

Supporting Information

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SI Materials and Methods

Antibodies. The phospho-specific antibodies human Sororin pT115 and pS164 were raised against synthetic phosphopeptides ATPST(PO₄)PVPNP (pep 3147) and PGRRS(PO₄)CFGFE (pep 3148), respectively, in rabbits. The resulting antisera were subjected to sequential affinity purification on phosphopeptide and nonphosphopeptide columns. Rabbit antihuman Sgo1 antibody, anti-*Xenopus* Lamin LIII, anti-*Xenopus* XCAP-E, and anti-*Xenopus* Bub1 were kindly provided from Y. Watanabe (Institute of Molecular and Cellular Biosciences, Tokyo, Japan), K. Ohsumi (Nagoya University, Nagoya, Japan), and A. Losada (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain), respectively. Polyclonal antibodies to human pSA2 (pS1261), *Xenopus* Pds5A, human Scc1, human SA1/2, human Sororin, human Wapl, human Smc2, and mouse monoclonal antiacetyl-Smc3 antibody have been described (1–7). Antihuman Pds5A (Scc-112) and Smc3 (Bethyl Laboratories; A300-089A and A300-060A, respectively), histone H3, and PCNA (Santa Cruz Biotechnology; C-16 and PC10 for immunoblotting, respectively), GFP (Abcam; ab290 for immunofluorescence and Roche; 11814460001 for immunoblotting) and CREST (Cortex Biochem) were commercially available.

HeLa Cell Culture, RNAi, and Chromosome Spreads. HeLa cells stably expressing mouse Sororin with a C-terminal localization and affinity purification (LAP) tag (single cell clone A4) have been described (3). To generate HeLa cells stably expressing human Sororin^{WT} or Sororin^{11A} with a C-terminal GFP tag, plasmids pEGFP-N1-Sororin^{WT} or Sororin^{11A} were used in transfections using Lipofectamine 2000 reagent (Life Technologies). Stably expressing cell clones were selected in DMEM (Dulbecco's Modified Eagle Medium) containing 500 µg/mL G418 and verified by fluorescence microscopy and immunoblotting. For live cell imaging, H2B-mCherry was transfected into Sororin^{WT}- or Sororin^{11A}-GFP-expressing cells and the stably expressing cells were selected with 0.25 µg/mL puromycin.

siRNAs were premixed with RNAiMax (Life Technologies) according to the manufacturer's instructions, added directly to cells (final concentration, 30 nM for single RNAi experiments), and incubated for 24 h (Sgo1 RNAi) or 72 h. To inhibit Plk1, Aurora B, or Cdk1, cells were first treated with nocodazole at a concentration of 330 nM for 10 h and then treated for 2 h with 250 nM BI 4834 (1), 100 nM Hesperadin (8), and 10 µM RO3306 (9).

Chromosome spreading and Giemsa staining were performed as described (8). Before fixation, nocodazole was added to 330 nM in the medium. Synthetic siRNA oligonucleotides were purchased from Ambion. The following sequences were described previously: Sgo1 (10), Wapl (2), and Sororin (4).

HeLa Cell Extracts, Immunoprecipitation, and Immunoblotting. Cell pellets were resuspended in extraction buffer [25 mM Tris-HCl pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 0.2% Nonidet P-40, 10% glycerol (vol/vol), complete protease inhibitor mix (Roche)] and lysed on ice by passing through a hypodermic needle. To produce total cell extracts, a fraction of the homogenate was incubated with benzonase (0.1 units/µL; Novagen) for 10 min on ice. For identification of phosphorylation sites of Sororin by MS, mouse Sororin-LAP was immunoprecipitated from total cell extracts of nocodazole-arrested mitotic cells or S/G2 cells 6 h after release from double thymidine arrest, and eluted by 0.1 M glycine HCl (pH 2.0). The eluates were neutralized with 1M Tris and analyzed by MS for the identification of proteins and phosphosite, as

previously described (1). To determine interaction partners of Sororin-LAP purified from HeLa cells by MS, tandem affinity purification using the LAP tag was performed as previously described (11). To obtain a chromatin-bound fraction, the homogenate was spun at 1,300 × *g* and washed three times with extraction buffer. The resulting material was centrifuged, and pellets were resuspended in SDS sample buffer, heated to 95 °C, sonicated, and passed through a 0.45-µm filter. All of the samples were run on SDS polyacrylamide gels and transferred to PVDF membranes using a semidry blotting apparatus. After blocking with 5% skimmed milk for 15 min, the membranes were incubated with primary antibodies at room temperature for 1 h or for at 4 °C for 12 h, and with secondary antibodies at room temperature for 1 h. Signals were detected using ECL (GE Healthcare).

In Vitro Wapl Removal Assay and Sororin Dephosphorylation Assay.

The in vitro Wapl removal assay was performed as described (3). In this assay, we used human Pds5A protein lacking N terminus amino acids 1–40. For the Sororin dephosphorylation assay, the Sgo1-PP2A complex was immunoprecipitated from total cell extracts as described (12). Briefly, the cells were extracted with immunoprecipitation (IP) buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20, 10% glycerol, 10 mM β-mercaptoethanol), spun at 250,000 × *g* in ultracentrifuge for 10 min, and the supernatant was incubated with anti-Sgo1 antibody-conjugated Affi-Prep protein A beads (Bio-Rad) at 4 °C for 2 h. The Sgo1-bound beads were washed three times in extraction buffer and once in phosphatase assay buffer (50 mM Hepes, 100 mM NaCl, 2 mM DTT). His-tagged recombinant human Sororin^{WT} was incubated in mitotic *Xenopus* egg extract at 22 °C for 30 min. The Sororin^{WT} was immunoprecipitated and eluted using antigen peptide as described (3), the eluate was mixed with Sgo1-bound beads, and incubated at room temperature for 30 min.

HeLa Cell Immunofluorescence Microscopy. Cells grown on coverslips were preextracted with 0.1% Triton X-100 for 2 min as previously described (13), PBS-washed, and fixed with 4% paraformaldehyde in PBS for 20 min. After fixation, cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and then blocked with 10% goat serum in PBS containing 0.01% Triton X-100. Cells were incubated with primary and secondary antibodies (Alexa 488, Alexa 568, and Alexa 647; Life Technologies). DNA was counterstained with DAPI or Hoechst 33342. Coverslips were mounted onto slides with ProLong Gold (Life Technologies). Images were taken on a Zeiss Axioplan 2 or Nikon Ni-E equipped with a CoolSnapHQ2 charge-coupled device (CCD) camera (Photometrics), Nikon TE2000 equipped with a spinning-disk confocal unit CSU-X1 (Yokogawa), or Zeiss LSM780 microscope with 63× or 100× Plan-Apochromat objective lenses.

Time-Lapse Microscopy. Cells were grown in a chambered coverglass (IWAKI). For imaging, medium was changed to DMEM high glucose without phenol red (Gibco/Life Technologies) supplemented with 10% FCS and antibiotics. Time-lapse microscopy was performed with a 40× PlanFluor objective lens on the TE2000 microscope (Nikon) equipped with a spinning-disk confocal unit CSU-X1 (Yokogawa), an electron-multiplying CCD camera (ImagEM; Hamamatsu) and a CO₂ incubation chamber maintained at 37 °C. For H2B-mCherry cells, six z-steps were acquired every 5 min. For quantification of the duration of mitosis, nuclear envelope breakdown and anaphase were chosen as frames marking the beginning and the end, respectively.

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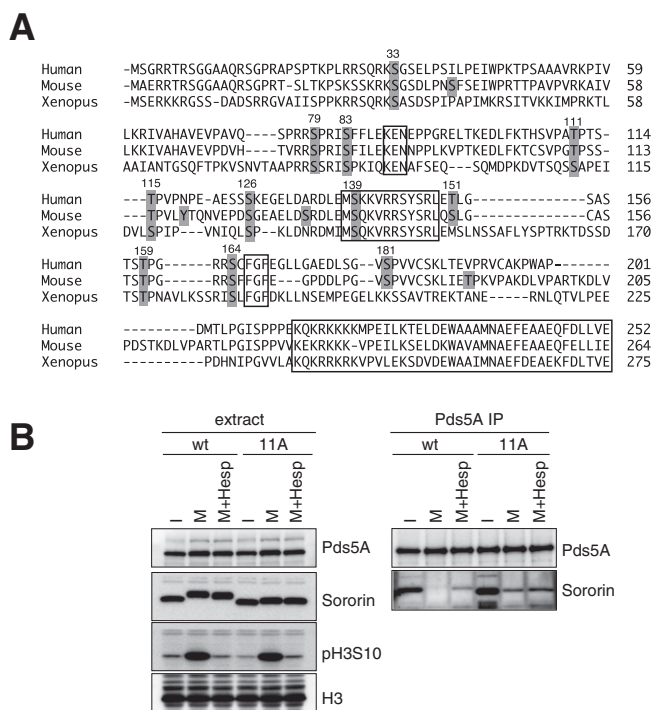


Fig. S1. Identification of mitosis-specific phosphorylation sites in Sororin and the binding ability of the nonphosphorylatable mutant to Pds5. (A) Alignment of Sororin orthologs from human, mouse, and *Xenopus*. Mitosis-specific phosphorylation residues in mouse Sororin identified by MS, and their conserved residues in *Xenopus* and/or human are shaded in gray. The amino acid numbers of each conserved residue in human Sororin are shown above gray shading. Boxes indicate well-conserved domains including the KEN box, internal basic region, FGF motif, and Sororin domain (3). (B) Pds5 binds to Sororin^{11A} in mitotic *Xenopus* egg extracts. Recombinant human Sororin^{WT} or Sororin^{11A} protein was incubated in either interphase (I) or mitotic (M) *Xenopus* egg extract (extract) in the presence or absence of Hesperadin (Hesp) and Pds5A was immunoprecipitated (Pds5A IP) and the bound proteins were analyzed by immunoblotting. Whereas Sororin^{WT} dissociated from endogenous Pds5A in mitosis and the association was partially restored by Hesperadin, Sororin^{11A} could associate with Pds5A even in mitosis and the binding was not increased significantly by Hesperadin.

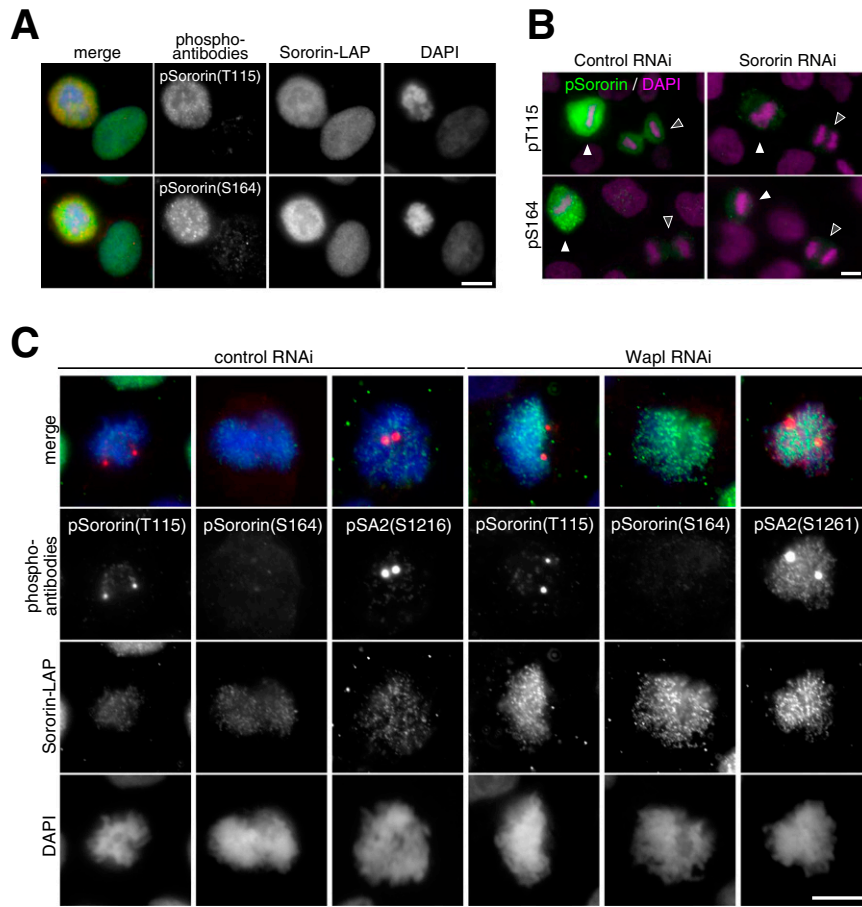


Fig. S2. Neither pSororin (T115) nor pSororin (S164) localizes on chromosomes in mitosis. (A) Both pSororin (T115) and pSororin (S164) antibodies stain only mitotic cells. Sororin-LAP cells were fixed and stained for phospho-Sororin (pT115 and pS164) or Sororin-LAP (GFP). DNA was counterstained with DAPI. Both pT115 and pS164 were detected only in mitotic cells distinguished by condensed DNA. (B) Phospho-Sororin signals are diminished by Sororin RNAi. HeLa cells were treated with either control or Sororin siRNA and stained for phospho-Sororin (pT115 and pS164). In control RNAi cells, prometaphase/metaphase cells (white arrowheads) were stained more strongly than anaphase/telophase cells (gray arrowheads) for both phosphoantibodies, whereas prometaphase/metaphase signals were decreased to the level of anaphase/telophase in Sororin RNAi cells, indicating that these phosphoantibodies detect specifically for phosphorylated Sororin. (C) Both pSororin (T115) and pSororin (S164) are not detectable on mitotic chromosomes even in the absence of Wapl. HeLa cells were treated with control or Wapl siRNA for 3 d. The cells were preextracted, fixed, and stained using the antibodies indicated. DNA was counterstained with DAPI. (Scale bar, 10 μ m.)

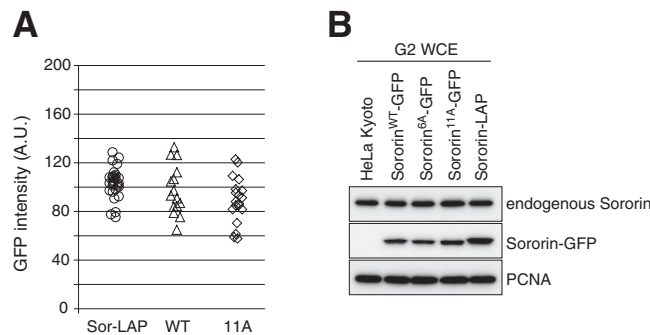


Fig. S3. Expression levels are similar between Sororin^{WT}- and Sororin^{11A}-expressing cells. (A and B) Expression levels of Sororin-LAP, Sororin^{WT}-, Sororin^{6A}-, and Sororin^{11A}-GFP were similar. HeLa cells stably expressing Sororin-LAP (Sor-LAP), Sororin^{WT}-GFP (WT), and Sororin^{11A}-GFP (11A) were fixed, stained with GFP antibody, and the total fluorescence intensities of the cells were plotted (A). HeLa cells stably expressing Sororin-LAP, Sororin^{WT}-GFP, Sororin^{6A}-GFP, and Sororin^{11A}-GFP were synchronized in G2 phase by single thymidine arrest and release, and whole-cell extracts (G2 WCEs) were analyzed by immunoblotting (B). The results from both A and B indicate that the expression levels of all Sororin-GFP constructs are similar to Sororin-LAP, which is regulated by a native promoter, thus generating an endogenous level of expression. PCNA was shown as a loading control.

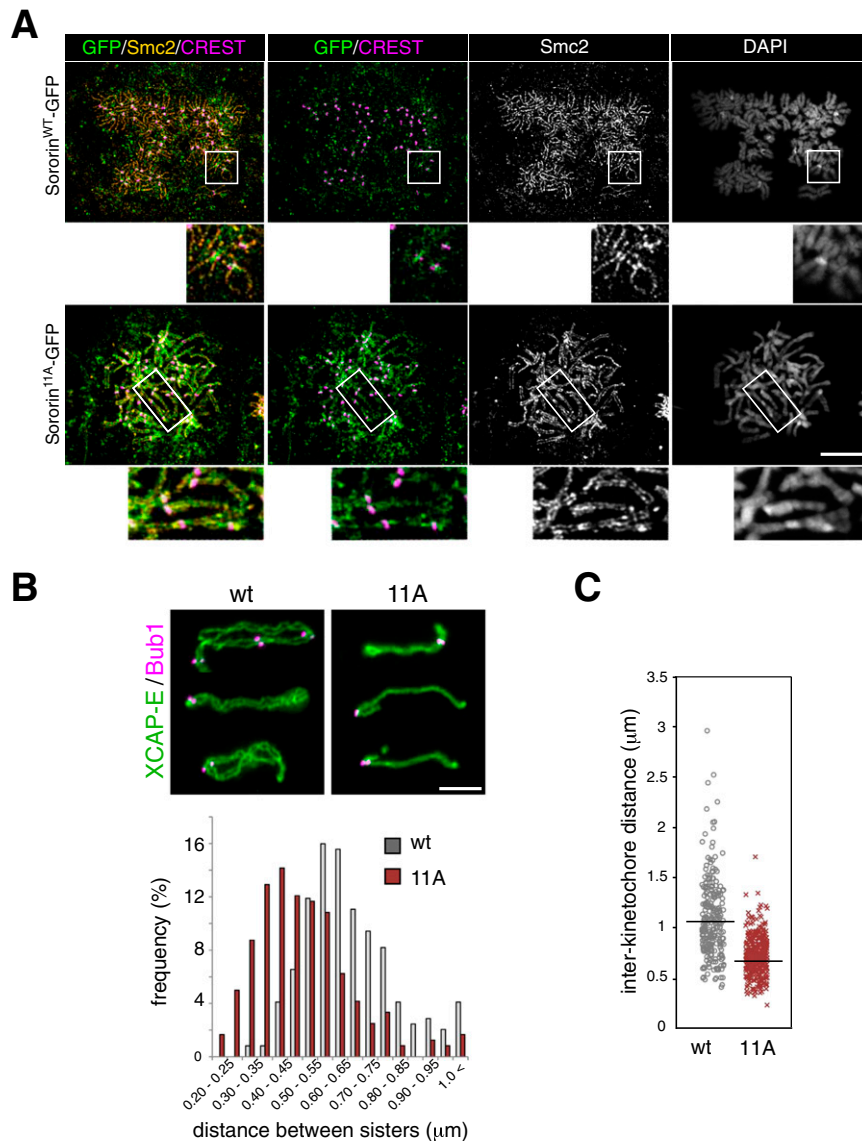


Fig. 54. Sororin^{11A} causes overcohesion phenotype in HeLa cells and *Xenopus* egg extracts. (A) Localization of Sororin-GFP in prometaphase cells. HeLa cells stably expressing Sororin^{WT}-GFP or Sororin^{11A}-GFP were treated for 2 h with nocodazole and mitotic cells were shaken off and immunostained with the antibodies indicated. DNA was counterstained with DAPI. The outlined regions were magnified and are shown *Lower*. (Scale bar, 10 μ m.) (B) Mitotic chromosomes were assembled in *Xenopus* egg extracts prepared as in Fig. 4F. Chromosomes were isolated 120 min after Δ 90CycB addition and stained for XCAP-E (Smc2 in frog; green) and Bub1 (magenta) (*Upper*). The distance between the two arms of sister chromatid pairs stained by XCAP-E in each extract is shown in the histogram (*Lower*). (Scale bar, 5 μ m.) (C) The distance between pairs of kinetochores stained by Bub1 in each extract in B is plotted. The bar indicates the mean interkinetochore distances of $n > 250$ chromosomes.

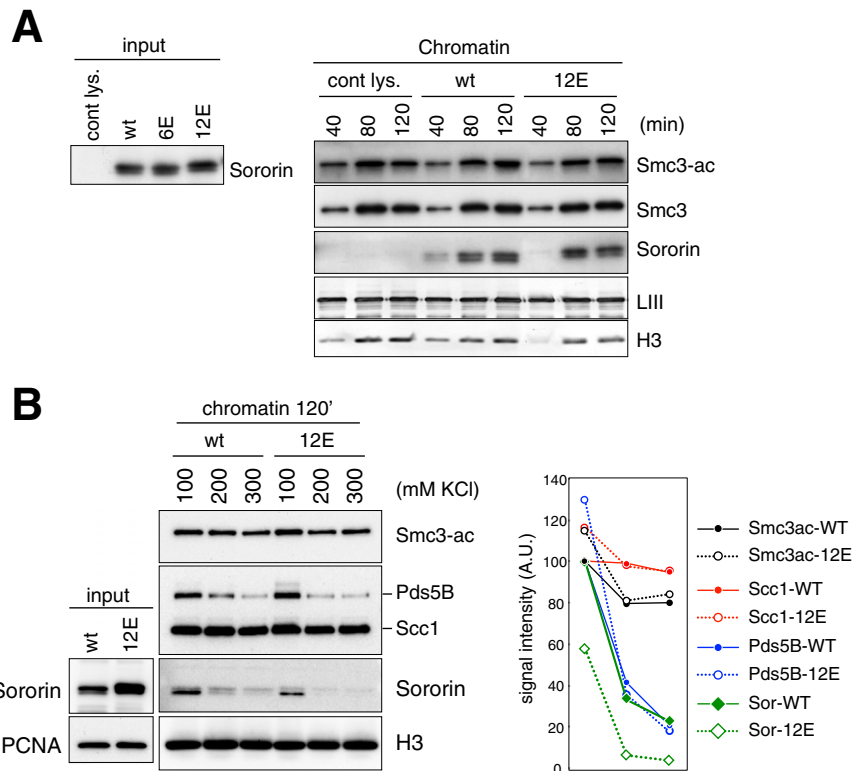


Fig. S5. Sororin^{12E} is less stably bound to chromatin in interphase. (A) Sororin^{12E}, in which all of the Ser or Thr residues mutated in Sororin^{11A} plus Ser181 were changed to Glu to mimic phosphorylation, is loaded onto chromatin with normal kinetics during S phase. Sororin^{WT}, Sororin^{6E}, and Sororin^{12E} were transcribed and translated in reticulocyte lysates *in vitro* (input). The lysates (control, or expressing Sororin^{WT} or Sororin^{12E}) were added to interphase *Xenopus* egg extract supplemented with demembrated sperm nuclei. At the indicated time points after addition of sperm nuclei, chromatin was isolated and binding proteins were analyzed by immunoblotting with the antibodies indicated. (B) Sororin^{12E} is more sensitive to high salt than Sororin^{WT}. The chromatin fraction was isolated from the extract prepared as in A 120 min after the addition of sperm nuclei, washed in a buffer including 100, 200, or 300 mM KCl, and then chromatin fractions were analyzed by immunoblotting. Signal intensity of each protein normalized by histone H3 signal are shown (Right).

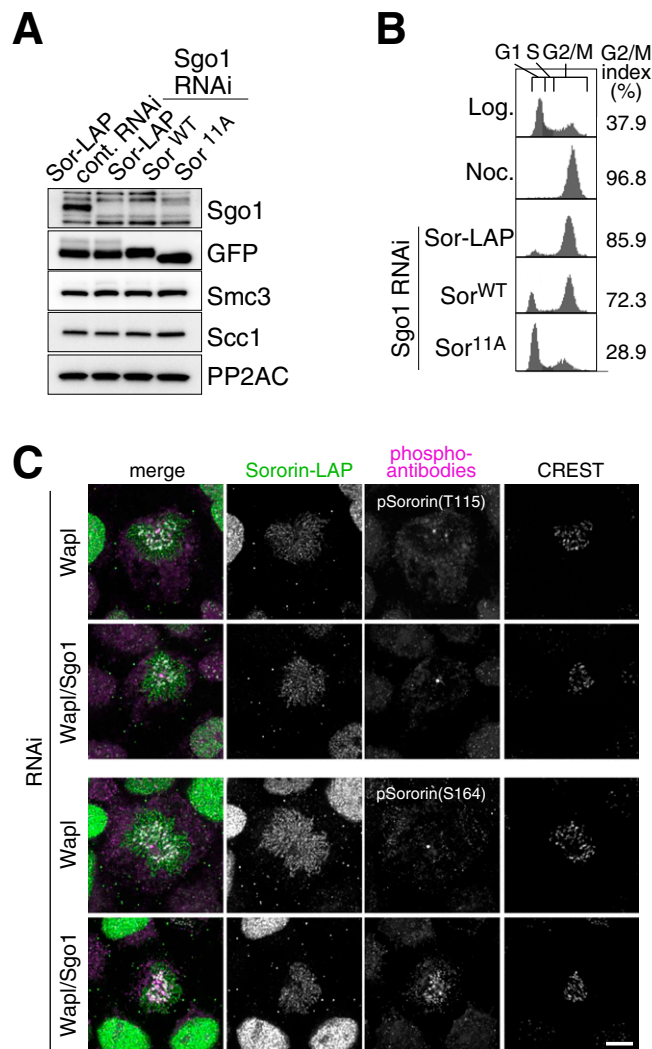


Fig. S6. Sgo1–PP2A antagonizes phosphorylation of Sororin at centromere. (A and B) Sororin^{11A} overcomes the cohesion defect in Sgo1-depleted cells. Depletion of Sgo1 in Sororin-LAP, Sororin^{WT}, or Sororin^{11A}-GFP cells. Sororin-LAP, Sororin^{WT}, or Sororin^{11A}-GFP stably expressing cells were transfected with either control or Sgo1 siRNAs and nocodazole-arrested mitotic cells were analyzed by immunoblotting (A). Logarithmically proliferating cells, nocodazole-arrested cells, or Sgo1 siRNA-treated cells expressing Sororin-LAP, Sororin^{WT}, or Sororin^{11A} was stained with propidium iodide and analyzed by flow cytometry (B). (C) Depletion of Sgo1 increases phosphorylation of Sororin on Ser164 at centromere. HeLa cells stably expressing Sororin-LAP were treated with either Wapl siRNA alone or Wapl and Sgo1 siRNAs for 72 h. The cells were immunostained with the antibodies indicated. pS164 signals became detectable in Wapl/Sgo1 RNAi cells around centromeres. (Scale bar, 10 μm.)

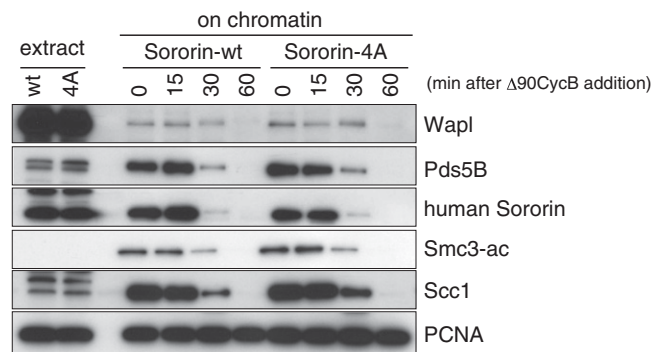


Fig. S7. Sororin^{4A} does not protect cohesin on chromatin in entry into mitosis. Sororin^{WT} or Sororin^{4A} mutant, where Ser156, Thr157, Ser158, and Thr159 were replaced by Ala, was added to interphase *Xenopus* egg extracts supplemented with demembrated sperm nuclei, and incubated for 90 min to allow DNA replication and the association of Sororin with chromatin. Subsequently, nondegradable cyclin B (Δ90CycB) was added to induce mitosis. At the indicated time points after addition of Δ90CycB, chromatin was isolated and its binding proteins were analyzed by immunoblotting. Cohesin subunits and Sororin were dissociated from chromatin in the similar kinetics.

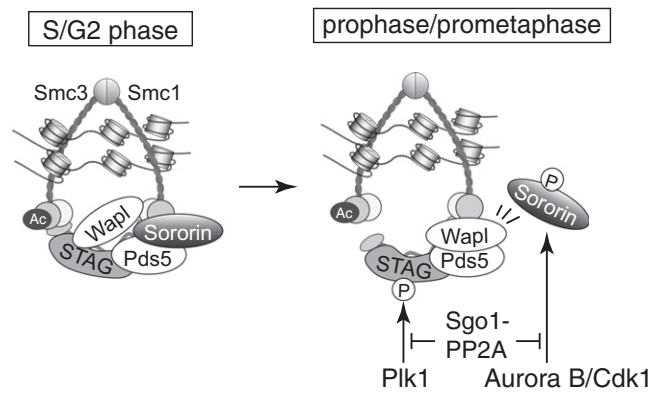


Fig. S8. Model for the prophase pathway. When cells enter mitosis, Sororin on acetylated cohesin is phosphorylated by Aurora B and Cdk1, thereby dissociating from chromatin. This brings about the formation of a Wapl-Pds5 heterodimer, which is required for Wapl's ability to dissociate cohesin from chromatin. STAG (SA1/2) is independently phosphorylated by Plk1, facilitating the opening of the "exit gate." Both the phosphorylation of STAG and Sororin are antagonized by Sgo1-PP2A at centromeres, so that acetylated cohesin maintains cohesion at that location.