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Wapl Controls the Dynamic Association of Cohesin with Chromatin

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SUMMARY

Cohesin establishes sister-chromatid cohesion from S phase until mitosis or meiosis. To allow chromosome segregation, cohesion has to be dissolved. In vertebrate cells, this process is mediated in part by the protease separase, which destroys a small amount of cohesin, but most cohesin is removed from chromosomes without proteolysis. How this is achieved is poorly understood. Here, we show that the interaction between cohesin and chromatin is controlled by Wapl, a protein implicated in heterochromatin formation and tumorigenesis. Wapl is associated with cohesin throughout the cell cycle, and its depletion blocks cohesin dissociation from chromosomes during the early stages of mitosis and prevents the resolution of sister chromatids until anaphase, which occurs after a delay. Wapl depletion also increases the residence time of cohesin on chromatin in interphase. Our data indicate that Wapl is required to unlock cohesin from a particular state in which it is stably bound to chromatin.

INTRODUCTION

Sister-chromatid cohesion is required for chromosome biorientation on the mitotic and meiotic spindle and for DNA-damage repair during G2 phase (Lee and Orr-Weaver, 2001). Cohesion is mediated by cohesin, a ring-shaped protein complex composed of the ATPases Smc1 and Smc3 and the kleisin Scc1/Mdc1/Rad21 (Guacci et al., 1997; Michaelis et al., 1997; Losada et al., 1998; Haering et al., 2002). Scc1 is bound to a fourth subunit, called Scc3 in yeast, that exists in different isoforms in vertebrate somatic cells, called SA1 and SA2 (Losada et al., 2000; Sumara et al., 2000). Cohesion also depends on Pds5/BimD/Spo76 (Hartman et al., 2000; Panizza et al., 2000; Tanaka et al., 2001; Losada et al., 2005). Vertebrate cells contain also two isoforms of this protein, Pds5A and Pds5B, both of which physically interact with cohesin (Sumara et al., 2000; Losada et al., 2005).

How cohesin binds to chromatin and how it connects replicated DNA molecules remains unknown, but it has been proposed that cohesin links sister chromatids by embracing them as a ring (Haering et al., 2002). According to this hypothesis, the cohesin ring would have to be opened to either generate or dissolve the interaction between cohesin and DNA. Alternatively, it is possible that cohesin interacts with DNA directly (Akhmedov et al., 1998; Hirano and Hirano, 2006; Huang et al., 2005). Fluorescence recovery after photobleaching (FRAP) experiments revealed that the majority of cohesin associates with chromatin reversibly throughout interphase. However, in G2 cells, a second population of cohesin binds to chromatin very stably. This population is only found when DNA has been replicated and may thus represent cohesin molecules that have established cohesion (Gerlich et al., 2006).

In budding yeast, most cohesin is destroyed at anaphase onset by Scc1 cleavage, which is mediated by the protease separase (Uhlmann et al., 2000). In contrast, vertebrate cells remove the majority of cohesin from chromosome arms during prophase without Scc1 cleavage (Losada et al., 1998; Sumara et al., 2000; Waizenegger et al., 2000). The activity of this "prophase pathway" is reduced if Plk1, Aurora B, or condensin I is inactivated, or if a nonphosphorylatable SA2 mutant (SA2-12xA) is expressed. Under these conditions sister-chromatid arms cannot be resolved (Losada et al., 2002; Sumara et al., 2002; Gimenez-Abian et al., 2004; Hirota et al., 2004; Hauf et al., 2005). At centromeres, a small amount of cohesin is protected from the prophase pathway by Sgo1 (Kitajima et al., 2004; Salic et al., 2004; Tang et al., 2004; McGuinness et al., 2005), and these cohesin complexes can only be removed from chromosomes by separase-mediated Scc1 cleavage (Hauf et al., 2001).

We have identified an ortholog of the *Drosophila* protein Wapl (wings-apart like) as a protein that is specifically associated with cohesin and that controls the dynamic association of cohesin with chromatin. Wapl is highly conserved among metazoan species, essential for viability in *Drosophila* and mice, and has been implicated in heterochromatin formation, chromosome segregation, and tumorigenesis (Verni et al., 2000; Dobie et al., 2001; Kwiatkowski et al., 2004; Oikawa et al., 2004), but Wapl's molecular functions were unknown.

We show that human Wapl interacts with cohesin throughout the cell cycle via cohesin's Scc1 and SA1/SA2 subunits and that Wapl forms a subcomplex with Pds5A. Like cohesin, Wapl is associated with chromatin from telophase until prophase of the next mitosis. Depletion of Wapl by RNA interference (RNAi) inhibits the dissociation of cohesin from chromosomes during prophase, prometaphase, and metaphase; although Plk1 and Aurora B are active, condensin I associates with mitotic chromosomes and SA2 is phosphorylated. This defect in cohesin dissociation does also not depend on the presence of Sgo1. The resolution of chromosome arms is severely impaired in Wapl-depleted cells until anaphase, which occurs eventually after a delay, implying that separase can still be activated in the absence of Wapl. The depletion of Wapl also results in increased levels of cohesin on chromatin in interphase, and FRAP experiments show that cohesin remains bound to chromatin longer in the absence of Wapl. These results indicate that Wapl is required for the release of cohesin from both interphase chromatin and mitotic chromosomes, perhaps by facilitating opening of the cohesin ring or by modulating direct interactions between cohesin and DNA. Wapl is thus a regulator of cohesin's association with chromatin, whose function is not restricted to but particularly important during the early stages of mitosis.

RESULTS

Human Cohesin Is Associated with the Wapl Protein

To identify cohesin-associated proteins we immunoprecipitated cohesin from lysates of logarithmically proliferating HeLa cells with antibodies to Smc3, SA1, or SA2 and analyzed the bound proteins by insolution digest and tandem mass spectrometry (MS). Besides Smc1, Smc3, Scc1, SA1/SA2, Pds5A, and Pds5B there was one additional protein reproducibly identified in all samples (Figure 1A). This protein is called Wapl (Oikawa et al., 2004) due to its orthology with *Drosophila* Wapl (Figure 1B) and is also known as FOE (Kwiatkowski et al., 2004) and KIAA0261 (Nagase et al., 1996). Wapl was also detected in Pds5A and Pds5B immunoprecipitates (IPs; Figure 1A) but could not be found in IPs of other proteins such as anaphase promoting complex/cyclosome (APC/C) or condensin (data not shown). Wapl further copurified with cohesin isolated with myc antibodies from HeLa cells that stably express Scc1-myc, but no Wapl was detected in myc IPs from regular HeLa cells (data not shown).

In immunoblot experiments, two Wapl peptide antibodies (986 and 987) were able to react with in vitro translated Wapl, and in HeLa extracts the antibodies recognized a 180 kDa band that could be depleted by transfection of HeLa cells with Wapl siRNAs (Figure 1C). When Wapl IPs were analyzed by MS, all cohesin subunits, Pds5A, and Pds5B could be detected (Figure 1A). Immunoblot experiments confirmed that cohesin subunits were present in Wapl IPs and that Wapl was present in cohesin but not in condensin samples (Figure 1E). When cohesin and Wapl IPs were compared by SDS-PAGE and silver staining, strikingly similar protein patterns were observed. Both samples contained bands that correspond to cohesin subunits, Pds5A, Pds5B, and Wapl (Figure 1D).

The association between cohesin and Wapl could be detected in interphase and mitotic HeLa cells, and Wapl was identified by immunoblotting in cohesin samples isolated from mouse embryonic fibroblasts (data not shown). We conclude that Wapl is specifically associated with cohesin throughout the cell cycle in mammalian cells.

Wapl Is an Evolutionary Conserved Helical Repeat Protein that Is Distantly Related to Budding Yeast Rad61

The sequence of Wapl is highly conserved among metazoan species, in particular in a C-terminal region (amino acid residues 1141–1667 of *Drosophila* Wapl; Figures 1B and S2). Secondary structure predictions indicate that this "Wapl domain" is predominantly α-helical. It hits, although subsignificantly, to hidden Markov models of helical repeat domains like Armadillo and HEAT repeats (data not shown). In iterative NCBI-PSI-BLAST searches with the *Drosophila* Wapl domain we identified the hypothetical *Neurospora crassa* protein emb/CAD70983.1 (E-value 8e-07) and proteins of unknown function from *Schizosaccharmoyces pombe* and other fungi (E-values < 1e-4; Figure S2 and Supplemental Experimental Procedures). We used the Wapl domains of the *S. pombe* and *Yarrowia lipolytica* proteins to perform iterative PSI-BLAST searches in the proteomes of Saccharomycetales and identified proteins in *Eremothecium gossypii* (AAR187C), *Klyveromyces lactis* (KLA-CDS1440.1), and *Saccharomyces cerevisiae* (Rad61; E-values < 6e-05). These proteins are all members of one protein family, but their overall similarity to Wapl is low. Interestingly, Rad61 has been implicated in DNA repair, cohesion, and chromosome segregation (Game et al., 2003; Warren et al., 2004; Measday et al., 2005). It is therefore possible that Wapl and Rad61 are related.

The Association of Wapl with Cohesin Depends on Scc1 and SA1/SA2

To understand if Wapl is required for cohesin assembly and to address which cohesin subunit is needed for the Wapl interaction, we isolated Wapl and cohesin from HeLa cells that had been depleted of Smc3, Scc1, or Wapl by RNAi and analyzed the IPs by immunoblotting. All cohesin core subunits could be coprecipitated when Wapl had been depleted, indicating that Wapl is not essential for cohesin assembly or stability (Figure 1F and data not shown).

When Smc3 had been depleted, Scc1 could still be detected in Wapl IPs. However, when Scc1 had been depleted, Smc3 did not coprecipitate with Wapl, although Smc3 was still associated with Smc1 (Figure 1E). The interaction between Wapl and the Smc1/Smc3 heterodimer therefore depends on Scc1. The depletion of SA1 and/or SA2 also reduced the association of Wapl with Smc1

A

	SA1 IP		SA2 IP		Smc3 IP		Pds5A IP		Pds5B IP		Wapl IP	
	score	cov. (%)	score	cov. (%)	score	cov. (%)	score	cov. (%)	score	cov. (%)	score	cov. (%)
SA1	929	23	-	-	758	14	147	2	182	2	-	-
SA2	-	-	1355	27	1112	22	663	15	680	17	393	10
Scc1	493	28	1197	28	1068	43	721	31	584	26	380	18
Smc1	803	15	2217	36	2088	33	1101	21	1036	18	1165	20
Smc3	1122	22	1864	30	2425	37	1147	22	1217	20	1109	21
Pds5A	83	1	551	12	642	14	2565	40	-	-	1240	24
Pds5B	664	13	664	13	51	1	-	-	2536	35	197	4
Wapl	165	3	832	16	216	4	2067	41	854	19	2923	53

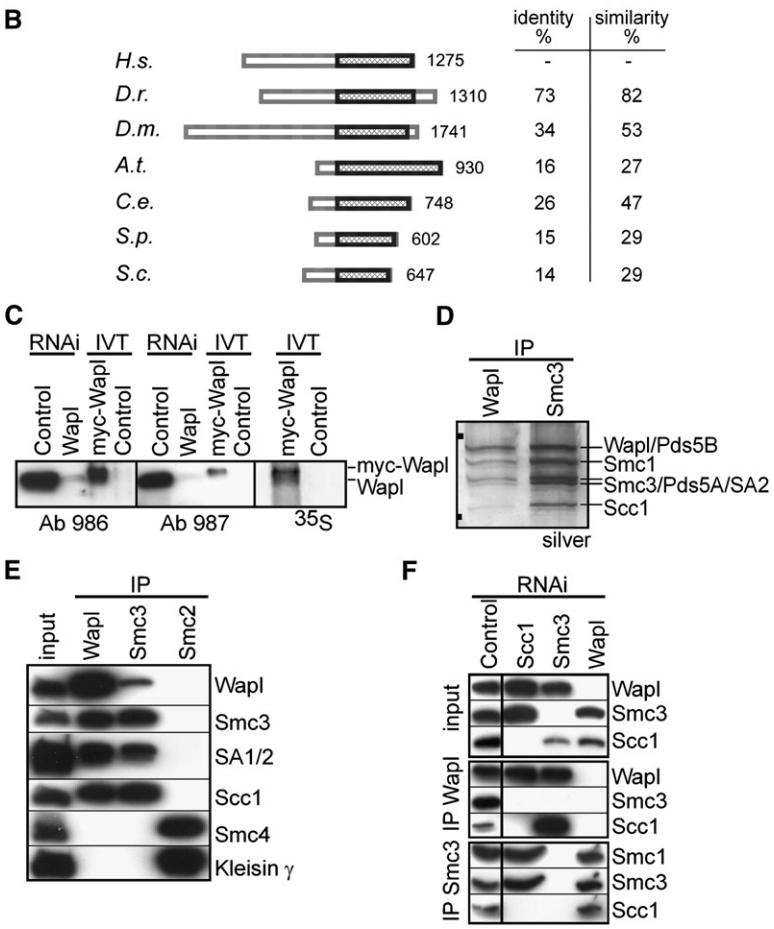

Figure 1. Wapl Is Associated with the Cohesin Complex

(A) Cohesin and Wapl were immunoprecipitated from HeLa extracts, and proteins analyzed by insolution digest and MS. Score, Mascot score; cov., sequence coverage.

(B) Domain structure of Wapl orthologs. The conserved Wapl domain is shown as boxes. Sequence identities and similarities to the Wapl domain of the human protein are shown. *A.t.*, *A. thaliana*; *C.e.*, *C. elegans*; *D.m.*, *D. melanogaster*; *D.r. D. rerio*; *H.s., H. sapiens*; *S.c., S. cerevisiae*; *S.p.*, *S. pombe*.

(C) Characterization of Wapl (986, 987) antibodies in immunoblots, using extracts from HeLa cells transfected with control or Wapl siRNAs. Myc-Wapl was in vitro translated (IVT) and detected by phosphorimaging (S^{35}).

(D) IPs obtained with Wapl (987) or cohesin (Smc3) antibodies were analyzed by silver staining. Black squares indicate 200 kDa and 116 kDa marker proteins.

(E) IPs obtained with Wapl, cohesin, and condensin (Smc2) antibodies were analyzed by immunoblotting using the indicated antibodies.

(F) HeLa cells were transfected with control, Scc1, Smc3, or Wapl siRNAs, and 48 hr post-transfection proteins were analyzed by immunoprecipitation and immunoblotting as indicated.

(Figure S1A). Wapl may thus interact with the part of cohesin that contains Scc1 and SA1/SA2.

Wapl Forms a Subcomplex with Pds5A

When we separated HeLa extract by sucrose density gradient centrifugation and analyzed the fractions by immunoblotting, we observed that the majority of Wapl was detected in 8S fractions, and only a small amount was detected after long exposures in 14S fractions where cohesin sediments (Figure 2A and data not shown). The interaction between Wapl and cohesin may therefore not be stable enough to persist during the 18 hr centrifugation step. Pds5A also sediments corresponding to 8S (Sumara et al., 2000; Figure 2A), and we therefore addressed if Wapl interacts with Pds5A. Consistent with this possibility we detected more Pds5A in Wapl IPs than in cohesin IPs by MS and immunoblotting (Figures 1A and 2B). When we peptide eluted proteins bound to Wapl antibodies and separated them in density gradients, we also found that Wapl and Pds5A cosedimented in 8S fractions (Figure 2C). Finally, we observed that Pds5A could be immunoprecipitated with Wapl antibodies from 8S fractions, whereas small amounts of cohesin subunits were precipitated with Wapl antibodies from 14S fractions (Figure 2D). These

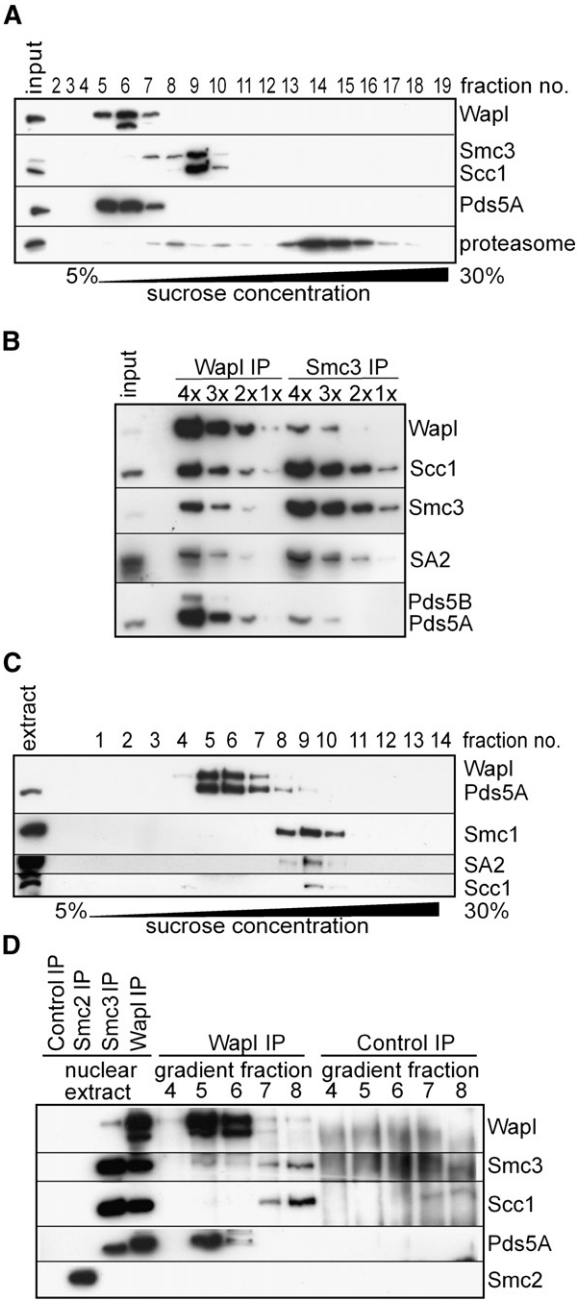

Figure 2. Wapl and Pds5A Form a Subcomplex

(A) HeLa cell extracts were separated by sucrose density gradient centrifugation, and fractions were analyzed by immunoblotting with the indicated antibodies.

(B) Serial dilutions of Wapl, and Smc3 IPs were compared by immunoblotting.

(C) Proteins in Wapl IPs were eluted by antigenic peptide and separated on a sucrose gradient. Fractions were tricholoro acetic acid (TCA) precipitated and analyzed by immunoblotting.

(D) HeLa nuclear extracts were separated in a sucrose gradient and fractions 4–8 were used for immunoprecipitation, with Wapl and control antibodies and analyzed by immunoblotting.

results indicate that Wapl and Pds5A form a subcomplex whose interaction with cohesin is less stable than the interactions among the core cohesin subunits. However, the formation of this subcomplex appears to be dependent on the core cohesin subunits because depletion of Scc1 by RNAi greatly reduced the ability of Wapl antibodies to immunoprecipitate Pds5A (Figure S1B). Although we could not reliably detect Pds5B in all experiments, our data indicate that Wapl also interacts with Pds5B (Figure 2A).

Wapl Is a Chromatin-Associated Protein that Dissociates from Chromosomes from Prophase until Telophase

To analyze where Wapl is located we stably expressed N-terminally myc-tagged Wapl under control of the regulatable "Tet on" promoter in HeLa cells. We compared myc-Wapl and cohesin localization in these cells by staining them with myc and Scc1 antibodies, respectively. Both proteins were mainly nuclear in interphase, became cytoplasmic from nuclear envelope breakdown (NEBD) until anaphase, and reaccumulated in nuclei in telophase (Figure 3A). Similar staining patterns were observed when soluble proteins were removed by preextraction (Figure 3B), indicating that a fraction of Wapl molecules is chromatin bound in interphase, as is cohesin. No Wapl could be detected on chromosomes from prometaphase until anaphase. Like cohesin, Wapl thus dissociates from mitotic chromosomes.

We confirmed these results in fractionation experiments. HeLa cells were synchronized in S phase by release from double thymidine treatment, enriched in mitosis by subsequent addition of nocodazole, and then released again (Figure 3C). At different time points lysates were generated, separated into soluble cytoplasmic and insoluble chromatin fractions, and analyzed by immunoblotting. In S and G2 cells, Wapl could be detected both in chromatin and supernatant fractions (Figure 3C and data not shown), but in mitotic cells only small amounts of Wapl were detected in chromosome pellets (Figure 3C).

To test if binding of Wapl to chromosomes depends on cohesin, Scc1-depleted HeLa cells were arrested in S phase because these cells would otherwise arrest in mitosis, where Wapl is not chromatin bound. Cell lysates were then analyzed by fractionation and immunoblotting as above. In the absence of Scc1, Wapl could not be detected in chromatin fractions (Figure 3D), indicating that the association of Wapl with chromatin depends on cohesin.

Wapl Is Required for Resolution of Sister-Chromatid Arms

To address if Wapl regulates cohesin or sister-chromatid cohesion we transfected HeLa cells with either one of two different Wapl siRNAs, which caused depletion of Wapl in both cytoplasmic and chromatin fractions beyond immunoblot detection levels (Figure 4A and Figure S1C). When these cells were preextracted and analyzed by immunofluorescence microscopy (IFM), cohesin was nevertheless detected in interphase nuclei, implying that

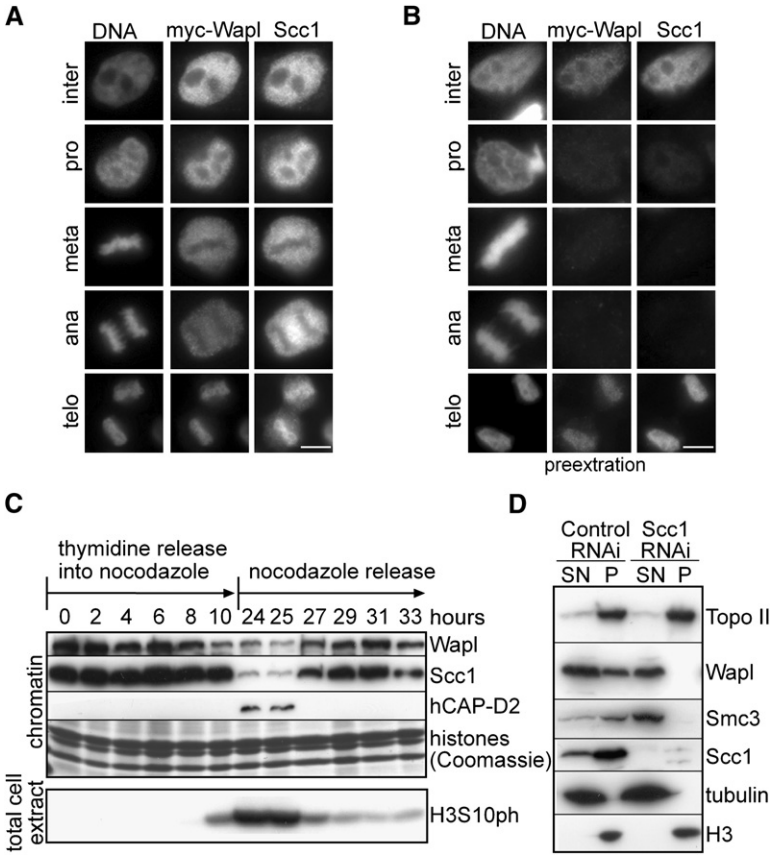

Figure 3. Wapl Dissociates from Chromosomes in Mitosis

(A) Myc-Wapl expression was induced for 48 hr with 2 μg/ml doxycycline, and cells were fixed and analyzed by IFM using myc and Scc1 antibodies. Size bar 10 μm.

(B) IFM as in (A), but cells were extracted with 0.1% Triton X-100 prior fixation.

(C) Synchronized HeLa cells were harvested at the indicated time points, and chromatin-associated proteins and total extracts were analyzed by immunoblotting. Histones were stained with Coomassie.

(D) HeLa cells were transfected with Scc1 siRNAs and, 12 hr later, treated with thymidine for 24 hr. The lysates were prepared and separated into supernatant (SN) and pellet (P) fractions and analyzed by immunoblotting.

cohesin binding to chromatin does not depend on Wapl (Figure 5C and data not shown). FACS analyses of cells synchronized by double thymidine treatment indicated that DNA replication was likewise normal after Wapl depletion (data not shown).

To address whether Wapl depletion causes cohesion defects we enriched cells in mitosis by a 30 min nocodazole treatment, collected them by shake off, and analyzed their chromosomes by spreading and Giemsa staining. Under these conditions, chromosomes from control cells showed the typical x-shape with clearly resolved sister-chromatid arms and a tight connection of sister chromatids at the centromere (Figure 4B). However, chromosomes from Wapl-depleted cells showed a strikingly different morphology. In 88% of cells transfected with Wapl siRNAs, individual sister chromatids were hardly visible within spread chromosomes (Figure 4B). These chromosomes were nevertheless composed of two sister chromatids because the chromosome width was larger than that of single chromatids and two closely opposed chromatids could be recognized in higher magnification images of some chromosomes (Figure 4C). The aberrant morphology of chromosomes from Wapl-depleted cells was thus not due to replication defects or to precocious loss of cohesion but to defects in sister-chromatid resolution. The arms of control chromosomes were on average 0.9 ± 0.3 μm apart when cells were analyzed after 30 min of nocodazole treatment, whereas chromosomes from Wapl-depleted cells had an average arm-to-arm distance of only 0.4 ± 0.2 μm (Figure 4D). Staining of fixed cells with CREST sera revealed that also interkinetochore distances were slightly decreased by Wapl depletion (Figures S3A and S3B). When control and Wapl-depleted cells were treated for 3 hr with nocodazole, 50% of all Wapl-depleted cells still contained chromosomes whose sister chromatids had not been resolved, whereas such chromosomes were only seen in 11% of control cells (Figure 4C). Wapl depletion thus causes severe defects in sister-chromatid resolution, even when mitosis is artificially prolonged.

The opposite effect was seen when myc-Wapl was overexpressed. When uninduced myc-Wapl cells were analyzed by chromosome spreading, about 5% of mitotic cells contained separated sister chromatids. After induction of myc-Wapl expression with doxycycline, however, 25% of mitotic spreads showed separated sister chromatids (Figure S3C). Wapl overexpression therefore causes cohesion defects.

Wapl Is Required for Normal Progression through Mitosis

To understand if the abnormal chromosome morphology in Wapl-depleted cells causes defects in mitotic progression, we synchronized control and Wapl-depleted cells by double thymidine treatment and analyzed them by IFM. After

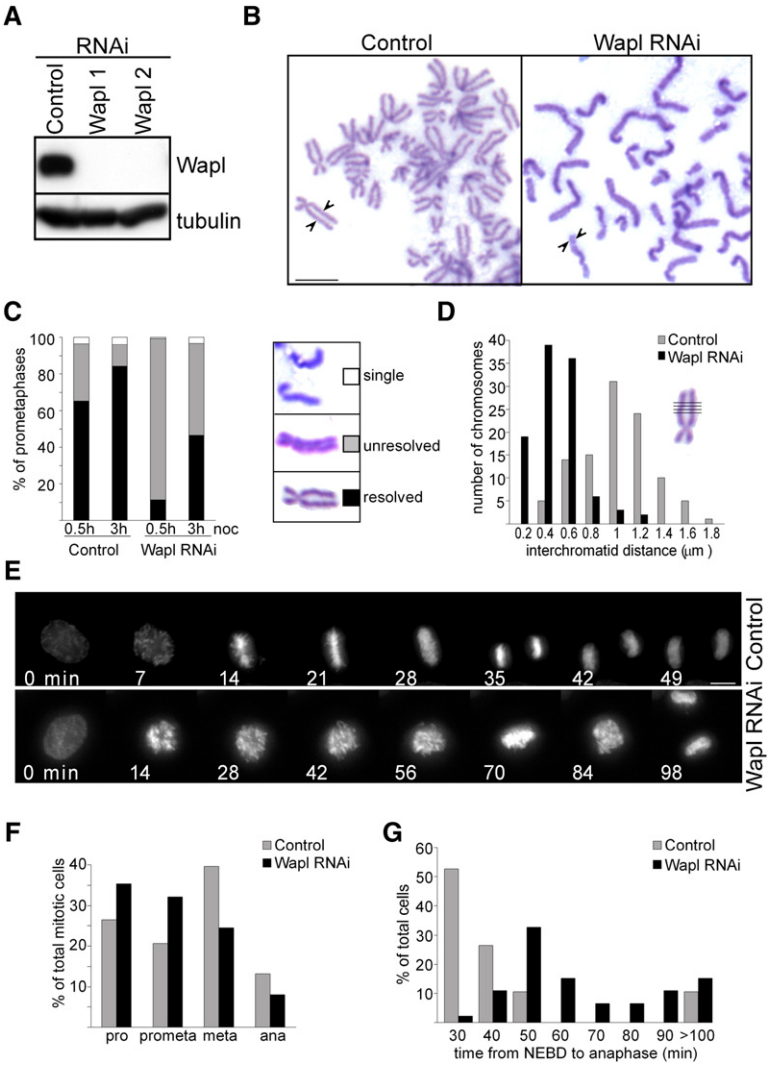

Figure 4. Wapl Is Required for Sister-Chromatid Resolution and for Mitotic Progression

(A) HeLa cells were transfected using control or two different Wapl siRNAs (Wapl1 and Wapl2), and, 2 days later, cell extracts were analyzed by immunoblotting.

(B) HeLa cells were transfected using control or Wapl siRNAs. Forty-eight hours later, cells were treated with nocodazole (noc) for either 30 min or 3 hr, and mitotic cells were collected by shake off and analyzed by hypotonic spreading and Giemsa staining. Size bar, 5 μm.

(C) Prometaphases obtained as in (B) were classified according to their chromosome morphology (n = 200).

(D) Distances between sister chromatids were measured in five chromosomes in >20 cells.

(E) HeLa cells stably expressing H2B-GFP were control transfected or transfected using Wapl siRNAs, and, 48 hr later, cells were filmed for 10 hr. Size bar, 10 μm.

(F) HeLa cells were control transfected or transfected using Wapl siRNAs, synchronized by double thymidine treatment, and fixed at different time points after the second release with formaldehyde, and cell-cycle stages were analyzed by Hoechst 33342 and H3S10ph staining (n > 250, values totaled over all time points).

(G) Cells were filmed as in (E) and time from NEBD to anaphase onset was quantified (n = 19, control; n = 44, Wapl RNAi).

Wapl depletion, more cells were found in prophase and prometaphase, whereas metaphases and anaphases were reduced (Figure 4F). In prometaphase cells, Mad2 was enriched on many kinetochores and in 88% of these cells, cyclin B had not been degraded (control cells 90%), indicating that the spindle-assembly checkpoint had been activated (Figures S3D and S3E). We also filmed Wapl-depleted and control cells that stably expressed GFP-tagged histone H2B by time-lapse microscopy and measured the time from NEBD to anaphase onset. Control cells needed on average 43 min for this period, whereas Wapl-depleted cells needed 67 min (Figures 4E and 4G). However, eventually, most Wapl-depleted cells entered anaphase and separated sister chromatids. Wapl depletion therefore delays progression through the early stages of mitosis.

Wapl Is Required for Dissociation of Cohesin from Chromosomes in Prophase

Because cohesin dissociation is required for resolution of sister chromatids, we analyzed if the resolution defect in Wapl-depleted cells may be caused by defects in cohesin dissociation. First, we depleted Wapl in HeLa cells that stably express SA2-myc, enriched cells in prometaphase by a 30 min nocodazole treatment, harvested them by shake off, and analyzed their chromosomes by spreading and IFM with myc and condensin antibodies. After control treatment, myc staining was enriched at centromeres in 90% of all prometaphase cells that expressed SA2-myc (n = 100), and only 5% of these showed in addition staining on chromosome arms. However, after Wapl depletion, 64% of all myc-positive prometaphase cells (n = 100) contained chromosomes with equally intense centromere and arm staining. The staining intensity of these chromosomes was higher than that of control chromosomes (Figure 5A and data not shown). Wapl depletion thus causes a defect in cohesin dissociation from chromosome arms.

We tested next if endogenous cohesin can also be detected on chromosomes after Wapl depletion. It is important to note that we have previously not been able to detect endogenous cohesin on prometaphase or

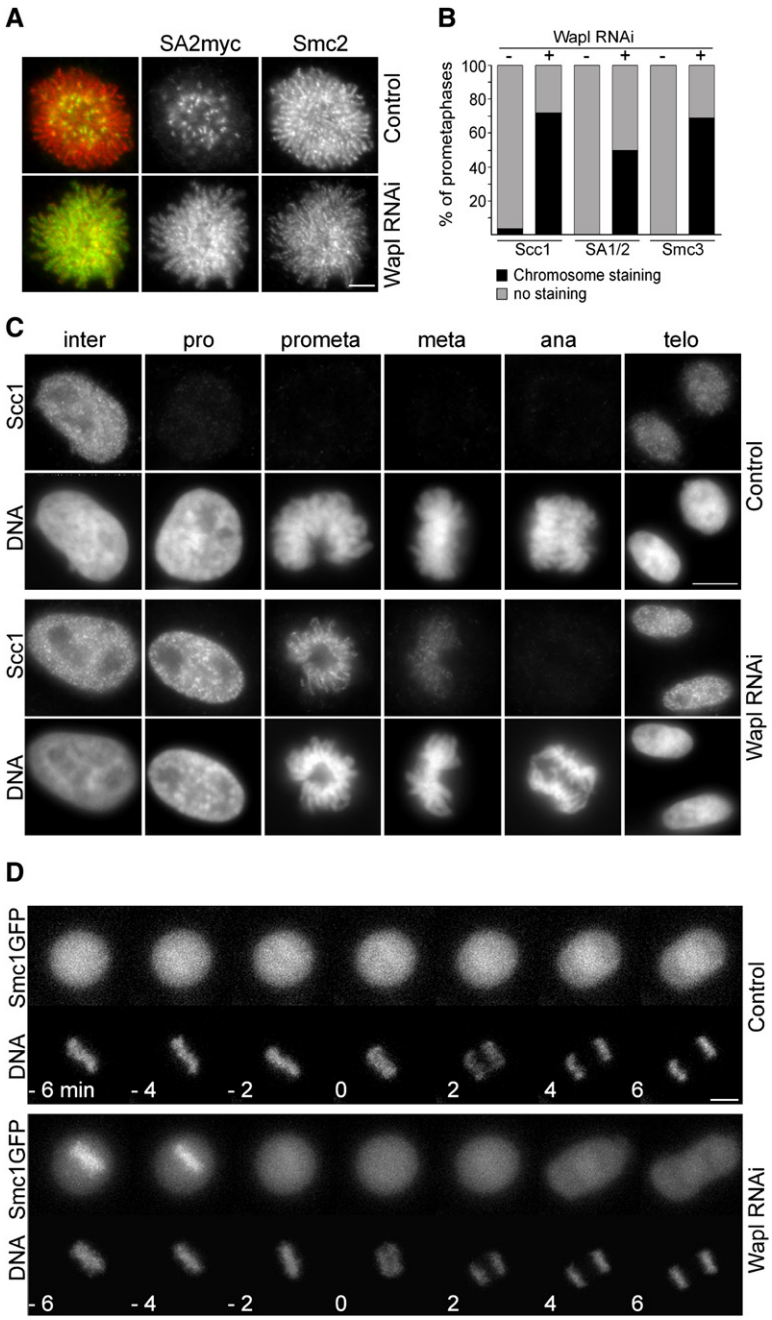

Figure 5. Wapl Is Required for Dissociation of Cohesin from Chromosomes in Early Mitosis

(A) HeLa SA2myc cells were transfected using control or Wapl siRNAs, and SA2 expression was induced with 1 μg/ml doxycycline. After 2 days, cells were treated for 30 min with nocodazole, harvested by mitotic shake off, spun onto glass slides, and analyzed by IFM.

(B and C) Cells were transfected with control or Wapl siRNAs and, 48 hr later, cells were preextracted, fixed with paraformaldehyde, and stained with Scc1, Smc3, or SA1/2 antibodies. Two hundred prometaphases and metaphases were analyzed.

(D) HeLa cells expressing Smc1-EGFP were control transfected or transfected using Wapl siRNAs and analyzed by live cell imaging 48 hr later. DNA was stained with 0.1 μg/ml Hoechst 33342. Every 2 min, five stacks were taken and projected using maximum intensities. Anaphase onset was set as the 0 time point. Size bars, 10 μm.

metaphase HeLa chromosomes by IFM. Even when components of the prophase pathway were inactivated, cohesin could only be detected on mitotic chromosomes by expression of tagged cohesin subunits (Sumara et al., 2002; Gimenez-Abian et al., 2004; Hirota et al., 2004; Figure S4A). This is at least in part due to the fact that many cohesin complexes can still dissociate from chromosomes when the prophase pathway is inactivated (Hauf et al., 2005). It was thus surprising for us to see that antibodies to Scc1, Smc3, and SA1/SA2 could all stain prophase and prometaphase chromosomes in Wapl-depleted cells, in many cases as intensely as interphase chromatin (Figure 5C, S4B, and S4C). Depending on the cohesin antibodies used, between 50% and 72% of all prometaphase cells were clearly stained when Wapl was depleted, whereas few if any stained prometaphase cells could be detected in control samples (4% with Scc1 antibodies and none for SA1/SA2 and Smc3 antibodies; Figure 5B). The cohesin staining that was seen in Wapl-depleted cells was located at centromeres and on chromosome arms, where it was mainly found between sister-chromatid axes (data not shown).

Importantly, cohesin staining was never seen on sister chromatids in anaphase, suggesting that Wapl is not required for the separase-mediated cleavage of cohesin at the metaphase-anaphase transition.

To confirm these results in living cells, we generated a cell line that stably expresses Smc1-EGFP (Figure S5). Life-cell imaging showed that this protein is located mainly in the nucleus in interphase, becomes cytoplasmic in mitosis, but cannot be detected on mitotic chromosomes (Figure 4D), like endogenous cohesin. However, when Wapl was depleted, Smc1-EGFP showed a strikingly different behavior. In this case the EGFP signal remained associated with chromosomes until metaphase and disappeared reproducibly 2–3 min before sister chromatids began to separate (Figure 4D).

Wapl Is Not Required for Activation of Known Components of the Prophase Pathway

Because Wapl is required for removal of cohesin from chromosomes in early mitosis, we analyzed if Wapl is required for activation of known components of the prophase pathway. Plk1 is required for association of γ-tubulin with centrosomes, for generation of a centrosomal epitope that is recognized by phospho-Apc6 antibodies and for formation of bipolar spindles (Lane and Nigg, 1996; Kraft et al., 2003). In IFM experiments we could not detect defects in these processes in Wapl-depleted cells (Figure S6A and data not shown). Likewise, the levels of H3S10ph were not detectably reduced in Wapl-depleted cells, indicating that Aurora B activation was also not compromised (data not shown), and condensin I antibodies stained sister-chromatid axes of prometaphase cells with similar intensities in Wapl-depleted and control cells (Figure S6B). Wapl is therefore not required for activation of known components of the prophase pathway.

It remained possible, however, that Wapl is specifically required for SA2 phosphorylation. To test this possibility we raised antibodies to a mitotic phospho-site on SA2, serine 1224 (Hauf et al., 2005). In immunoblot experiments, these antibodies reacted specifically with SA2 in mitotic HeLa extracts (Figure 6A). However, these antibodies did neither recognize proteins in interphase extracts, nor nonphosphorylatable SA2-12xA, nor mitotic cohesin IPs that had been incubated with protein phosphatase (data not shown), indicating that the antibodies are specific for mitotically phosphorylated SA2. When mitotic control cells were separated into soluble and chromatin fractions, the pS1224-SA2 antibodies recognized SA2 only in the soluble fraction, whereas pS1224-SA2 could also be detected on chromatin from Wapl-depleted cells (Figure 6B).

Similar results were obtained in IFM experiments. The pS1224-SA2 antibodies stained nuclei in prophase and the cytoplasm from prometaphase to anaphase. The signal was reduced in cells in which SA2 had been depleted by RNAi (data not shown). Centrosomes were also labeled throughout mitosis, but these signals were not abolished by SA2 RNAi, indicating that they were caused by cross reactions with different proteins. However, when Wapl-depleted cells were analyzed, the pS1224-SA2 antibodies stained prophase and prometaphase chromosomes very clearly, and this signal colocalized with SA2 staining (Figure 6C). Wapl is therefore not required for SA2 phosphorylation on serine 1224.

To address whether chromatin bound SA2 was also phosphorylated on other sites in Wapl-depleted cells, we fractionated mitotic cells under conditions that resolve slower migrating forms of SA2 that are only generated when SA2 is phosphorylated on multiple sites (Hauf et al., 2005; Kitajima et al., 2006). Indeed, slower migrating forms of SA2 were observed on chromatin in Wapl-depleted mitotic cells (Figures 6D and S6C). Taken together, these results indicate that cohesin is not released from chromosomes in Wapl-depleted cells although SA2 is phosphorylated.

The Effect of Wapl Depletion on Cohesin Dissociation Is Not Mediated by Scc2/Scc4 or Sgo1

The cohesin loading complex Scc2/Scc4 dissociates from chromosomes in mitosis (Watrin et al., 2006). We tested if Wapl depletion interferes with this process because the persistence of Scc2/Scc4 on mitotic chromosomes could result in constant reloading of cohesin onto chromosomes, which could explain the abnormal distribution of cohesin in Wapl-depleted cells. However, IFM experiments showed that Wapl depletion did not change the location of either Scc2 or Scc4, i.e., both proteins were present on interphase chromatin but undetectable on mitotic chromosomes, although Scc1 remained on them after Wapl depletion (Figure S7A and data not shown). The persistence of cohesin on mitotic chromosomes can thus not be due to effects of Wapl depletion on Scc2/Scc4 localization.

It has been shown that redistribution of Sgo1 from centromeres to chromosome arms (induced by depletion of Bub1) coincides with an increase of cohesin on chromosome arms (Kitajima et al., 2005). Wapl depletion could therefore alter the behavior of cohesin by affecting the distribution of Sgo1 on chromosomes. When we analyzed Sgo1 distribution by IFM, we indeed found that Wapl depletion resulted in slightly increased amounts of Sgo1 on chromosome arms. However, when Wapl and Sgo1 were depleted simultaneously, the intensity of cohesin staining on chromosomes remained as high as in cells only lacking Wapl (Figures 6D and 6E). Sgo1 is therefore not required for the persistence of cohesin on chromosome arms in Wapl-depleted cells.

This notion was also supported by the following observations: we confirmed that Bub1 depletion increases the association of Sgo1 with chromosome arms, and we observed that depletion or inactivation of Aurora B has the same effect (Figure 6E). However, in these cells endogenous cohesin could not be detected on prometaphase chromosomes, despite the fact that the effects of Bub1 or Aurora B depletion on Sgo1 localization were stronger than the effect of Wapl depletion (Figure 6E). These

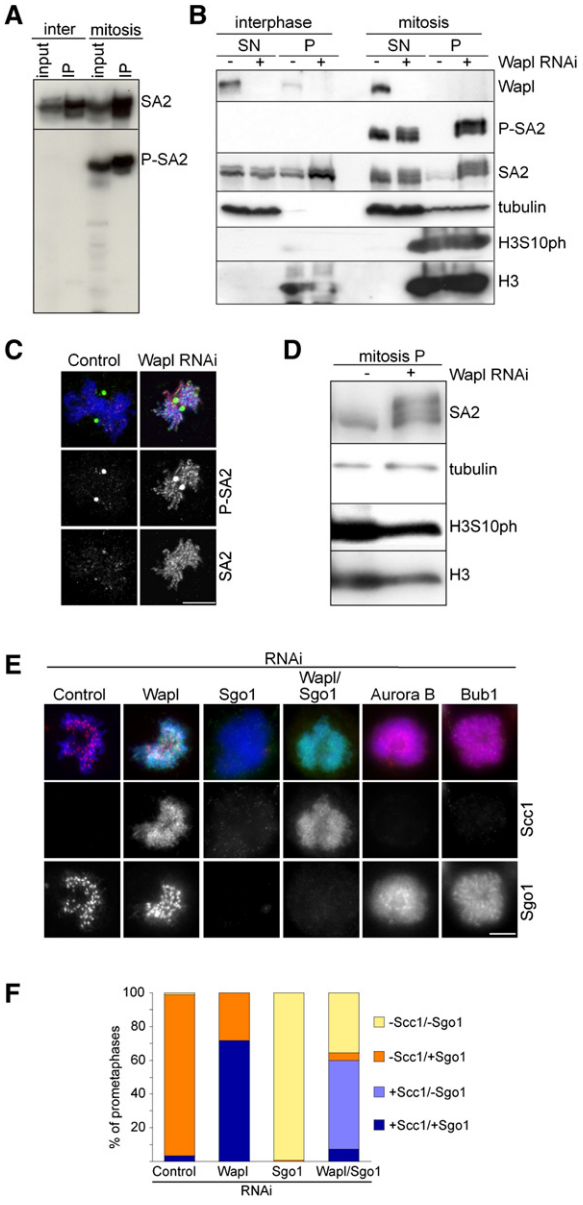

Figure 6. Wapl Is Dispensable for SA2 Phosphorylation, and the Cohesin Dissociation Defect in Wapl-Depleted Cells Does Not Depend on Sgo1

(A) Characterization of pS1224-SA2 antibodies. Cohesin was immunoprecipitated with SA2 antibodies from extracts of interphase and mitotic (nocodazole arrested) HeLa cells, and inputs and IPs were analyzed by immunoblotting with pS1224-SA2 and SA2 antibodies.

(B) HeLa cells transfected with control or Wapl siRNAs were synchronized by double thymidine treatment. When cells started to enter mitosis, nocodazole was added for 3.5 hr, mitotic cells were harvested by shake off, and the attached cells were used as interphase samples. Cell lysates were separated into soluble (SN) and pellet (P) fractions and analyzed by immunoblotting. Eight times more mitotic chromatin was loaded to allow detection of nonphosphorylated SA2 in control samples.

(C) Two days after transfection with control or Wapl siRNAs, HeLa cells were extracted with 0.1% TritonX-100 and stained with pS1224-SA2 and SA2 antibodies.

observations imply that the low levels of Sgo1 on chromosome arms in Wapl-depleted cells cannot be sufficient to maintain the high levels of cohesin at these sites.

Wapl Depletion Suppresses the Mitotic Arrest Caused by Depletion of Sgo1

As previously reported (Salic et al., 2004; Tang et al., 2004; Kitajima et al., 2005; McGuinness et al., 2005), we found that depletion of Sgo1 causes precocious separation of sister chromatids and an arrest in prometaphase. Remarkably, samples of cells in which both Sgo1 and Wapl had been depleted contained almost normal number of anaphases (Figures S7B and S7C). This observation implies that the cohesion defect caused by Sgo1 depletion is reverted by depletion of Wapl, as it is by expression of nonphosphorylatable SA2-12xA (McGuinness et al., 2005). The precocious loss of cohesion in Sgo1-depleted cells thus depends on Wapl.

Wapl Is Required for the Dynamic Association of Cohesin with Chromatin in Interphase

Our data so far indicated that Wapl depletion has strong effects on cohesin dissociation from mitotic chromosomes without affecting any of the known mitotic regulators of cohesin. We therefore considered the possibility that the function of Wapl may not be restricted to mitosis. First, we analyzed if Wapl depletion increases the amount of cohesin that is associated with chromatin in interphase. To rule out differences in specimen preparation, we mixed cells that had been transfected with either Wapl or control siRNAs, seeded them together on coverslips, analyzed them by IFM with different cohesin antibodies, and quantified signal intensities in automatically acquired images. To be able to differentiate the two cell populations, we used a cell line that stably expresses a marker protein (CENPA-EGFP) in 99% of all cells for transfection with Wapl siRNAs and regular HeLa cells for control transfections (Figure 7A). When we analyzed the total nuclear amounts of Scc1, no differences between Wapl-depleted and control cells could be seen (Figure 7B). However, when chromatin bound Scc1 was measured in preextracted cells, an increase in signal intensity of 26% ± 7% was seen after Wapl depletion in three independent experiments (Figure 7B). Similar data were obtained with Smc3 and SA1/SA2 antibodies and when Wapl was depleted from regular HeLa cells and the CENPA-EGFP cells were used for control transfections (data not shown). Increased amounts of cohesin in chromatin fractions could

(D) Immunoblot analysis as in (B), but more $MgCl_2$ was added to cell extracts to visualize the SA2 phospho-shift (see Experimental Procedures).

(E) HeLa cells were released from a thymidine arrest for 6 hr, transfected with the indicated siRNAs, arrested again for 24 hr with thymidine, released for 12 hr, extracted as in (C), and stained with Sgo1 and Scc1 antibodies.

(F) Prometaphase cells shown in (E) were classified for Sgo1 and cohesin staining (n > 100). Size bars, 10 μm.

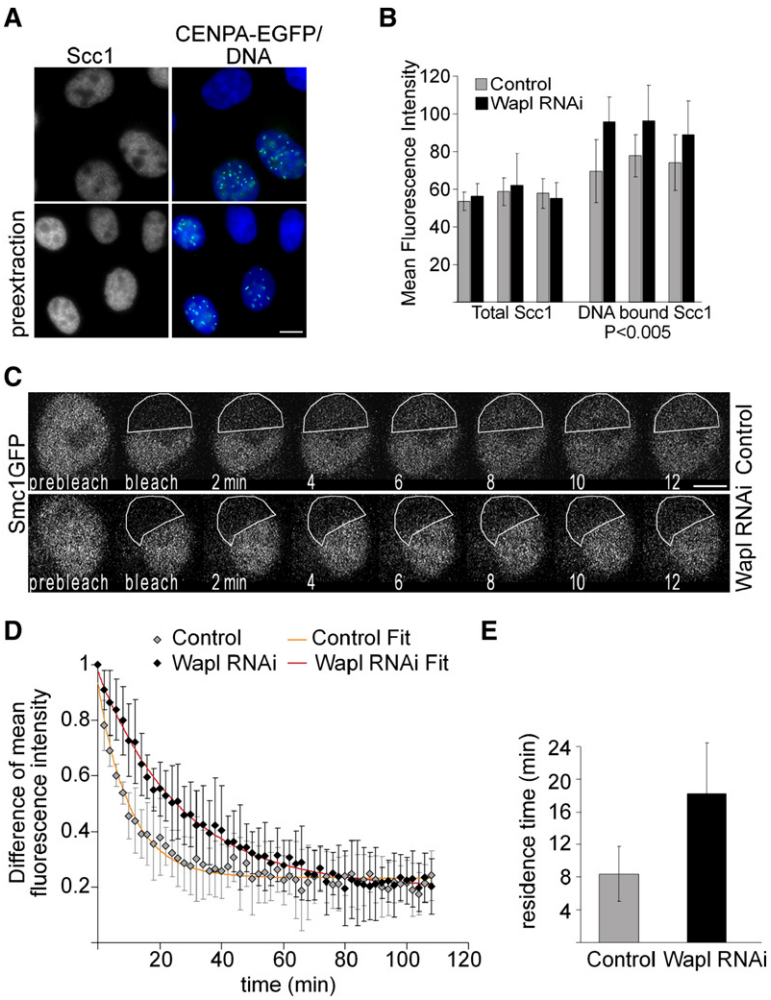

Figure 7. Wapl Is Required for the Dynamic Association of Cohesin with Chromatin in Interphase

(A) Regular HeLa cells and HeLa cells stably expressing CENPA-EGFP were transfected using control or Wapl siRNAs. Two days after transfection, cells were trypsinized, mixed 1:1, and seeded onto coverslips. 12 hr later, cells were extracted with 0.1% Triton X-100 or not and stained for Scc1.

(B) Scc1 fluorescence intensities were quantified in cells obtained as in (A), using Definies Developer/Cellenger (Definies). Three independent experiments were analyzed ($n > 200$; mean values and SD are shown; P values were calculated with nonpaired t test).

(C–E) Smc1-EGFP cells transfected with control or Wapl siRNAs were synchronized by double thymidine treatment, released for 6 hr from the second thymidine block, and analyzed by FRAP. Images were acquired using a Zeiss LSM 510 confocal microscope. After prebleach scans, half of the nucleus and the cytoplasm were bleached, and single stack images acquired subsequently. Bleached areas are circled. In (D), fluorescence recovery and decay were measured and individual data sets fitted to a single exponential function. In (E), average residence times were calculated from fitted curves ($n = 14$; mean values and SD are shown; $P < 0.0001$; calculated with nonpaired t test). Size bars, 10 μm.

also be detected by immunoblotting in Wapl-depleted cells (Figure 6B, compare lanes 3 and 4).

To understand if the increased cohesin levels on interphase chromatin were caused by changes in the dynamics of cohesin association with chromatin, we analyzed the mobility of EGFP-tagged cohesin by FRAP experiments, using assays that have recently been developed for normal rat kidney (NRK) cells (Gerlich et al., 2006). We released HeLa cells that stably express Smc1-EGFP for 6 hr from a thymidine arrest to allow entry into G2, photobleached one half of the nucleus in each cell, and followed both loss of the EGFP signal from the unbleached half and signal recovery in the bleached half for 110 min (Figure 7C). Some of the fluorescence intensity in the unbleached region decreased rapidly after bleaching, representing 30% of nuclear cohesin that is soluble. We analyzed the redistribution kinetics of the remaining chromatin bound cohesin by plotting the difference between loss and recovery curves and fitting them with exponential functions (Figure 7D). This analysis showed that in G2 also HeLa cells, like NRK cells (Gerlich et al., 2006), contain two populations of chromatin-associated cohesin: one that has a relatively short residence time on chromatin of 8.4 ± 3.3 min (80% of bound cohesin; Figure 7E) and another one (20% of bound cohesin) that binds to chromatin so stably that its residence time could not be measured during the observation period (reflected by the plateau of the curve around 0.2).

When we analyzed Smc1-EGFP in Wapl-depleted G2 cells we could also detect two populations of chromatin bound cohesin, and their ratio was not detectably changed (Figure 7D). As in control cells, the residence time of the "slow" cohesin population on chromatin could not be determined, but the residence time of the "fast" cohesin population was significantly increased to a value of 18.2 ± 6.2 min (Figure 7E). The ability of cohesin to dissociate from interphase chromatin with normal kinetics therefore depends on Wapl.

DISCUSSION

Cohesin complexes have to be able to interact with chromatin for long enough to maintain cohesion from S phase until the subsequent mitosis or meiosis, which can occur

many hours, or in the case of vertebrate meiotic cells, even years after DNA replication has been completed. Cohesin may therefore associate with DNA in a particularly stable manner that cannot easily be reverted. However, once chromosomes have been bioriented on the spindle, cohesion has to be dissolved rapidly to allow sister-chromatid separation in anaphase. For these reasons, it is essential that the association of cohesin with chromatin is tightly regulated.

By searching for cohesin-associated proteins, we have identified Wapl as a protein that controls the interaction between cohesin and chromatin. In early mitosis, where the large bulk of cohesin normally dissociates from chromosome arms, Wapl depletion inhibits cohesin dissociation, and in Wapl-depleted interphase cells, cohesin also remains bound to chromatin longer than normally. Wapl may therefore be a protein that facilitates, through direct physical interaction with cohesin, the release of cohesin from chromatin, perhaps by "unlocking" cohesin from a particular state or conformation in which it interacts with DNA.

Has the Function of Wapl Been Conserved during Evolution?

Wapl is highly conserved among metazoan species from plants to mammals. In mice, it is essential for viability and can, when overexpressed, promote tumorigenesis (Oikawa et al., 2004). The first *wapl* gene was identified in *Drosophila*, where its mutation causes larval lethality (except in a few "escapers," which develop into adults whose wings are abnormally apart [Gvozdev et al., 1975]). Genetic and cytological observations imply that *Drosophila* Wapl is required for the formation of heterochromatin (Perrimon et al., 1985; Verni et al., 2000). Wapl has furthermore been identified in a screen for *Drosophila* mutants with defects in chromosome segregation (Dobie et al., 2001). In Wapl mutant neuroblasts, the largely heterochromatic chromosomes 4 and Y lose cohesion precociously, but the other chromosomes maintain cohesion along their entire length even when cells are arrested in prometaphase (Verni et al., 2000). The latter phenotype is consistent with the possibility that *Drosophila* Wapl mutants have a defect in dissociating cohesin from chromosome arms. We therefore speculate that Wapl is also required to release cohesin from chromosome arms during the early stages of mitosis in *Drosophila* and other metazoan species.

When during the Cell Cycle does Wapl Function?

Depletion of Wapl in human cells significantly prolongs the association of cohesin with chromatin in both interphase and mitosis. Since the mitotic phenotype is much stronger than the one seen in interphase, it is formally possible that the absence of Wapl in mitosis leads indirectly to effects in interphase. However, all cohesin is eventually removed from chromosomes when Wapl-depleted cells enter anaphase, presumably due to activation of separase, implying that the increased amounts of cohesin on interphase chromosomes does not result from earlier mitotic defects. Conversely, the 26% increase in chromatin bound cohesin that is seen in Wapl-depleted interphase cells is presumably also too small to explain the mitotic phenotype that is caused by Wapl depletion. It is therefore more plausible to think that the function of Wapl is needed in both interphase and mitosis to facilitate the release of cohesin from chromosomes.

Is Wapl Part of the Prophase Pathway of Cohesin Dissociation?

The mitotic phenotype that is caused by Wapl depletion resembles the effects on cohesin that are seen when Plk1 or Aurora B is inactivated, condensin I is depleted, or when nonphosphorylatable SA2 is expressed. However, in all the latter cases, small amounts of cohesin persist on chromosome arms for long periods of time, but the bulk of cohesin still dissociates from chromosome arms. The previously identified components of the prophase pathway may therefore only be essential for the removal of a subset of cohesin complexes, perhaps those that have established cohesion (Hauf et al., 2005). The notion that different cohesin complexes may require different unloading mechanisms is consistent with the hypothesis that cohesin complexes that contribute to cohesion bind to chromatin much more stably than cohesin complexes that do not have this function (Gerlich et al., 2006).

Wapl differs from the previously known components of the prophase pathway because it is required for the dissociation of most cohesin complexes from chromosome arms in mitosis. This observation and the finding that arm cohesion persists longer in Wapl-depleted than in control cells implies that Wapl is required for the dissociation of both types of cohesin complexes: those that have established cohesion and those that have not. The fact that our FRAP experiments have only revealed an effect of Wapl depletion on the fast cohesin pool is not inconsistent with this hypothesis, because for technical reasons we were unable to measure the residence time of the slow cohesin pool in our experiments. It is therefore possible that Wapl also regulates the chromatin association of the slow cohesin population, and Wapl may perform this function "downstream" of the previously known components of the prophase pathway.

How Does Wapl Facilitate Release of Cohesin from Chromatin?

How Wapl contributes to the dissociation of cohesin from chromatin remains unknown, in part because it is still unclear how cohesin interacts with DNA. It has recently been proposed that cohesin and other structural maintenance of chromosomes (SMC) complexes interact with DNA via the "hinge" dimerization-domains of their SMC subunits, either stably (Hirano and Hirano, 2006) or transiently to allow entry of the DNA into the cohesin ring (Gruber et al., 2006). Wapl appears to interact with Smc1/Smc3 via cohesin's opposite end, where Scc1 and SA2 are bound to the ATPase domains of Smc1/Smc3 and where the

prophase pathway promotes release of cohesin from DNA by phosphorylating SA2 (Hauf et al., 2005). If Wapl had any effect on the hinge domains of Smc1/Smc3, such an effect might therefore be indirect, for example by influencing the ATPase activity of these proteins. Alternatively, Wapl could promote the opening of an "exit gate" for DNA at the other end of the cohesin ring, where Scc1 is bound.

Wapl forms a subcomplex with Pds5A and possibly also an alternative subcomplex with Pds5B. Depletion of either Pds5A or Pds5B causes mild cohesion defects in HeLa cells, but codepletion of both Pds5A and Pds5B from *Xenopus* egg extracts does surprisingly not decrease cohesion but instead increases the amount of cohesin on mitotic chromosomes (Losada et al., 2005). One possible explanation for the latter effect is that immunodepletion of Pds5 proteins from *Xenopus* extracts could result in codepletion of Wapl and might thereby increase the amounts of cohesin on chromatin indirectly. It is also interesting to note that depletion of Wapl and Pds5 proteins has opposite effects on cohesion in HeLa cells. It will therefore be interesting to address if these proteins control the association of cohesin with chromatin through antagonistic mechanisms.

EXPERIMENTAL PROCEDURES

Immunoprecipitation and Mass Spectrometry Analysis

Ten microliters of FLAG agarose (SIGMA) or protein A beads (BioRad) coupled to antibodies were incubated with 3 mg of HeLa extract for 1 hr at 4°C, washed 3× with TBS-Tween and 2× with TBS and eluted with 1.5 bead volume of 0.2 M glycine (pH2). Eluates were adjusted immediately to pH > 7 using Tris buffer (pH 9). For peptide elution, beads were incubated with 1.5 bead volume 1 mg/ml antigenic peptide in TBS. For MS, glycine eluates or bands cut from silver-stained gels were trypsinized overnight (Hauf et al., 2005). Proteolytic peptides were applied to a precolumn (PepMAP C18, 0.3 × 5 mm, Dionex) and eluted onto an analytical column (PepMAP C18, 75 μm × 150 mm, Dionex). The eluted peptides were introduced via a nanospray ion source interface (Proxeon) into an ion trap mass spectrometer (Finnigan LTQ). The mass spectrometer cycled through seven scans—one full mass scan followed by six tandem mass scans of the six most intense ions. Sequenced peptides were put onto an exclusion list for 1 min. All tandem mass spectra were searched against the human nonredundant protein database by using algorithms included in MASCOT 2.1 (Matrix Science).

Fractionation and Sucrose Gradient Centrifugation

For fractionation, cells were synchronized by double thymidine block, released for 7 hr, arrested overnight in nocodazole, and then released from the mitotic arrest into fresh, prewarmed medium by gentle shake off. Cells were harvested by trypsinization. Cell pellets were lysed 1:1 in lysis buffer (20 mM Tris pH7.5, 100 mM NaCl, 1.5 mM $MgCl_2$, 20 mM β-glycerophosphate, 10% glycerole, 0.5 mM DTT, 0.2% NP-40, and protease inhibitors) by douncing and separated into high-speed supernatant and pellet. Pellets were washed 4× with excess of lysis buffer, resuspended in SDS sample buffer, and solubilized by sonication. To visualize phosphorylated forms of SA2, lysis buffer was supplemented with 2 mM orthovanadate and 1 μM okadaic acid, and $MgCl_2$ was adjusted to 5 mM.

Immunofluorescence Microscopy

Cells were either grown on 18 mm coverslips in 12-well plates or spun onto glass slides using a Cytospin centrifuge (Shandon brand, available from Thermo Electric) and fixed with 4% PFA. Where indicated, cells were extracted using 0.1% Triton X-100 before fixation (Hauf et al., 2005). Antibodies were used at a concentration of 2 μg/ml in 3% BSA, DNA was counterstained with Hoechst 33342, and slides were mounted using Vectashield Mounting Medium (H1000, Vector Laboratories). Image acquisition was performed as described (Waizenegger et al., 2000). Giemsa staining and interchromatid distance measurements were performed as described (Hauf et al., 2005).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental Referencse, and seven figures and can be found with this article online at http://www.cell.com/cgi/content/full/127/5/955/DC1/.

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