of the

causality or feedback between cenRNA transcription and overall transcriptional change after chromosome mis-segregation and CIN. In addition, little is known about the effects of these cenRNAs on the E3 ligase of CENP-A, including how these transcriptional changes and regulation are related to the function of E3 ligase. Although, it is essential to study specific physiological functions of each E3 ligase, the physiological phenotype of budding yeast is limited (e.g., growth, cell death, etc.), thus naturally there is a limit in the discussion of the results in the budding yeast model. Thus, studies of an E3 ligase in CENP-A in higher eukaryotes, mammals, or humans are essential for translational research and informing future therapy, and these topics are described in the next chapter.

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Conflict of interest

The authors declare no conflict of interest.

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