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Scm3 Is a Centromeric Nucleosome Assembly Factor*[S]

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The Cse4 nucleosome at each budding yeast centromere must be faithfully assembled each cell cycle to specify the site of kinetochore assembly and microtubule attachment for chromosome segregation. Although Scm3 is required for the localization of the centromeric H3 histone variant Cse4 to centromeres, its role in nucleosome assembly has not been tested. We demonstrate that Scm3 is able to mediate the assembly of Cse4 nucleosomes *in vitro*, but not H3 nucleosomes, as measured by a supercoiling assay. Localization of Cse4 to centromeres and the assembly activity depend on an evolutionarily conserved core motif in Scm3, but localization of the CBF3 subunit Ndc10 to centromeres does not depend on this motif. The centromere targeting domain of Cse4 is sufficient for Scm3 nucleosome assembly activity. Assembly does not depend on centromeric sequence. We propose that Scm3 plays an active role in centromeric nucleosome assembly.

The centromere is a cis-acting chromosomal region that provides all living cells with the ability to transfer their genetic material faithfully during mitotic and meiotic cell divisions. The centromere is the location for the assembly of the kinetochore, a multiprotein complex that enables the attachment of chromosomes to the spindle microtubule and ensures the equal segregation of chromosomes to the daughter cells. The budding yeast kinetochore is composed of more than 65 proteins, many of which are evolutionarily conserved from yeast to man (1, 2). The inner kinetochore or DNA-binding layer is comprised of several proteins, including Mif2, the CBF3 complex (Ndc10, Cep3, Skp1, and Ctf13), a centromeric histone H3 variant Cse4, and Scm3, all of which are essential for kinetochore function (3–8). The CBF3 complex binds specifically to the budding yeast centromere sequence. Budding yeast centromeres consist of ~125 bp divided into three DNA elements: CDE I (14 bp), CDE II (87–88 bp), and CDE III (11 bp) (9, 10). The Ndc10 subunit of CBF3 is critical to nucleate kinetochores (11, 12). Cse4, Ndc10, and Scm3 are dependent on each other for efficient localization to centromeres (6).

Although the sequence composition of centromeres is highly variable among organisms, centromeres in all eukaryotes are universally marked by the presence of a centromere-specific histone H3 variant, termed CENP-A in humans, Cse4 in budding yeast, and CID in *Drosophila melanogaster* (13). The centromere targeting domain (CATD), consisting of loop 1 and helix 2 of the histone fold domain, is required for centromere loading of centromeric histone variants (14, 15). Canonical nucleosomes, the basic module of chromatin, consist of 146 bp of DNA wrapped around an octamer of four core (H3/H4/H2A/H2B) histones (16). At the centromeric nucleosome, Cse4 replaces canonical H3 (4). Although the *Saccharomyces cerevisiae* genome contains ~70,000 nucleosomes (17), a single Cse4 nucleosome defines the centromere on each chromosome (18, 19). The histone fold domain of Cse4 is >60% identical to H3 (20), raising the question of how Cse4 is specifically targeted to the centromere sequence.

Histones are often associated with specific chaperones/nucleosome assembly factors that assist their interaction with DNA, both deposition and removal. Nucleosome assembly factors can be defined as factors that associate with histones and stimulate a reaction involving histone transfer. Some histone variants have specific chaperones that play an important function in their deposition (21). For instance, Chz1 is a histone chaperone that has preference for H2AZ and can deliver H2AZ for SWR1-dependent histone replacement (22). Nucleosome assembly factors also play an important role in assembly of histone H3.1 and H3.3, in a replication-dependent and -independent manner, respectively, thereby differentially marking the active and inactive regions of the genome (23, 24). It is unknown whether there is a specific assembly factor involved in Cse4 deposition at centromeres. One candidate for a Cse4-specific assembly factor is Scm3 (Suppressor of Chromosome Missegregation 3). Scm3 and its orthologs in *Schizosaccharomyces pombe* (Scm3^{sp}) and humans (HJURP) are required for localization of the centromeric histone variant at centromeres (6, 25, 26). In addition to its role at the centromere sequence, Scm3 is required to deposit Cse4 at the stable partitioning locus within the 2-μ plasmid (27). HJURP has been shown to facilitate the association of CENP-A/H4 tetramers with DNA *in vitro* (28).

In budding yeast, Scm3 has been shown to bind to both Cse4 and Ndc10 and is required for their efficient localization to centromeres, leading to the hypothesis that Scm3 serves as a molecular link between a centromere-specific DNA binding complex (CBF3) and the centromeric histone variant (6). Herein, we provide evidence that Scm3 is more than a simple adapter and possesses unique nucleosome assembly activity. The assembly activity depends on an evolutionarily conserved core motif shared with Scm3^{sp} and HJURP. The assembly activ-

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ity is specific for Cse4 but independent of DNA sequence. Furthermore, assembly activity depends on the CATD of Cse4. We conclude Scm3 plays an active role in the assembly of centromeric nucleosomes.

EXPERIMENTAL PROCEDURES

Yeast Strains—The *S. cerevisiae* strains used in this study are listed in supplemental Table 1 and were constructed in the W303 background.

Co-immunoprecipitation—Whole cell extracts were obtained by beadbeating in the presence of lysis buffer (100 mM Tris (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 10% glycerol, and protease inhibitors). Co-immunoprecipitations were performed with anti-FLAG M2 affinity gel (Sigma-Aldrich). Beads were washed several times with lysis buffer and proteins were eluted with 10 mM Tris (pH 8.0)/1 mM EDTA/1% SDS.

ChIP and Quantitative (q)PCR—ChIPs were performed with biological replicates as described previously (6). ChIP lysates were sonicated to obtain sheared DNA fragments ~300 bp in length. α-Myc (9E10; Santa Cruz Biotechnology) was used at 1:2500. ChIPs were harvested by incubation with protein G-Sepharose (Amersham Biosciences). qPCR[2] was performed for eluted ChIP samples on an iCycler real-time PCR machine with IQ SYBR Green Supermix (Bio-Rad). Specific primer sets used were centromere 1 (forward, 5′-TGACATTGAACTTCAAAACCTTT-3′ and reverse, 5′-GGCGCTTGAAATGAAAGCTC-3′) and centromere 3 (forward, 5′-GATCAGCGCCAAACAATATGG-3′ and reverse, 5′-AACTTCCACCAGTAAACGTTTC-3′) as described previously (6, 29). PCR of ChIP DNA was quantified for biological replicates by comparing immunoprecipitates and total chromatin.

FACS—FACS analysis was performed to confirm cell cycle arrest on cells fixed in 70% ethanol. Cells were washed with FACS buffer (50 mM sodium citrate), treated with RNase, stained with Sytox Green (1 mM final), and analyzed by using a Cyan cytometer (Dako Cytomation).

Purification of Recombinant Proteins, Octamers, and Scm3/Cse4/H4 Complex—Yeast recombinant histones (H3, H4, H2A, H2B, and Cse4) were individually expressed in *Escherichia coli* and purified from inclusion bodies as described previously (30). His_6-Scm3 and its lethal mutants were purified using nickel-nitrilotriacetic acid metal-affinity agarose and standard His tag protein purification protocols (31). Assembly of histone octamers was carried out as in Ref. 32. Assembly of the Scm3/Cse4/H4 complex was performed as described previously (33). For constructing the H3/Cse4 hybrid, the region from Ala^{76} through Ile^{113} of H3 was replaced by the corresponding region containing the loop 1 and α2 helix of Cse4 (Thr^{166} through Leu^{206}) based on previous studies (15).

In Vitro Chromatin Assembly—The assembly of nucleosomes was performed as described previously (19, 34). Briefly, 0.2 μg of plasmid was relaxed with topoisomerase I. Purified canonical or Cse4 containing octamers (H3 or Cse4/H2A/H2B/H4) or Scm3/Cse4/H4 complex and His_6-Scm3 or lethal mutants were added and incubated for 2 h at room temperature in the presence of topoisomerase I with 8.3 mM HEPES (pH 7.4), 0.5 mM EGTA, 0.65 mM $MgCl_2$, 1.7% glycerol, 0.005% Nonidet P-40, 33 mM KCl, 0.33 mM DTT, and 0.02 mg/ml BSA. Plasmid DNA was deproteinized and purified by standard methods, and then topoisomers were resolved in agarose gels. Recombinant topoisomerase I was a kind gift from S. Venkatesh, Stowers Institute. Two plasmid were used: (i) pG5E4-5S containing five repeats of 5S flanking each side of an E4 core promoter downstream of five Gal4-binding sites (gift from the Workman laboratory, Stowers Institute) and (ii) pCEN1-10X containing 10 tandem repeats of the centromere 1 sequence.

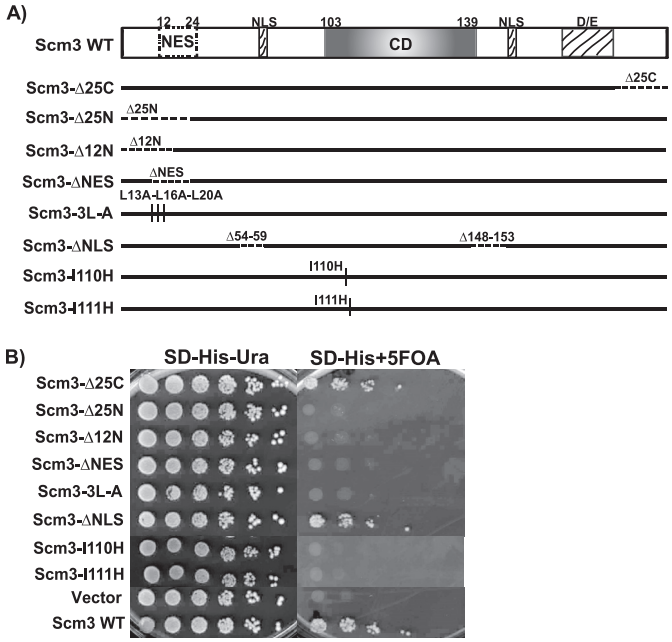

FIGURE 1. **Mutational analysis of Scm3.** *A*, schematic diagrams of wild-type Scm3 and site-directed Scm3 mutants. The NES, conserved motif (CD), potential NLS and C-terminal acidic (D/E) regions are *boxed*. *B*, plasmid shuffle complementation tests of mutants shown in *A*. Growth on 5-fluroorotic acid (*FOA*) medium indicates the respective mutant allele provides Scm3 function.

RESULTS

Scm3 Contains Two Essential Motifs—Scm3 is a relatively small protein (~25 kDa) containing several motifs (Fig. 1*A*). At the N terminus from amino acids 13–24, there is a putative leucine nuclear export sequence (NES) (7). There are two short patches of basic residues, similar to bipartite nuclear localization sequences (NLSs) found at positions 54–59 and 148–153 (35, 36). At its center, Scm3 has an evolutionarily conserved core motif. This motif resembles a coiled-coil domain in that it has repeating heptad units with hydrophobic residues occupying the fourth position and polar residues in the first position (37, 38). The C-terminal 58 amino acids are acid-rich (40% Asp + Glu).

To identify essential motifs, we carried out site-directed mutagenesis for each of these motifs and tested the mutant proteins for function using a plasmid shuffle assay (Fig. 1*B*). Deletion of either the C-terminal 25 amino acids (Scm3-Δ25C) or the bipartite NLS (Scm3-ΔNLS) did not result in a loss of growth. In contrast, mutations in evolutionarily conserved res-

[2] The abbreviations used are: qPCR, quantitative PCR; NES, nuclear export sequence; NLS, nuclear localization sequence.

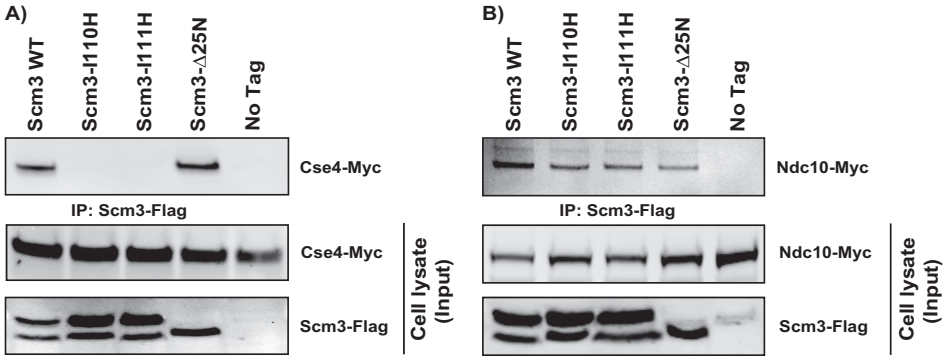

FIGURE 2. **Co-immunoprecipitation of Scm3 mutants with Cse4 and Ndc10.** Immunoprecipitations of Scm3-FLAG mutants were performed in a background containing Cse4-Myc or Ndc10-Myc. *No Tag* indicates that Scm3 does not have the FLAG tag. Western blotting was carried out with anti-Myc antibody. Full-length Scm3-FLAG often runs as a doublet for reasons that are currently unclear. *A*, evolutionarily conserved motif is required for Cse4 interaction. Point mutants in this motif no longer interact with Cse4. When the 25 N-terminal amino acids of Scm3 are deleted, this protein still pulls down Cse4. *B*, both point mutants in the conserved motif and a mutant with a deletion of 25 amino acids from N terminus still co-immunoprecipitate with Ndc10.

idues in the central motif or deletion of the N-terminal 25 amino acids (Scm3-Δ25N) were lethal. To define further the essential portion of the N-terminal region, we deleted residues 2–12, 13–24 (which contains the NES motif), or mutated the leucines in the NES motif. All of these mutations were lethal, suggesting that the NES as well as the amino acids upstream are essential, consistent with previous mutational analysis of Scm3 (7). We conclude that Scm3 has two essential motifs, the N-terminal 25 amino acids and the conserved core motif.

Conserved Motif of Scm3 Is Essential for Interaction with Cse4 and Its Localization to Centromeres—Scm3 physically associates with Cse4 and Ndc10 (6, 7, 33). We tested the proficiency of the lethal mutants for interactions with Cse4 and Ndc10 *in vivo*. For this study we have used a Gal-*SCM3* conditional allele (pGal$_{1-10}$-3HA-*SCM3*) so that we can shut off the wild-type chromosomal copy of Scm3 by switching to glucose medium for 2 h. This switch eliminates Scm3 as measured by Western blotting (6). Mutant versions of Scm3 are FLAG-tagged on a plasmid under the control of the endogenous promoter. Results shown in Fig. 2 reveal that point mutations (I110H, I111H) in the conserved motif of Scm3 disrupt the interaction with Cse4 whereas deletion of the N terminus of Scm3 (Scm3-Δ25N) does not disrupt this interaction (Fig. 2*A*). However, all of the lethal mutants interact with Ndc10 (Fig. 2*B*). These results demonstrate that the conserved motif is important for interaction with Cse4.

By using a similar strategy as above, we tested whether Cse4 is present at the centromere in these lethal mutants by ChIP/qPCR (Fig. 3). Interestingly Cse4 is present at CEN1 with the Scm3-Δ25N protein, but in the case of Scm3-I110H, Cse4 is not localized to the centromere (Fig. 3, *A* and *B*). Although Scm3-Δ25N can interact with Ndc10 and Cse4 and can apparently localize Cse4, this mutation is still lethal. Although the conserved motif appears to be essential for localization of Cse4 at centromeres, the essential function of the N terminus is not clear at present.

The Two Essential Motifs of Scm3 Cannot Be Differentiated by Point of Execution in the Cell Cycle—Cse4 appears to load at centromeres during S phase (39, 40). Without Cse4, the kinetochore will be defective, leading to a spindle checkpoint arrest (6, 41). In previous work we showed that when Scm3 was depleted in the G_1 phase of the cell cycle, the spindle check point was not activated. In contrast, if Scm3 was depleted in S phase or G_2/M, the spindle checkpoint is activated (6). Because Scm3 cannot be depleted in G_2/M without activating the checkpoint, Scm3 function appears to be required even after centromeric chromatin is formed. We performed the same type of point-of-execution experiments with the Scm3 lethal mutants, to determine whether one motif was more critical for checkpoint signaling.

We conducted arrest-deplete-release experiments. Cells were grown in galactose medium and synchronized in the G_1, S, and G_2/M phase of the cell cycle with α factor, hydroxyurea, and nocodazole, respectively. At this point, wild-type Scm3 either continued to be expressed (galactose) or was depleted by transfer of cells to glucose-containing medium (glucose). Then cells were released into the cell cycle. Cultures were monitored by flow cytometry. When we deplete wild-type Scm3 and express the mutant proteins at G_1 phase, there is a decrease in cells with 4 N DNA content compared with the total absence of Scm3 (supplemental Fig. 1*A*). In the Scm3-null background we could visualize a population of cells containing 4 N DNA content compared with the two lethal mutants. We have also visualized DNA by DAPI staining to verify the presence of multiple DNA masses in a single cell (data not shown). Taken together, these results suggest that the spindle checkpoint is activated more efficiently in the *scm3* lethal mutants compared with the null background. When wild-type Scm3 was depleted in early S or G_2/M phase, there were no significant differences between the null case and the two lethal mutants in terms of DNA content (supplemental Fig. 1, *B* and *C*). In all cases, cells arrest with 2 N DNA content and are large budded with a single DAPI mass (data not shown), suggesting that the spindle checkpoint is efficiently activated.

Next, we tested whether Cse4 and Ndc10 were present at centromeres in G_2/M by ChIP/qPCR when Scm3 was depleted in a G_1 arrest. Cse4 was not detected at the centromere in the Scm3-I110H background but was present at the centromere in the Scm3-Δ25N background (Fig. 3, *C* and *D*). However, Ndc10 was present at centromeres in both the Scm3-I110H and Scm3-Δ25N backgrounds (Fig. 3, *E* and *F*), consistent with the result

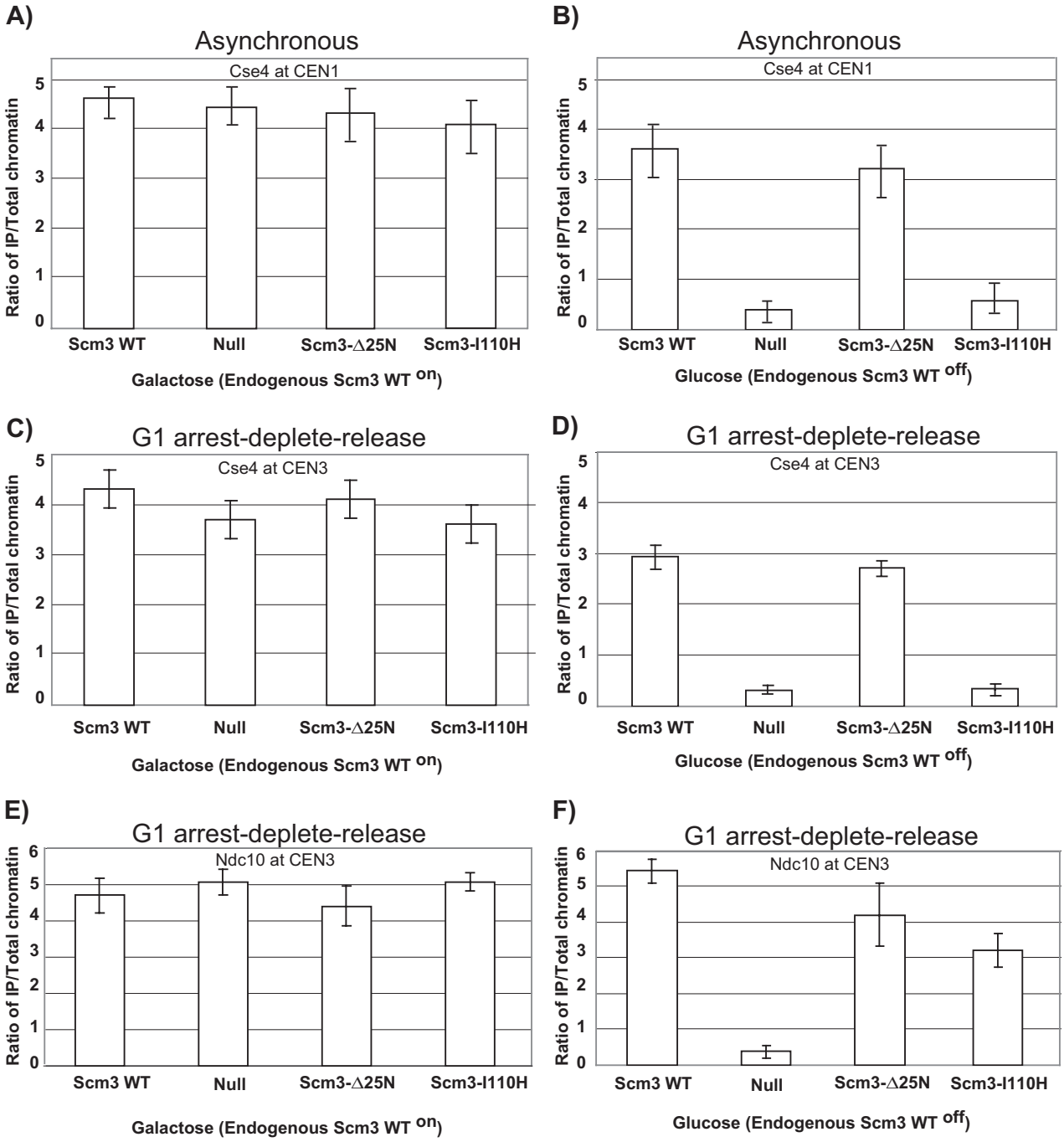

FIGURE 3. **Evolutionarily conserved core motif of Scm3 is required to load Cse4 at the centromere but not Ndc10.** Strains were constructed in which the endogenous copy of Scm3 was under control of the Gal promoter, a plasmid contained another source of Scm3, and Cse4 was tagged with 12myc epitopes. In galactose-containing medium, Scm3 is expressed (*A*, *C*, and *E*) but in glucose (*B*, *D*, and *F*) the only source of Scm3 is the plasmid. The ChIP/qPCR from the galactose cultures serves as a control. ChIP/qPCR shows that Cse4 is not present at CEN1 in the Scm3-I110H mutant background in glucose in either asynchronous cultures (*B*) or at CEN3 in G_1-arrested and released (4 h) cultures (*D*). *Error bars* represent ± the average deviation of biological replicates. A control ChIP omitting antibody was performed for each sample; all values were below 0.01 (ratio of no antibody/total chromatin). *E* and *F*, ChIP/qPCR for Ndc10 in G_1-arrested and released (4 h) cultures shows that Ndc10 is present at CEN3 in both the lethal mutant backgrounds. See also supplemental Fig. 1 for cytometry profiles.

that both of these mutants can interact with Ndc10 (Fig. 2*B*). Ndc10 is necessary for activation of the spindle checkpoint (42). The efficient localization of Ndc10 in the mutants compared with the null is the most likely explanation for the difference in checkpoint activation. Taken together, these results suggest that Scm3 has two distinct functions: (i) Cse4 deposition, which requires the evolutionarily conserved motif, and (ii) recruitment of Ndc10 to activate the spindle assembly checkpoint. The point of execution for each of the two essential motifs cannot be differentiated with respect to Ndc10 recruitment/checkpoint function.

Both Scm3 Lethal Mutants Can Separate H2A/H2B Dimers from Cse4 Octamer—It was reported previously that when Scm3 was added to Cse4-containing octamers, H2A/H2B

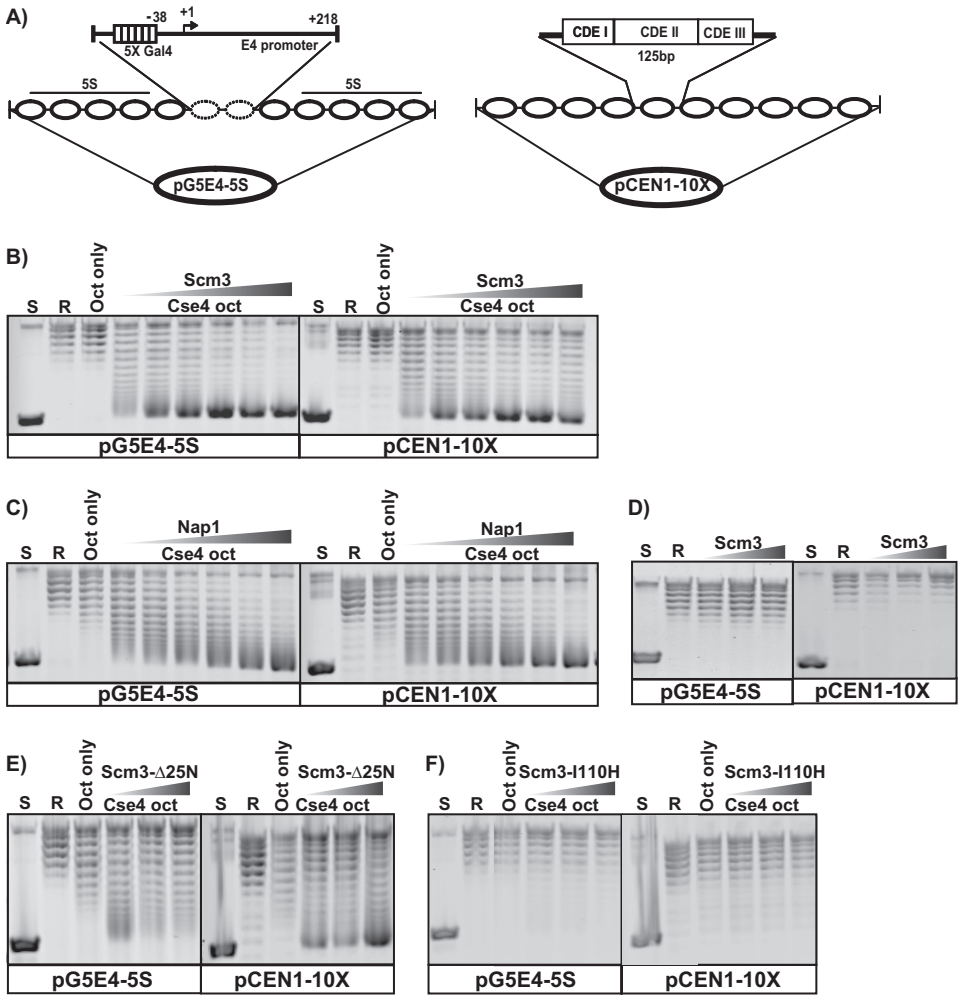

FIGURE 4. **Scm3 can assemble Cse4-containing nucleosomes *in vitro*.** Nucleosome assembly activity of Scm3 was studied with a plasmid supercoiling assay. Supercoiled plasmids were purified from *E. coli* (*S*) and relaxed by addition of topoisomerase I (*R*). Octamers alone (*Oct only*) was included as a control for each assembly experiment. *A*, schematic diagram of plasmid construct pG5E4-5S and pCEN1-10X plasmids is shown. *B*, chromatin assembly was performed by incubating the relaxed plasmids with purified His_6-Scm3 and Cse4 octamers. DNA and Cse4 octamer amounts are held constant at a ratio of 1:1, and Scm3 is added at a ratio of 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6. *C*, Nap1 was incubated with Cse4 octamers. DNA and Cse4 octamer amounts are held constant at a ratio of 1:1, and Nap1 is added at a ratio of 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0. *D*, control assembly reaction with only His_6-Scm3 at a ratio to DNA of 0.6 and 0.8 does not yield any supercoils on either of the plasmids. Higher amounts also had no effect. *E* and *F*, conserved core of Scm3 is necessary for chromatin assembly. Chromatin assembly reactions were performed by incubating the relaxed pG5E4-5S or pCEN1-10X plasmid with Cse4 octamers and either Scm3-Δ25N (*E*) or Scm3-I110H (*F*).

dimers were evicted, and a Scm3/Cse4/H4 complex was formed (33). We analyzed how recombinant Scm3 lethal mutant proteins behaved with respect to octamer splitting. Interestingly, when each lethal mutant was incubated with Cse4 octamers, the octamers were split into two distinct populations, one that contained Scm3/Cse4/H4 and one that contained Scm3/H2A/H2B as measured by gel filtration chromatography (supplemental Fig. 2, *C* and *D*). We did not find any difference between Scm3 lethal mutants with respect to H2A/H2B eviction since both mutants were able to split the octamers. We further tested whether Scm3 is present in a complex with Cse4/H4 or H2A/H2B by pulldowns from the fractions containing the split species. As shown previously (33), Scm3 interacts with Cse4/H4, but not H2A/H2B (supplemental Fig. 2*F*).

Conserved Core Motif Is Necessary for de Novo Scm3/Cse4/H4 Complex Formation—It was shown previously that recombinant Scm3, Cse4, and histone H4 form a stoichiometric complex with a molecular weight consistent with a hexamer (33). We tested whether the lethal Scm3 mutants could make a *de novo* Scm3/Cse4/H4 complex when mixed with recombinant Cse4 and H4. To address this question, wild-type or mutant Scm3 was mixed with Cse4 and H4 in 2 M NaCl. Scm3-WT and Scm3-ΔN25 were able to form a Scm3/Cse4/H4 complex with Cse4 and H4; Scm3-I110H could not (supplemental Fig. 3*A*). This result is consistent with the inability of the Scm3-I110H protein to co-immunoprecipitate with Cse4. Previously, a Scm3 mutant protein consisting of amino acids 93–143 was shown to be sufficient for Scm3/Cse4/H4 complex formation (33). Together these results suggest the conserved motif is essential to interact with Cse4.

Scm3 Assembles Nucleosomes in Vitro—Scm3 is necessary for the localization of Cse4 to centromeres *in vivo*. We tested whether Scm3 could facilitate the assembly of nucleosomes *in vitro*. To measure the chromatin assembly activity of Scm3 *in vitro*, we used a plasmid supercoiling assay in which the wrapping of DNA around the histone core particle induces super-

coiling in relaxed, closed, circular DNA. We tested two plasmids (Fig. 4*A*), one containing 10 copies of a 5 S nucleosome positioning sequence (pG5E4-5S, a gift from the Workman laboratory) and one containing 10 tandem copies of a yeast centromere 1 (CEN1) repeat unit. Following the assembly reaction, DNA was deproteinized, and plasmid topoisomers were resolved by agarose gel electrophoresis. Incubation of purified His_6-Scm3 and Cse4 octamers with either pG5E4-5S or pCEN1-10X resulted in the induction of several supercoils compared with controls (Fig. 4*B*), demonstrating that Scm3 can assemble Cse4 containing chromatin on both plasmids. With both plasmids we observed an increase of supercoils in a dose dependent manner to a certain level, followed by a decrease that may be related to the precipitation of Scm3 at higher concentrations. Nap1, a well studied histone chaperone (43), was also able to induce supercoils with Cse4 octamers on both plasmids (Fig. 4*C*). These experiments demonstrate that Scm3 can induce supercoils with Cse4 chromatin irrespective of DNA sequence. Scm3 alone does not mediate the supercoiling reaction (Fig. 4*D*).

Conserved Core of Scm3 Is Necessary for Chromatin Assembly—Because the conserved motif, but not the N-terminal motif of Scm3, is necessary for localization of Cse4 *in vivo*, we wanted to address whether these motifs are required for chromatin assembly *in vitro*. To address this we used purified recombinant Scm3 lethal mutant proteins (Scm3-Δ25N and Scm3-I110H) in chromatin assembly assays. Scm3-Δ25N was able to induce supercoils on both the pG5E4-5S and pCEN1-10X plasmid (Fig. 4*E*), but Scm3-I110H could not (Fig. 4*F*). Therefore, the conserved motif is essential for nucleosome assembly.

Scm3 Is a Cse4-specific Nucleosome Assembly Factor and Requires the Cse4 CATD for Nucleosome Assembly Activity—To test the specificity of Scm3 for Cse4, assembly reactions were carried out with H3 octamers. Strikingly, there was no addition of topoisomers when we use canonical octamers on either the CEN plasmid or the 5S plasmid (Fig. 5*A*). These H3 octamers could be assembled into chromatin using the chaperone Nap1 (Fig. 5*B*).

We next aimed to identify the motif/sequence in Cse4 that is required for Scm3 nucleosome assembly activity. Cse4 has two domains: (i) a divergent N-terminal essential domain and (ii) a highly conserved histone fold domain (44). The centromere targeting domain (CATD), consisting of loop 1 and helix 2 of the histone fold domain, is required for centromeric loading of centromeric histone variants (14). The CATD is a key regulator of Cse4 protein stability (45). HJURP, the human ortholog of Scm3, binds to CENP-A through its CATD domain, and this interaction occurs via the TLTY box of HJURP (28). Scm3 lacks a TLTY motif, which is conserved only in vertebrates (28). To test whether Scm3 nucleosome assembly activity requires the CATD domain of Cse4, we made octamers containing a H3/Cse4 chimeric protein in which H3 contains the Cse4 CATD domain (15). Although H3 octamers are not assembled into nucleosomes by Scm3, octamers containing this chimeric H3/Cse4 protein were assembled into nucleosomes (Fig. 5*C*), suggesting that the CATD domain is sufficient for Scm3 nucleosome assembly activity.

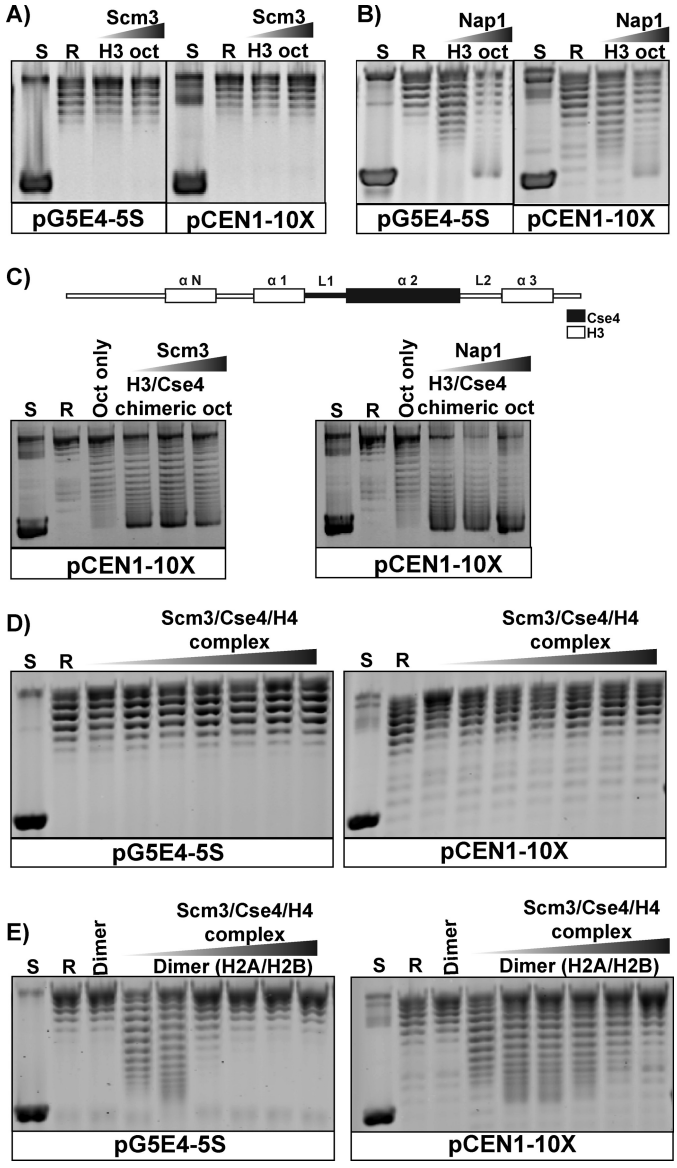

FIGURE 5. **Scm3 is a Cse4-specific nucleosome assembly factor.** *A*, Scm3-mediated chromatin assembly reaction was performed by incubating the relaxed plasmids and yeast canonical octamers at a ratio of 1:1 with increasing amounts of Scm3 (ratio of 1.0, 1.2). Higher amounts also had no effect. *B*, chromatin assembly was performed on relaxed plasmids with Nap1 and yeast canonical octamers as in *A* (ratio of 1.0, 1.2). *C*, diagram of the H3/Cse4 chimeric protein is shown. Chromatin assembly reactions were performed on the relaxed pCEN1-10X plasmid with increasing amounts of reconstituted octamers containing H3/Cse4 chimeric protein and Scm3 or Nap1. *D* and *E*, H2A/H2B are critical for Scm3 to induce supercoiling. Chromatin assembly reactions were performed under the conditions in Fig. 4*B* by incubating the relaxed pG5E4-5S and pCEN1-10X plasmid with (*D*) increasing amounts of reconstituted Scm3/Cse4/H4 complex alone (His_6-Scm3/Cse4/H4) or (*E*) Scm3/Cse4/H4 complex with an equivalent molar ratio of H2A/H2B dimers. In the lane labeled *dimer*, H2A/H2B dimers were added at a ratio of 0.8 to the DNA. See also supplemental Figs. 2 and 3.

H2A/H2B Dimers Are Critical for Scm3 to Induce Supercoiling—The composition of centromeric nucleosomes has been hotly debated (19, 46, 47). At present there are three models for the composition of the budding yeast centromeric nucleosome. One model suggests that Cse4 replaces H3 in an octameric nucleosome that contains Cse4, H2A, H2B, and H4 (19). Octameric nucleosomes containing human CENP-A can be reconstituted *in vitro* (48, 49). A second model proposes that

centromeric nucleosomes contain a single molecule each of CenH3, H2A, H2B, and H4, which forms a tetrameric structure called a "hemisome" (47). This hemisomal complex was purified from interphase *Drosophila* S2 cells by cross-linking and immunoprecipitation of CID. Hemisomes appear half as tall as canonical nucleosomes when analyzed by atomic force microscopy and are predicted to contain <120 bp of DNA (47). A third model is a "hexameric nucleosome," composed of two copies each of Scm3, Cse4 and H4 (33). Given these models, we decided to test whether addition of the Scm3/Cse4/H4 complex (supplemental Fig. 3*A*) would induce supercoiling in our assembly assay. We did not observe supercoiling on either type of plasmid (Fig. 5*D*). Recently, Vishnapuu and Greene reported that they reconstituted nucleosomes using the Scm3/Cse4/H4 complex and linear λDNA (50), but we were not able to replicate this outcome on a circular plasmid. Interestingly, addition of H2A/H2B dimers results in some supercoiling (Fig. 5*E*) on both plasmids, suggesting H2A/H2B are necessary for the assembly reaction.

DISCUSSION

Although Scm3 is an essential inner kinetochore protein, its precise molecular function has remained poorly understood. Herein, we have shown that Scm3 appears to be a bona fide Cse4-specific chaperone. We further show that the chaperone activity depends on the evolutionarily conserved motif of Scm3 and the CATD of Cse4, but not centromeric DNA sequence. The conserved motif of Scm3 is required for interaction with and deposition of Cse4, but not Ndc10, at centromeres, arguing for two separable functions for Scm3. The deposition of Ndc10, and therefore the ability to activate the spindle assembly checkpoint, depends on Scm3, but not its chaperone function. Furthermore, this result suggests that Ndc10 recruitment and spindle checkpoint activation do not depend on Cse4 deposition at the centromere.

The chaperone activity of Scm3 requires the CATD domain of Cse4 and the evolutionarily conserved motif of Scm3. Recently, it has been shown that Psh1 is an E3 ubiquitin ligase that targets Cse4 (45, 51), and Scm3 appears to protect Cse4 from Psh1 (51). Consistent with this proposal, Psh1 requires the CATD domain of Cse4 to target Cse4 (45). Thus, it seems likely that Scm3 has an active function in Cse4 protein maintenance and nucleosome assembly that depends on the CATD domain. HJURP, a putative human ortholog of Scm3, also possesses assembly activity for CENP-A/H4 complexes with DNA *in vitro* (28). HJURP interacts with CENP-A through its TLTY box, a highly conserved motif across vertebrates (28). However, Scm3 lacks a TLTY motif. Instead, Cse4 interaction and assembly activity depend on the evolutionarily conserved core motif of Scm3.

One of the proposed structures for centromeric nucleosomes is a hexasome, which does not contain H2A/H2B (33, 50). We have used a supercoiling assay to test whether the Scm3/Cse4/H4 complex would induce topoisomers. Although the Scm3/Cse4/H4 complex fails to induce supercoiling, addition of H2A/H2B dimers along with Scm3/Cse4/H4 complex did result in supercoiling, suggesting that H2A and H2B are critical for nucleosome formation. The requirement for H2A *in vitro* is consistent with previous data suggesting that H2A is present in Cse4 nucleosomes (19) and is required for proper centromere function (52). Thus, our results are most consistent with models for the centromeric nucleosome that contain H2A and H2B.

The assembly of nucleosomes by Scm3 can occur on either centromeric DNA sequences or a canonical nucleosome positioning sequence. Thus, the reactions we have conducted *in vitro* lack sequence specificity. This result is consistent with a recent report in which it was shown that Scm3 is required to load Cse4 at a noncentromeric sequence, the stable partitioning locus within the 2-μ plasmid (45). We speculate that sequence specificity *in vivo* is achieved by the CBF3 protein complex, which is a sequence specific binding complex found in point centromere-containing organisms. In future studies it will be interesting to determine whether the addition of CBF3 or other components will increase the specificity for the centromere sequence. However, our observations suggest that Scm3 alone cannot provide sufficient DNA sequence specificity to restrict Cse4 nucleosomes to centromeres.

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