Supplemental Material

A high sensitivity phospho-switch triggered by Cdk1 governs chromosome morphogenesis during cell division

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Supplemental Materials and Methods:

**Yeast strains and growth conditions.** Standard yeast culture conditions have been used (Guthrie and Fink 1991). *Saccharomyces cerevisiae* strains used in this study are derivative of K699 and K700 strains (Nasmyth et al. 1987) and their genotype is summarized in Table S1. Synchronization of cell cultures was performed using α-factor (50 ng/ml for 180 min; G1 arrest), nocodazole (30 μg/ml for 150 min; metaphase arrest), NM-PP1 (500 nM for 150 min; G2/M arrest in *cdc28-as1* background), or hydroxyurea (0.2 M for 120 min; S phase arrest) at 23 °C, 25 °C or 37.5 °C (Bishop et al. 2000; St-Pierre et al. 2009). Treatment of yeast with indole-3-acetic acid (IAA) was performed according to published procedures (Nishimura et al. 2009). The growth properties of yeast mutants were determined by serial dilution on solid medium, as described previously (Ratsima et al. 2011). For photobleaching experiments, yeast cultures were grown in low fluorescence synthetic media (i.e., using yeast nitrogen base without folic acid and riboflavin) (Sheff and Thorn 2004). The cyclin-defective yeast mutant (*clb1Δ clb3Δ clb4Δ clb2-ts* and *clb5Δ clb6Δ*), and the strain carrying *mCherry*-tagged *HTA1* were kind gifts from A. Amon and F.R. Cross, respectively. *Schizosaccharomyces pombe* culture conditions were as described previously (Moreno et al. 1991).

**Plasmid and mutant construction.** Phospho-site mutations in *SMC4* were incorporated into *YCplac111*-derived plasmids carrying a *SMC4::3xSTII::T*<sub>ADH1</sub>::*HIS3MX6* insert. The following alleles of *SMC4* and of *YCS4* were created in part by custom gene synthesis (Bio Basic Inc.): *smc4-7A*(601); *smc4-7E*(757); *smc4-7EE*(879); *smc4-7AA*(969); *smc4-7AE*(1061); *smc4-7QQ*(1075); and *ycs4-6A*. Manual site-directed mutagenesis (using Quickchange Multi-
Mutagenesis kit, Stratagene) and/or subcloning strategies were used to create other combinations of mutations in the \textit{YCplac111-smc4-x::3xSTII::T\textsubscript{ADH1}::HIS3MX6} plasmid backbone, as well as in \textit{YCplac33-YCS4::13MYC::T\textsubscript{ADH1}::kanMX6, YCplac111-YCG1::T\textsubscript{ADH1}::TRP1, YCplac22-SMC2::T\textsubscript{ADH1}::caURA3MX6} plasmids. Condensin alleles created in this study carry the following mutations: \textit{smc4-7A(601)}: S4A, T43A, T60A, S109A, S113A, S117A and S128A; \textit{smc4-7E(757)}: S4E, T43E, T60E, S109E, S113E, S117E and S128E; \textit{smc4-7EE(879)}: SP\[4-5\]EE, TP\[43-44\]EE, TP\[60-61\]EE, SP\[109-110\]EE, SP\[113-114\]EE, SP\[117-118\]EE and SP\[128-129\]EE; \textit{smc4-7AA(969)}: SP\[4-5\]AA, TP\[43-44\]AA, TP\[60-61\]AA, SP\[109-110\]AA, SP\[113-114\]AA and SP\[128-129\]AA; \textit{smc4-7AE(1061)}: SP\[4-5\]AE, TP\[43-44\]AE, TP\[60-61\]AE, SP\[109-110\]AE, SP\[113-114\]AE, SP\[117-118\]AE and SP\[128-129\]AE; \textit{smc4-7QQ(1075)}: SP\[4-5\]QQ, TP\[43-44\]QQ, TP\[60-61\]QQ, SP\[109-110\]QQ, SP\[113-114\]QQ, SP\[117-118\]QQ and SP\[128-129\]QQ; \textit{smc4-10A(702)}: S4A, T43A, T60A, S109A, S113A, S117A, S128A, T757A, T847A and T1345A; \textit{smc4-927*}: TP\[757-758\]EE, TP\[847-848\]EE and TP\[1345-1346\]EE; \textit{smc4-1051}: SP\[113-114\]EE, SP\[117-118\]EE and SP\[128-129\]EE; \textit{smc4-929*}: TP\[757-758\]EE, TP\[847-848\]EE and TP\[1345-1346\]EE; \textit{smc4-1002}: SP\[113-114\]EE and SP\[117-118\]EE; \textit{smc4-1002}: SP\[109-110\]EE, SP\[113-114\]EE and SP\[117-118\]EE; \textit{smc4-997}: SP\[109-110\]EE and SP\[117-118\]EE; \textit{smc4-996}: SP\[109-110\]EE and SP\[113-114\]EE; \textit{smc4-995}: SP\[113-114\]EE; \textit{smc4-984*}: SP\[4-5\]EE; \textit{smc4-930*}: TP\[847-848\]EE and TP\[1345-1346\]EE; \textit{smc4-1013}: S4A, T43A, T60A, SP\[109-110\]EE, SP\[113-114\]EE, SP\[117-118\]EE, SP\[128-
129]EE, T757A, T847A and T1345A; \textit{smc4-82}: S40P, T43A, S54A, T60A, S67A, S68A, Y106F, S109A, S113A, S117A, L525F, L526P, E557G, V697D, L744V, and L1240P; \textit{smc4-7EE-1}: SP[4-5]EE, TP[43-44]EE, TP[60-61]EE, SP[109-110]EE, SP[113-114]EE, SP[117-118]EE and SP[128-129]EE, L526P, E557G, V697D, L744V; \textit{smc4-\Delta NT(1011)}: [1-153]\Delta-L154M; \textit{ycs4-6A}: S41A, S48A, T66A, T186A, S305A and S988A; \textit{ycg1-4A}: S128A, T591A, T608A and S734A; \textit{smc2-2A}: S91A and S98A. To insert the phospho-mutant alleles of condensin subunits at their endogenous loci, diploid yeast strains were transformed with linearized plasmids containing the appropriate mutations. Integration of the desired mutations was subsequently confirmed by sequencing the relevant genomic loci. Some alleles of \textit{smc4} (marked with an asterisk above) were obtained through partial integration of phospho-mutations contained within mutagenic constructs. Yeast strains carrying mutations in multiple genes were created by conventional mating and dissection of sporulated heterozygous diploid strains.

To create the \textit{S. pombe cut3-T19V} allele, the open reading frame (ORF) of the gene plus 200 bp of its promoter sequence were subcloned into a pFA6a-kanMX6 series plasmid (Bahler et al. 1998) to create the pFA6a-\textit{P}_{cut3}::\textit{cut3::T}\textit{ADH1::kanMX6} vector. Site-directed mutagenesis was used to create the pFA6a-\textit{P}_{cut3}::\textit{cut3-T19V::T}\textit{ADH1::kanMX6} mutant plasmid. Both plasmids were subsequently used as templates to amplify \textit{cut3}-targeting constructs (encompassing the entire ORF plus 200 bp of adjacent sequence) by PCR. The amplification products were transformed into a wild-type diploid yeast strain, and transformants were selected for their ability to grow on solid YES-A medium containing G418. Homologous integration at the \textit{cut3} locus was confirmed by PCR and the entire ORF of the mutant and wild-type alleles were sequenced to confirm the absence of unexpected mutation.
Strains expressing \( SMC4 \) from an ectopic locus were generated by integration at the \( URA3 \) locus of a linearized plasmid carrying the appropriate allele of \( SMC4 \) under the control of the \( GAL1 \) promoter (overexpression) or its own promoter. For each strain, we confirmed by PCR that the \( URA3 \) locus contained a single copy of the integrative plasmid. To create the \( CLB2::SMC4 \) fusion construct, we amplified a version of \( CLB2 \) lacking its destruction and KEN boxes and subcloned it upstream of \( SMC4 \) coding sequence in \( YCplac111-\text{SMC}4::3x\text{STII}::T_{\text{ADH}1}::\text{HIS}3\text{MX}6 \) plasmid, thereby creating \( YCplac111-\text{PSMC}4-CLB2::\text{SMC}4::3x\text{STII}::T_{\text{ADH}1}::\text{HIS}3\text{MX}6 \). This cloning step also introduced a flexible linker between \( CLB2 \) and \( SMC4 \). The \( CLB2::\text{smc}4-7A \) fusion construct was obtained by subcloning the N-terminal part of \( \text{smc}4-7A \) from \( \text{YIplac}211-\text{PGAL1-smc}4-7A::3x\text{STII}::T_{\text{ADH}1}::\text{URA}3 \) plasmid into the \( YCplac111-\text{PSMC}4-CLB2::\text{SMC}4::3x\text{STII}::T_{\text{ADH}1}::\text{HIS}3\text{MX}6 \). To create the \( \text{CycBox}\Delta \) variant of this construct, we introduced 2 new \( Stul \) restriction sites in the nucleotide sequence encoding residues RF291-292 and MN362-363 of \( CLB2 \). \( YCplac111-\text{PSMC}4-\text{clb}2-\text{CycBox}\Delta::\text{SMC}4::3x\text{STII}::T_{\text{ADH}1}::\text{HIS}3\text{MX}6 \) was obtained after removal of the \( Stul \)-fragment and re-ligation of the plasmid. This removes the sequence encoding residues F292 to N363 ( inclusively), thereby creating a version of Clb2 analogous to the \( \Delta 241-313 \) mutant of human Cyclin A2, which is incapable of binding to Cdc2/Cdk1 (Lees and Harlow 1993). Wild-type, \( \text{smc}4-7A \) and \( \text{CycBox}\Delta \) versions of the \( CLB2::\text{SMC}4 \) plasmids were then linearized and transformed into a wild-type diploid strain. To create the \( \text{smc}4-\DeltaNT(1011) \)-targeting construct, we introduced a new BamHI restriction site at the sequence encoding L154 of \( SMC4 \) in \( YCplac111-\text{PSMC}4-\text{BamHI-SMC}4::3x\text{STII}::T_{\text{ADH}1}::\text{HIS}3\text{MX}6 \). The \( YCplac111-\text{PSMC}4-\text{BamHI-smc}4-\DeltaNT::3x\text{STII}::T_{\text{ADH}1}::\text{HIS}3\text{MX}6 \) construct was obtained after removal of the BamHI fragment and re-ligation of the parental plasmid. This removes the sequence M1 to
R153 (inclusively) and substitutes the L154 into a start codon. To create strains expressing smc4-AID, we subcloned the auxin-inducible degron sequence (Nishimura et al. 2009) downstream of SMC4 coding sequence in YCplac111-SMC4::3xSTII::T<sub>ADH1</sub>::HIS3MX6, thereby creating YCplac111-SMC4::3xSTII::AID::HIS3MX6. This plasmid was then linearized and transformed into a strain expressing Oryza sativa (Os) TIR1 gene (Nishimura et al. 2009). Selection of the transformed yeast on SC-histidine medium and PCR screening revealed clones with smc4-AID integrated at its endogenous locus. Single copy integration of smc4-aid was confirmed by qPCR analysis of the amplification signal obtained from genomic DNA isolated from a control wild-type strain and from the smc4-aid strain. The SMC4 amplification signal was essentially identical (relative to TRP1) in both strains, thereby indicating the presence of a single copy of SMC4 gene in the smc4-aid strain (data not shown). The plasmid containing the AID degron and the yeast strain expressing OsTIR1 were obtained from the Yeast Genetic Resource Center (YCGR, Japan). Strains expressing Smc4-NT/Smc4-7A-NT/Smc4-rxl-NT from centromeric plasmids were constructed as follows. A new restriction site was introduced by site-directed mutagenesis into YCplac111-SMC4::13MYC::T<sub>ADH1</sub>::HIS3MX6 to allow removal of the C-terminal part of Smc4 (i.e., from residue 143 to 1418). After the removal was completed, the sequence encoding SV40 large T antigen nuclear localization signal (PKKKRK) (Kalderon et al. 1984) was inserted between the sequence encoding SMC4-[1–142] and the 13xMYC tag to ensure the correct localization of the reporter construct. YCplac111-smc4-7A(601)[1-142]::NLS::13MYC::T<sub>ADH1</sub>::HIS3MX6 was obtained by subcloning the N-terminal part of YCplac111-smc4-7A(601)::13MYC::T<sub>ADH1</sub>::HIS3MX6 into YCplac111-SMC4[1-142]::NLS::13MYC::T<sub>ADH1</sub>::HIS3MX6. YCplac111-smc4[1-142]-[RxL#1/2->AAA]::NLS::13MYC:
\[ T_{ADH1}::HIS3MX6 \] was obtained by site-directed mutagenesis of \textit{SMC4} sequence in \textit{YCplac111-SMC4[1-142]}::\textit{NLS}::13\textit{MYC}::\textit{TADH1}::HIS3MX6 plasmid (using Quickchange Multi-Mutagenesis kit, Stratagene). These single-copy plasmids were transformed into \textit{cdc15-2, clb1\Delta clb3\Delta clb4\Delta clb2-VI, clb5\Delta clb6\Delta, cdc5-99}, and \textit{cdc28-4 cdc5-99} mutants, or in a wild-type strain and maintained in yeast using selective medium. The plasmid expressing \textit{SMC4-7EE-NT} from the \textit{GAL1} promoter was obtained by subcloning the N-terminal part of \textit{YCplac111-smc4-7EE[1-142]}::13\textit{MYC}::\textit{TADH1}::HIS3MX6 into \textit{Ylplac211-smc4-7EE::3xSTII::TADH1}. The plasmid used to express and purify the N-terminal extension of Smc4 in bacteria was created by subcloning a PCR-amplified fragment encoding the first 163 amino-acid residues of \textit{SMC4} into the pET30a plasmid. The resulting construct expresses Smc4-[1-163] as a fusion protein with a C-terminal hexahistidine tag. In all plasmid constructs, the sequences of condensin subunits correspond to those present in the wild-type K699 strain background. For the overexpression and purification of the Cdc28-Clb2 complex, \textit{P\textit{GAL1-2xStreptagII-CLB2}} and \textit{P\textit{GAL10-9xHIS-CDC28}} were subcloned in a 2\mu \textit{URA3 leu2-d} plasmid, resulting in p711. Key residues within the destruction box of \textit{CLB2} were removed (\textit{i.e.}, R25A, L28A) to ensure maximal expression of the protein in yeast. Maps of most plasmids used in this study are available and will be provided upon request.

**Western blotting.** Cell lysates were prepared using the TCA/glass bead method (Foiani et al. 1994). To visualize the phosphorylation-induced gel shift of Smc4-NT, lysates were separated on 8\% Phos-tag gels (Kinoshita et al. 2006) following the manufacturer’s instructions. Note that Smc4-NT and Smc4-7A-NT migrate at nearly identical positions on gel after phosphatase treatment (data not shown). To detect full-length Smc4, yeast lysates
were separated by SDS-PAGE using gels containing 7.5% Next Gel acrylamide (St-Pierre et al. 2009) (Amresco). All gels were transferred using the iBlot system (Invitrogen). Membranes were probed with the following antibodies: mouse monoclonal 9E-10 (from GeneTex; at 1:1000 dilution) or 9E-11 (gift from A. Verreault; at 1:1000 dilution); rabbit polyclonal anti-Swe1 (gift from A.D. Rudner; at 1:1000 dilution in Fig. 3A); rat monoclonal 18D9 and 15F2 antibodies against Swe1 (from Medimabs; both at 1:150 dilution in Fig. 3D); mouse monoclonal anti-StrepTagII (from Qiagen; at 1:1000 dilution); mouse monoclonal 11H12 and 4F10 against Cdc5 (from Medimabs; both at 1:500 dilution); and mouse monoclonal 22C5 (from Abcam; at 1:10,000 dilution) in 2% milk and 1% BSA. The rabbit polyclonal anti-phospho-serine 128 and anti-phospho-serine 4 antibodies were generated by Genscript using a phosphorylated peptide covering the amino-acid sequence surrounding Ser128 or Ser4 in Smc4 and used at a 1:250 and 1:1000 dilutions in 5% BSA respectively. The rat monoclonal antibodies 18D9 and 15F2 were generated against full-length recombinant Swe1 expressed in bacteria and recognize different epitopes on the protein. Secondary antibodies used were HRP-conjugated anti-mouse or anti-rabbit IgG antibodies (1:10,000, Amersham/GE Healthcare). Protein-antibody conjugates were revealed by chemiluminescence (Western Lightning Plus ECL; Perkin-Elmer).

**Fluorescent in situ hybridization (FISH).** For all experiments, cells were fixed and harvested in 0.1M KPO₄ buffer pH 6.4 containing 3.7% formaldehyde for 2hrs at 23 °C. We used published procedures to reveal the morphology of the rDNA locus by FISH (Guacci et al. 1994; Lavoie et al. 2004). Probes were generated by PCR amplification of a section of the rDNA locus cloned within plasmid p333, which contains a ~9.1 kb Xmal fragment.
encompassing a complete rDNA repeat. The amplified fragment was purified after electrophoretic separation using Qiagen’s Gel Extraction kit and labelled with digoxigenin using the BioNick Labelling System (Invitrogen). The digoxigenin labelling protocol is similar to that described for biotin labelling, except that 1 µl digoxigenin DNA labelling mixture (Roche) and 5 µl 10X nick translation buffer (0.5 M Tris-HCl pH 7.8, 50 mM MgCl, 100 mM beta-mercaptoethanol, 100 µg/ml Bovine Serum Albumin [BSA]) are substituted for 5 µl 10X dNTP mix from the BioNick Labelling kit. The digoxigenin-labelled probe was detected using a mouse anti-DIG antibody from Roche, and FITC-conjugated goat anti-mouse IgG (Jackson Immunoresearch) and Alexa Fluor 488-conjugated rabbit anti-goat IgG antibodies (Jackson Immunoresearch). All three antibodies were diluted 1:250 using 10% horse serum immediately prior to use. Nuclei were counterstained with propidium iodide (PI; Sigma) in 5 mg/ml of p-phenylenediamine (Sigma).

**Microscopy.** Procedures to monitor mitotic spindle length, nucleus morphology and spindle pole body position in cells have been described previously (Ratsima et al. 2011). Live cell imaging and visualization of rDNA morphology were performed on a DeltaVision microscope using the softWoRx software (Applied Precision). The microscope was equipped with a 100X/NA 1.4 Plan APO objective (Olympus) and a CoolSnap HQ2 camera (Photometrics). Images were acquired at 1x1 binning. Final images represent maximum intensity projections of multiple image stacks taken at 0.2 µm intervals. Deconvolution (softWoRx) was applied to images shown in Figures 1A and 4G to represent accurately the morphology of the rDNA loops observed under the microscope.
Photobleaching. Cells were grown until exponential phase at 25 °C in modified synthetic medium. Photobleaching experiments were performed on a Zeiss 710 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) attached to an AxioObserver inverted microscope stand. Images were collected and photobleaching was performed using a 63x/1.4 NA plan-apochromat oil immersion objective lens. Single images of cells in metaphase (i.e., with a large bud and the nucleus close to the bud neck) were collected at 10x zoom and at low laser power (2% of the 488 nm laser line of a 25 mW argon ion laser) in DIC and in fluorescence before and after each photobleaching experiment. Cells were then centered in the field of view. Photobleaching was performed using the line scan feature and a bleach line of 512 pixels across the center of the cell was selected. Total of 20,000 line scans were collected with 3.15 μs pixel dwell times for each cell, PMT gain at 700 digital gain at 1, digital offset at 10, pinhole at 2 Airy unit, no line averaging and 20% laser power. Emission from the sample was collected from 493 nm to 598 nm. Experiments were done on at least twelve budded cells for each strain. Fixed cell experiments were performed the same way on cells fixed with 3.7% formaldehyde for 5 min at room temperature. Two rectangular regions of interest were selected on XT line scan plots, one for background intensity and one for EGFP fluorescence intensity measurements. Intensity measurements were then corrected line-by-line for the background intensity (from an area of the XT line scan image where no cell was located). Corrected intensities were normalized to maximal for each photobleaching trace. Normalized data from three separate experiments were averaged and standard deviations were calculated from the three experimental averages. Final normalized curves for each strain were plotted and fit to a double exponential decay with an offset (i.e., Equation 1). Graphs were generated and fit
using SigmaPlot software (Systat Software Inc., Chicago, IL). Equation 1: \( y = y_0 + a \cdot e^{-x/b} + c \cdot e^{-x/d} \).

**Protein purification.** To identify Cdk1 phosphorylation sites on condensin, the complex was immunopurified from nocodazole-arrested cell lysates and processed for mass spectrometry analysis, as previously described (St-Pierre et al. 2009). For the purification of Smc4 N-terminus from bacteria, plasmid p792 expressing the first 163 residues of Smc4 fused to a hexahistidine tag was transformed into *Escherichia coli* BL21 strain. Cells were inoculated at an optical density \((A_{600})\) of 0.225 in LB medium containing all the necessary antibiotics and grown for 2 hr at 30 °C. Overexpression was induced by the addition of 1mM IPTG for 1 hr. Cells were collected by centrifugation and suspended in buffer A (50 mM KPO₄ pH 8.0, 500 mM NaCl, 10 mM imidazole, plus protease inhibitor cocktail [0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 10 μM Pepstatin A and 7.5 μM E64]) containing 300 μg/ml lysozyme. After a 30 min incubation at 4 °C, cells were lysed by sonication using a Misonix sonicator 3000 (3 pulses of 10 sec at output level 3) and centrifuged 15 min at 13000 rpm. The supernatant was further clarified by filtration with a 0.45 μm syringe-filter. The recombinant Smc4-[1-163] protein was purified on a FPLC system equipped with a HisTrap column (GE Healthcare) and fractions containing the purified protein were concentrated by ultrafiltration using Amicon Ultra filtration units (3K Nominal Molecular Weight Limit [NMWL]; Millipore). All purification steps were carried out at 4 °C. For kinase assays, condensin and Cdc28-Clb2 were purified according to published procedures (St-Pierre et al. 2009; Ratsima et al. 2011).
**In vitro kinase assay.** 3 µg of purified condensin or 0.5 µg of histone H1 were diluted into a final volume of 15 µl of kinase reaction buffer (25 mM Tris-HCl pH 7.5, 2 mM DTT, 10 mM MgCl₂, 0.5 mM EDTA, 100 µM ATP) supplemented with protease/phosphatase inhibitors (1 mM AEBSF, 10 µM E-64, 10 µM pepstatin A and 200 µM tungstate), and 2 µCi [γ32P] ATP, and 1.86 µg purified Cdc28-Clb2. Phosphorylation reactions were performed for 30 minutes at 30 °C and stopped by addition of SDS-PAGE sample loading buffer. Proteins were denatured 5 minutes at 100 °C and separated on a 4-12% gradient gel (Biorad) before being revealed by Coomassie staining (Biorad). 32P-labelled bands were detected in dried gels by autoradiography using Amersham Hyperfilm ECL films (GE Healthcare). For kinase reactions with Smc4-[1-163], 0.5 µg of purified substrate was phosphorylated using the same conditions as described above, except that radio-labelled ATP was omitted from the reaction. Phosphorylated proteins were separated on 14% SDS-polyacrylamide gels and revealed by coomassie staining.

**Immunoprecipitation and dephosphorylation.** Cell lysates were prepared in lysis buffer (50 mM Tris-HCl pH7.5, 100 mM KCl, 100 mM NaF, 10% glycerol, 0.1% tween 20, 1 mM tungstate, 1 mM DTT, 10 µM AEBSF, 10 µM pepstatin A, 10 µM E-64), as previously described (St-Pierre et al. 2009). For each immunoprecipitation reaction, 5 µg of anti-Myc 9E-10 or anti-Strep-tag antibody were bound to GammaBind Plus Sepharose beads (GE Healthcare). Dephosphorylation reactions were performed at 30 °C for 30 minutes in λ-phosphatase buffer (NEB) in presence or absence of purified λ phosphatase.
Other experimental procedures and statistical analyses. Determination of budding index by light microscopy and DNA content by flow cytometry were performed using standard procedures (Ratsima et al. 2011). For budding index, 100 cells were scored per time-point. Except for mass spectrometry analysis of purified condensin subunits (i.e., Supplementary Fig. 2a), all experiments described in this study have been performed 3 (or more) independent times. Error bars in graphs represent standard deviation (S.D.). Triple star symbols in graphs indicate statistically significant differences, as determined by \( t \)-test (paired, one tailed distribution). \( P \)-values are provided in the legend of figures.

Supplemental Figure Legends:

Figure S1. Microtubule, nucleus, and rDNA morphology in cells arrested at different stages of interphase and mitosis. (A) Schematic representation of the experimental set up. Cells carrying \( clb1 \ clb3 \ clb4 \ clb2-VI \) mutations (Amon et al. 1993) (i.e., \( clb-ts \) mutants) were arrested in G1 with \( \alpha \)-factor, split into four cultures, and released from the arrest. The first culture was shifted at 37 \( ^\circ \)C and allowed to grow for 135 min to generate a prophase-like \( clb-ts \) arrest. The second culture was grown at 23 \( ^\circ \)C for 105 min to allow most cells in the culture to reach mitosis naturally. The third culture was released into medium containing nocodazole for 150 min at 23 \( ^\circ \)C to generate a metaphase-like arrest. The fourth culture was harvested immediately after the G1 release. Samples of each culture were collected and processed for rDNA FISH analysis or indirect immunofluorescence to detect \( \alpha \)-tubulin. It was necessary to perform FISH and immunofluorescence staining in different cells because the conditions for staining the rDNA and tubulin are incompatible. (B) Morphology of the rDNA locus as detected by FISH. A representative micrograph of the dominant rDNA
species for each condition is shown. Propidium iodide (PI; red) and fluorescein isothiocyanate (FITC; green) were used to label the nucleus and rDNA locus, respectively. (C) Cell morphology (differential interference contrast [DIC] microscopy), nuclei morphology (4’,6-diamidino-2-phenylindole; DAPI) and mitotic spindle length/tubulin staining have been determined in the cells described above. (D–E) Quantification of the rDNA and spindle/tubulin morphologies observed in cells described in panels B and C, respectively (n = 3; error bars represent S.D.). At least, 100 nuclei or cells were counted per condition. The categories "bright spot" and "dot" in panel e refer to duplicated and unduplicated SPB, as previously described (Fitch et al. 1992; Yin et al. 2002).

Figure S2. The intertwist rDNA morphology is revealed in cells undergoing chromosome morphogenesis at low temperatures. (A–C). Cells were synchronized in G1 using α-factor and released into a fresh medium containing nocodazole at 16°C for 240 minutes. Samples were collected every 60 minutes and processed for visualization of the budding index, DNA content analysis and rDNA morphology. (A) Budding index of the cell population examined during the time-course. At least 100 cells were scored per time point (B) Flow cytometry analysis of cellular DNA content at various time points during the experiment. (C) Morphology of the yeast rDNA locus revealed by FISH. Quantification of each rDNA species is shown on the top. At least one hundred nuclei were counted per condition. (n=3 and error bars represent S.D.). Representative micrographs of the most prominent rDNA morphology observed during the time course is shown below the graph. PI (red) and FITC (green) were used to label the nucleus and rDNA locus, respectively.
Figure S3. The formation of the intertwist rDNA morphology requires cohesin activity. clb-ts or clb-ts mcd1-1 cells growing asynchronously at 23°C were arrested in early mitosis by shifting the culture at 37°C for 135 min. Samples were then fixed and the morphology of the rDNA locus was monitored by FISH. Representative micrographs of the most prominent rDNA morphology observed for each condition is shown on the left. PI (red) and FITC (green) were used to label the nucleus and rDNA locus, respectively. Quantification of each rDNA species is shown on the right. At least one hundred nuclei were counted per condition. (n=3 and error bars represent S.D.).

Figure S4. Cdk1 targets the evolutionarily-conserved N-terminus of Smc4. (A) Mass spectra of Cdk1 phosphorylation sites identified in Smc4 immuno-purified from mitotic cell extracts. Spectra are shown for phospho-residues Thr43, Thr60, Ser109, Ser113 and Ser128 in the N-terminal extension of Smc4. The blue box at the bottom right corner of this panel highlights the amino-acid sequence surrounding the phosphorylation sites. Most of the phospho-sites conform to the optimal consensus sequence for Cdk1 phosphorylation (i.e., Ser/Thr-Pro-X-Arg/Lys) (Koivomagi et al. 2011), whereas one conforms to the core Cdk1 consensus (i.e., Ser/Thr-Pro; Thr43) (Rudner and Murray 2000; Holt et al. 2009). Note that several of those sites, together with Ser117, were found to be phosphorylated in a Cdk1-dependent manner in proteome-wide screens that aimed to identify mitotic phosphorylation sites (Holt et al. 2009; Bazile et al. 2010; Kao et al. 2014). Interestingly, Cdk1 phospho-sites were not identified in other condensin subunits in the course of our mass spectrometry analysis. (B) Schematic representation of the SMC subunits of cohesin (Smc1 and Smc3) and condensin (Smc2 and Smc4) complexes from budding yeast. The
conserved functional domains of SMC family members are drawn to approximate scale. 

Note that only Smc4 carry an N-terminal extension among this group of SMC proteins. 

Conservation of the N-terminal extension among Smc4 family members in eukaryotes. A multiple local alignment of the primary amino-acid sequence corresponding to the N-terminal portion of *H. sapiens* (Hs), *S. pombe* (Sp) and *S. cerevisiae* (Sc) Smc1-4 homologs is shown. The blue arrow indicates the start of the ATPase domain, whereas the boxed sequences correspond to the N-terminal extension specific to the Smc4 family members.

Known phospho-sites that conform to the Cdk1 consensus sequence in the N-terminal extensions of budding yeast, fission yeast and human Smc4 are highlighted in green (Sutani et al. 1999; Holt et al. 2009; Bazile et al. 2010; Kao et al. 2014).

**Figure S5.** Cdk1 regulation of Smc4 phosphorylation in mitosis. (A) Budding index and mitotic spindle length of the cell populations examined in the time-courses presented in Figures 3D. (B) Validation of antibodies against phosphoserine 4 (pSer4) of Smc4. Protein samples from interphase (G1) and mitotic (M) cells of the indicated genotypes were probed by immunoblot analysis for the presence of phosphorylated Smc4. The Smc4-3xSTII signal serves as loading control (C) Budding index and mitotic spindle length of the cell populations examined in the time-courses presented in Figures 3E. (D) Smc4 phosphorylation is delayed and reduced in strains carrying *clb5Δ clb6Δ* mutations. Cells expressing the Smc4-NT fragment were released from a G1 arrest into fresh medium containing nocodazole at 37 °C. Samples were collected every 30 minutes and processed for immunoblotting. The intensity of the signal for the electrophoretically-retarded phospho-species of Smc4 was quantified using ImageJ software, and is shown below the
gels. A red arrow indicates the peaks corresponding to the most retarded phospho-species of Smc4. These species usually appear 60 minutes after release in the control wild-type situation, but is significantly delayed and reduced in the clb5Δ clb6Δ mutant. Percentages of budded cells are shown below the gels. We noticed, as previously observed (Schwob and Nasmyth 1993), that clb5 clb6 mutants show delayed kinetics of cell cycle entry and that this may indirectly affect the appearance of Smc4 phospho-species in these mutants. This limitation can be addressed by testing for Smc4 phosphorylation in a mutant lacking its RxL docking sites, as shown in Figure 3G. These motifs are used by early B-type cyclins to selectively target their substrates in vivo, and removal of RxL motifs has been shown to reduce phosphorylation of Clb5 substrates in yeast (Archambault et al. 2005; Loog and Morgan 2005). (E-F) Budding indexes of the cell populations examined in the time-courses presented in Figures 3F and G, respectively. (G-H) Smc4 phosphorylation is not dependent on Cdc5 activity. (G) Cells expressing the Smc4-NT fragment were released from a G1 arrest at 23°C into fresh medium at 37°C. Samples were collected every 30 minutes and processed for immunoblot analysis and budding index. Pgk1 serves as loading control. (H) Cells expressing the Smc4-NT fragment were arrested in metaphase (nocodazole) at 23°C, shifted to 37°C maintaining nocodazole arrest for 1 hour. Samples were collected every 30 minutes and processed for immunoblot analysis and budding index. Pgk1 serves as loading control. Note how removal of Cdk1 activity in the cdc5-99 cdc28-4 double mutant leads to a rapid dephosphorylation of Smc4 in dividing cells. In contrast, the cdc5-99 single mutant maintains full Smc4-NT phosphorylation under the same conditions.
**Figure S6.** Phenotype of yeast carrying single-mutations in condensin phosphosites. (A) Loss of individual Cdk1 sites in Smc4 does not affect cell proliferation. Five-fold dilution series of wild-type and smc4 mutant yeast strains defective in single Cdk1 phosphorylation sites were spotted on solid medium to evaluate growth at different temperatures. Plates were grown 2-3 days until individual colonies were clearly visible. (B) Morphology of the yeast rDNA locus at 37 °C in the smc4-S128A mutant as revealed by FISH. Representative micrographs of the most prominent rDNA morphology observed for each condition is shown on the left. Propidium iodide (PI; red) and fluorescein isothiocyanate (FITC; green) were used to label the nucleus and rDNA locus, respectively. Quantification of each rDNA species is shown on the right. At least one hundred nuclei were counted per condition. (n=3 for all experiments and error bars represent S.D.) The ycg1-2 mutant was used as positive control to induce a typical chromosome condensation defect (Lavoie et al. 2002). The lack of condensation defect in our FISH experiment with the smc4-S128A single mutant is consistent with a previous analysis showing no increase in cells showing decondensed DNA (i.e., “2-dot phenotype” using the dual LacO repeats system) in the absence of Ser128 phosphorylation (Kao et al. 2014). We note that the modest ~20% increase in “3-dot phenotype” previously linked with loss of Ser128 phosphorylation (Kao et al. 2014) likely reflects difference in the timing of sister-chromatid segregation at different loci, as previously shown (Straight et al. 1997). Indeed, condensin is a known regulator of chromosome arm cohesion (Lam et al. 2006), and the simplest interpretation of a 3-dot phenotype in this context is that it reflects a cohesion defect. Interestingly, the strong condensin mutant smc2-8 (Freeman et al. 2000) did not exhibit any chromosome condensation defects in the assays performed by Kao and collaborators.
Figure S7. Genetic analysis of phosphorylation mutants of Smc4/Cut3 in budding and fission yeasts. (A) Inactivation of the protein quality control pathway does not rescue the temperature-sensitive (ts) growth phenotype of smc4-7A mutants. Five-fold dilution series of SMC4, SMC4-3xSTII and smc4-7A-3xSTII strains carrying either san1Δ or wild-type SAN1 were spotted on solid medium to evaluate growth at different temperatures. Loss of the protein quality control pathway in san1Δ cells did not suppress the ts phenotype of the smc4-7A-3xSTII mutant, thereby indicating that the phenotype of this strain is not due to protein instability (Gardner et al. 2005). (B) A heterozygous diploid fission yeast strain carrying the cut3-T19V:Tadh1:kanMX6 phosphomutant allele was induced to sporulate and the viability of the resulting haploid spores was determined after 3 days of growth on solid medium. Three typical tetrads of spores are shown. The genotype of spores was deduced using the kanMX6 marker associated with the cut3 locus (i.e., + sign indicate spores that have inherited the wild-type cut3+ allele). Note that the cut3-T19V allele also has a weak meiotic effect, thereby causing some tetrads to exhibit less than 2 live spores. A heterozygous wild-type cut3/cut3:Tadh1:kanMX6 strain was included as control. Haploid G418-resistant (i.e., kanMX6 positive) spores were never recovered after sporulation of the mutant, whereas the kanMX-linked wild-type cut3 allele segregated in a perfect Mendelian manner after sporulation. (C) Five-fold dilution series of the cut3-T19V:Tadh1:kanMX6/cut3 heterozygous diploid and control strains were spotted on solid YES-A medium to evaluate growth at different temperatures. Note the absence of dominant growth defect associated with the cut3-T19V:Tadh1:kanMX6 allele.
**Figure S8.** Constitutive phosphorylation of Smc4 – Structural implications and effects.

Secondary structure analysis of Smc4 N-terminal extension. Smc4 primary amino-acid sequence was analyzed *in silico* to determine the position and nature of structural landmarks in the protein. The presence of extended antiparallel coiled-coil regions in Smc4 was determined using the COILS program (Lupas et al. 1991), whereas the propensity of particular regions of the protein to adopt an unstructured conformation was determined using IUPred (Dosztanyi et al. 2005). (A) The graph on top shows the regions of Smc4 predicted to adopt a coiled-coil structure. (B) The secondary structure prediction is consistent with the known boundaries of the ATPase and hinge domains of SMC family members (Hirano and Mitchison 1994; Saitoh et al. 1994; Saka et al. 1994; Strunnikov et al. 1995), as highlighted in the protein schematic. (C) This graph shows the propensity of the various regions of Smc4 to adopt an unstructured conformation. According to this analysis, the N-terminal extension of Smc4 shows a very high probability of being unstructured relative to other parts of the protein. This property correlates well with the observation that most Cdk1 sites in cells are found within unstructured parts of proteins (Holt et al. 2009). (D) The lethality of Smc4 charge-mimetic mutations is not due to lower protein abundance. A culture of heterozygous diploid cells expressing Smc4-7EE-3xSTII/Smc4 where grown at 23 °C and split into three subcultures. The first subculture was grown for an additional 120 min at 23 °C and corresponds to an exponential population of cells (Exp). The second subculture was blocked in metaphase using nocodazole for 150 min (NZ). The third subculture was blocked in S phase using 0.2M hydroxyurea for 120 min (HU). Samples were taken and analysed by immunoblot with an anti-STII antibody. Cdc5 serves as marker for mitotic cells and Pgk1 levels are used as loading control.
Figure S9. Phenotypes of phospho-defective and charge-mimetic mutants of Smc4. (A) Quantification of the fraction of tetrads showing a 2:2 lethality phenotype co-segregating with smc4 fusion alleles. Tetrads were derived from the dissection of heterozygous diploid strains expressing Clb2-Smc4 fusion derivatives that drives constitutive phosphorylation of Smc4 by Cdk1 (i.e., CLB2::SMC4::HIS3MX6/SMC4). The clb2-CycBoxΔ::SMC4::HIS3MX6 and CLB2::smc4-7A::HIS3MX6 alleles are used as negative controls since the former lacks part of the cyclin box region necessary for its interaction with Cdk1 (Lees and Harlow 1993), whereas the latter lacks the N-terminal Cdk1 phospho-sites of Smc4. The ability of cells to grow on solid medium lacking histidine (i.e., HIS+ phenotype) was used to score the segregation of the fusion alleles after meiosis and sporulation. The genotype of unviable spores was deduced based on Mendelian inheritance of SMC4 alleles. At least 70 tetrads were counted for each genotype. (B–E) Overexpression of the charge-mimetic mutant of Smc4 does not induce unscheduled condensation of chromosomes in interphase. Strains containing inducible ectopic copies of SMC4 or smc4-7EE were synchronized in G1 with alpha-factor in raffinose-containing media. Overexpression of SMC4 or smc4-7EE was induced by adding 2% galactose in medium for 90 min. (B) FISH analysis of rDNA locus morphology after SMC4 overexpression. Representative micrographs of G1-arrested cells are shown after 90 min of overexpression of smc4-7EE or SMC4. (C) Quantification of rDNA locus morphology after smc4-7EE or SMC4 overexpression. Most cells show the typical uncondensed “puff” rDNA morphology in G1 despite SMC4 overexpression. (D) Immunoblot analysis of Smc4 abundance before and after induction with galactose in G1. Nsp1 protein levels are used as loading control. (E) Flow cytometry analysis of cellular
DNA content after 90 min overexpression of smc4-7EE or SMC4. Most cells show a typical 1C DNA content under these experimental conditions.

**Figure S10.** (A) Impact of the smc4 charge mimetic mutant on the intertwist morphology. *clb-ts* cells expressing smc4-7EE (or an empty vector) from an ectopic locus were grown asynchronously at 23°C before being arrested in early mitosis by shifting the culture at 37°C for 135 min. The morphology of the yeast rDNA locus was revealed by FISH. Representative micrographs of the most prominent rDNA morphology observed for each condition is shown on the left. PI (red) and FITC (green) were used to label the nucleus and rDNA locus, respectively. Quantification of each rDNA species is shown on the right. At least one hundred nuclei were counted per condition. (n=3 and error bars represent S.D.). (B) Turnover of phosphorylation sites on condensin regulates rDNA condensation. Quantification of rDNA morphologies in yeast phosphatase mutants grown at non-permissive temperature. Cells were grown asynchronously at 23°C until exponential phase and subsequently shifted at 37°C for 150 min. Nocodazole was used to block cells in metaphase. At least 100 cells have been counted for each genotype in 3 independent experiments (error bars are S.D.).

**Figure S11.** (A) Loss of Cdk1 phosphorylation in the smc4-7A phosphomutant affects its interaction with chromatin. Decay of fluorescence after photobleaching of cells expressing Smc4-3xGFP. Histogram showing the calculated decay constant \( d \) of wild-type Smc4, Smc4-7A mutant and cytosolic GFP (c.f., Equation 1 above). Data for this decay constant comes from the experiment shown in Fig. 7. Triple star symbols indicate a significant difference in
decay constant for cells expressing Smc4-3xGFP relative to cells expressing cytosolic GFP or the Smc4-7A-3xGFP mutant (**p < 0.001). Error bars indicate S.D. over three independent experiments (n≥12 cells per experiment). (B) Expanded model for the role of Cdk1 in the activation of condensin during early mitosis. During interphase, the condensin complex is not targeted for phosphorylation by cyclin-dependent kinases and remains inactive. Upon mitotic entry, the progressive activation of Cdk1 activity leads to Smc4 phosphorylation and initial formation of condensed chromosomes (Figs. 1–3). We propose that condensin responds to Cdk1 activity in a sensitive and proportional manner due to the multisite nature of this phosphoregulatory event (Figs. 2, S4A, S6A). The relative hypersensitivity of condensin to low levels of Cdk1 activity explains why the process of chromosome condensation occurs earlier in the mitotic program than other processes that require more Cdk1 activity for completion (Figs. 1, 6, S1). From a molecular perspective, the phosphorylation of N-terminal part of Smc4 by Cdk1 leads to a change in condensin dynamic interaction with chromatin. The phosphorylation slows down the interactions between condensin and chromatin, effectively stabilizing condensin on chromosomes for longer periods of time (Fig. 7). Ultimately, this increased residency time allows condensin to act on its substrate more effectively, thereby triggering chromosome condensation.

**Figure S12.** Conservation of the C-terminal extensions of CAP-D2 family members from various eukaryotes. Schematic representation of CAP-D2/Ycs4 proteins from different species. Clusters of HEAT domains conserved in all eukaryotic members of the CAP-D2 family (Onn et al. 2007) are shown in red. The light blue box marks the boundaries of the C-terminal extension in CAP-D2 homologs, whereas the dark blue box indicates the presence
of condensin II/CAP-D3 in the selected species. Known or putative (Holt et al. 2009) Cdk1 phosphorylation sites located in the C-terminal extension of CAP-D2 homologs are represented as green circles above the protein schematics. In human CAP-D2, Thr1339 is a putative Cdk1 site whereas Thr1384 and Thr1389 are likely Cdk1 phosho-sites (Bazile et al. 2010), while in drosophila, Thr1328 is a putative Cdk1 site. HEAT domain positions and phospho-residue locations within CAP-D2 homologs are drawn to approximate scale. Note the absence of putative Cdk1 consensus sites in the C-terminal extensions of a number of important exemplar species found within 4 kingdoms of the eukaryotic domain. In some of these species, condensin is monomorphic as evidenced by the absence of CAP-D3 homologs (Hirano 2012). Species names are abbreviated as follows: Hs, Homo sapiens; Dm, Drosophila melanogaster; Sc, Saccharomyces cerevisiae; Sp, Schizosaccharomyces pombe; Os, Oryza sativa; Si, Setaria italica; Li, Leishmania infantum; and Tb, Trypanosoma brucei brucei.

Table S1. Yeast strains used in this study. Relevant parts of the genotypes of yeast strains used in this study are described next to yeast designations.

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Table 1. Yeast strains used in this study

| Figure | Name | Relevant genotype |
|--------|------|-------------------|
| Fig. 3A | MATa CDC28 | SMC4/smc4-928::3xSTII::HIS3MX6 | YCG1::3xFLAG::kanMX6 cdc15-2-253294 Table1_Robellet |
| Fig. 3C | MATa SMC4::3xSTII::HIS3MX6 | SMC4/smc4-927::3xSTII::HIS3MX6 MATα/MATα | YCG1::3xFLAG::kanMX6 cdc15-2 |
| Fig. 3E | MATa SMC4::3xSTII::HIS3MX6 YCG1::3xFLAG::kanMX6 cdc15-253294 Table1_Robellet | MATa smc4-7A(601)::3xSTII::URA3 NET1::3xGFP::kanMX6 | YCG1::3xFLAG::kanMX6 cdc15-2 |
| Fig. 3G | MATa SMC4::3xSTII::HIS3MX6 | MATa smc4-7A(601)::3xSTII::URA3 NET1::3xGFP::kanMX6 | YCG1::3xFLAG::URA3 | 253294 Table1_Robellet |
| Fig. 4A | MATa smc4-7A(601)::3xSTII::HIS3MX6 | MATa smc4-7A(601)::3xSTII::URA3 NET1::3xGFP::kanMX6 | YCG1::3xFLAG::URA3 | 253294 Table1_Robellet |
| Fig. 4C | MATa SMC4::3xSTII::HIS3MX6 | MATa SMC4::3xSTII::YCG1 | MATa YCG1::3xFLAG::kanMX6 cdc15-253294 Table1_Robellet |
| Fig. 4E | MATa SMC4::3xSTII::HIS3MX6 | MATa SMC4::3xSTII::YCG1 | MATa YCG1::3xFLAG::kanMX6 cdc15-253294 Table1_Robellet |
| Fig. 4G | MATa SMC4::3xSTII::HIS3MX6 | MATa SMC4::3xSTII::YCG1 | MATa YCG1::3xFLAG::kanMX6 cdc15-253294 Table1_Robellet |
| Fig. 4H | MATa SMC4::3xSTII::HIS3MX6 | MATa SMC4::3xSTII::YCG1 | MATa YCG1::3xFLAG::kanMX6 cdc15-253294 Table1_Robellet |
| Fig. 5A | MATa CDC28 | SMC4/smc4-928::3xSTII::HIS3MX6 | YCG1::3xFLAG::kanMX6 cdc15-2 |
| Fig. 5C | MATa YCG1::3xFLAG::kanMX6 | MATa smc4-7A(601)::3xSTII::URA3 NET1::3xGFP::kanMX6 | YCG1::3xFLAG::URA3 | 253294 Table1_Robellet |
| Fig. 5E | MATa YCG1::3xFLAG::kanMX6 | MATa smc4-7A(601)::3xSTII::URA3 NET1::3xGFP::kanMX6 | YCG1::3xFLAG::URA3 | 253294 Table1_Robellet |
| Fig. 6 | MATa SMC4::3xSTII::HIS3MX6 | MATa SMC4::3xSTII::HIS3MX6 | MATa YCG1::3xFLAG::kanMX6 cdc15-253294 Table1_Robellet |
