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Sororin Mediates Sister Chromatid Cohesion by Antagonizing Wapl

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SUMMARY

Sister chromatid cohesion is essential for chromosome segregation and is mediated by cohesin bound to DNA. Cohesin-DNA interactions can be reversed by the cohesion-associated protein Wapl, whereas a stably DNA-bound form of cohesin is thought to mediate cohesion. In vertebrates, Sororin is essential for cohesion and stable cohesin-DNA interactions, but how Sororin performs these functions is unknown. We show that DNA replication and cohesin acetylation promote binding of Sororin to cohesin, and that Sororin displaces Wapl from its binding partner Pds5. In the absence of Wapl, Sororin becomes dispensable for cohesion. We propose that Sororin maintains cohesion by inhibiting Wapl's ability to dissociate cohesin from DNA. Sororin has only been identified in vertebrates, but we show that many invertebrate species contain Sororin-related proteins, and that one of these, Dalmatian, is essential for cohesion in *Drosophila*. The mechanism we describe here may therefore be widely conserved among different species.

INTRODUCTION

In eukaryotic cells, sister chromatids remain physically connected from the time of their synthesis during DNA replication until their separation during mitosis or meiosis. This sister chromatid cohesion is essential for biorientation of chromosomes on the spindle and for DNA-damage repair (reviewed in Nasmyth and Haering, 2009; Onn et al., 2008; Peters et al., 2008). Cohesion is mediated by cohesin complexes. Three cohesin subunits, the ATPases Smc1 and Smc3 and the kleisin Scc1/Rad21/Mcd1, form triangular structures that have been proposed to mediate cohesion by embracing sister chromatids (Gruber et al., 2003; for an illustration of this "ring model," see Figure 6C below). Scc1 binds to a fourth core subunit, called Scc3 in yeast and stromal antigen (SA) in vertebrates, where somatic cells contain two SA paralogs (SA1 and SA2). Scc1 and SA proteins are further associated with a heterodimer of two proteins, called Wapl and Pds5, the latter of which also exists in two isoforms in vertebrates (Pds5A and Pds5B; Gandhi et al., 2006; Kueng et al., 2006).

Cohesin complexes are loaded onto DNA before replication (in animal cells already in telophase) and establish cohesion during replication. In the subsequent mitosis, cohesion is dissolved by removal of cohesin from chromosomes. In vertebrate cells, this process occurs in two steps (Waizenegger et al., 2000): the bulk of cohesin is removed from chromosomes in prophase by a mechanism that depends on Polo-like kinase 1 (Plk1/Plx1) and Wapl (Gandhi et al., 2006; Kueng et al., 2006). At centromeres, small amounts of cohesin are protected from the prophase pathway by Shugoshin, and these complexes can only be removed from chromosomes by the protease separase (reviewed in Sakuno and Watanabe, 2009). This process occurs only in metaphase because a surveillance mechanism called the spindle assembly checkpoint (SAC) prevents separase activation until all chromosomes have been bioriented. The SAC inhibits APC/C^{Cdc20} (anaphase-promoting complex/cyclosome associated with Cdc20), a complex whose ubiquitin ligase activity is required for separase activation (reviewed in Peters, 2006).

How cohesion is established and maintained is poorly understood. Fluorescence recovery after photobleaching (FRAP) experiments in mammalian cells revealed that cohesin binds to DNA much more stably after than before DNA replication, suggesting that cohesion depends on an unidentified event during DNA replication that stabilizes cohesin on DNA (Gerlich et al., 2006). The dynamic mode of cohesin binding to DNA might depend on Wapl because depletion of this protein from mammalian cells does not only interfere with the prophase pathway but also increases the residence time of cohesin on chromatin during interphase (Kueng et al., 2006).

The only molecular event during DNA replication that is known to be essential for cohesion establishment is acetylation of cohesin (Ben-Shahar et al., 2008; Unal et al., 2008; Zhang et al., 2008). This modification occurs on two lysine residues in the ATPase domain of Smc3 (K112/113 in budding yeast) and is catalyzed by the acetyltransferase Eco1. The lethality of yeast that is caused by deletion of the *ECO1* gene can be suppressed by changing K112/113 to residues that might functionally mimic

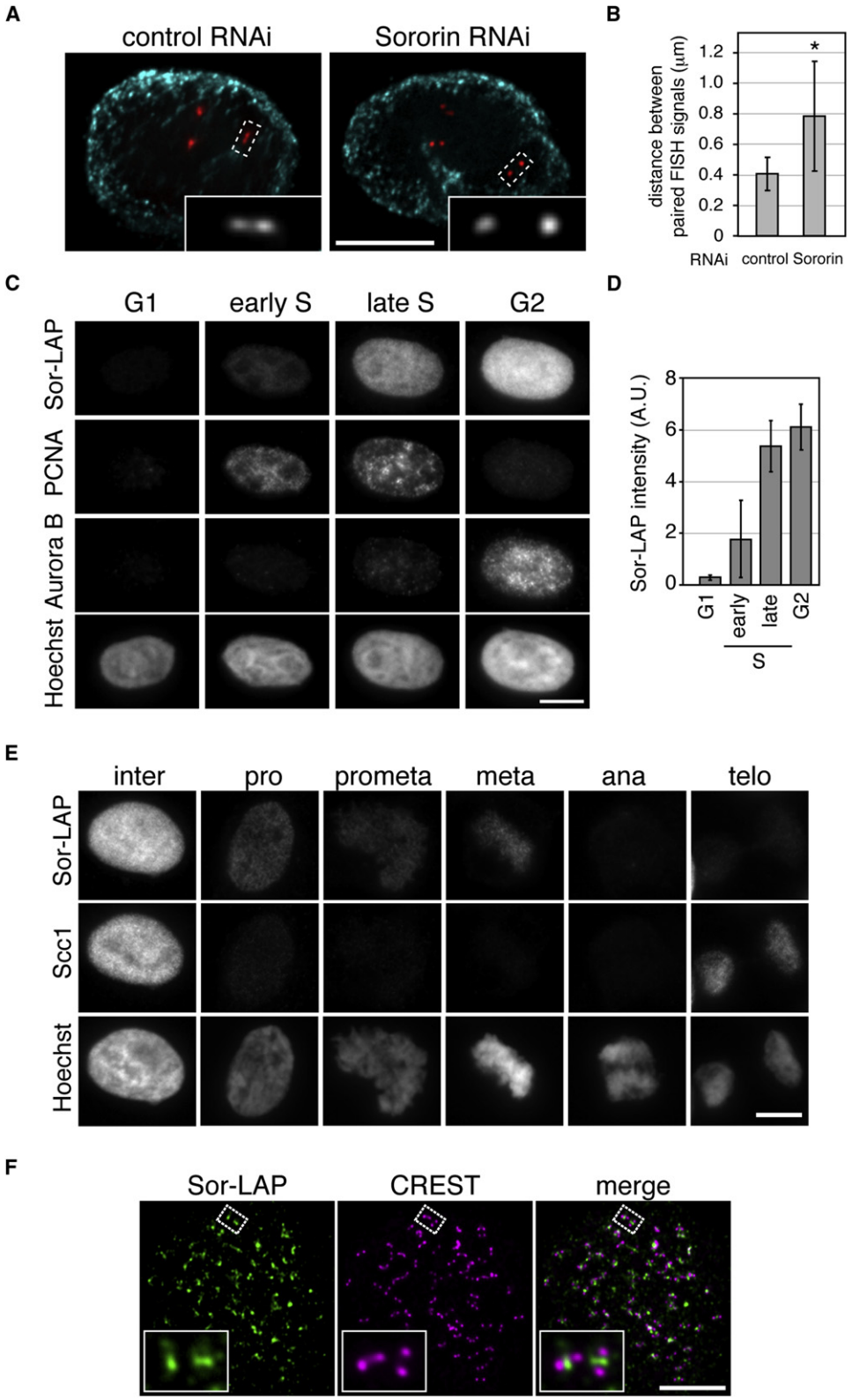


Figure 1. Sororin Is Required for Cohesion in S Phase

(A) FISH of Sororin-depleted S phase cells. HeLa cells were synchronized in S phase by double thymidine arrest and transfected with control or Sororin siRNA. Four hours after release from the second thymidine arrest, cells were labeled with BrdU for 15 min, pre-extracted, and subjected to FISH with a probe specific for

acetylated lysine but also by deletion of the *WPL1/RAD61* gene, which encodes a Wapl ortholog, and by mutations in *Pds5* (Ben-Shahar et al., 2008; Rowland et al., 2009; Sutani et al., 2009; Unal et al., 2008). Cohesin is also acetylated in mammalian cells on Smc3 residues K105/106 (Zhang et al., 2008), where two Eco1 orthologs exist, called Esco1 and Esco2 (Hou and Zou, 2005).

In vertebrate cells, cohesin-DNA interactions are also regulated by Sororin. This protein was identified as a substrate of APC/C^{Cdh1}, a form of the APC/C that is active during mitotic exit and G1 phase, and Soronin was found to be essential for cohesion in mammalian cells (Rankin et al., 2005). Interestingly, Sororin depletion also reduces the number of cohesin complexes that bind stably to DNA during G2 phase, indicating that Sororin is required for the formation of stable cohesin-DNA interactions (Schmitz et al., 2007). However, it is unknown how Sororin performs this function, and whether the role of Sororin is related to the function of cohesin acetylation. Furthermore, it is unknown how widespread the role of Sororin is because Sororin has only been identified in vertebrates.

Here we provide evidence that Sororin is recruited to chromatin-bound cohesin complexes in a manner that depends on DNA replication and Smc3 acetylation, that Sororin causes a conformational rearrangement within cohesin by displacing Wapl from Pds5, and that these molecular events stabilize cohesin on DNA by antagonizing Wapl's ability to dissociate cohesin from DNA. Furthermore, we show that distant orthologs of Sororin exist in many metazoan species, and that one of these proteins, Dalmatian, is required for cohesion in *Drosophila*. We therefore propose that sister chromatid cohesion depends on stabilization of cohesin on DNA by Sororin-related proteins.

RESULTS

Sororin Is Required for Cohesion during S Phase

We had previously shown that Sororin is required for cohesion in G2 phase (Schmitz et al., 2007). To address whether Sororin's function is already needed during S phase, we used RNA interference (RNAi) to deplete Sororin from HeLa cells that had been synchronized in the cell cycle and pulse-labeled these cells with bromodeoxyuridine (BrdU). Cells in S phase were identified by immunofluorescence microscopy (IFM) using BrdU antibodies, and the distance between sister chromatids was measured by DNA fluorescence in situ hybridization (FISH) using a probe for an arm region on chromosome 21. On average, FISH signals were twice as far separated in BrdU-positive, Sororin-depleted cells than in control cells (Figures 1A and 1B), indicating that Sororin is already required for cohesion during S phase. At variance with these results, it has been reported that Sororin-depleted cells only lose cohesion during metaphase and that Sororin is therefore not required for cohesion in early mitosis (Diaz-Martinez et al., 2007). However, in time-lapse microscopy experiments we observed that most Sororin-depleted cells failed to congress chromosomes, consistent with the existence of cohesion defects before metaphase (Figures S1A–S1D available online). The function of Sororin is therefore not restricted to mitosis and is instead already needed during or shortly after DNA replication.

Sororin Associates with Chromatin during the Period of the Cell Cycle Where Cohesion Exists

We next analyzed the intracellular distribution of Sororin. Previous IFM and fractionation experiments had shown that Sororin associates with chromatin in interphase, but Sororin could not be detected on mitotic chromosomes (Rankin et al., 2005). Because our antibodies could not detect Sororin in IFM experiments, we tagged Sororin at its carboxy-terminus with a localization-affinity purification (LAP) tag that contains green fluorescent protein (GFP; Figure S1E). We modified the *Sororin* gene on a bacterial artificial chromosome (BAC), enabling gene expression from the endogenous promoter (Poser et al., 2008). We used a mouse BAC for these experiments to enable RNAi "rescue" experiments and generated clonal HeLa cell lines that had stably integrated this BAC. The LAP-tagged version of mouse Sororin could substitute for the cohesion function of endogenous human Sororin when this was depleted by RNAi (Figures S1F and S1G), and in tandem affinity purification experiments mouse Sororin-LAP was found associated with human cohesin (Figures S1H and S1I), indicating that this tagged version of Sororin behaves similarly to endogenous Sororin. We therefore analyzed by IFM the intracellular distribution of Sororin-LAP, using antibodies to GFP. We stained proliferating cell nuclear antigen (PCNA) and Aurora B in the same cells as markers for S and G2 phases, respectively. Cellular Sororin-LAP levels were low in G1, accumulated between early S and G2 phases in the nucleus, and became dispersed in the cytoplasm following nuclear envelope breakdown (Figures S1J–S1L). When we analyzed cells from which soluble proteins had been extracted before fixation, we observed that Sororin-LAP accumulated on chromatin between early S phase and G2 phase, whereas most Sororin-LAP disappeared from chromosomes in prophase (Figures 1C–1E). At this stage, the cellular levels of Sororin were still high (Figure S1L), indicating that the removal of Sororin from prophase chromosomes is caused by dissociation, not degradation. Biochemical fractionation experiments confirmed this notion (Figure S1M). Importantly, however, small amounts of Sororin-LAP could still be detected by IFM on

the trisomic *tff1* locus on chromosome 21. BrdU-labeled nuclei (blue) with three pairs of FISH signals (red) are shown. Higher-magnification images are shown in the insets. Bar: 5 μm.

(B) Quantification of the distance between paired FISH signals in (A) (mean ± standard deviation [SD]; $n \geq 30$ per condition, $^*p < 0.01$).

(C) Sororin-LAP cells were pre-extracted prior to fixation and stained for Sor-LAP (GFP), PCNA, and Aurora B. DNA was counterstained with Hoechst. Bar: 10 μm.

(D) Quantification of chromatin-bound Sororin-LAP levels in (C) (mean ± SD; $n \geq 50$ per class).

(E) Sororin-LAP cells were synchronized in mitosis, pre-extracted prior to fixation, and stained for Sor-LAP (GFP), Scc1, and DNA (Hoechst). Bar: 10 μm.

(F) Sororin-LAP localizes to centromeres in mitosis. Sororin-LAP cells were pre-extracted prior to fixation and stained for Sor-LAP (GFP), kinetochores (CREST), and DNA (DAPI). Insets show magnified views. Bar: 10 μm.

See also Figure S1.

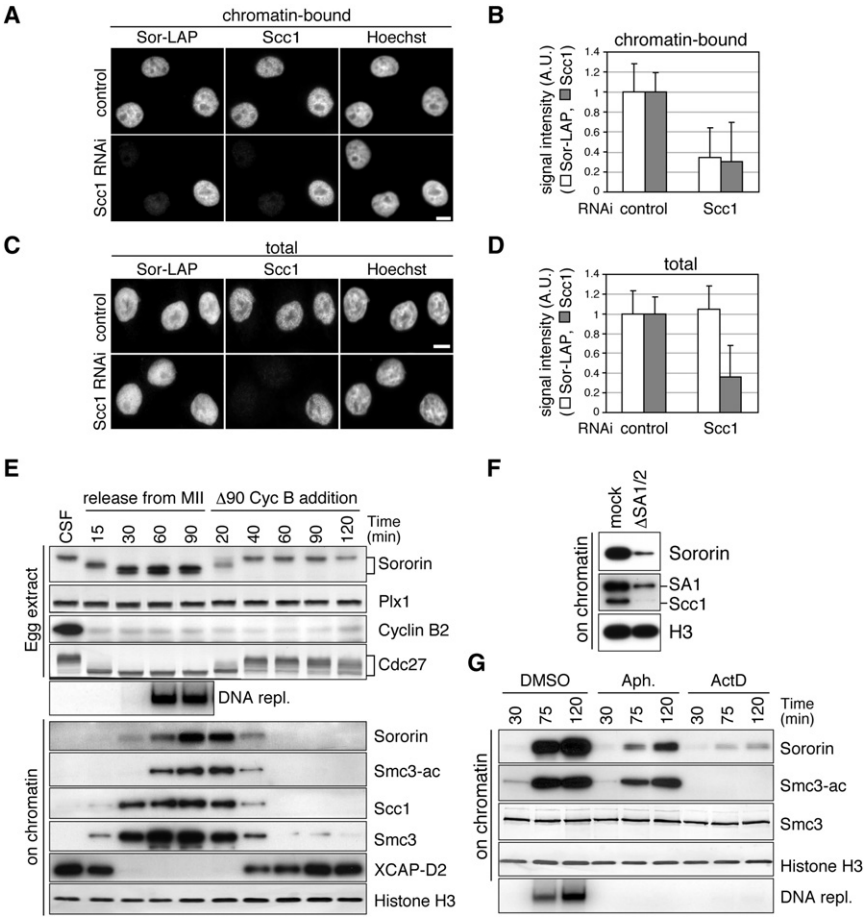


Figure 2. Association of Sororin with Chromatin Depends on Cohesin and DNA Replication

(A–D) Sororin-LAP cells were transfected with siRNAs and synchronized in G2 phase. Cells were fixed (C and D) or pre-extracted prior to fixation (A and B) and stained for Sor-LAP (GFP), Scc1, and DNA (Hoechst). Bar: 10 μm. Quantification of Sororin-LAP levels in (A) and (C) is shown in (B) and (D), respectively (mean ± SD; n ≥ 110 (B) and n ≥ 130 (D) per condition).

(E) Sororin is stably present throughout the cell cycle but associates with chromatin during S phase in *Xenopus* egg extracts. $CaCl_2$ and cycloheximide were added to meiotic metaphase II (MII) arrested CSF extract to induce meiotic exit. At 90 min after $CaCl_2$ addition, Δ90 Cyclin B was added to induce mitosis. Samples were taken at indicated time points after $CaCl_2$ addition (release from MII) or Δ90 Cyclin B addition (Δ90 Cyc B addition). DNA replication (DNA repl.) was monitored by incorporation of [α-^{32}P]dCTP into sperm chromatin. Chromatin-bound proteins in the same extracts are also shown. Chromatin was preincubated for 30 min in CSF extracts.

(F) Sororin association with chromatin depends on cohesin. *Xenopus* interphase extracts were subjected to mock or SA1/2 immunodepletion. Two hours after sperm chromatin addition, chromatin fractions were analyzed by immunoblotting.

(G) Sororin association with chromatin depends on DNA replication. Interphase extracts were incubated for indicated times with sperm chromatin. DMSO, aphidicolin (Aph.), or actinomycin D (ActD) was added to the extracts 25 min after sperm addition. Chromatin fractions were analyzed by immunoblotting.

See also Figure S2.

chromosomes in prophase, prometaphase, and metaphase, but not in anaphase or telophase (Figure 1E). Like cohesin (Waizenegger et al., 2000), Sororin-LAP was enriched at centromeres in prometa/metaphase (Figure 1F). Sororin therefore associates with chromatin from S phase until metaphase, i.e., as long as cohesion exists.

The Association of Sororin with Chromatin Depends on Cohesin

Because Sororin binds to cohesin and, like cohesin, is removed from mitotic chromosomes in two steps, during prophase and at the metaphase-anaphase transition, we tested whether the association of Sororin with chromatin depends on cohesin. Scc1 depletion reduced the intensity of Sororin-LAP staining on chromatin without affecting the cellular levels of Sororin-LAP (Figures 2A–2D), indicating that Sororin can only efficiently associate with chromatin in the presence of cohesin. Biochemical experiments in *Xenopus* egg extracts confirmed this notion (see Figure 2F below). The presence of Sororin on mitotic chromosomes also depends on cohesin, as depletion of either Scc1 or Shugoshin-like 1 (Sgo1) reduced chromosomal Sororin-LAP staining, whereas depletion of Wapl or inhibition of Plk1 increased the amounts of Sororin on chromosome arms (Figure S2A).

Although the intracellular distribution of Sororin and cohesin is similar from prophase to anaphase, the two proteins behave differently in telophase. Whereas cohesin reassociates with chromatin at this stage, little if any Sororin-LAP could be detected on chromatin in telophase (Figure 1E). This difference was not due to lower sensitivity in the detection of Sororin than cohesin because Sororin-LAP could easily be observed on early mitotic chromosomes, where endogenous cohesin cannot be detected (due to its low abundance; Waizenegger et al., 2000). The absence of Sororin on telophase chromatin was also not caused by APC/C^{Cdh1}-mediated degradation of all cellular Sororin because Sororin-LAP could be observed in fixed telophase cells (Figure S1L). Time-lapse microscopy of living cells showed that Sororin levels begin to decrease in anaphase when APC/C^{Cdh1} becomes active but revealed that most Sororin degradation occurs after telophase, i.e., during G1, as is typical for APC/C^{Cdh1} substrates (Figures S2B–S2E). The absence of Sororin on chromatin in telophase is therefore not simply due to the complete degradation of Sororin.

Efficient Association of Sororin with Chromatin Depends on DNA Replication

The absence of Sororin on telophase chromatin could be caused by local APC/C^{Cdh1}-mediated degradation on chromatin, or the

association of cohesin with chromatin could be required but not sufficient for Sororin binding to chromatin. To distinguish between these possibilities, we analyzed the chromatin association of Sororin in *Xenopus* eggs, which do not contain Cdh1 and where Sororin is therefore predicted to be stable during mitotic exit. If cohesin was sufficient for recruiting Sororin to chromatin, both proteins would be expected to associate with chromatin simultaneously in *Xenopus* egg extracts. To test this possibility, we isolated two *Xenopus* Sororin cDNAs (Sororin-A and -B), which encode closely related 35 kDa proteins. *Xenopus* Sororin antibodies recognized both Sororin isoforms in immunoblots (visible as a doublet of bands; see for example Figure 2E) and could deplete both proteins from egg extracts (see Figure 4A below). Immunodepletion experiments also revealed that the chromatin association of *Xenopus* Sororin proteins depends on cohesin (Figure 2F) and that these proteins are required for cohesion (see Figure 4B below), even though their amino acid sequences are only 38% identical to the sequence of human Sororin. The two *Xenopus* proteins characterized here (hereafter collectively called *Xenopus* Sororin) are therefore functionally related to mammalian Sororin.

To address when Sororin and cohesin associate with chromatin, we released *Xenopus* egg extracts from a cytostatic factor (CSF) arrest in metaphase of meiosis II into interphase by addition of Ca^{2+}, which leads to activation of APC/C^{Cdc20}, degradation of mitotic Cyclins, and mitotic exit (Figure 2E). As a source of chromatin, demembranated sperm nuclei were added. DNA replication was monitored by incorporation of [α-^{32}P]dCTP into DNA and occurred within 60 min after Ca^{2+} addition. After 90 min, we added a recombinant form of nondegradable Cyclin B (Δ90 Cyc B) to induce entry of the extract into a mitotic state. At different time points, proteins in the chromatin fraction or the total extract were analyzed by immunoblotting (Figure 2E). As expected, Ca^{2+} addition led to rapid degradation of Cyclin B2 (a substrate of APC/C^{Cdc20}), but the levels of the APC/C^{Cdh1} substrates Sororin and Plx1 remained largely unchanged (only the electrophoretic mobility of Sororin was reduced by phosphorylation in CSF and mitotic extracts). Importantly, even though Sororin was present throughout all stages of the cell cycle, it began to associate with chromatin only 60 min after addition of Ca^{2+}, i.e., when DNA replication was initiated. In contrast, the cohesin subunits Scc1 and Smc3 could be detected on chromatin at least 30 min earlier. The association of Sororin with chromatin was abolished by Geminin (Figure S2F), a protein that inhibits cohesin loading onto DNA (Gillespie and Hirano, 2004; Takahashi et al., 2004), indicating that our assay reflected physiological binding of Sororin to chromatin. These observations suggest that local APC/C^{Cdh1}-mediated degradation of Sororin on chromatin cannot explain why Sororin associates with chromatin later than cohesin. Instead, our results indicate that the presence of cohesin on chromatin is not sufficient for recruitment of Sororin.

Because Sororin associates with chromatin during S phase in *Xenopus* extracts and in somatic cells (Figure 1C and Figure 2E), we tested whether DNA replication is required for recruitment of Sororin to chromatin. We prevented replication in *Xenopus* extracts by addition of aphidicolin or actinomycin D. Aphidicolin allows initiation of DNA replication but leads to the stalling of replication forks from which the replicative MCM helicase is uncoupled, whereas actinomycin D inhibits progression of both DNA polymerase and helicase (Pacek and Walter, 2004). In our assays, aphidicolin reduced association of Sororin with chromatin partially, and actinomycin D inhibited this process largely, even though Smc3 levels on chromatin were not reduced (Figure 2G). DNA replication is therefore required for efficient recruitment of Sororin to chromatin. However, because aphidicolin and actinomycin D inhibited DNA replication more efficiently than Sororin binding, it is possible that some Sororin can associate with chromatin in the absence of DNA replication. Similar observations were made in HeLa cells where inhibition of DNA replication by thymidine also reduced the Sororin-LAP levels on chromatin (Figures S2G and S2H).

Cohesin Acetylation Facilitates but Is Not Sufficient for the Association of Sororin with Chromatin

Because Sororin associates with chromatin during DNA replication, i.e., when cohesin is known to be acetylated, we analyzed whether Smc3 acetylation and Sororin binding depend on each other. To detect Smc3 acetylation, we used a monoclonal antibody that specifically recognizes Smc3 singly acetylated on K106 or doubly acetylated on K105 and K106 (Figure S3A). We observed that Sororin binding to chromatin and Smc3 acetylation occurred at the same time (Figure 2E) and that inhibition of DNA replication had similar effects on both events, supporting the notion that the two events are linked (Figure 2G). However, depletion of Sororin from *Xenopus* extracts or from HeLa cells affected neither the kinetics nor the degree of Smc3 acetylation, suggesting that Sororin is not required for cohesin acetylation (Figures S3B and S3C).

To test whether Smc3 acetylation is required for the chromatin association of Sororin, we depleted Esco1 and Esco2 from HeLa cells. Only depletion of both enzymes reduced Smc3 acetylation, indicating that Esco1 and Esco2 can both acetylate cohesin (Figure 3A). To analyze whether depletion of Esco1 and Esco2 affects the association of Sororin with chromatin, we synchronized cells in S phase by double thymidine arrest-release and measured the amount of Sororin-LAP on chromatin by immunoblotting and IFM. We also depleted endogenous Sororin in these experiments to ensure that Sororin-LAP was analyzed under conditions where it is functional. To rule out that reduced chromatin binding of Sororin was caused indirectly by a delay in DNA replication, we labeled cells with BrdU and quantified Sororin-LAP IFM signals only in cells that had similar amounts of BrdU incorporated. Both by immunoblotting and IFM we observed a reduction in Sororin on chromatin (Figures 3B–3D). Depletion of Esco1 and Esco2 also reduced the amount of endogenous Sororin that was associated with chromatin-bound cohesin (Figures S3D and S3E). Esco1 and Esco2 are therefore required for efficient binding of Sororin to cohesin on chromatin. It is possible that the residual amounts of Sororin on chromatin that were seen in our assays were due to incomplete depletion of Esco1 and Esco2.

To address whether Esco1 and Esco2 regulate Sororin by acetylating Smc3, we mutated K105 and K106 in Smc3 to either glutamine (Smc3^{QQ}), arginine (Smc3^{RR}), or alanine (Smc3^{AA}) residues. Smc3^{QQ} has been proposed to mimic acetylated and

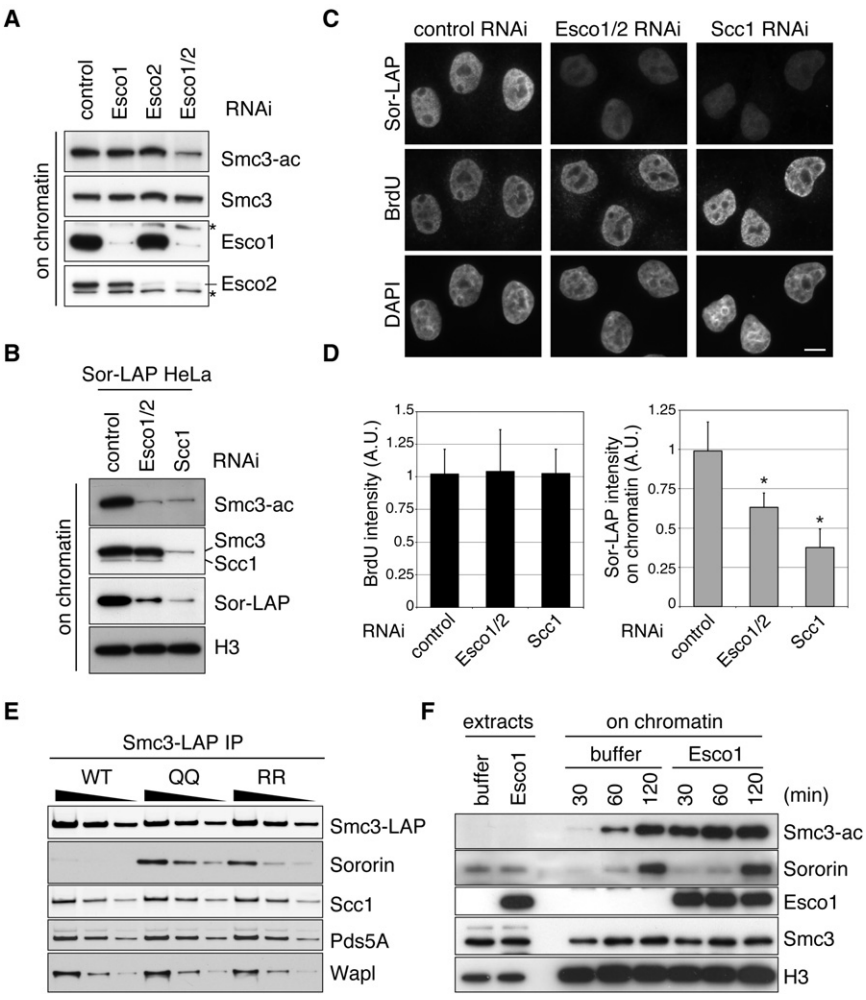


Figure 3. Acetylation of Smc3 Facilitates but Is Not Sufficient for the Association of Sororin with Chromatin

(A) RNAi against both Esco1 and Esco2 causes a decrease in Smc3 acetylation. HeLa cells were transfected with siRNAs and harvested at S phase. Chromatin-bound proteins were analyzed by immunoblotting. Asterisks indicate nonspecific signals.

(B) Reduction of Smc3 acetylation causes a decrease of Sororin on chromatin. Sororin-LAP HeLa cells were synchronized at S phase and chromatin fractions were analyzed by immunoblotting.

(C) Cells in (B) were treated with BrdU after the second thymidine release, pre-extracted, and costained for BrdU, Sor-LAP (GFP), and DNA (DAPI). Bar: 10 μm.

(D) Quantification of chromatin-associated Sororin-LAP signal in cells with similar levels of BrdU incorporation. Cells described in (C) with similar BrdU intensities (left) were analyzed for Sor-LAP intensity (right) (mean ± confidence interval; *$p < 0.01$).

(E) Soluble $Smc3^{QQ}$ and $Smc3^{RR}$ proteins stably bind to Sororin in HeLa cells. HeLa cells expressing $Smc3^{WT}$-, $Smc3^{QQ}$-, or $Smc3^{RR}$-LAP were synchronized in G2 phase, Smc3-LAP was immunoprecipitated from the soluble fraction of cells, and the coprecipitated proteins were analyzed by immunoblotting using a 2-fold serial dilution.

(F) Acetylation of Smc3 is not sufficient for Sororin binding to chromatin. Interphase *Xenopus* egg extracts were incubated with sperm chromatin in the presence (Esco1) or absence (buffer) of Esco1 for indicated times. Chromatin fractions were analyzed by immunoblotting (on chromatin). Extracts without sperm chromatin were incubated for 120 min in the presence or absence of Esco1 (extracts).

See also Figure S3.

$Smc3^{RR}$ and $Smc3^{AA}$ to mimic nonacetylated Smc3. We mutated a LAP-tagged version of the *Smc3* gene on a BAC, stably integrated the modified BACs into HeLa cells, purified the wild-type and mutant forms of Smc3 either from soluble extracts or from chromatin, and analyzed their interaction partners by immunoblotting and mass spectrometry. For wild-type Smc3-LAP, these experiments confirmed that Sororin only associates with cohesin on chromatin but not, or only to a small degree, with soluble cohesin (Figure S3G). However, when $Smc3^{QQ}$-LAP was purified, Sororin could also reproducibly be found in association with soluble cohesin, consistent with the possibility that Smc3 acetylation promotes binding of Sororin to cohesin (Figure 3E and Figure S3G). This interaction was abolished by depletion of Scc1, indicating that $Smc3^{QQ}$ does not simply represent a misfolded protein to which Sororin binds nonspecifically (Figure S3H). Unexpectedly, similar results were also obtained when $Smc3^{RR}$ and $Smc3^{AA}$ were analyzed (Figures 3E, Figure S3F, and Figure S3G). This suggests that Sororin-cohesin interactions can be stabilized not only by mutations that might chemically mimic acetylation but also by other mutations that alter K105 and K106 (for possible interpretations of these results, see Discussion).

We also attempted to generate acetylated cohesin in vitro by using recombinant purified Esco1 (Figure S3I). We observed that Esco1 could acetylate Smc3 when cohesin was associated with chromatin in a *Xenopus* extract, but not in extract lacking chromatin or when Esco1 was incubated with purified soluble cohesin (Figure 3F and data not shown). Esco1 may therefore only be able to modify cohesin on chromatin. Consistent with this possibility, endogenous acetylated forms of Smc3 could only be detected by immunoblotting in chromatin fractions (Figure S3J), and quantitative mass spectrometry indicated that the fraction of acetylated Smc3 relative to total Smc3 is 97-fold higher for chromatin-bound than for soluble cohesin (data not shown).

When we added Esco1 to *Xenopus* extract containing chromatin, we observed that Smc3 acetylation was advanced by at least 30 min, but Esco1 had no effect on the chromatin association of Sororin (Figure 3F), indicating that Smc3 acetylation is not sufficient for recruitment of Sororin to chromatin. In support of

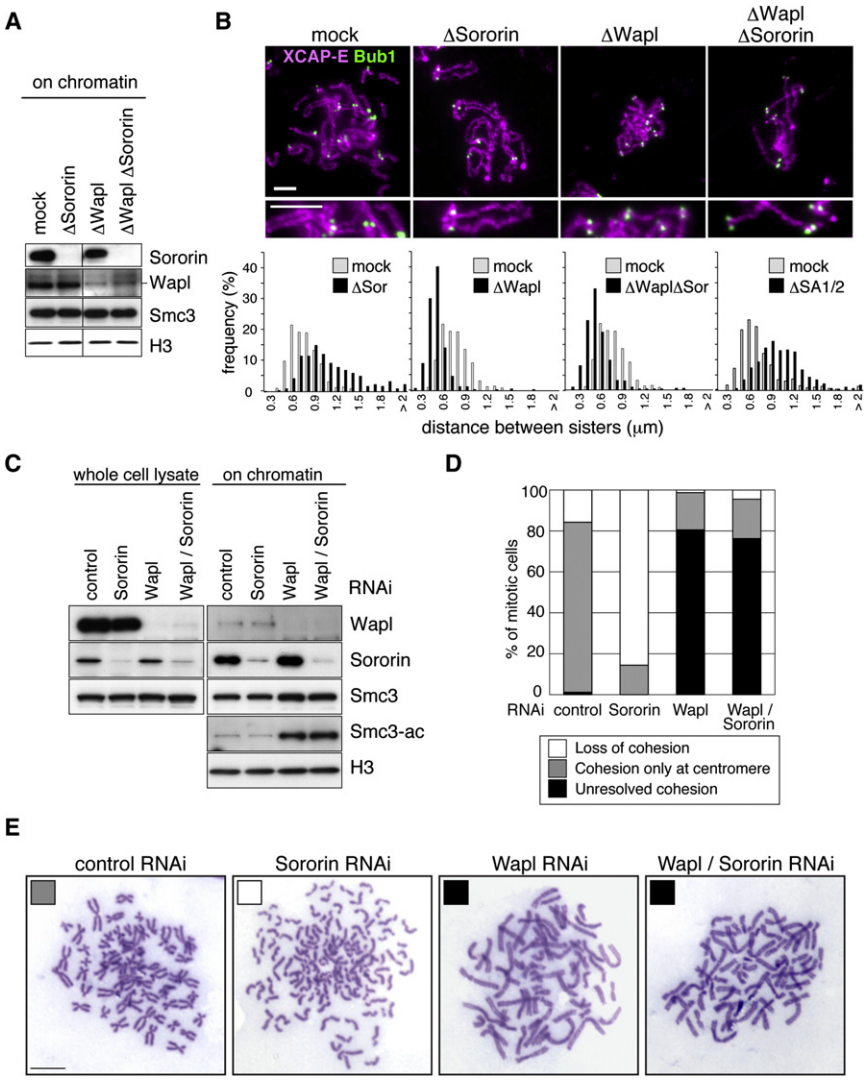


Figure 4. Sororin Is Dispensable for Cohesion in the Absence of Wapl

(A) Chromatin fractions from mock-, Sororin-, Wapl-, and Wapl- and Sororin-depleted interphase extracts were analyzed by immunoblotting.

(B) Δ90 Cyclin B was added to the extracts shown in (A) and mitotic chromosomes were assembled. Chromosomes were isolated 120 min after Δ90 Cyclin B addition and stained for XCAP-E (magenta) and Bub1 (green). Higher-magnification images are shown in lower panels. Distance between two chromosome arms stained by XCAP-E in each extract is shown in a histogram as a comparison to the mock-depleted extract. Depletion of SA1/2 is shown as an example of cohesin depletion. Bar: 5 μm.

(C) Codepletion of Sororin and Wapl in HeLa cells. Cells were transfected with the indicated siRNAs and treated with nocodazole. After mitotic shake-off for chromosome spreads (D and E), residual cells were harvested for immunoblotting. See also Figure S4A.

(D) Analysis of chromosome spreads after Sororin and Wapl depletion. Mitotic cells harvested as in (C) were examined by chromosome spreading and Giemsa staining. Five hundred cells per RNAi experiment were classified into three categories.

(E) Representative pictures of the most prominent phenotype class upon RNAi depletion in the Giemsa spread analysis. Color code is shown in (D). Bar: 10 μm.

this hypothesis, we found that the association of Sororin with Smc3^{QQ} was still partially dependent on DNA replication (Figure S3K). Taken together, these results indicate that Smc3 acetylation is required but not sufficient for efficient recruitment of Sororin to chromatin-bound cohesin.

Sororin Is Dispensable for Cohesion in the Absence of Wapl

Several previous observations are consistent with the possibility that Sororin and Wapl have antagonistic functions: depletion of Sororin and Wapl has opposite effects on cohesion (increased and decreased proximity between sister chromatids, respectively) and on the stability of cohesin-DNA interactions (decreased and increased residence times of cohesin on chromatin, respectively). Likewise, addition of excess Sororin to *Xenopus* extracts mimics the "overcohesion" phenotype caused by depletion of Wapl, and overexpression of Wapl causes cohesion defects, as does loss of Sororin (Gandhi et al., 2006; Kueng et al., 2006; Rankin et al., 2005; Schmitz et al., 2007; Shintomi and Hirano, 2009).

To understand the functional relationship between Sororin and Wapl we depleted both proteins either individually or simultaneously from *Xenopus* extracts and analyzed cohesion in mitotic chromosomes. We analyzed chromosome morphology by staining the condensin subunit XCAP-E and Bub1 as markers for sister chromatid arms and kinetochores, respectively. Depletion of Sororin alone increased the distance between sister chromatids, indicating a partial cohesion defect (Figures 4A and 4B). This defect was similar in magnitude to the defect that was observed after simultaneous depletion of the cohesin subunits SA1 and SA2, suggesting that also in *Xenopus* extracts Sororin is similarly important for cohesion as cohesin itself (Figure 4B). As expected, depletion of Wapl had the opposite effect, i.e., resulted in tightly connected chromatids. Remarkably, depletion of both proteins caused a phenotype that was very similar to the phenotype caused by depletion of Wapl alone. Similar results were obtained when Sororin and Wapl were depleted singly or simultaneously by RNAi from HeLa cells and mitotic chromosomes were analyzed by Giemsa staining (Figures 4C–4E). Also in this case, the phenotype obtained after codepletion of Sororin and Wapl was nearly identical to the phenotype obtained after depletion of Wapl alone, i.e., in the majority of mitotic cells sister chromatids were more tightly associated with each other

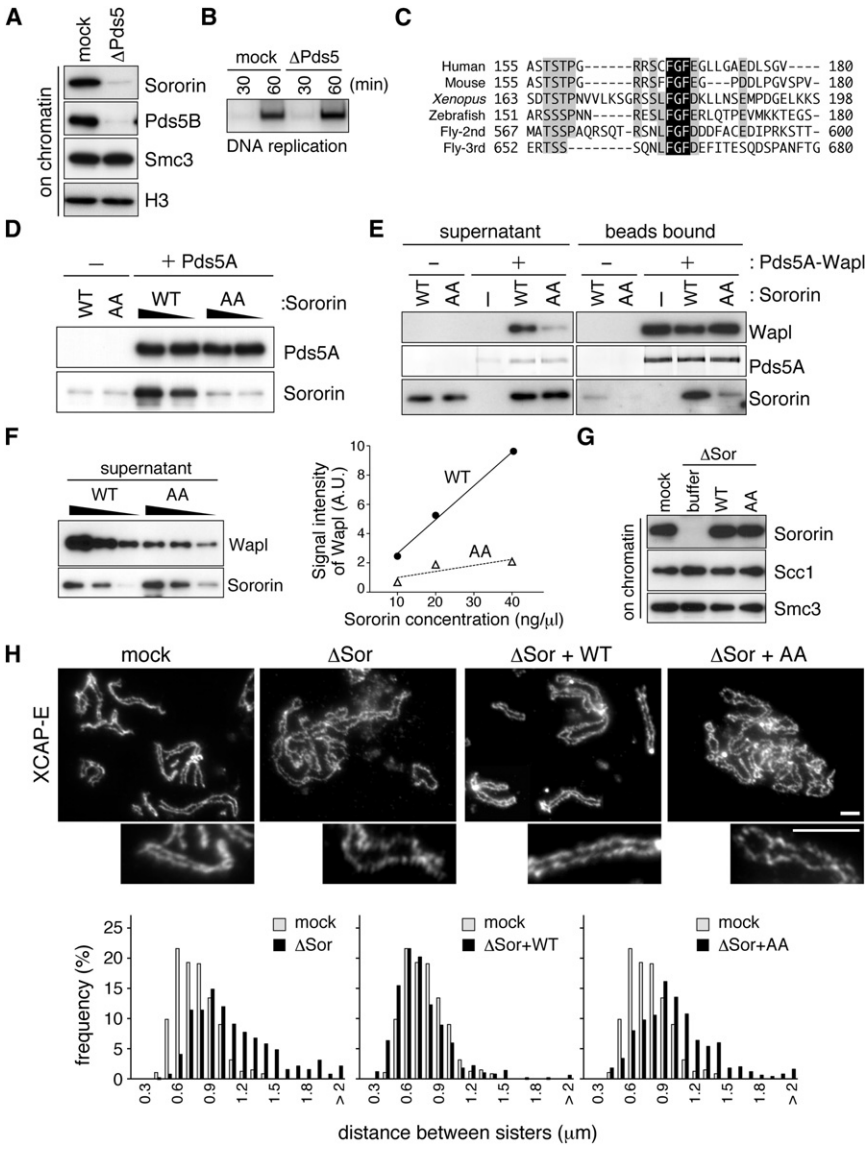


Figure 5. The FGF Motif of Sororin Is Required for Cohesion

(A and B) Pds5 is required for Sororin association with chromatin. Interphase *Xenopus* egg extracts were subjected to mock or Pds5A and B immunodepletion. Two hours after sperm chromatin addition, chromatin fractions were analyzed by immunoblotting (A). DNA replication in the extracts shown in (A) was monitored for 30 or 60 min by incorporation of [α-^{32}P]dCTP into sperm chromatin (B).

(C) Sequence comparison in the region including FGF motifs of vertebrate Sororin and fly Dalmatian. Identical and similar residues are shaded in black and gray, respectively. In *Xenopus*, Sororin-A is shown. In fruit fly, the latter two of three FGF motifs are shown (see also Figure 7A).

(D) Anti-Pds5A antibody beads were incubated with Sororin-WT or -AA mutant in the presence or absence of Pds5A protein. Beads-bound proteins were analyzed by immunoblotting.

(E) Anti-Pds5A antibody beads were incubated with Sororin-WT or -AA mutant in the presence or absence of the Pds5A-Wapl heterodimer. Beads-bound proteins were separated from the supernatant and were analyzed by immunoblotting.

(F) Wapl removal activity of Sororin is increased in a dose-dependent manner. Increasing amounts (10–40 ng/μl) of Sororin-WT or -AA mutant were used in the experiment shown in (E), supernatant fractions were analyzed by immunoblotting (left), and signal intensity of Wapl was quantified (right).

(G) Sororin-depleted interphase extracts were combined with buffer, Sororin-WT, or -AA mutant. Two hours after sperm chromatin addition, chromatin fractions were analyzed by immunoblotting.

(H) Δ90 Cyclin B was added to the extracts shown in (G) and mitotic chromosomes were assembled. Chromosomes were isolated 120 min after Δ90 Cyclin B addition and stained for XCAP-E. Magnified images are shown in lower panels. Distance between two chromosome arms stained by XCAP-E is shown in lower histogram as a comparison to mock-depleted extract. Bar: 5 μm. See also Figure S4.

than normally. These observations indicate that Sororin is only required for cohesion in the presence of Wapl, and they therefore suggest that Sororin's key function is to antagonize Wapl.

We also observed in these experiments that Wapl depletion greatly increased the degree of Smc3 acetylation (Figure 4C and Figure S4A). Wapl depletion could cause this effect by increasing the residence time of cohesin on DNA, but it is also possible that Wapl inhibits cohesin acetylation and that this function is required for Wapl's ability to allow cohesin dissociation from DNA.

Sororin Interacts with Pds5 via a Conserved FGF Motif and Can Displace Wapl from Pds5

When we isolated Sororin-LAP via tandem affinity purification, we identified cohesin core subunits and Pds5A and Pds5B, indicating that Sororin can directly bind to these proteins (Figure S1I and data not shown). Sororin antibodies also immunoprecipitated Pds5A and Pds5B from solubilized chromatin of HeLa cells (Figure S4B), and when we immunodepleted Pds5A and Pds5B from *Xenopus* extracts the binding of Sororin to chromatin was greatly reduced (Figure 5A). The latter effect was not caused by a delay in DNA replication because [α-^{32}P]dCTP incorporation into sperm DNA was unaffected by depletion of Pds5 proteins (Figure 5B). These observations are consistent with the possibility that the association of Sororin with cohesin depends on Pds5 proteins.

To address directly whether Sororin interacts with Pds5 proteins or Pds5-Wapl heterodimers, we purified recombinant forms of human Sororin, Pds5A and Wapl. As predicted, Wapl bound efficiently to Pds5A, either when expressed simultaneously in Baculovirus-infected insect cells or when incubated with each other as individually purified proteins (Figure S4C

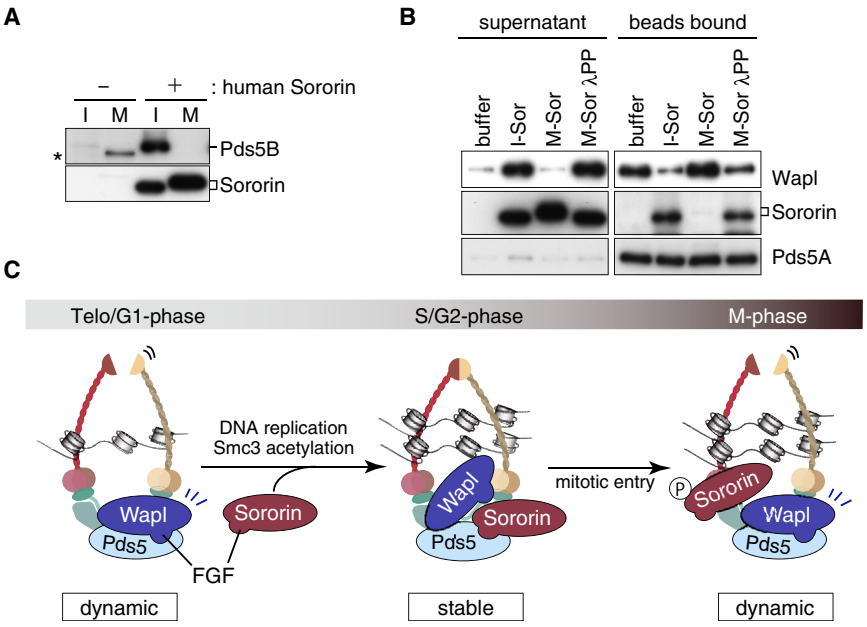


Figure 6. Phosphorylated Sororin Is Unable to Dissociate Wapl from Pds5

(A) Sororin is dissociated from Pds5 in mitosis. Sororin-WT was incubated in either interphase (I) or mitotic (M) *Xenopus* egg extracts and immunoprecipitated, and the precipitates were analyzed by immunoblotting. Asterisk indicates nonspecific signal.

(B) Wapl removal activity is abolished by phosphorylation of Sororin. Wapl-Pds5A heterodimer on anti-Pds5A antibody beads was incubated with either buffer, Sororin preincubated in interphase egg extract (I-Sor) or mitotic egg extract (M-Sor), or λ-protein phosphatase-treated M-Sor (M-Sor λ-PP). Beads-bound proteins were separated from the supernatant and analyzed by immunoblotting.

(C) Model for the role of Sororin in sister chromatid cohesion. The cohesin complex is loaded onto chromatin during telo/G1 phase, where Wapl-Pds5 destabilizes cohesin binding to chromatin in the absence of Sororin. During DNA replication in S phase, Sororin associates with chromatin depending on cohesin and this association is facilitated by acetylation of Smc3. Sororin binds to Pds5 through its FGF motif and thereby can antagonize the function of Wapl by modulating the topology of Wapl and Pds5 so that stable cohesion is maintained. Upon entry into mitosis, phosphorylation of Sororin abolishes the ability to inhibit Wapl, allowing cohesin removal in prophase.

and data not shown). The interaction between Pds5 and Wapl depends on two sequence elements composed of phenylalanine-glycine-phenylalanine (FGF) residues in Wapl (Shintomi and Hirano, 2009), and we noticed that a similar FGF motif is also present at a conserved position in all known Sororin sequences (Figure 5C and see Figure S5B). We therefore also generated a Sororin mutant in which the two phenylalanine residues in this motif were changed to alanines (hereafter called "Sororin-AA"). Wild-type Sororin associated with Pds5A, whereas the AA mutant bound less well (Figure 5D). Also, when added to *Xenopus* extracts, wild-type Sororin associated with cohesin and Pds5B more efficiently than the AA mutant (Figure S4D). When we performed Sororin-binding experiments with Pds5A-Wapl, we observed, remarkably, that Sororin displaced some Wapl from the Pds5A-Wapl heterodimers. Also, this effect was reduced when the AA mutant was used (Figures 5E and 5F). These observations raised the possibility that Sororin regulates cohesin by interacting with the Pds5-Wapl heterodimer.

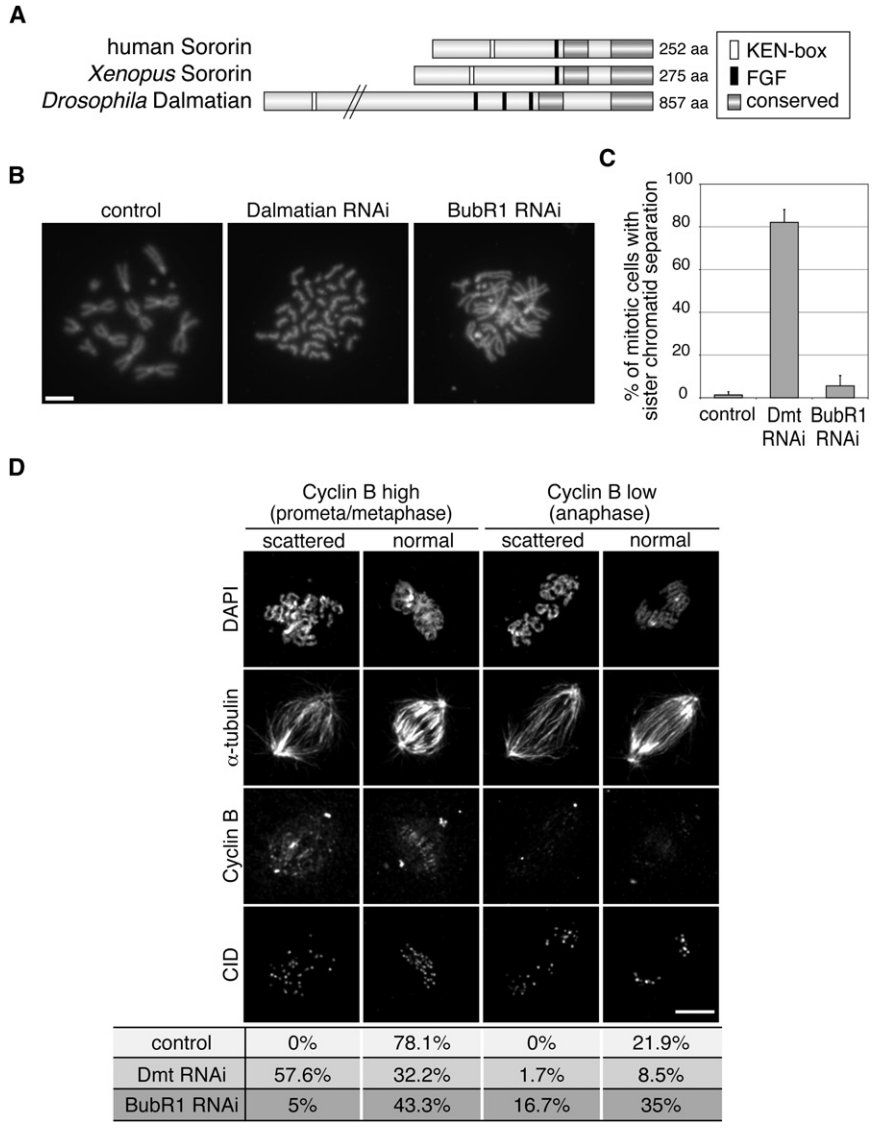
The FGF Motif of Sororin Is Essential for Its Cohesion Function

To address whether Sororin's ability to displace Wapl from Pds5 is of functional relevance, we replaced Sororin in *Xenopus* extracts by the Sororin-AA mutant and analyzed its effect on cohesion. We immunodepleted Sororin from interphase egg extracts, added either buffer, recombinant wild-type Sororin, or the AA mutant, and analyzed mitotic chromosomes as above. Importantly, the cohesion defect observed after Sororin depletion could be restored by wild-type Sororin but not by the AA mutant (Figures 4G and 4H). Similar results were obtained when excess Sororin was added to *Xenopus* extracts from which the endogenous protein had not been depleted: in this assay wild-type Sororin causes an "overcohesion" phenotype (Rankin et al., 2005), but the AA mutant had no effect (Figure S4E). These results show that the FGF motif of Sororin is required for its function in cohesion, and they suggest that Sororin might execute this function by displacing Wapl from Pds5.

However, we could not obtain evidence that the Sororin-dependent displacement of Wapl from Pds5 results in the dissociation of Wapl from chromatin. Addition of recombinant Sororin to *Xenopus* extracts increased, and did not decrease, the amount of Wapl and Pds5A on chromatin, as if Sororin stabilized the interactions between Pds5A-Wapl and cohesin, rather than dissociating Wapl from cohesin (Figure S4F). It is therefore possible that the Sororin-mediated displacement of Wapl from Pds5A causes a rearrangement in the topology of cohesin-associated proteins and does not lead to dissociation of Wapl from cohesin.

Sororin Is Inactivated by Phosphorylation in Mitosis

The prophase pathway of cohesin dissociation depends on Wapl (Gandhi et al., 2006; Kueng et al., 2006). It is therefore conceivable that Sororin has to be inactivated at the onset of mitosis to relieve Wapl from its inhibition by Sororin. We therefore analyzed whether Sororin's ability to dissociate Wapl from Pds5 proteins is cell cycle regulated. Consistent with this possibility, recombinant Sororin could associate with Pds5B in *Xenopus* interphase extracts but not in mitotic extracts where Sororin is phosphorylated (Figure 6A). Furthermore, we observed that Sororin could bind to recombinant purified Wapl-Pds5A



control	0%	78.1%	0%	21.9%
Dmt RNAi	57.6%	32.2%	1.7%	8.5%
BubR1 RNAi	5%	43.3%	16.7%	35%

Figure 7. Dalmatian Is an Ortholog of Sororin in *Drosophila*

(A) Schematic sequence comparison of human and *Xenopus* Sororin and *Drosophila* Dalmatian. The conserved regions are shaded in gray and KEN-box and FGF motifs are depicted with white and black boxes, respectively.

(B) Dalmatian (Dmt) RNAi causes premature sister chromatid separation in S2 cells. Cells were transfected with dsRNA against Dmt or BubR1 or were left untransfected (control). Chromosome spreads were stained with DAPI. Representative images are shown. Bar: 5 μm.

(C) Cells described in (B) were quantified for loss of cohesion. Error bars denote standard deviations between three independent experiments.

(D) Mitotic defects in Dalmatian knockdown cells. Cells were transfected with dsRNA against Dmt or BubR1 or were untransfected (control) and costained for α-tubulin and Cyclin B to define mitotic stages, CID (Cenp-A in *Drosophila*) to assess centromere pairing, and DAPI (upper panel). The lower table summarizes the observed phenotype over all mitotic cells (n > 59 per condition). Bar: 5 μm.

See also Figure S5 and Table S1.

heterodimers and dissociate Wapl from Pds5A when Sororin was preincubated in *Xenopus* interphase extracts but not when Sororin had been incubated in a mitotic extract (Figure 6B). The Wapl dissociation activity of mitotic Sororin was fully restored when Sororin was first dephosphorylated by λ-protein phosphatase. These results suggest that Sororin phosphorylation in mitosis relieves Wapl from inhibition by Sororin (Figure 6C; for further discussion of this model see below).

Dalmatian Is a *Drosophila* Ortholog of Sororin

Wapl orthologs exist in species from yeast to human (Kueng et al., 2006), but Sororin has only been identified in vertebrates (Rankin et al., 2005). To address whether inhibition of Wapl by Sororin could also be required for cohesion in nonvertebrate species, we searched for Sororin-related sequences in invertebrate genomes (Table S1). BLAST searches identified Sororin sequences in vertebrates and one distantly related protein in the mollusc *Lottia gigantea*. We used the C-terminal portion of these sequences, where the highest degree of similarity is found, to perform iterative rounds of similarity searches in invertebrate proteome databases. We identified a single sequence with significant similarity to Sororin in 18 different metazoan species belonging to different taxa, including cephalochordates, echinoderms, insecta, cnidaria, and placozoa. All of these proteins contain sequences related to the C terminus of Sororin, which we therefore call the "Sororin domain" (Figure S5A). Furthermore, 17 of these proteins also contain an FGF sequence motif (Figure S5B), or sometimes several of these motifs (Figure 7A).

Of the 18 hypothetical proteins, only one has previously been characterized. This is a 95 kDa protein called Dalmatian, which is required for development of the *Drosophila* embryonic peripheral nervous system (Prokopenko et al., 2000). Recent RNAi screens have shown that depletion of Dalmatian causes defects in mitotic spindle assembly, chromosome alignment, and cell division (Goshima et al., 2007; Somma et al., 2008). Dalmatian inactivation also causes precocious sister chromatid separation in the presence of colchicine, a compound that activates the SAC. It has therefore been proposed that Dalmatian is required for the SAC (Somma et al., 2008).

Because Dalmatian shares sequence similarity with Sororin, we tested whether Dalmatian is required for cohesion. If this

were the case, Dalmatian depletion would be predicted to cause precocious sister chromatid separation, to activate the SAC, and thus to cause an increase in mitotic index, whereas inactivation of a SAC protein would shorten mitosis and cause a decrease in mitotic index. We observed a modest increase in mitotic index from 3.2% in control *Drosophila* S2 cells to 5.3% in Dalmatian RNAi cells, whereas depletion of BubR1, a protein required for the SAC (Perez-Mongiovi et al., 2005), decreased the mitotic index to 1.4% (data not shown). Chromosome spreading revealed that cohesion had been lost in 82% of all mitotic Dalmatian RNAi cells, but only in less than 6% of mitotic control or BubR1 RNAi cells (Figures 6B and 6C). In IFM experiments, we observed that Dalmatian depletion caused chromosome congression defects (''scattered chromosomes'') in 57.6% of prometa/metaphase cells (Figure 6D). Many of the scattered chromosomes were single sister chromatids, as judged by staining of the centromere protein centromere identifier (CID), and Cyclin B levels were similarly high in cells with scattered chromatids as in control prometaphase cells. Because SAC defects would lead to precocious APC/C^{Cdc20} activation and Cyclin B degradation, these results indicate that Dalmatian depletion does not inactivate the SAC. Instead, our results suggest that Dalmatian is a distant ortholog of Sororin that is required for cohesion.

DISCUSSION

Although establishment and maintenance of sister chromatid cohesion are essential for chromosome segregation, it is poorly understood how cohesin generates cohesive structures during DNA replication and how these are maintained for hours, or in the case of mammalian oocytes even for years. Recent studies have revealed that both the stability of cohesin-DNA interactions (Gerlich et al., 2006) and the acetylation state of cohesin change during DNA replication (Ben-Shahar et al., 2008; Rowland et al., 2009; Unal et al., 2008; Zhang et al., 2008), suggesting that cohesion is not simply established by doubling the number of sister chromatids inside otherwise unchanged cohesin rings. Our results further extend this view by showing that also the composition of cohesin complexes changes during DNA replication through the recruitment of Sororin, and importantly our data suggest that only Sororin-associated cohesin complexes are able to mediate cohesion. Consistent with this view, we find that Sororin is the only known protein whose presence on chromatin coincides precisely with the periods of the cell cycle during which cohesion exists (from initiation of DNA replication to metaphase), whereas cohesin binds to DNA long before cohesion is established.

Based on our results, we propose the following model for how Sororin enables cohesin to become ''cohesive'' (Figure 6C): Smc3 acetylation and possibly other unidentified events during DNA replication promote the recruitment of Sororin to chromatin-bound cohesin. These events might occur directly at replication forks because Eco1 has been detected at these sites (Lengronne et al., 2006), Smc3 can only be acetylated on chromatin (Unal et al., 2008; this study), and actinomycin D, a compound that inhibits DNA polymerase and MCM helicase progression (Pacek and Walter, 2004), prevents both Smc3 acetylation and Sororin recruitment. Because Smc3 acetylation and Sororin recruitment are blocked less efficiently by aphidicolin and thymidine, in whose presence helicase progression can still occur, it is possible that Smc3 acetylation and Sororin binding are coupled to helicase progression. Within the cohesin complex, Sororin binds to Pds5 via an FGF sequence motif that is shared between Sororin and Wapl. Sororin displaces Wapl from Pds5, but not from cohesin, suggesting that Sororin induces a rearrangement in the topology of these cohesin-associated proteins. We propose that these changes inhibit Wapl's ability to dissociate cohesin from DNA, and that the resulting stable interaction of cohesin with DNA enables cohesin to mediate cohesion. Our data further indicate that in prophase, Sororin is inactivated by phosphorylation, enabling Wapl to dissociate cohesin from mitotic chromosomes. Later in telophase and G1, APC/C^{Cdh1} targets Sororin for degradation. The function of this process remains to be understood, but it might ensure that Sororin associates with cohesin only after the initiation of DNA replication once APC/C^{Cdh1} has been inactivated.

This model makes a number of important predictions: (1) If Sororin is an antagonist of Wapl, one would expect that Sororin orthologs can be identified in species where Wapl exists. We show that this is indeed the case for many metazoans, including species from evolutionarily old taxa such as cnidaria (jellyfish) and placozoa, the simplest known metazoa. Our observation that depletion of the *Drosophila* member of this protein family (Dalmatian) causes cohesion defects suggests that these proteins are also functionally related to Sororin. We have so far not been able to identify Sororin-related proteins in worms or yeast. It therefore remains to be seen whether Sororin is required for cohesion in all eukaryotes, or whether some species have evolved cohesion mechanisms that are independent of Sororin.

(2) If the key function of Sororin is to inhibit Wapl, then Sororin is expected to be dispensable in the absence of Wapl. Our results indicate that this is indeed the case. An interesting implication of this result is that Sororin might not be essential for the initial entrapment of sister chromatids by cohesin rings, i.e., for cohesion establishment, at least in the absence of Wapl. It is therefore possible that Sororin's main function is to prevent dissociation of cohesin from DNA, rather than enabling opening and closure of the ring around DNA. However, the situation could be different in yeast where deletion of *WAPL/RAD61* does not result in accumulation of cohesin on DNA but has the opposite effect, a reduction of cohesin on chromatin (Rowland et al., 2009; Sutani et al., 2009). If a Sororin-related Wapl/Rad61 antagonist exists in yeast, such a protein (or protein domain) might therefore instead be needed for cohesion establishment by having to overcome the proposed ''anti-establishment'' activity of Wapl/Rad61 (Rowland et al., 2009; Sutani et al., 2009).

(3) If the stable postreplicative association of cohesin with DNA was due to inhibition of Wapl by Sororin, depletion of Wapl should enable cohesin to bind to DNA also stably before Sororin has been recruited to cohesin, i.e., in G1 phase. At variance with this prediction, we observed previously that depletion of Wapl from HeLa cells increased the residence time of dynamically bound cohesin complexes only modestly, from 8 min in control cells to 18 min (Kueng et al., 2006), and not to many hours, as is normally seen for cohesin complexes in G2 phase (Gerlich et al., 2006). However, we have in the meantime

measured the residence time of cohesin on chromatin in mouse embryonic fibroblasts from which the *Wapl* gene has been deleted, and in which therefore a more complete depletion of Wapl can be achieved than by RNAi. In these cells the residence time of cohesin on chromatin is increased from minutes to several hours even before S phase (A. Tedeschi, personal communication), indicating that it is indeed the presence of Wapl that enables cohesin to interact with DNA dynamically before replication. This result supports the hypothesis that inhibition of Wapl by Sororin enables stable binding of cohesin to DNA in postreplicative cells.

Our model also raises several important new questions. One of them is whether the essential function of Smc3 acetylation is to recruit Sororin, or whether this modification has other important effects, for example on the ATPase activity of Smc3. The absence of Sororin in yeast would suggest that cohesin acetylation must have other essential functions, but given the low sequence similarity among Sororin orthologs it cannot be excluded that Sororin-related proteins also exist in yeast. A related important question is how Smc3 acetylation might promote recruitment of Sororin. As Pds5 proteins are required for the recruitment of Sororin to cohesin, and Sororin binds to Pds5 proteins, we suspect that Smc3 acetylation promotes Sororin binding indirectly, possibly by causing changes in how Pds5 or Wapl interact with cohesin or each other. Likewise, it is unclear why replacement of K105/106 to not only glutamine (which is believed to mimic acetylated lysine) but also to arginine or alanine residues can stabilize cohesin-Sororin interactions. It is possible that it is not the presence of acetyl residues on K105/106 that creates a binding site, for example for a cohesin subunit, but that any mutation that removes lysines at these positions will destroy a binding site or pocket, which would lead to subunit rearrangements that would facilitate Sororin recruitment. A more detailed characterization of how cohesin interacts with Wapl, Pds5, and Sororin will be required to address these questions.

EXPERIMENTAL PROCEDURES

Immunodepletion and Monitoring of DNA Replication in *Xenopus* Egg Extracts

For immunodepletion of *Xenopus* egg extracts, affinity-purified antibody (70 μg anti-Sororin, mixture of 40 μg anti-Pds5A and 25 μg anti-Pds5B, 200 μg anti-Wapl, or 250 μg anti-SA1/2) was conjugated to 30 μl Affi-Prep Protein A Matrix (Bio-Rad), mixed with 100 μl interphase extracts, incubated for 30 min for Sororin depletion or 1 hr for Pds5A/B, Wapl, and SA1/2 depletions on ice, and beads were removed by centrifugation. For add-back experiments, Sororin wild-type or AA mutant (F166A, F168A) was added to Sororin-depleted extracts at 6.5 nM.

DNA replication was monitored by the incorporation of [α-^{32}P]dCTP into DNA. Demembranated sperm nuclei (2000 nuclei/μl) were added to egg extract containing [α-^{32}P]dCTP (3.7 kBq/μl), incubated at 22°C, and the reaction stopped by addition of 2 volumes of stop solution (8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 5% SDS, 0.2% bromophenol blue, 80 mM Tris-HCl pH 8.0). The mixture was incubated with 2 mg/ml Proteinase K for 30 min at 37°C and analyzed by agarose gel electrophoresis followed by autoradiography.

Preparation of *Xenopus* Chromatin Fractions

Sperm nuclei were incubated in extracts at a concentration of 2000 nuclei/μl. Thirty microliters of extract was diluted 10-fold with ice-cold extract buffer (EB; 5 mM $MgCl_2$, 100 mM KCl, HEPES-KOH pH 7.5) containing 0.25% Triton X-100, overlaid onto a 30% sucrose/EB cushion, and spun at 15,000 *g* for 10 min. The pellets were washed with EB containing 0.25% Triton X-100 and resuspended in SDS sample buffer.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.cell.2010.10.031.

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