Structure of cohesin subcomplex pinpoints direct shugoshin-Wapl antagonism in centromeric cohesion

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Orderly termination of sister-chromatid cohesion during mitosis is critical for accurate chromosome segregation. During prophase, mitotic kinases phosphorylate cohesin and its protector sororin, triggering Wapl-dependent cohesin release from chromosome arms. The shugoshin (Sgo1)–PP2A complex protects centromeric cohesin until its cleavage by separase at anaphase onset. Here, we report the crystal structure of a human cohesin subcomplex comprising SA2 and Scc1. Multiple HEAT repeats of SA2 form a dragon-shaped structure. Scc1 makes extensive contacts with SA2, with one binding hotspot. Sgo1 and Wapl compete for binding to a conserved site on SA2–Scc1. At this site, mutations of SA2 residues that disrupt Wapl binding bypass the Sgo1 requirement in cohesin protection. Thus, in addition to recruiting PP2A to dephosphorylate cohesin and sororin, Sgo1 physically shields cohesin from Wapl. This unexpected, direct antagonism between Sgo1 and Wapl augments centromeric cohesin protection.

The ring-shaped cohesin complex maintains genome integrity through regulating sister-chromatid cohesion, DNA repair and transcription in eukaryotes1–6. Dysregulation of cohesin and its regulators has been implicated in human cancers and developmental diseases7,8. Cohesin consists of an Smc1–Smc3 heterodimer and a non-Smc heterodimer1,9. The Smc1 and Smc3 ATPases heterodimerize through their hinge domains (Fig. 1a). Scc1 binds to the ATPase domains of Smc1 and Smc3 through its C- and N-terminal winged-helix domains, respectively, forming a ring. In human somatic cells, Scc1 binds through its central region to either SA1 or SA2, two homologous huntingtin, elongation factor 3, A subunit and TOR (HEAT) repeat–containing proteins.

The dynamics and mode of cohesin association with chromatin are tightly regulated by a set of cohesin regulators2,3,10,11. In telophase, cohesin is loaded onto chromatin by the cohesin loader Scc2–Scc4 (refs. 12–15). In G1, cohesin association with chromatin remains dynamic, and the chromatin-bound cohesin can be released by the cooperative actions of the adaptor protein Pds5 and the cohesin inhibitor Wapl (refs. 16–18). During S phase, the cohesin protector sororin binds cohesin in part through Pds5 and antagonizes Wapl to establish cohesin19–21. During prophase in human somatic cells, the mitotic kinases Cdk1, Plk1 and Aurora B collaborate to phosphorylate cohesin and sororin, triggering Wapl-dependent cohesin release from chromosome arms22–24. A pool of cohesin at centromeres is protected by the Sgo1–PP2A complex25–27, which keeps cohesin and sororin in a hypophosphorylated state and maintains centromeric cohesion28. At the metaphase–anaphase transition, the proper kinetochore-microtubule attachment creates tension across sister kinetochores and silences the spindle checkpoint29,30, thus leading to separase activation. Kinetochore tension also triggers a redistribution of Sgo1 from centromeres to kinetochores31,32 that is thought to inactivate Sgo1 and allow cohesin cleavage by active separase. Inactivation of Sgo1-mediated protection of centromeric cohesion leads to premature sister-chromatid separation and spindle checkpoint–dependent mitotic arrest33,34.

The SA2–Scc1 heterodimer mediates the binding between cohesin and its regulators, including Scc2–Scc4, Pds5, Wapl and Sgo1 (refs. 28,35,36). The mechanisms by which this non-Smc cohesin subcomplex coordinates these crucial molecular interactions are poorly understood. To gain structural insights into these interactions, we have determined the crystal structure of human SA2 bound to the SA2-binding region of Scc1 and have mapped the binding sites of Sgo1 and Wapl. Our results establish a competition between Sgo1 and Wapl in cohesin binding and implicate a role for this direct antagonism in centromeric cohesion protection.

RESULTS

Structure of human SA2 bound to Scc1

We coexpressed human SA2 and the SA2-binding region of Scc1 in insect cells, purified the resulting cohesin subcomplex and determined its crystal structure (Fig. 1 and Table 1). SA2 and Scc1 form a simple 1:1 heterodimer. SA2 contains a helical N-terminal domain (N domain) followed by 17 HEAT repeats (termed R1–R17). The SA2 structure is shaped like a dragon. Two long helices of R1 and
R2 resemble the snout of the dragon. The N domain, comprising seven helices, packs against R1 and resembles the head of the dragon. The body of the dragon bends sharply at R9 and R10. This bend divides SA2 into N and C halves. Scc1 consists of four short helices (termed αA–αD) and two long extended segments. These elements interact extensively with SA2 at four major sites as Scc1 sits astride the dragon (Fig. 1 and Fig. 2a). As expected, many residues at the SA2–Scc1 interface are highly conserved in metazoans.

At site I, the N-terminal loop of Scc1 makes mainly electrostatic interactions with SA2 residues in the N domain and at the ridge of R1 and R2 (Fig. 1b). There are four salt bridges formed between R322, D327, K330 and E331 of Scc1 and E154, R213, D209 and R298 of SA2, respectively. In addition, Scc1 L324 packs against T149, F152 and V212, forming hydrophobic interactions. At site II, the αA helix of Scc1 packs against R2–R4 of SA2 (Fig. 1c). The side chain packing between Scc1 and SA2 at this site appears to be suboptimal. For example, even though I337 and L341 of Scc1 are located in the vicinity of Y297 and W334 of SA2, there is minimal contact between their side chains. The interaction between Scc1 and SA2 at this site appears to be suboptimal. For example, even though I337 and L341 of Scc1 are located in the vicinity of Y297 and W334 of SA2, there is minimal contact between their side chains. The interaction between Scc1 and SA2 at this site appears to be suboptimal. For example, even though I337 and L341 of Scc1 are located in the vicinity of Y297 and W334 of SA2, there is minimal contact between their side chains. The interaction between Scc1 and SA2 at this site appears to be suboptimal. For example, even though I337 and L341 of Scc1 are located in the vicinity of Y297 and W334 of SA2, there is minimal contact between their side chains. The interaction between Scc1 and SA2 at this site appears to be suboptimal. For example, even though I337 and L341 of Scc1 are located in the vicinity of Y297 and W334 of SA2, there is minimal contact between their side chains.

Identification of a binding hotspot between SA2 and Scc1

To ascertain the functional importance of the observed SA2–Scc1 interface, we created about 50 SA2 and Scc1 mutants targeting conserved, surface-exposed residues at or near the SA2–Scc1 interface. Strikingly, among these mutants, only one, SA2 D793K, abolished the binding between SA2 and Scc1 (Fig. 2b and Supplementary Fig. 1a–d). SA2 D793 is located at site IV. Its side chain forms hydrogen bonds with the backbone amides of A377 and Q378 of Scc1, which reside in the loop connecting αC and αD (Fig. 2c). In agreement with this result, deletion of P376 and A377 in this loop, deletion of αD or mutation of the three hydrophobic residues in αD abolished Scc1 binding to SA2 (Supplementary Fig. 1e). Moreover, ectopic expression of Myc–SA2 wild type (WT), but not D793K, in HeLa cells rescued the premature sister-chromatid separation and mitotic arrest caused by co-depletion of SA1 and SA2 (Fig. 2d–g). Therefore, SA2 and Scc1 interact through an extensive interface, with site IV being a binding hotspot. Interestingly, in the prefusion state of hemagglutinin (HA) of the influenza virus, an aspartate side chain of the fusion peptide forms similar hydrogen bonds to the backbone amides of residues A377 and Q378 of Scc1.

Table 1 Data collection and refinement statistics

| Data collection | Native | SeMet | SeMet |
|-----------------|--------|-------|-------|
| Space group     | P2₁₂₁₂₁ | P2₁₂₁₂₁ | P₂₁₂₁₂₁ |
| Cell dimensions | a, b, c (Å) | 78.455, 107.275, 78.606, 108.752, 78.733, 108.045, 180.103, 181.791, 180.835 | 78.455, 107.275, 78.606, 108.752, 78.733, 108.045, 180.103, 181.791, 180.835 |
| Wavelength (Å)  | 0.97918 | 0.97918 | 0.97924 |
| Resolution (Å)  | 2.95 | 3.05 | 2.85 |
| R syn            | 0.111 (0.959) | 0.102 (1.00) | 0.098 (1.00) |
| I / σ I         | 19.0 (1.8) | 19.9 (1.5) | 20.1 (1.5) |
| Completeness (%) | 100.0 (99.8) | 100.0 (99.9) | 99.9 (99.9) |
| Redundancy       | 6.2 (6.2) | 10.0 (8.9) | 8.8 (8.2) |

| Refinement       | Native | SeMet | SeMet |
|------------------|--------|-------|-------|
| Resolution (Å)   | 47.68–2.95 | 45.45–3.05 | 41.71–2.85 |
| No. reflections  | 32,803 | 30,393 | 36,729 |
| R work / R free (%) | 18.8 / 25.1 | 19.5 / 23.8 | 20.9 / 22.5 |
| No. atoms        | 7,974 | 7,909 | 7,982 |
| Protein          | 12 | 0 | 12 |
| Ligand/ion       | 0 | 22 | 14 |
| Water            | – | 50.3 | 29.8 |
| r.m.s. deviations| Bond lengths (Å) | 0.009 | 0.007 | 0.005 |
| Bond angles (°)  | 1.21 | 1.03 | 0.87 |

Data sets were collected with one native crystal with MES in the crystallization buffer, one selenomethionine (SeMet) crystal without MES and one SeMet crystal with MES. Values in parentheses are for highest-resolution shell.

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in a pocket on HA\textsuperscript{37}. Disruption of this interaction is a rate-limiting step in viral-membrane fusion.

**Identification of a functional Sgo1-binding site on cohesin**

Human Sgo1 associates with cohesin during mitosis, and phosphorylation of Sgo1 at T346 enhances this association\textsuperscript{28}. It is unclear, however, whether the cohesin-Sgo1 interaction is direct. Through systematic deletion mutagenesis (data not shown), we mapped the cohesin-binding region of Sgo1 to a 41-residue conserved motif containing T346. Both the unphosphorylated Sgo1 and phospho-T346 Sgo1 (pSgo1) peptides bound purified recombinant SA2–Scc1 (Fig. 3a). pSgo1 bound more tightly than unphosphorylated Sgo1 did. As determined by isothermal titration calorimetry (ITC), unphosphorylated Sgo1 bound SA2–Scc1 with a $K_D$ of $618 \pm 80$ nM (mean $\pm$ s.d. of three independent measurements), whereas pSgo1 had a $K_D$ of $109 \pm 35$ nM (Fig. 3b). Both peptides bound SA2–Scc1 with a stoichiometry of 1:1, results indicating that SA2–Scc1 has a single Sgo1-binding site. Thus, Sgo1 binds directly to the SA2–Scc1 cohesin subcomplex, and Sgo1 phosphorylation enhances, but is not required for, this binding.

Despite numerous attempts, we failed to obtain diffracting crystals of the SA2–Scc1–pSgo1 complex. The structural basis of phosphorylation-enhanced Sgo1 binding to cohesin remains to be established. We thus sought to define the Sgo1-binding site on SA2–Scc1 through mutagenesis. Among the SA2 and Scc1 mutations, SA2 Y297A, R298E, D326K, K330E, Y331A, W334A, D793K and K870E greatly diminished Sgo1 or pSgo1 binding in vitro (Fig. 3c and Supplementary Fig. 2a,b). The SA2 D793K mutant lost binding to Sgo1 because it could not bind Sccl. Another mutant, K870E, also had weakened Sgo1 binding. K870 is located at site IV, in proximity to D793. Mutation of this residue probably affects Sgo1 binding indirectly through affecting Sccl binding. Aside from D793 and K870, all other residues critical for Sgo1 binding, including Y297, R298, D326, K330, Y331 and W334, are clustered near site II of the SA2-Scc1 interface (Fig. 3d). When coexpressed with GFP-Sgo1 in HeLa cells, mutants targeting these residues also exhibited weaker binding to GFP-Sgo1 (Supplementary Fig. 2c). Moreover, the Sgo1 binding–deficient SA2 mutants, Y297A, R298E, Y331A and W334A, failed to rescue the mitotic-arrest phenotypes of HeLa cells depleted of SA1 and SA2 (Fig. 3e,f and Supplementary Fig. 2d,e). Consistently with this, Y331A and W334A also failed to prevent premature sister-chromatid separation in these cells (Fig. 3g). Collectively, these results establish site II of SA2–Scc1 as a functional Sgo1-binding site. The notion that Sgo1 binds near the SA2–Scc1 interface is also consistent with the fact that Sgo1 binds to only the SA2–Scc1 complex and does not associate with either SA2 or Scc1 alone (data not shown).

We noticed that a 2-(N-morpholino)ethanesulfonic acid (MES) molecule from the crystallization solution bound to the Sgo1-binding site (Fig. 3d and Supplementary Fig. 3a,b). In particular, the sulfate group of MES makes hydrogen bonds and favorable electrostatic interactions with Y297 and R298 and is in the vicinity of Y331. Y297 and R298 belong to a signature FHTRYD motif conserved in SA proteins in eukaryotes from yeast to humans. SA2 Y297F and Y331F were defective in Sgo1 binding (Supplementary Fig. 2c), thus implicating their hydroxyl groups in engaging in nonhydrophobic interactions with Sgo1. Thus, one intriguing possibility is that MES might mimic phospho-T346 of Sgo1. Future structural studies are needed to rigorously test this possibility.

The conserved FHTRYD motif is involved in Scc2–Scc4 binding in fission yeast\textsuperscript{35}. Mutations of this motif cause a partial defect in topological cohesin loading to circular DNA in vitro and a cohesin defect in yeast cells\textsuperscript{35}. We tested whether this motif in human SA2 was also required for cohesin loading in human cells. GFP-SA2 Y297A and R298E localized to chromatin in telophase HeLa cells as efficiently as GFP-SA2 WT (Supplementary Fig. 3c,d). As a negative control, the Sccl binding–deficient D793K mutant did not associate

![Diagram](image-url)

**Figure 2** Identification of a binding hotspot between SA2 and Scc1. (a) Cartoon diagram of the structure of human SA2–Scc1, with SA2 and Scc1 colored blue and pink, respectively, in an orientation rotated 180° relative to that in Figure 1a. The N and C termini of both proteins and the four helices of Scc1 are labeled. The four SA2–Scc1 contact sites are boxed. (b) Anti-Myc and anti-Scc1 immunoblots of anti-Myc immunoprecipitates of HeLa cells transfected with the indicated Myc-SA2 plasmids. WT, wild type. Uncropped blots are shown in Supplementary Figure 6. (c) Zoomed-in view of contact site IV, with SA2 and Sccl residues in yellow and gray sticks, respectively. Sccl residues are labeled in red. The dashed red lines indicate hydrogen bonds. (d) Anti-SA2 and anti-β-tubulin immunoblots of lysates of HeLa cells transfected with the indicated short interfering RNAs (siRNAs) and plasmids. WT, wild type; endo, endogenous. Uncropped blots are included in Supplementary Figure 6. (e) Quantification of the mitotic indices (defined as the percentage of MPM2-positive cells with 4n DNA content) of cells in d. Error bars, s.d. (n = 4 independent experiments). (f) Four major types of metaphase spreads of cells in d. Spreads were stained with DAPI (blue) and the kinetochore marker CREST (red). Selected sister chromatids are magnified and shown in insets. Scale bar, 5 μm. (g) Quantification of the percentage of cells in d with type III and IV chromosome morphologies as in f. Error bars, s.d. (n = 4 independent experiments).
with chromatin. Therefore, we do not have evidence to indicate a role for the FVHYRD motif of human SA2 in cohesin loading. Because Scc2–Scc4 interacts with multiple cohesin subunits, disruption of a single interaction surface might not block cohesin association with chromatin. Our results thus do not rule out a role of this motif in the N-terminal region of Wapl is critical for its function in human cells. Because both Sgo1 and Wapl interact with SA2–Scc1, we tested whether Sgo1 and Wapl-M competed for binding to SA2–Scc1. A GST-Wapl fragment containing Wapl-M pulled down recombinant SA2–Scc1 purified from insect cells (Fig. 4d). Addition of the pSgo1 peptide reduced SA2–Scc1 binding to GST–Wapl-M in a dose-dependent manner (Fig. 4d,e). As expected, the unphosphorylated Sgo1 peptide was less effective in the competition. As a control, a shorter pSgo1 peptide (with five residues flanking pT346 on either side) showed no competition.

We then mapped the Wapl-binding site on SA2–Scc1. We focused our analysis on the conserved patch of residues at the Sgo1-binding site (Fig. 4f,g). Indeed, mutations of K290, D326 and K330 abolished Myc-SA2 binding to endogenous Wapl in human cells (Fig. 4h). Mutations of Y331 and W334 in SA2 diminished (but did not abolish) Wapl binding in human cells. Four of the five Wapl-binding-deficient SA2 mutants, including D326K, K330E, Y331A and W334A were also defective in Sgo1 binding. Mutation of SA2 Y328 had no effect on Wapl or Sgo1 binding. We further confirmed that the K290E, D326K and K330E mutations greatly reduced the binding of SA2–Scc1 to GST–Wapl-M in vitro (Supplementary Fig. 4c,d). Therefore, Sgo1 and Wapl compete with each other for cohesin binding, and they bind to overlapping sites on SA2–Scc1.

SA2 mutations or high concentrations of pSgo1 do not completely block Wapl binding to SA2–Scc1, thus suggesting that Wapl might bind SA2–Scc1 through multiple interfaces. In addition, the Wapl- and Sgo1-binding residues on SA2 are highly conserved in all metazoans, whereas the cohesin-binding motifs of Sgo1 and Wapl are conserved in only vertebrates and not in Drosophila or Caenorhabditis elegans. It is possible that certain conserved structural features of Sgo1 and Wapl cannot be easily gleaned from amino acid sequences alone. Alternatively, these SA2 residues are conserved for other purposes, such as binding Scc2–Scc4.

**Figure 3** A conserved, functional Sgo1-binding site of SA2–Scc1. (a) Coomassie-stained SDS-PAGE gel of recombinant SA2–Scc1 (input) and the same complex bound to the indicated beads. Uncropped images are shown in Supplementary Figure 6. (b) ITC curves of the binding between SA2–Scc1 and unphosphorylated or phospho-T346 Sgo1 peptides, with $K_d$ and binding stoichiometry ($N$) indicated. DP, differential power. (c) Autoradiograph of $^{35}$S-labeled SA2–Scc1 proteins (input) and the same proteins bound to beads coupled to unphosphorylated or phospho-T346 Sgo1 peptides. WT, wild type. Uncropped images are shown in Supplementary Figure 6. (d) The Sgo1-binding site of SA2–Scc1. Color and labeling schemes are as in Figure 1c. A MES molecule bound at this site is shown in sticks. (e) Anti-SA2 and anti-β-tubulin immunoblots of lysates of HeLa cells transfected with the indicated siRNAs and Myc-SA2 plasmids. The positions of the endogenous and Myc-SA2 are indicated. WT, wild type. Uncropped blots are shown in Supplementary Figure 6. (f) Quantification of the mitotic indices (defined as the percentage of MPM2-positive, 4n cells) of cells in e. Error bars, s.d. (n = 4 independent experiments). (g) Quantification of the percentages of mitotic cells in e with separated sister chromatids (type III and IV metaphase spreads). Error bars, range (n = 2 independent experiments).
Direct Sgo1-Wapl antagonism strengthens cohesion protection

An established function of cohesin-bound Sgo1 is to recruit PP2A to cohesin and enable PP2A to protect sororin from phosphorylation by Cdk1 (ref. 28). Hypophosphorylated sororin remains bound to Pds5 to counteract Wapl, thereby maintaining centromeric cohesion. Expression of the nonphosphorylatable sororin 9A mutant with all Cdk1 sites mutated has been shown to bypass the requirement for Sgo1 in cohesion protection during normal mitotic progression24,28. As shown above, pSgo1 binds to SA2–Scc1 with high affinity (around 868 VOLUME 21  NUMBER 10  OCTOBER 2014 Sgo1 in cohesion protection during normal mitotic progression 24,28.  Cdk1 sites mutated has been shown to bypass the requirement for Sgo1 or both are colored magenta, green and yellow, respectively. (h) Anti-Myc and anti-Wapl immunoblots of anti-Myc immunoprecipitates of HeLa cells transfected with the indicated plasmids. WT, wild type. Uncropped blots are included in Supplementary Figure 6.

We first revisited the phenotypes of sororin 9A–expressing cells depleted of Sgo1. As shown previously28, in the presence of nocodazole (which arrested cells in prometaphase, with all kinetochores not experiencing spindle-pulling force or tension), expression of sororin 9A in HeLa cells prevented premature sister-chromatid separation caused by Sgo1 depletion (Fig. 5a). In stark contrast, in cells treated with the proteasome inhibitor MG132 (which arrested cells at metaphase, with persistent spindle-pulling force and kinetochore tension), Sgo1 depletion caused massive premature sister-chromatid separation even in cells expressing sororin 9A (Fig. 5a). Thus, in the absence of the spindle-pulling force at kinetochores, hypophosphorylated sororin alone presumably suffices to protect centromeric cohesion. When kinetochores are under persistent tension, both Sgo1 and hypophosphorylated sororin are required to maintain sister-chromatid cohesion. This result suggests that, in addition to promoting sororin dephosphorylation, Sgo1 has other roles in cohesion protection.

We hypothesized that the direct competition between Sgo1 and Wapl for cohesin binding might allow Sgo1 to shield cohesin from Wapl, Sgo1 or both are colored magenta, green and yellow, respectively. (h) Anti-Myc and anti-Wapl immunoblots of anti-Myc immunoprecipitates of HeLa cells transfected with the indicated plasmids. WT, wild type. Uncropped blots are included in Supplementary Figure 6.

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We hypothesized that the direct competition between Sgo1 and Wapl for cohesin binding might allow Sgo1 to shield cohesin from Wapl and account for the sororin-independent function of Sgo1 in cohesion protection. A strong prediction of this hypothesis was that cohesin containing Wapl binding–deficient SA2 mutants should alleviate the need for Sgo1 protection. Indeed, overexpression of SA2 K290E, D326K or K330E mutants that lost Wapl binding in HeLa cells partially rescued the premature sister-chromatid separation (Supplementary Fig. 5a–c) and mitotic arrest (Fig. 5b) caused by Sgo1 depletion. As controls, expression of SA2 mutants that retained partial or full Wapl binding, including Y328A, Y331A and W334A, failed to bypass Sgo1 requirement in cohesion protection. Expression
of other SA2 mutants targeting a conserved patch of residues in the C half or the Scc1 binding-deficient D793K mutant also had no effect. Finally, overexpression of SA2 D326K or K330E even rescued the gradual loss of cohesion in the presence of pro-AC, acetyl group.

**DISCUSSION**

In this study, we have determined the crystal structure of the SA2–Scc1 cohesin subcomplex, which is the interaction hub for cohesin regulators. Further biochemical and functional analyses have uncovered direct competition between the cohesion protector Sgo1 and the cohesion inhibitor Wapl for cohesin binding and have demonstrated the relevance of this competition in cohesion protection.

Sgo1 forms a homodimer through its N-terminal coiled-coil domain that binds PP2A27. One Sgo1 dimer binds to one PP2A complex43. We have shown that the pSgo1 peptide binds with 1:1 stoichiometry to SA2–Scc1. In principle, the two monomers of the Sgo1 dimer could each bind one cohesin complex, thus bridging two cohesin rings. We do not have evidence that this type of Sgo1-dependent cohesin cross-linking occurs in human cells. More importantly, the SA2 D326K and K330E mutants, which are defective in Sgo1 binding, can bypass the requirement for Sgo1 in cohesion, by virtue of their inability to interact with Wapl. Because these mutants cannot physically link cohesin rings, the putative Sgo1-mediated cohesin cross-linking (even if it does occur) is unlikely to directly contribute to sister-chromatid cohesion.

The results presented herein, along with previously published results, suggest the following model for cohesion establishment and maintenance in human cells (Fig. 5c). During telophase and G1, cohesin is loaded on chromosomes but undergoes Wapl-dependent dynamic release from chromosomes. This release involves an opening of the cohesin ring at the Smc3–Scc1 juncture44,45 and requires an interaction between Pds5 and the N-terminal region of Wapl (Wapl-N) and interactions between cohesin and the middle region and C-terminal domain of Wapl. The mechanism by which Wapl–Pds5

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**Supplementary Fig. 5d.** These results suggest that a critical function of Sgo1 in cohesion protection is to directly shield cohesin from Wapl through binding to SA2–Scc1.
removal of mitotic cohesin from centromeres, when the Sgo1–cohesin interaction is not disrupted properly31,32. Interestingly, the two separase-cleavage sites in human Scc1 flank the central SA2-binding region of Scc1 delineated in our structure49. In the future, it will be interesting to test whether SA2 contributes to Scc1 cleavage by providing a docking site for separase, and if it does so, to test whether Sgo1 binding to SA2–Scc1 also shields cohesin from separase.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors of SeMet derivatives of SA2–Scc1, SA2–Scc1–MES and native SA2–Scc1–MES complexes have been deposited in the Protein Data Bank under accession codes 4PJU, 4PJW and 4PK7, respectively.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

K.H. crystallized SA2–Scc1 and determined its structure with the help of J.C. and D.R.T. and performed the in vitro binding assays with SA2 and Scc1 mutants. G.Z. performed the functional cellular assays. Q.Q. performed the cohesin binding assay. H.L. performed the metaphase spread assay between SA2–Scc1 and Wapl. Z.O. first observed competition between Wapl and Sgo1 and performed the functional cellular assays. H.Y. performed the metaphase spread assay of sororin 9A–expressing cells depleted of Sgo1. H.Y. supervised the project and wrote the paper with input from all authors.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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1. Nasmyth, K. & Haering, C.H. Cohesin: its roles and mechanisms. Annu. Rev. Genet. 43, 525–558 (2009).
2. Peters, J.M., Tedeschi, A. & Schmitz, J. The cohesin complex and its roles in chromosome segregation. Cell 124, 105–129 (2006).
3. Onn, I., Heidinger-Pauli, J.M., Guacci, V., Unal, E. & Koshland, D.E. Sister chromatid cohesion, and mitotic progression. Curr. Biol. 23, 564–585 (2013).
4. Murayama, Y. & Uhlmann, F. Biochemical reconstitution of topological DNA binding by shugoshin. Nature 421, 636–641 (2003).
5. Wu, N. Chromosome organization: Wapl-mediated removal of cohesin protects against segregation errors and anaphase. Curr. Biol. 23, 2071–2077 (2013).
6. Yu, H. Cohesin biology. Wapl spreads its wings. Curr. Biol. 23, R923–R925 (2013).
7. Daum, J. & Cdk1 mediated Wapl activation and release of acetylated cohesin from chromosomes by phosphorylating Sororin. Proc. Natl. Acad. Sci. USA 108, 13404–13409 (2013).
8. Kitajima, T.S. et al. Shugoshin collaborates with protein phosphatase 2A to protect cohesin. Nature 441, 56–52 (2006).
9. Nasmyth, K. Cohesin’s binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol. Cell 5, 243–254 (2000).
10. Solomon, D.A. et al. Mutational inactivation of STAG2 causes aneuploidy in human cancer. Science 333, 1039–1043 (2011).
11. Shiomi, K. & Hiraora, T. Sister chromatid resolution: a cohesin releasing network and beyond. Chromosoma 119, 459–467 (2010).
12. Shiomi, K. & Hiraora, T. Sister chromatid resolution: a cohesin releasing network and beyond. Chromosoma 119, 459–467 (2010).
13. Takahashi, T.S. & Jallepalli, P.V. Sister acts: coordinating DNA replication and cohesin establishment. Genes Dev. 24, 2723–2731 (2010).
14. Shintomi, K. & Hiraora, T. Sister chromatid resolution: a cohesin releasing network and beyond. Chromosoma 119, 459–467 (2010).
15. Ciosk, R. et al. Cohesin’s binding to chromosomes depends on a separate complex consisting of Scc2 and Scc4 proteins. Mol. Cell 5, 243–254 (2000).
16. Wapl. Curr. Biol. 23, 2071–2077 (2013).
17. Daum, J. & Cdk1 mediated Wapl activation and release of acetylated cohesin from chromosomes by phosphorylating Sororin. Proc. Natl. Acad. Sci. USA 108, 13404–13409 (2013).
18. Kueng, S. et al. Wapl controls the dynamic association of cohesin with chromatin. Cell 127, 957–967 (2006).
19. Rankin, S. et al. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol. 3, e59 (2005).
20. Ouyang, Z. et al. Structure of the human cohesin inhibitor Wapl. Proc. Natl. Acad. Sci. USA 101, 653–658 (2004).
21. Tedeschi, A. et al. Wapl-mediated removal of cohesin protects against segregation errors and anapholess. Curr. Biol. 23, 2071–2077 (2013).
22. Hauf, S. et al. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol. 3, e59 (2005).
23. Kueng, S. et al. Wapl controls the dynamic association of cohesin with chromatin. Cell 127, 957–967 (2006).
24. Rankin, S. et al. Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol. 3, e59 (2005).
25. Ouyang, Z. et al. Structure of the human cohesin inhibitor Wapl. Proc. Natl. Acad. Sci. USA 101, 653–658 (2004).
26. Riedel, C.G. et al. Protein phosphatase 2A protects centromeric sister chromatid cohesion during meiosis I. Nature 441, 53–61 (2006).
27. Tonkin, E.T., Wang, T.J., Lisgo, S., Bamshad, M.J. & Strachan, T. NIPBL, encoding a docking site for separase, and if it does so, to test whether Sgo1 binding to SA2–Scc1 also shields cohesin from separase.

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**ONLINE METHODS**

**Protein expression and purification.** The cDNAs of human SA2 (residues 80–1060) and Scc1 (residues 281–420) were separately cloned into the FseI and Ascl sites of a modified pFastBac HT vector (Invitrogen). The final constructs encoded an N-terminal His6-Flag–tagged SA2 and an N-terminal His6-streptag–tagged Scc1, with a tobacco etch virus (TEV) protease cleavage site between the Flag tag and SA2, and a PreScission protease cleavage site between the strep tag and Scc1. Recombinant SA2 and Sccl baculoviruses were constructed with the Bac-to-Bac system (Invitrogen) according to the manufacturer's protocols. For large-scale production of recombinant proteins, Hi5 insect cells (Sigma-Aldrich) were infected with both the SA2 and Sccl baculoviruses and harvested at about 50 h after infection. Cells were resuspended in lysate buffer (50 mM Tris-HCl, pH 7.7, 150 mM KCl, 0.1% (v/v) Triton X-100 and a protease-inhibitor cocktail). After sonication and centrifugation, the supernatant was applied onto Ni2+-NTA resin (Qiagen) equilibrated with lysis buffer, and incubated at 4 °C overnight. The resin was washed with wash buffer I (50 mM Tris-HCl, pH 7.7, 1.2 M KCl and 10 mM imidazole) and then washed with wash buffer II (20 mM HEPES, pH 7.4, 100 mM NaCl and 20 mM imidazole). The SA2–Sccl complex was eluted with the elution buffer (20 mM HEPES, pH 7.4, 100 mM NaCl and 50–200 mM imidazole), and incubated with the TEV and PreScission proteases at 4 °C overnight. The complex with the tags removed was applied onto a Hitrap Q HP column (GE Healthcare) equilibrated with the QA buffer (50 mM Tris-HCl, pH 8.5) with an AKTA chromatography system (GE Healthcare). SA2–Sccl was eluted with a linear salt gradient from 0 to 600 mM NaCl. SA2–Sccl was further purified onto a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare) equilibrated with the purification buffer (20 mM Tris-HCl, pH 7.7, 300 mM NaCl and 5 mM TCEP). Purified SA2–Sccl was concentrated to 7 mg/ml flash frozen in liquid nitrogen and stored at −80 °C for crystallization or binding assays.

The SeMet-labeled SA2–Sccl complex was expressed in Hi5 cells according to the manufacturer's protocols. Briefly, Hi5 cells cultured in the ESF92 medium (Expression Systems) were infected with both SA2 and Sccl baculoviruses. The cells were incubated for 16 h and then supplied with 100 µg/ml-selenomethionine. After an additional 48 h, the cells were harvested, and the SeMet SA2–Sccl complex was purified as described above.

**Crystalization and data collection.** All crystallization experiments were performed at 20 °C. Initial screening for the SA2–Sccl–MES complex was carried out by sitting-drop vapor diffusion with a Phoenix crystallization robot (Art Robbins Instruments), with commercially available screen kits from Hampton Research, Qiagen and Molecular Dimensions. Conditions were further optimized with the hanging-drop vapor-diffusion method. SA2–Sccl–MES crystals suitable for X-ray-diffraction experiments appeared within 1 week with a reservoir solution consisting of 0.12 M Morpheus Divalent Mix, 0.1 M Morpheus Buffer System 1 and 27–30% (v/v) Morpheus EOD_PK8 (Molecular Dimensions). Similarly, the SA2–Sccl crystals were grown in 1 week with a reservoir solution consisting of 0.12 M Morpheus Divalent Mix, 0.1 M imidazole–HCl, pH 6.5, and 27–30% (v/v) Morpheus EOD_PK8. All crystals were cryoprotected with a reservoir solution including 30% (v/v) Morpheus EOD_PK8 before being flash frozen.

All X-ray diffraction data were collected at 100 K at the Structural Biology Center (Beamline 19ID) at Argonne National Laboratory. SA2–Sccl–MES (Native-MES), SeMet-labeled SA2–Sccl–MES (SeMet-MES), and SeMet-labeled SA2–Sccl without MES (SeMet) crystals diffraction to 2.95 Å, 2.85 Å and 3.05 Å, respectively (Table 1). Diffraction data were processed with the HKL package.30

**Structure determination and model refinement.** Initial phases for the SeMet-labeled SA2–Sccl were obtained by single-wavelength anomalous dispersion (SAD) with AutoSol in the PHENIX package.31,32 Model building of the SA2–Sccl–MES complex was carried out with COOT.27,33 The structure was refined with PHENIX. Structures of the Native-MES and SeMet-MES complexes were solved by molecular replacement with PHENIX, with the SeMet structure (without MES) as the search model. Statistics for refinement are summarized in Table 1. The final model of the native SA2–Sccl–MES complex (Native-MES) contains residues 83–254, 261–438, 455–505, 512–543, 547–748, 752–805, 807–836, 853–933, 936–959, 965–986, 988–990, 993–1035 and 1037–1047 from SA2, residues 321–396 from Sccl and one MES molecule. The final model of the SeMet SA2–Sccl–MES complex (SeMet-MES) contains residues 83–91, 93–253, 260–438, 455–505, 513–543, 548–748, 753–836, 853–959, 965–991, 994–1035 and 1037–1048 from SA2, residues 321–395 from Sccl and one MES molecule. The final model of the SeMet SA2–Sccl complex (SeMet) contains residues 83–253, 261–438, 456–505, 513–543, 546–746, 777–748, 753–804, 808–836, 852–932, 938–959, 965–991, 994–1049 from SA2, and residues 321–394 from Sccl. Remaining residues of SA2 and Sccl are disordered. All structure drawings in this study were created with PyMOL (http://www.pymol.org/) and depicted the SeMet-MES complex.

**In vitro binding assays.** The cDNAs encoding full-length human SA2, full-length human Sccl and the Sccl fragment (residues 211–420) were cloned into the FseI and Ascl sites of a modified pCS2-Myc vector. The SA2 and Sccl1 mutants were constructed with the QuikChange Site-directed Mutagenesis Kit (Stratagene). The pCS2-Myc-SA2 (wild type and mutants) or pcS2-Myc-Sccl1 (wild type and mutants) vectors were added either alone or as a mixture of 3:1 ratio to the TNT Quick Coupled Transcription Translation System (Promega) and incubated in the presence of 135 mM methionine at 30 °C for 90 min to produce 3S-labeled MYC-SA2, MYC-Sccl or the MYC-SA2–Sccl1 complex.

To assay the binding between SA2 and Sccl, GST-SA2 (residues 80–1060) and GST-Sccl (residues 211–420) were expressed in bacteria and purified with the glutathione–Sepharose 4B resin (GE Healthcare). The glutathione–Sepharose beads bound to GST-SA2 or GST-Sccl were incubated with 3S-labeled Myc-Sccl1 (wild type and mutants) or Myc-SA2 (wild type and mutants), respectively, at 4 °C overnight, and washed four times with TBS containing 0.05% Tween 20. Beads bound to GST were used as negative controls in both cases. The bound proteins were separated by SDS-PAGE. The gels were stained with Coomassie blue, dried and analyzed with a phosphorimager (Fujifilm). Intensities of bound proteins were quantified with ImageJ.

To assess the interaction between Sgo1 and SA2–Sccl, synthetic Sgo1 (residues 313–353) and phospho-T346 Sgo1 (pSgo1) peptides (each with an extra C-terminal cysteine) were coupled to beads with the SulfoLink Immobilization Kit and Coupling Resin (Thermo Scientific) according to the manufacturer's instructions. Recombinant purified 35S-labeled wild-type and mutant MYC-SA2–Sccl1 complexes were incubated with Sgo1- or pSgo1-coupled beads at 4 °C overnight, and washed four times with TBS containing 0.05% Tween 20. The bound proteins were separated by SDS-PAGE and stained with Coomassie blue. For the assays with radioactive proteins, the gels were dried and analyzed with a phosphorimager (Fujifilm). Intensities of bound proteins were quantified with ImageJ.

To assay the binding between Wapl and SA2–Sccl, GST-Wapl410–590 and GST-Wapl410–590 (wild-type and mutants) were expressed in bacteria and purified with glutathione–Sepharose 4B beads. Beads bound to GST or GST-Wapl peptides were incubated with recombinant purified 35S-labeled wild-type and mutant MYC-SA2–Sccl1 complexes in the presence of increasing concentrations of Sgo1 or pSgo1 peptides. The beads were washed with TBS containing 0.05% Tween 20. The bound proteins were analyzed and quantified as described above.

**Isothermal titration calorimetry (ITC).** ITC was performed with a MicroCal iTC200 (GE Healthcare) at 20 °C. Calorimetric measurements were performed with purified SA2–Sccl and synthetic unphosphorylated or phospho-T346 Sgo1 peptides containing residues 313–353. For each titration, 300 µl of 23 µM SA2–Sccl1 in a buffer containing 20 mM Tris-HCl, pH 7.7, 100 mM NaCl and 5 mM TCEP was added to the calorimeter cell. The Sgo1 or pSgo1 peptide (350 µM) in the same buffer was injected with 18 portions of 2 µl with an injection syringe. Binding parameters were evaluated with the Origin package provided with the instrument.

**Cell culture, transfection and synchronization.** HeLa Tet-On cells were grown in DMEM (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine. Plasmid transfection was performed when cells reached a confluency of 50% with the Effectene reagent (Qiagen) according to the manufacturer's protocols. For siRNA transfection, cells were transfected with Lipofectamine RNAiMAX (Invitrogen) at 20–40% confluency according to the manufacturer's protocols, and analyzed at 24–48 h after transfection. The siRNA oligonucleotides targeting human Wapl (siWapl; 5′-CCGAAGCTACCCGCTAGCAGAAA-3′), Sgo1 (siSgo1;
SGO1 is required for cohesin retention at kinetochores during mitosis.

**Antibodies, immunoblotting and immunoprecipitation.** The anti-Wapl antibody was generated against a C-terminal fragment of human Wapl (residues 601–1190) as described. Rabbit polyclonal anti-GFP antibodies were raised against purified recombinant eGFP at Zymoz Antibodies. The following antibodies were purchased from the indicated commercial sources: anti-Myc (Roche, 11667203001), anti-SA2 (Santa Cruz, Biotechnology, sc-81852), anti-Scc1 (Bethyl Laboratories, A300-080A), anti-β-tubulin (Sigma, T4026) and MP12 (Millipore, 05-368). The antibodies to Myc and Scc1 have been validated for immunoblotting and immunoprecipitation applications. The antibody to MP12 has been validated for flow cytometry. The relevant validation information and references can be found on the manufacturer’s websites. The antibody to SA2 is validated for immunoblotting in this study (Fig. 2d), and the anti-GFP antibody is validated for both immunoblotting and immunoprecipitation (Supplementary Fig. 2c).

**Flow cytometry.** Cells were grown with trypsinization and fixed in 70% ice-cold ethanol overnight. After being washed with PBS, cells were permeabilized with PBS containing 0.25% Triton X-100 on ice for 5 min. Then cells were incubated with the antibody to MP12 in PBS containing 1% BSA for 3 h at room temperature, and this was followed by an incubation with a fluorescent secondary antibody (Invitrogen) for 30 min. After being washed with PBS, cells were resuspended in PBS containing 0.1% Triton X-100, RNase A and propidium iodide, and then analyzed with a flow cytometer. Data were processed with FlowJo.

**Immunofluorescence and metaphase spreads.** After synchronization, mitotic HeLa Tet-On cells were collected by shake-off. Cells were washed once with PBS, treated with 55 mM KCl hypotonic solution at 37°C for 15 min and spun onto microscope slides with a Shandon Cytospin centrifuge. Cells on the slides were first permeabilized with the PHEM buffer (25 mM HEPES, pH 7.5, 10 mM EGTA, 10 mM Na3VO4, 10 mM β-glycerophosphate, 1 mM DTT, protease inhibitor mixture (Roche) and 50 units/ml Turbo Nuclease (Accelagen)). After a 1-h incubation on ice and a 10-min incubation at 37°C, all lysates were centrifuged at 4°C at 20,817g for 20 min. The supernatants were incubated with the desired antibody beads for 3 h at 4°C. The beads were then washed three times with the lysis buffer containing mouse NaCl. Proteins bound to beads were dissolved in SDS sample buffer, separated by SDS-PAGE and blotted with the appropriate antibodies.

**Immunofluorescence.** Cells on microscope slides were washed three times with PBS containing 0.1% Triton X-100 for 2 min each time, and were incubated with CREST in PBS containing 3% BSA and 0.1% Triton X-100 at 4°C overnight. The cells were then washed three times with PBS containing 0.1% Triton X-100 for 2 min each time, and were incubated with fluorescent secondary antibodies (Molecular Probes) in PBS containing 3% BSA and 0.1% Triton X-100 for 1 h at room temperature. Cells were again washed three times with PBS containing 0.1% Triton X-100 and then stained with 1 μg/ml DAPI for 2 min. After the final washes, the slides were sealed with nail polish and viewed with a 100× objective on a DeltaVision fluorescence microscope (GE Healthcare). Image processing and quantification were performed with ImageJ.

For the cohesin-loading assay, cells were plated in four-well chamber slides (LabTek). Cells were transfected with GFP-S2A plasmids for 8 h, and this was followed by siSA1 and siSA2 transfection for another 36 h. Cells were first extracted with PHEM buffer containing 0.5% Triton X-100 for 5 min and then were fixed in 2% paraformaldehyde for 15 min. After being washed with PBS, cells were blocked in PBS containing 2% BSA for 1 h, and were incubated with the anti-GFP and anti-tubulin antibodies in PBS containing 0.2% Triton X-100 and 3% BSA overnight at 4°C. After being washed three times with PBS containing 0.05% Tween 20, cells were incubated with fluorescent secondary antibodies in PBS containing 0.2% Triton X-100 and 3% BSA for 1 h at room temperature. The cells were again washed three times with PBS containing 0.05% Tween 20 and stained with 1 μg/ml DAPI in PBS for 2 min. After final washes, slides were mounted and viewed with a 100× objective on a DeltaVision deconvolution fluorescence microscope. A series of z-stack images were captured at 0.2 µm intervals, deconvolved and projected. Image processing and quantification were performed with ImageJ.

50. Ołtweński, Z. & Minor, W. Processing X-ray diffraction data collected in oscillation mode. Methods Enzymol. 276, 307–326 (1997).
51. Adams, P.D. et al. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221 (2010).
52. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132 (2004).