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Supplemental Information

Nonhistone Scm3 Binds to AT-Rich DNA to Organize Atypical Centromeric Nucleosome of Budding Yeast

Hua Xiao, Gaku Mizuguchi, Jan Wisniewski, Yingzi Huang, Debbie Wei, and Carl Wu

Inventory of Supplemental Information

Supplemental Information (Figures and Movies)

Movie S1, Continuous presence of Scm3 at centromeres during anaphase, related to Figure 5.

Movie S2, Continuous presence of Cse4 at centromeres during anaphase, related to Figure 5.

Supplemental Experimental Procedures

Supplemental References

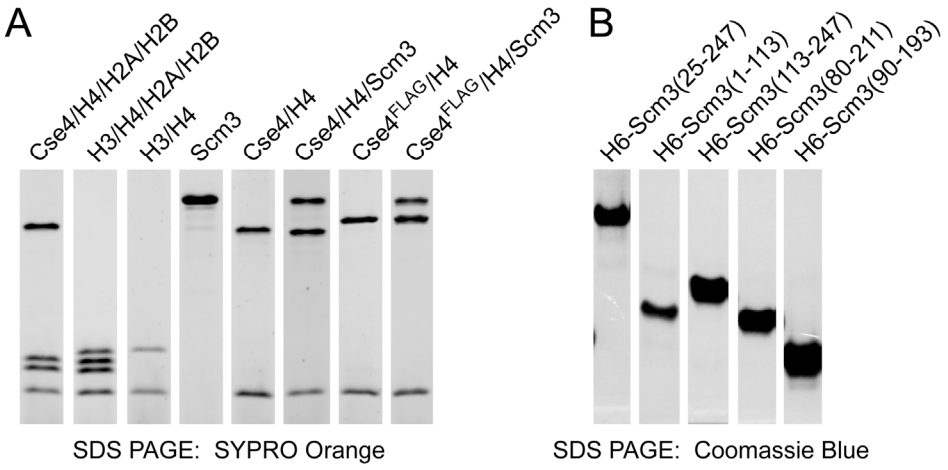

Figure S1. Purified proteins and protein complexes used in this study. *(A)* Cse4, Scm3 and histone complexes. Bacterial expression, purification of individual proteins and reconstitution of protein complexes were performed as described in Supplemental Experimental Procedures. Purified complexes were analyzed by SDS PAGE and SYPRO Orange staining. *(B)* Purified His_6-tagged Scm3 and Scm3 fragments analyzed by SDS PAGE and Coomassie Blue staining.

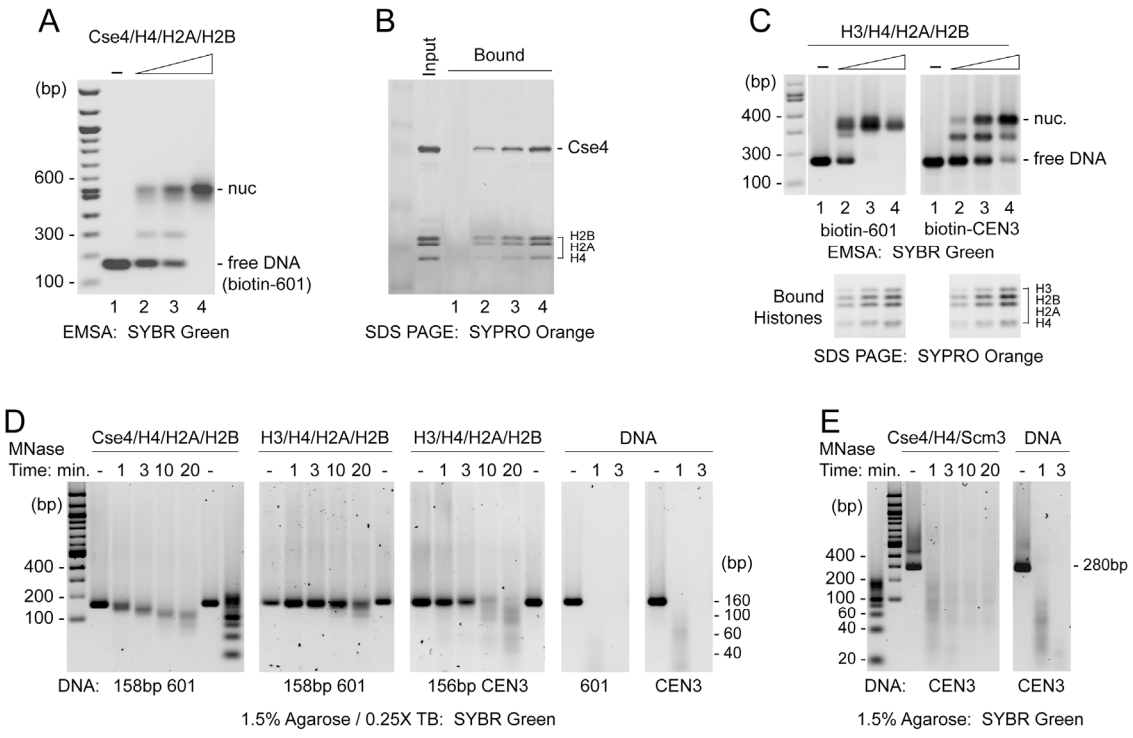

Figure S2. Analysis of Cse4 nucleosome, conventional H3 nucleosome and Cse4/H4/Scm3 nucleoprotein complexes reconstituted on *601* and CEN3 DNA fragments. *(A)* Cse4 nucleosomes reconstituted by salt dialysis with $[Cse4/H4/H2A/H2B]_2$ octamers (in ascending order: 1.25, 2.5, 5.0 µg) and 3 µg of biotinylated *601* DNA in 50 µl volume, analyzed by EMSA and SYBR Green staining. *(B)* Cse4 nucleosomes contain all four histones. Histones on biotinylated DNA were pulled down with strepavidin beads and analyzed by SDS-PAGE and SYPRO Orange staining. Lanes 1-4 correspond to samples in (A). *(C)* Conventional H3 nucleosomes were reconstituted with $[H3/H4/H2A/H2B]_2$ octamers and biotinylated *601* and *CEN3* DNA fragments. Histones on biotinylated DNA were pulled down with strepavidin beads and analyzed by SDS-PAGE and SYPRO Orange staining. *(D)* MNase digestion of Cse4 nucleosomes and conventional H3 nucleosomes

reconstituted on *601* and *CEN3* DNA fragments. Samples corresponding to lane 4 in *A* and *C* were digested with 0.5 U of MNase for the indicated time (min). Digested samples were treated to 1% SDS, and DNA analyzed by EMSA and SYBR Green staining. *(E)* MNase digestion of Cse4/H4/Scm3 nucleoprotein complexes reconstituted on 280bp *CEN3* DNA (r_m = 1.65) and analyzed as in *D*.

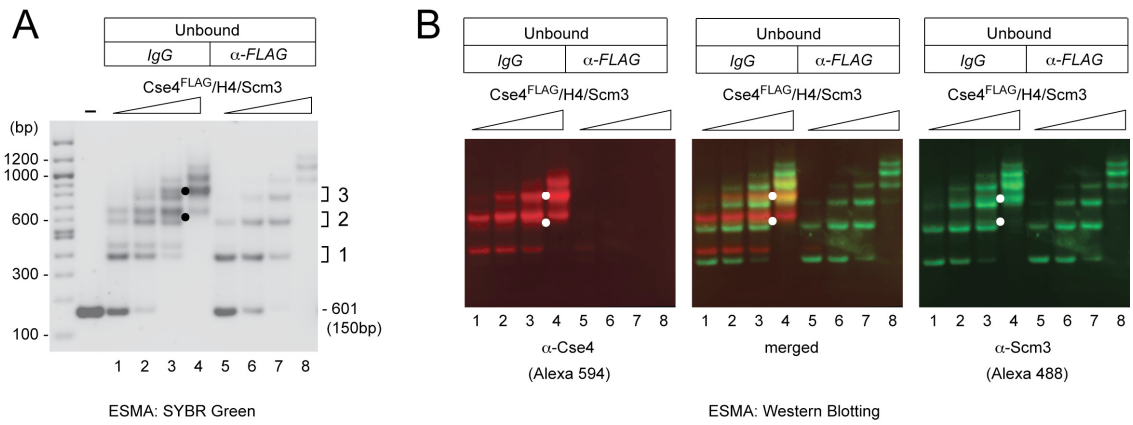

Figure S3. Reconstitution of Cse4^{FLAG}/H4/Scm3 nucleoprotein complexes on *601* DNA fragment. (*A*) Complexes were reconstituted by salt gradient dialysis using 150 bp *601* DNA and Cse4^{FLAG}/H4/Scm3 (in ascending order r_m = 0.44, 0.88, 1.32 and 1.76). Reconstitutes were depleted for Cse4^{FLAG}-containing species by IP with α-*FLAG*, and unbound complexes (containing only Scm3) were analyzed by EMSA and SYBR Green staining (lanes 5-8). (*B*) Cse4- and Scm3-containing complexes confirmed by Western blot analysis of a replicate gel as in (*A*), probing with α-*Cse4* (rabbit) and α-*Scm3* (guinea pig) antibodies, and visualized by fluorescent secondary antibodies, Alexa Fluor 594 goat-anti-rabbit and Alexa Fluor 488 goat-anti-guinea pig, respectively. The dots mark distinctly resolved complexes that are depleted by α-*FLAG* and therefore contain both Scm3 and Cse4^{FLAG}. *IgG* was used as a depletion control (lanes 1-4). The reconstitution efficiency of band #3 normalized to input DNA is 1.5-fold lower for 601 than for CEN3 (Figure S3A, lane 3 versus Figure 2E, lane 3).

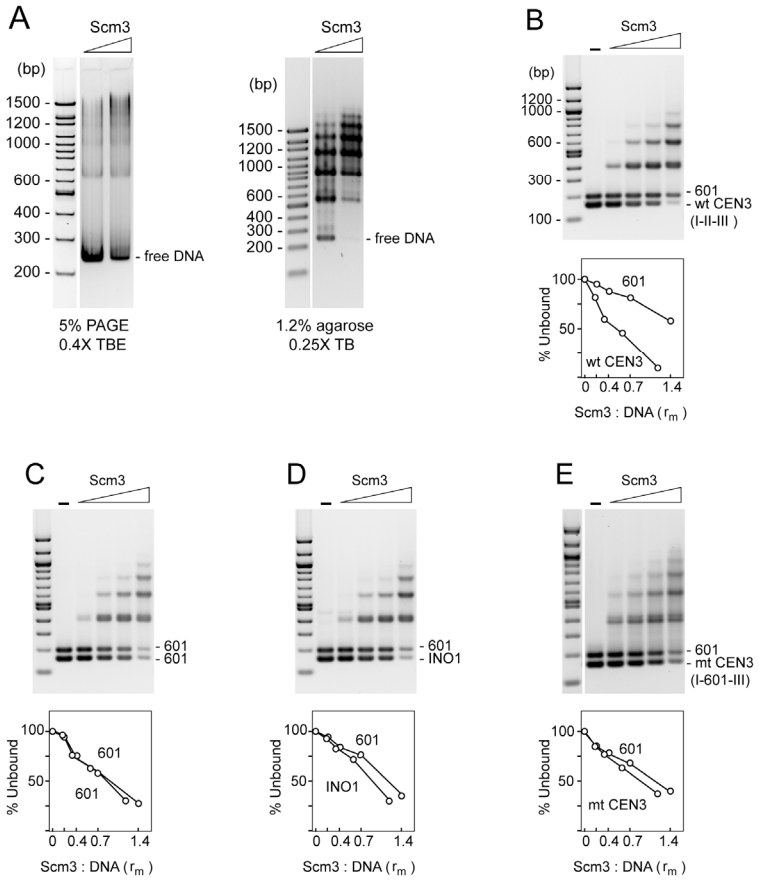

Figure S4. Scm3 binds nonspecifically to DNA, but prefers AT-rich centromere sequences. (*A*) Scm3-DNA complexes are unstable and poorly resolved on a non-denaturing polyacrylamide gel, but clearly detected on an agarose gel. Equivalent samples are compared. *(B-E)* Mixtures of various DNA fragments were incubated with increasing concentrations of purified Scm3 (r_m in ascending order: 0.2, 0.4, 0.7, 1.4) and complexes were analyzed by EMSA and SYBR Green staining. (*B*) Mixture of *601* (194 bp) and wild type *CEN3*: CDE I-II-III (148 bp) DNA fragments; (*C*) mixture of *601* (194 bp) and *601* (150 bp) DNA fragments; (*D*) mixture of *601* (194 bp) and *INO1* (150 bp) DNA fragments; and (*E*) mixture of *601* (194 bp) and mutant *CEN3*: CDE I-601-III (148 bp) DNA fragments.

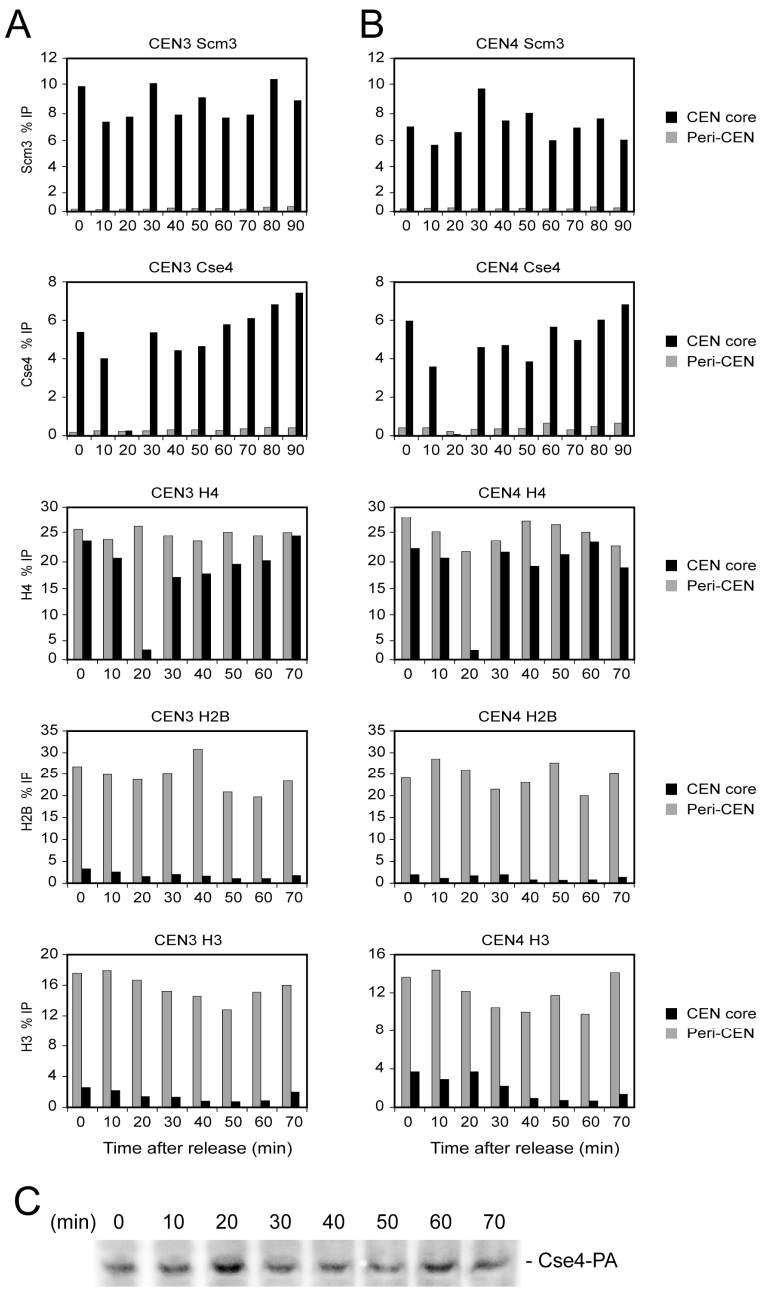

Figure S5. Quantification of histone and nonhistone protein occupancy at centromeres. *(A, B)* Bar graphs showing the % IP of each PCR fragment relative to input DNA for *CEN3* and *CEN4*, respectively, derived from raw data of Figure 6. *(C)* Steady-state levels of Cse4-PA through the cell cycle, shown by Western blotting using α-PA antibody.

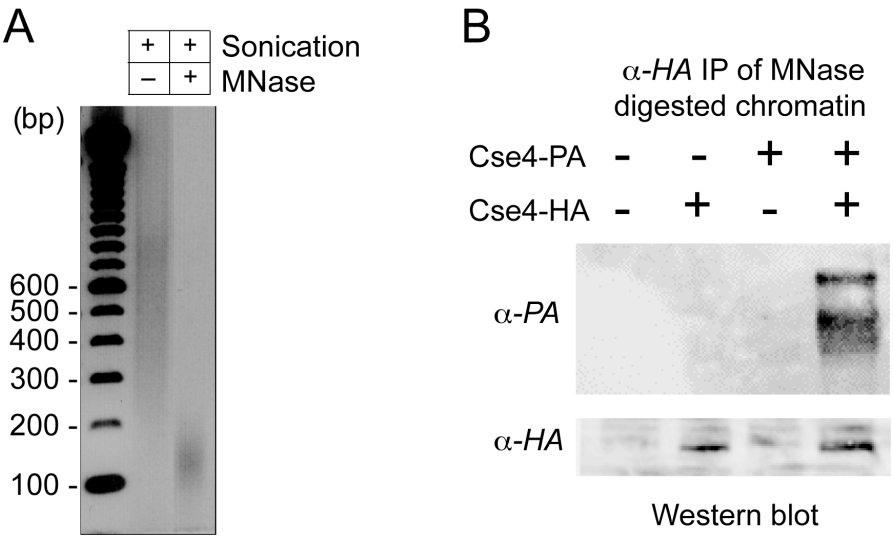

Figure S6. Centromeric nucleosome contains at least two molecules of Cse4. (*A*) Agarose gel electrophoresis showing DNA lengths of sonicated chromatin, with or without MNase digestion. Crude chromatin isolated from cross-linked diploid cells carrying the two distinct versions of Cse4 was sonicated and extensively digested with MNase. (*B*) Co-IP of protein A-tagged Cse4 (Cse4-PA) and HA-tagged Cse4 (Cse4-HA). MNase digested chromatin was immuno-precipitated with α-HA-3F10 antibodies. Bound proteins were de-crosslinked and analyzed by SDS-PAGE and Western blotting using α-PA or α-HA antibodies.

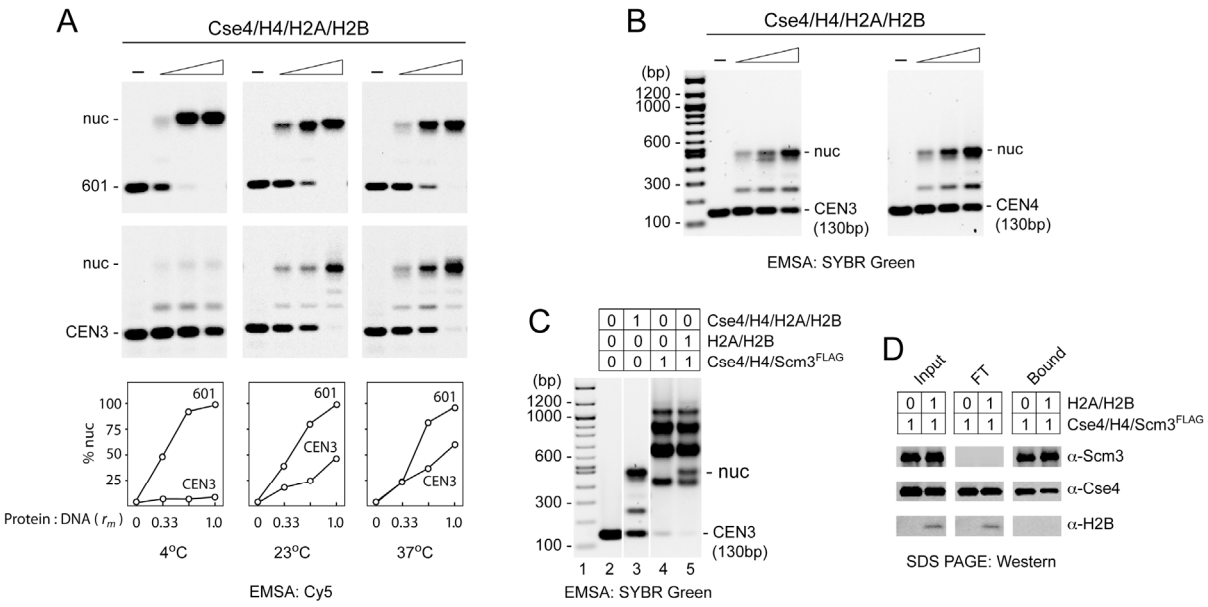

Figure S7. (*A*) Temperature effects on the reconstitution of Cse4 nucleosomes and Cse4/H4/Scm3 complexes on 601 and CEN DNA. Cy5-labeled 601 or CEN3 DNA tracer was mixed with unlabeled CEN3 or 601 DNA, respectively, followed by addition of $[Cse4/H4/H2A/H2B]_2$ octamers (protein : DNA molar ratio r_m in ascending order = 0.3, 0.6, 1.2) and salt gradient dialysis at indicated temperatures. The reactions were then analyzed by EMSA, and the efficiency of nucleosome reconstitution was expressed as % nucleosome of the input DNA signal (100%). (*B*) Reconstitution of Cse4 nucleosomes on 130bp CEN3 and CEN4 DNA fragments by salt gradient dialysis at 30°C. (*C*) Reconstitution of DNA-protein complexes in the presence of both Cse4/H4/Scm3 complex and H2A/H2B dimers. 130bp CEN3 DNA was mixed with both Cse4/H4/Scm3 complex and H2A/H2B dimer, followed by salt gradient dialysis at 30°C. Addition of H2A/H2B dimers results in a distinctly resolved EMSA species that co-migrates with the octameric Cse4 nucleosome (compare lanes 3 and 5). (*D*) Western blot analysis of α-*FLAG* pulldown of reconstituted Cse4/H4/Scm3FLAG + H2A/H2B on CEN3 as in *C*, lanes 4 and 5.

Movie S1. Continuous presence of Scm3 at centromeres during anaphase. Time-lapse movie assembled from fluorescent images of cells carrying *SCM3-GFP* as the sole copy of the gene. The cell at the center of the screen is undergoing anaphase. To limit phototoxicity and photobleaching, exposures (1 sec; EM gain=149) were acquired at a single focal plane every 5 seconds. 100x objective was used for imaging and image scale is 160nm/pixel (frame size, 24μm x 24μm). Duration of this movie is 520 sec in real time.

Movie S2. Continuous presence of Cse4 at centromeres during anaphase. Time-lapse movie assembled from fluorescent images of cells carrying *CSE4-GFP* as the sole copy of the gene. Lower cell at the center of the screen is undergoing anaphase. To limit phototoxicity and photobleaching, exposures (0.5 sec; EM gain=200) were acquired at a single focal plane every 5 seconds. 100x objective was used for imaging and image scale is 160nm/pixel (frame size, 24μm x 24μm). Duration of this movie is 520 sec in real time.

Supplemental Experimental Procedures

Plasmids for the Synthesis of Core Histones and Scm3 Proteins in E. coli

Cse4, Scm3 and other core histone genes were PCR-amplified from yeast genomic DNA (Novagen) and cloned into either pET15 or pET28 vectors where the expression of the cloned genes are driven by the T7 promoter (Mizuguchi et al., 2007). A FLAG-tagged Cse4 was synthesized and cloned into pET28. The H2A/H2B genes were cloned into the poly-cistronic vector pST39 (Tan, 2001) to co-express the H2A/H2B pair. In addition, His-tagged Scm3 and its sub-fragments were constructed for affinity purification and protein affinity chromatography. Details of the plasmid constructions are available on request.

Synthesis and Purification of Recombinant Core Histones and Scm3 proteins

Recombinant histone H3, histone H4, and histone variant Cse4 were expressed individually in *E. coli* and purified using established protocols (Dyer et al., 2004). Recombinant H2A/H2B dimers were synthesized and purified as described previously (Mizuguchi et al., 2007).

Full length Scm3 was purified using the standard core histone purification protocol (see above). His-tagged Scm3 and its sub-fragment derivatives were purified by Talon beads affinity chromatography, followed by size fractionation on a Superdex 200 gel filtration column.

Reverse Phase HPLC

Higher purities of core histones and Scm3 proteins were obtained by reverse phase HPLC purification. Proteins were first purified via ion exchange chromatography, and

then subject to reverse phase HPLC purification using 60% acetonitrile and water as mobile phase solvent. Proteins were eluted and lyophilized.

Reconstitution of Histone Octamers and Scm3/Cse4/H4 Complex

Core histone octamers and Scm3/Cse4/H4 complexes were reconstituted by using established protocols (Dyer et al., 2004). For both core histone octamers and Scm3/Cse4/H4 complexes, equimolar amounts of purified recombinant histones (H2A, H2B, H3 and H4, or H2A, H2B, Cse4 and H4, or Scm3, Cse4 and H4) were dissolved in unfolding buffer (7M guanidine-HCl, 20mM Tris-Cl, pH7.5, 10mM DTT) at 2mg/ml. The mixtures were dialyzed against four changes of two liters each of refolding buffer (10mM Tris-Cl, pH7.5, 1mM EDTA, 5mM β-mercaptoethanol, 0.1mM PSMF) containing 2M NaCl for two days at 4°C. The mixture was then centrifuged at 15,000 rpm in a Tomy MX-300 micro-centrifuge to remove any insoluble material. Reconstituted octamers were separated from H2A/H2B dimers and H3/H4 or Cse4/H4 tetramers by size fractionation on a Superdex 200 gel filtration column.

Preparation of DNA Fragments

The 194bp 601 DNA fragment was prepared by PCR amplification, followed by agarose gel electrophoresis and ethanol precipitation. The 150bp 601 DNA was first PCR amplified with an asymmetrical AvaI site at both ends and cloned into a modified pUC19 vector that contains an asymmetrical AvaI site. Various centromeric DNA fragments were synthesized with an asymmetric AvaI site and cloned into pUC19 or into pUC57 vectors. The cloned 601 and centromeric DNA fragments were isolated and ligated into tandem arrays through the asymmetrical AvaI site,

which were then cloned in a modified pUC19 or pUC57 vector that contains an asymmetrical AvaI site. After restriction digestion, fragments were purified by agarose gel electrophoresis and ethanol precipitation. Thus, it should be noted that such DNA fragments have a 4bp overhang at both ends of the fragments, and that for some applications these sticky ends were filled in with Biotin-dNTP (Roche), Cy3-dNTP or Cy5-dNTP (GE Healthcare) using Klenow polymerase. The CDEII and CDEI+CDEIII replacement constructs were made in which the corresponding regions were substituted with 601 or INO1 DNA of the same length, and DNA fragments were prepared as above (details of plasmid constructs are available upon request).

Reconstitution of Nucleosomes and Histone-DNA Complexes

Purified core histones and DNA were mixed in 50 µl of high salt buffer (2M NaCl, 10 mM Tris-Cl, pH7.5, 1 mM EDTA, 0.02% NP-40, 5mM β-mercaptoethanol) supplemented with BSA at 400 µg/ml. The mixture was transferred to a Slide-A-Lyzer MINI dialysis unit (Thermo Scientific). The dialysis unit was placed in a container with 600ml of high salt buffer, and dialyzed for 30 minutes. To start salt gradient dialysis, a low salt buffer (100mM NaCl, 10mM Tris-Cl, pH7.5, 1mM EDTA, 0.02% NP-40, 2mM β-mercaptoethanol) was pumped into the container at 3.5ml/min for 16 hours. The dialysis unit was then transferred to low salt buffer and dialyzed for 60 minutes. The dialysis was done at 4°C, unless otherwise noted. Reconstituted nucleosomes and protein-DNA complexes were analyzed by EMSA.

Electrophoresis Mobility Shift Assays (EMSA)

Reconstituted nucleosomes and protein-DNA complexes were analyzed by

electrophoresis at 120 V for 70-90 minutes on native agarose gels (Seakem ME, Lonza, ME) in a buffer containing 25 mM Tris, 25 mM boric acid (Huynh et al., 2004; Zimarino and Wu, 1987). After electrophoresis, the gel was stained with SYBR Green I (Invitrogen) and visualized with a Fujifilm LAS-3000 camera.

Affinity Pulldown of Associated Core Histones

To examine proteins associated with Biotin-labeled DNA fragments, strepavidin-coated-magnetic beads (Invitrogen) were added to reconstituted protein-DNA complexes, and incubated with gentle mixing for 20 minutes. Beads were washed with binding buffer containing 200 mM NaCl. Bound proteins were analyzed by SDS PAGE and SYPRO Orange staining.

Yeast Strains

All used strains were derived from S. cerevisiae W303.

JBY121: MATa ADE2 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 SCM3-GFP(S65T)-kanMX NUF2-mCherry-natMX

MBY507: MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 CSE4-GFP(S65T)

MBY512: MATa/Matα ade2-1/ade2-1 can1-100/can1-100 his3-11,15/his3-11,15 leu2-3/leu2-3,112 trp1-1/trp1-1 ura3-1/ura3-1 RAD5/RAD5 CSE4-6HA/CSE4-ProteinA

MBY 545: MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 SCM3-GFP(S65T)-kanMX

MBY558: MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 RAD5 SCM3-3HA-kanMX

Chip-Western

ChIP-Western assays of CSE4 (Figure S6) were performed with the yeast cells expressing *CSE4- PA* and *CSE4-HA*. The cells were treated with 3% paraformaldehyde, and then lysed with glass beads in FA140 buffer as described previously (Mizuguchi et al., 2007). Sonicated chromatin was then treated with micorcoccal nuclease (1 unit/µl) for 15 min at 37°C. The resulting mononucleosomal-size chromatin samples were incubated with IgG Dynabeads (Invitrogen), washed with FA500 buffer (Mizuguchi et al., 2007) five times, and boiled in SDS-PAGE sample buffer for 30 min to reverse crosslinking. The eluted fractions were subjected to western blot analysis with anti-Protein A (Sigma-Aldrich) or anti-HA antibody (Abcam).

Antibodies

Polyclonal antibodies against full-length Cse4 and Scm3 proteins for ChIP or western blotting were generated at Cocalico Biologicals Inc. using purified bacterially expressed proteins (Mizuguchi et al., 2007). Anti-Htz1 and anti-H2B antibody were described previously (Luk et al., 2007; Wu et al., 2005). Requests for antibodies should be directed to J.W. Commercial antibodies used for western blotting and ChIP included anti-HA (ab9110; abcam), anti-Flag M2 (Sigma-Aldrich), anti-H3 (ab1791; Abcam), anti-H4 (#05-858; Upstate). Secondary antibodies for immunofluorescence microscopy included donkey anti-mouse IgG, goat anti-rabbit IgG, and goat anti-guinea pig IgG conjugated to AlexaFluor 488 or 594 (Invitrogen).

Flow Cytometry

A FACS scan was used for flow cytometric analysis of cellular DNA content as described previously (Amberg et al., 2005; Breeden, 1997).

Microscopy and Imaging

For live cell imaging, yeast strains MBY507 (expressing Cse4-GFP as the sole source of Cse4), MBY545 (expressing Scm3-GFP as the sole source of Scm3) and JBY121 (expressing Scm3-GFP and Nuf2-mCherry as the sole source of the proteins) were grown in a shaker-incubator at 24°C in synthetic medium composed of 0.79 g/L CSM (Bio101 Systems, Vista, CA, USA), 6.7 g/L yeast nitrogen base without amino acids (Invitrogen, Carlsbad, CA, USA), 2% glucose and 400mg/L adenine-HCl. Cells were immobilized in Y2 or Y4 microfluidic culture plates (CellASIC, San Leandro, CA, USA) and maintained during imaging with constant flow of medium at 5 psi. For imaging we used Zeiss AxioObserver Z1 inverted microscope, equipped with Zeiss Plan-Apo 150x/1.35NA Gly Corr Vis-IR objective (or Zeiss EC Plan-NeoFluar 100x/1.3NA oil POL objective, when indicated), matching DIC sliders and custom narrowband transmitted light illuminator (containing 671nm/10nmFWHM filter and additional 700nm cutoff heat-mirror from Edmund Optics, Barrington, NJ), Colibri LED (474nm 28nm FWHM or 585nm/35nm FWHM) light sources (Zeiss Microimaging, Thornwood, NY, USA) and P-737 piezoelectric Z-stage (Physik Instrumente, Auburn, MA, USA). Images were acquired with Hamamatsu C9300-13 back-illuminated EMCCD camera (Hamamatsu Corp., Bridgewater, NJ, USA) with detector cooled to -94°C and operating at the highest precision readout (16-bit, 0.69MHz), typically in the regular CCD mode

(conversion factor = 1.49 electrons/ADU, readout noise = 11 electrons RMS, dark current = 0.005 electron per pixel x sec., typical exposure of 5 sec. per image for epifluorescence and 0.5 sec. for DIC) or, where indicated, in electron-multiplying mode (for example at EM gain = 100, conversion factor = 0.019 electron/ADU, readout noise = 0.345 electron RMS, dark current = 0.0013 electron per pixel x sec, typical exposure of 0.5 sec. or 1 sec.). Epifluorescence images were acquired for GFP through ET525/50 emission filter combined with T495LP beamsplitter (Chroma Technologies, Bellows Falls, VT, USA), while Chroma 615LP emission filter combined with Semrock FF605-DiO1 beamsplitter was used for mCherry (Semrock Inc., Rochester, NY, USA). Semrock FF01-750/SP filter was mounted in front of the camera window to block IR scattered from microscope encoders. Hamamatsu HC Image software was used for image acquisition and direct triggering of LED light source and piezoelectric stage movement. For Z-stacks, images were acquired at 9 stage positions separated by 0.50μm. Original 16-bit images were converted into 32-bit floating-point FITS files using Image J (NIH, Bethesda, MD – custom batch macro available upon request from J.W.) and calibrated with corresponding bias, thermal and flat frames (see Berry and Burnell, 2006) using AIP4WIN software (Willman-Bell, Richmond, VA, USA). For display as Figures, images were linearly scaled and converted into 8-bit grayscale TIF files. Time-lapse movies were assembled from such 8-bit images as uncompressed AVI files.

Supplemental References

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