

Cohesin-mediated DNA loop extrusion resolves sister chromatids in G2 phase

Paul Batty^{1,2,*} , Christoph CH Langer¹ , Zsuzsanna Takács¹ , Wen Tang³, Claudia Blaukopf¹, Jan-Michael Peters³  & Daniel W Gerlich^{1,**} 

Abstract

Genetic information is stored in linear DNA molecules, which are highly folded inside cells. DNA replication along the folded template path yields two sister chromatids that initially occupy the same nuclear region in an intertwined arrangement. Dividing cells must disentangle and condense the sister chromatids into separate bodies such that a microtubule-based spindle can move them to opposite poles. While the spindle-mediated transport of sister chromatids has been studied in detail, the chromosome-intrinsic mechanics preseggregating sister chromatids have remained elusive. Here, we show that human sister chromatids resolve extensively already during interphase, in a process dependent on the loop-extruding activity of cohesin, but not that of condensins. Increasing cohesin's looping capability increases sister DNA resolution in interphase nuclei to an extent normally seen only during mitosis, despite the presence of abundant arm cohesion. That cohesin can resolve sister chromatids so extensively in the absence of mitosis-specific activities indicates that DNA loop extrusion is a generic mechanism for segregating replicated genomes, shared across different Structural Maintenance of Chromosomes (SMC) protein complexes in all kingdoms of life.

Keywords chromosomes; cohesin; condensin; mitosis; sister chromatid resolution

Subject Categories Cell Cycle; Chromatin, Transcription & Genomics; DNA Replication, Recombination & Repair

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Introduction

Chromosomes reorganise extensively during the cell cycle to enable gene expression during interphase and chromosome segregation during cell division. This dynamic reorganisation of chromosomes during the cell cycle is regulated by various SMC protein complexes (van Ruiten & Rowland, 2018; Batty & Gerlich, 2019; Yatskevich

et al, 2019; Davidson & Peters, 2021; Mirny & Dekker, 2022), which establish topological linkages between DNA molecules (Gruber *et al*, 2003; Ivanov & Nasmyth, 2005) and extrude DNA into loops through a motor activity (Ganji *et al*, 2018; Davidson *et al*, 2019; Kim *et al*, 2019; Golfier *et al*, 2020).

The SMC protein complex cohesin was originally discovered as a factor linking sister chromatids along their arms (Guacci *et al*, 1997; Michaelis *et al*, 1997) through the topological embrace of two DNA molecules (Gruber *et al*, 2003; Ivanov & Nasmyth, 2005; Haering *et al*, 2008; Srinivasan *et al*, 2018). A distinct subset of cohesin complexes was later found to extrude DNA into dynamic loops (Davidson *et al*, 2019; Kim *et al*, 2019; Golfier *et al*, 2020) throughout interphase to fold chromosomes of vertebrate cells into topologically associating domains (TADs) (Gassler *et al*, 2017; Rao *et al*, 2017; Schwarzer *et al*, 2017; Wutz *et al*, 2017). Most cohesin dissociates from chromosome arms during mitotic entry in vertebrate cells (Waizenegger *et al*, 2000; Gerlich *et al*, 2006b; Liang *et al*, 2015) in a process that depends on the protein WAPL (Gandhi *et al*, 2006; Kueng *et al*, 2006). During mitosis, the structurally related SMC protein complexes, condensin I and condensin II, associate with chromosomes (Ono *et al*, 2003; Hirota *et al*, 2004; Gerlich *et al*, 2006a; Walther *et al*, 2018) to form DNA loops in mitotic cells that are larger than those formed by cohesin in interphase cells (Naumova *et al*, 2013; Gibcus *et al*, 2018). How these cell cycle-regulated activities of cohesin and condensin contribute to sister chromatid resolution, however, has remained unclear.

Imaging chromosomes with differentially labelled sister DNAs suggests that sister chromatids begin to resolve at the onset of mitotic prophase (Nagasaka *et al*, 2016) through a process that depends on condensins, topoisomerase II and WAPL (Gandhi *et al*, 2006; Kueng *et al*, 2006; Nagasaka *et al*, 2016; Eykelenboom *et al*, 2019; Chu *et al*, 2020). These observations support a model in which removal of arm cohesion combined with an increase in DNA loop-extruding activity by condensin in the presence of a DNA strand passing activity is necessary and sufficient to promote sister chromatid resolution (Nasmyth, 2001; Goloborodko *et al*, 2016a). Imaging fluorescently labelled genomic loci, however, showed that sister chromatids transiently split already during interphase, often as far apart as in mitotic cells (Ono *et al*, 2013; Stanyte

1 Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna BioCenter (VBC), Vienna, Austria

2 Vienna BioCenter PhD Program, Doctoral School of the University of Vienna and Medical University of Vienna, Vienna, Austria

3 Research Institute of Molecular Pathology (IMP), Vienna BioCenter (VBC), Vienna, Austria

*Corresponding author. Tel: +43 1 79044 4767; E-mail: paul.batty@imba.oeaw.ac.at

**Corresponding author. Tel: +43 1 79044 4760; E-mail: daniel.gerlich@imba.oeaw.ac.at

et al, 2018; Eykelenboom *et al*, 2019). Moreover, sister chromatid-sensitive chromosome conformation capture (scsHi-C) revealed that sister chromatids locally separate within TADs, despite abundant chromosome arm cohesion at this cell cycle stage (Mitter *et al*, 2020). Together, these findings raise the question as to how arm cohesion affects the resolution of sister chromatids, and how cohesin-mediated loop extrusion compares to condensin-mediated loop extrusion in its capacity to promote sister chromatid resolution. As previous structural analyses of sister chromatids were based on very harsh DNA denaturation and fixation procedures (Nagasaka *et al*, 2016) that are known to disrupt the structure of chromosomes (preprint: Beckwith *et al*, 2022), we aimed to develop improved methodologies to study the fine structure of sister chromatids in human cells not only in mitosis, but also during interphase.

Results

Cohesin resolves sister chromatids in G2

To implement an improved method for the detection of sister chromatid resolution, we used the nucleotide analogue F-ara-EdU, as it has low toxicity and can be visualised without DNA denaturation (Neef & Luedtke, 2011; Hao *et al*, 2021). To generate cells in which each replicated chromosome contains a single labelled sister chromatid, we cultured synchronised HeLa cells for one S phase in the presence of F-ara-EdU, before subsequently removing F-ara-EdU and letting the cells divide and progress further through one additional S phase (Figs 1A and EV1A). We then visualised F-ara-EdU by attaching a fluorophore using click-chemistry and imaged cells by AiryScan microscopy.

To validate our approach, we first assessed the efficiency and specificity of sister chromatid labelling. Due to the semiconservative

nature of DNA replication, F-ara-EdU should be incorporated into each of the newly synthesised DNA strands during S phase when cells are cultured in the presence of F-ara-EdU. Indeed, in the first G2 phase and mitosis after culturing cells in F-ara-EdU, we observed a strong overlap between F-ara-EdU fluorescence and the general DNA dye Hoechst 33342, such that mitotic chromosome arms were labelled on both sister chromatids (Fig 1B, two-sister labelled; Fig EV1B). Mitotic cells that had progressed through an additional S phase in the absence of F-ara-EdU, such that the newly replicated strands did not contain F-ara-EdU, had chromosome arms labelled only on a single sister (Fig 1B, one-sister labelled; Fig EV1C). Thus, our procedure specifically and completely labels one sister chromatid per chromosome.

To quantify the resolution of sister chromatids, we developed an automated analysis pipeline to calculate the pixel-wise correlation between F-ara-EdU fluorescence and Hoechst 33342 (Fig EV1D) (see Materials and Methods for details). We reasoned that although our approach allows specific labelling of only a single sister chromatid, by measuring the reduction in correlation between F-ara-EdU and Hoechst in cells labelled on one sister chromatid compared to reference cells labelled on both sisters, we would be able to quantitatively assess changes in sister chromatid resolution. In the first mitosis after labelling, where a high degree of colocalisation between Hoechst and F-ara-EdU is expected, the Spearman correlation coefficient was close to 1, demonstrating homogeneous and complete labelling of both sister chromatids. In the second mitosis, after cells had progressed through one S phase in the absence of F-ara-EdU, the Spearman correlation coefficient dropped to 0.5, as only the F-ara-EdU labelled chromatid colocalised with the Hoechst counterstain, indicating that sister chromatids were highly resolved (Fig EV1E–G). Based on these two reference conditions, we calculated a normalised sister chromatid separation score (Figs 1B–E and EV1H), thereby allowing

Figure 1. Cohesin resolves sister chromatids in G2.

Schematics of the synchronisation schemes used in these experiments can be found in the extended version figures as follows: Wild-type two or one-sister labelled cells (Fig EV1A), ΔSMC4 G2 cells (Fig EV3A), ΔNIPBL G2 cells (Fig EV4A). SMC4 was depleted in G1 through the addition of 5-Ph-IAA 1 h before the final release into S phase such that cells progressed through S and G2 phase in the absence of condensins. NIPBL was depleted in G2 phase after completion of DNA replication to avoid potential effects of NIPBL depletion on cohesion establishment.

- A Sister chromatid labelling assay. Cells presynchronised to the G1/S boundary are cultured for one cell cycle in the presence of F-ara-EdU and then for another cell cycle in nucleotide-free medium to obtain cells with one sister chromatid labelled on each replicated chromosome. Cells with two sister chromatids labelled per chromosome are generated by fixing the cells after culturing for one cell cycle in F-ara-EdU. For analysis, cells are arrested either in prometaphase by STLC or in G2 by RO-3306 and DNA is stained using Hoechst 33342. F-ara-EdU is visualised through conjugation of AF488.
- B Representative images from wild-type prometaphase cells labelled on one or two sister chromatids as indicated.
- C Representative images from wild-type G2 cells labelled on one or two sister chromatids as indicated.
- D Quantification of sister chromatid separation in one-sister labelled prometaphase cells for experimental conditions as indicated. Dots represent individual cells; red bars indicate the mean. Wild-type ($n = 83$ cells), ΔSMC4 ($n = 75$ cells) and ΔNIPBL ($n = 48$ cells) were analysed. Significance was tested using a two-tailed Mann–Whitney U test; $P = 2.33 \times 10^{-27}$.
- E Quantification of sister chromatid separation as in (D) for one-sister labelled G2 cells. Dots represent individual cells; red bars indicate the mean. Wild-type ($n = 69$ cells), ΔSMC4 ($n = 50$ cells) and ΔNIPBL ($n = 70$ cells) were analysed. Significance was tested using a two-tailed Mann–Whitney U test; $P = 6.55 \times 10^{-19}$.
- F Representative images from ΔSMC4 prometaphase cells labelled on one sister chromatid.
- G Representative images from ΔSMC4 G2 cells labelled on one sister chromatid.
- H Representative images from ΔNIPBL prometaphase cells labelled on one sister chromatid.
- I Representative images from ΔNIPBL G2 cells labelled on one sister chromatid.

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological replicates: (B–I) Two-sister labelled wild-type prometaphase ($n = 4$), one-sister labelled wild-type prometaphase ($n = 6$), ΔSMC4 prometaphase ($n = 4$), ΔNIPBL prometaphase ($n = 3$), wild-type G2 ($n = 4$), ΔSMC4 G2 ($n = 3$), ΔNIPBL G2 ($n = 5$). Technical replicates: (B–I) Two-sister labelled wild-type prometaphase ($n = 9$), one-sister-labelled wild-type prometaphase ($n = 14$), ΔSMC4 prometaphase ($n = 9$), ΔNIPBL prometaphase ($n = 6$), wild-type G2 ($n = 8$), ΔSMC4 G2 ($n = 5$), ΔNIPBL G2 ($n = 8$). All images show single Z-slices from 3D-stacks. Yellow boxes indicate inset regions. Scale bars large panels: 5 μm , insets: 1 μm .

Source data are available online for this figure.

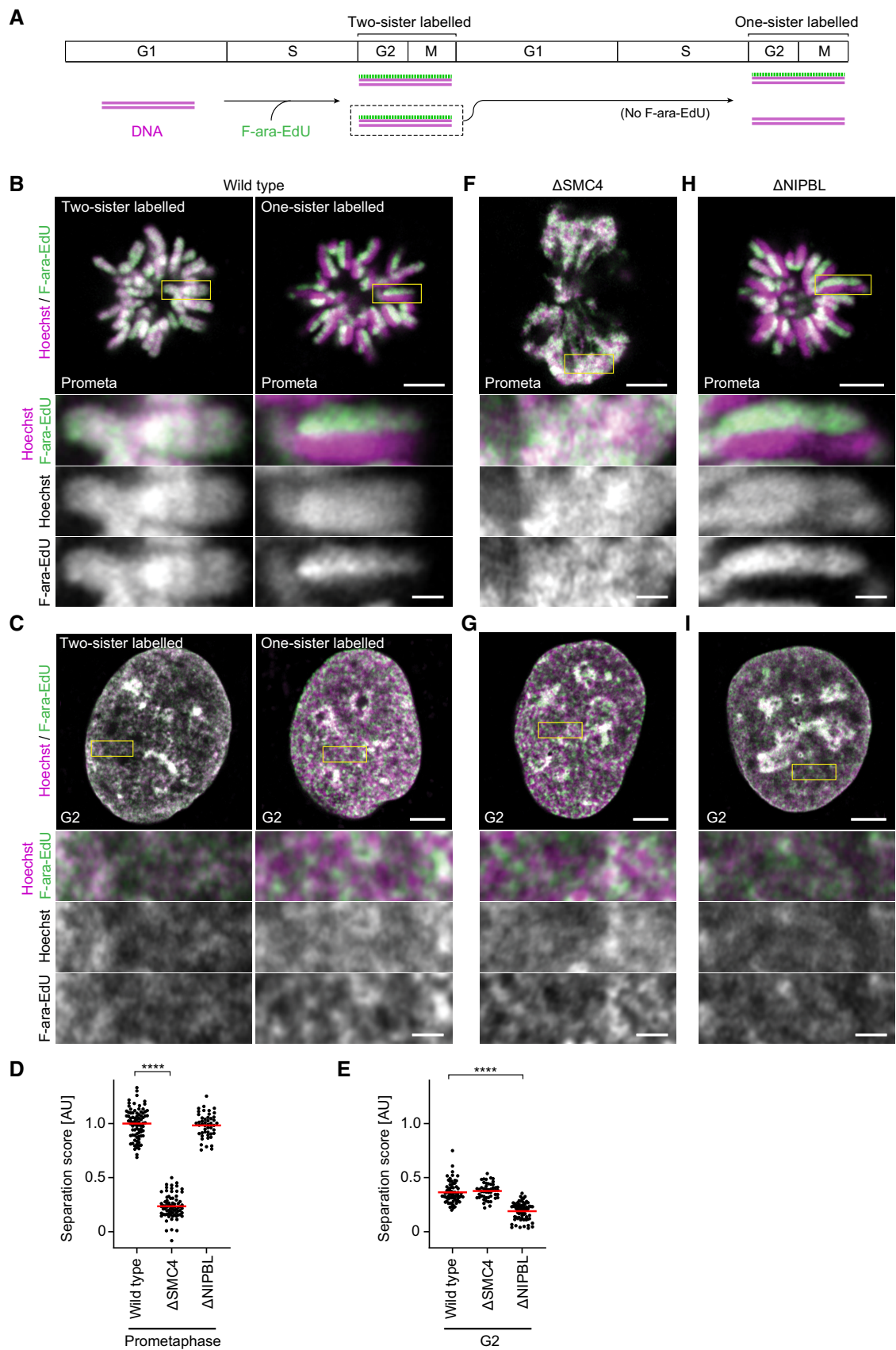


Figure 1.

investigation of sister chromatid resolution under various experimental conditions (Fig 1B–I).

We first assessed the organisation of sister chromatids in wild-type cells synchronised to G2 by the CDK1 inhibitor RO-3306 (Vassilev *et al*, 2006). Imaging cells with single sister chromatids labelled revealed substantial separation of F-ara-EdU and Hoechst 33342 that was not visible in G2 cells with both sister chromatids labelled (Figs 1C and E, and EV1I). Indeed, the separation score for one sister-labelled wild-type G2 cells was 0.36 ± 0.10 (mean $\pm$ SD), indicating that more than a third of the sister resolution observed in prometaphase cells occurs prior to mitotic entry (Fig 1D and E). By immunofluorescence detection of histone 3 phosphorylation and cyclin B1 localisation, we confirmed that these cells had not entered mitosis (Fig EV2A–D). Analysis of wild-type cells without RO-3306 treatment gave similar results (Fig EV2E and F), showing that such separation was not due to secondary effects resulting from CDK1 inhibition. Thus, even though sister chromatids do not form discrete bodies in interphase nuclei, they resolve extensively before cells enter mitosis.

The resolution of sister chromatids in G2 might be mediated by partial activation of condensin II, which binds to chromatin already prior to mitotic entry (Hirota *et al*, 2004; Gerlich *et al*, 2006a; Ono *et al*, 2013). To test whether the resolution of sister chromatids in G2 cells depends on condensin, we used auxin-induced degradation to deplete SMC4, an essential component of the condensin complex, such that cells progressed through S and G2 phase in the absence of condensins (Fig EV3A–D). Condensin depletion completely abrogated the formation of resolved cylindrical chromosomes in the following mitosis (Fig 1D and F), yet it had no detectable effect on the resolution of sister chromatids in G2 cells (Figs 1E and G, and EV3E–H). Thus, while our data confirm that condensin is an essential factor for sister chromatid resolution in mitosis (Nagasaka *et al*, 2016), the premitotic resolution of sister chromatids in G2 nuclei must be driven by other factors.

Given that cohesin continuously forms DNA loops throughout interphase (Gassler *et al*, 2017; Rao *et al*, 2017; Schwarzer *et al*, 2017; Wutz *et al*, 2017), we investigated its role in sister chromatid resolution. To determine the effect of cohesin-mediated DNA looping, we used auxin-mediated degradation to deplete NIPBL, a cofactor that is essential for cohesin-mediated loop extrusion *in vitro* (Davidson *et al*, 2019; Kim *et al*, 2019) and cohesin-mediated looping in cells (Schwarzer *et al*, 2017; Mitter *et al*, 2020). By adding the auxin analogue 5-Ph-IAA after cells had progressed through S phase, we induced NIPBL depletion to levels undetectable by Western blotting in G2-synchronised cells (Fig EV4A–D). While the AID-tagged cell line showed reduced NIPBL levels already prior to 5-Ph-IAA addition compared with wild-type cells, previous characterisation has shown that this residual background degradation does not impair sister chromatid cohesion (Mitter *et al*, 2020). The depletion of NIPBL substantially reduced sister chromatid resolution in G2 cells, yet in the subsequent mitosis sister chromatids were fully resolved (Figs 1D, E, H and I, and EV4E–H). Thus, an initial resolution of sister chromatids during interphase depends on cohesin-mediated DNA looping, whereas in the subsequent mitosis condensin takes over to promote more extensive resolution into cylindrical sister chromatids.

Hyperactivating cohesin's looping processivity results in mitosis-like sister chromatids in G2 phase

That sister chromatids resolve less in G2 compared with mitosis might be due to the different processivities by which cohesin and condensin extrude DNA loops. Loop-forming cohesin is released from interphase chromatin after about 10–20 min by the protein WAPL (Gerlich *et al*, 2006b; Tedeschi *et al*, 2013), limiting DNA loop size (Gassler *et al*, 2017; Haarhuis *et al*, 2017; Wutz *et al*, 2017). In contrast, condensin II stably associates with mitotic chromatin (Gerlich *et al*, 2006a; Walther *et al*, 2018) to form DNA loops that are substantially larger than those of interphase cells (Naumova *et al*, 2013; Gibcus *et al*, 2018), with individual loops strengthened by the binding of multiple condensins (Goloborodko *et al*, 2016a). To test whether cohesin's capacity to resolve sister chromatids in G2 can be further tuned to promote more extensive sister chromatid resolution, we therefore searched for ways to specifically increase cohesin-mediated DNA looping.

The size of DNA loops formed by cohesin during interphase can be increased by depleting WAPL (Gassler *et al*, 2017; Haarhuis *et al*, 2017; Wutz *et al*, 2017), which also relocalises cohesin to axial structures on chromatin (Tedeschi *et al*, 2013). By inhibiting cohesin release, WAPL depletion leads to an increase in the amount of chromatin-associated cohesin, which could also potentially increase the amount of cohesive cohesin if the depletion is induced before cells complete DNA replication. Prior analyses of cells depleted of WAPL by RNAi reported a lower degree of sister locus separation in interphase (Eykelboom *et al*, 2019) and less separation of whole sister chromatids in mitosis (Gandhi *et al*, 2006; Kueng *et al*, 2006; Nagasaka *et al*, 2016; Eykelboom *et al*, 2019), but the extent to which these phenotypes are caused by effects on cohesion or loop extrusion has remained unclear.

To investigate more specifically how increasing cohesin-mediated DNA looping affects the global resolution of sister chromatids in G2 cells, we adapted our synchronisation regime such that we could selectively deplete WAPL in G2 (Appendix Fig S1A). Using this synchronisation procedure, we degraded WAPL homozygously tagged with FKBP12^{F36V} (Nabet *et al*, 2018; here on referred to as dTAG) after cells had completed DNA replication (Appendix Fig S1B–D), thereby excluding potential effects of WAPL depletion on cohesion establishment, which can only occur during DNA replication (Uhlmann & Nasmyth, 1998). WAPL depletion in G2 cells increased the resolution of sister chromatids compared to control cells, while it also reorganised sister chromatids into parallel threads (Fig 2A–D). Codepletion of AID-tagged SMC4 together with dTAG-WAPL did not affect this organisation (Appendix Fig S1E–I; Fig EV5A–C), demonstrating that these structures form due to the activity of cohesin-mediated looping, independently of condensin. Furthermore, immunofluorescence-based detection of H3-S10 phosphorylation confirmed the G2 state of WAPL depleted cells with highly resolved sister chromatids (Fig EV5D and E). Thus, when its loop-extruding processivity is increased, cohesin has the remarkable ability to resolve interphase sister chromatids to an extent that normally only occurs during mitosis—in the absence of condensins and other mitotic activities.

Polymer simulations suggest that SMC-mediated loop extrusion organises DNA into a bottlebrush-like structure around a central axis of SMC protein complexes. Repulsion between the two

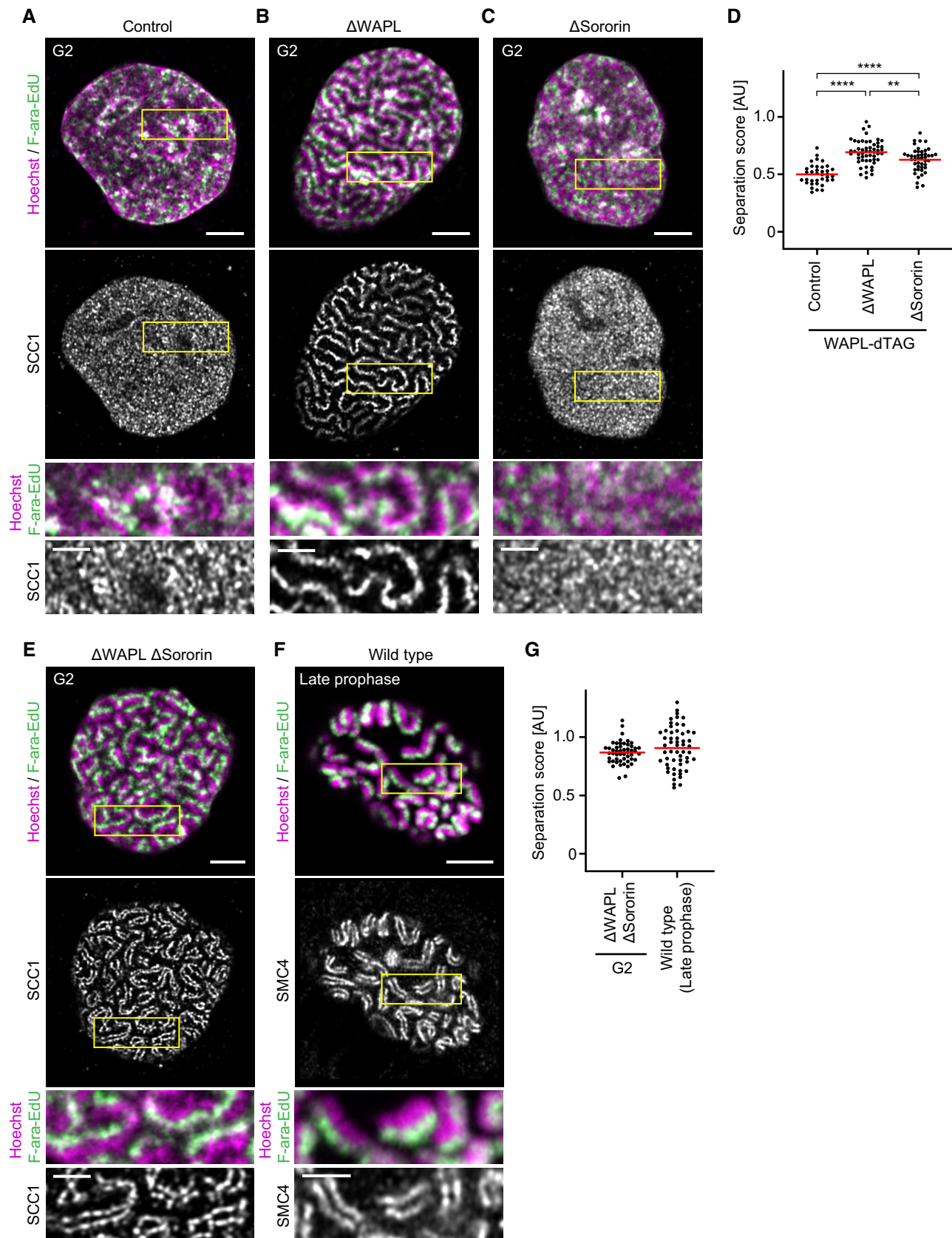


Figure 2.

Figure 2. Hyperactivating cohesin's looping processivity results in mitosis-like sister chromatids in G2 phase.

A schematic of the synchronisation scheme used to generate Δ WAPL G2 cells can be found in Appendix Fig S1A. WAPL was depleted in G2 phase after completion of DNA replication to avoid potential effects of WAPL depletion on cohesion establishment.

A–G One sister chromatid was labelled per chromosome as in Fig 1 and SMC4 (wild-type late prophase cells) or SCC1 (WAPL-dTAG cells) was visualised by immunofluorescence.

A Representative images of control WAPL-dTAG cells synchronised to G2 by RO-3306.

B Representative images of Δ WAPL cells synchronised to G2 by RO-3306.

C Representative images of Δ Sororin cells synchronised to G2 by RO-3306.

D Quantification of sister chromatid separation as in Fig 1D and E. Control WAPL-dTAG G2 ($n = 36$ cells), Δ WAPL G2 ($n = 53$ cells) and Δ Sororin G2 ($n = 45$ cells) cells were analysed. Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 3.89 \times 10^{-11}$ (Control G2, Δ WAPL G2), $P = 5.90 \times 10^{-7}$ (Control G2, Δ Sororin G2), $P = 2.75 \times 10^{-3}$ (Δ WAPL G2, Δ Sororin G2).

E Representative images of Δ WAPL Δ Sororin cells synchronised to G2 by RO-3306.

F Representative images of wild-type cells synchronised to late prophase by release from a RO-3306-mediated G2 arrest.

G Quantification of sister chromatid separation as in Fig 1D and E. Δ WAPL Δ Sororin G2 cells ($n = 51$ cells) and wild-type late prophase ($n = 55$ cells) cells were analysed. Dots represent individual cells; red bars indicate the mean.

Data information: (****) $P < 0.0001$; (**) $P > 0.01$; two-tailed Mann–Whitney U test. Biological replicates: (A–G) Control WAPL-dTAG G2 ($n = 3$), Δ WAPL G2 ($n = 3$), Δ Sororin G2 ($n = 3$), Δ WAPL Δ Sororin G2 ($n = 3$), wild-type late prophase ($n = 4$). Technical replicates: (A–G) Control WAPL-dTAG G2 ($n = 6$), Δ WAPL G2 ($n = 6$), Δ Sororin G2 ($n = 6$), Δ WAPL Δ Sororin G2 ($n = 6$), wild-type late prophase ($n = 7$). All images show single Z-slices from 3D-stacks. Yellow boxes indicate inset regions. To aid visualisation, the SCC1 channel is not contrast matched. Scale bars large panels: 5 μ m, insets: 2 μ m.

Source data are available online for this figure.

bottlebrushes of sister chromatids then promotes their resolution (Goloborodko *et al*, 2016a). Removal of cohesive linkages between sister chromatid arms is essential for efficient resolution to occur in such simulations (Goloborodko *et al*, 2016a). We therefore wondered how cohesin can resolve sister chromatids so extensively in WAPL-depleted G2 cells, which are expected to contain abundant arm cohesion. By visualising cohesin's core subunit SCC1 using immunofluorescence, we observed a diffuse localisation of cohesin in control G2 cells, whereas after depletion of WAPL, cohesin localised to a single axis on each chromosome (Fig 2A and B). The F-ara-EdU labelled chromatids consistently formed extended threads of DNA that localised adjacent to this cohesin axis, without intermixing across the axis with the sister chromatid not labelled by F-ara-EdU. These threads were often multiple microns in length, suggesting that adjacent DNA loops formed by cohesin consistently orient into the same direction over large genomic distances. Thus, in WAPL-depleted cells, cohesin resolves sister DNAs from a central axial location rather than forming two separate bottlebrush-like structures.

Reduced cohesion between sister chromatids might itself also function as a mechanism that promotes sister chromatid separation. To test how loss of cohesion affects the resolution of sister chromatids, we depleted Sororin, a protein required for the establishment and maintenance of cohesion (Rankin *et al*, 2005; Schmitz *et al*, 2007; Ladurner *et al*, 2016) but not for DNA looping (Davidson *et al*, 2019; Kim *et al*, 2019; Mitter *et al*, 2020) using RNAi (Fig EV5F). In the absence of Sororin, sister chromatid resolution was increased compared with control cells, but not to the extent seen in WAPL depleted cells, and sister chromatids did not form extended threads (Fig 2C and D). Thus, our data suggest that increased DNA looping processivity is a more powerful mechanism to resolve sister chromatids than removal of cohesion.

Cohesin's localisation at the interface between sister chromatids in WAPL-depleted G2 cells might result from steric constraints imposed by the cohesive pool of cohesin. To assess how cohesion affects the organisation of loop-forming cohesin, we let Sororin-depleted cells replicate in the presence of WAPL such that cohesion between sister DNAs was lost, before synchronising cells to G2 and

degrading WAPL. This perturbation reorganised cohesin into pairs of separate axes, with $86 \pm 3.1\%$ (mean $\pm$ SD) of cohesin axes split under these conditions (Fig 2E; Appendix Fig S2A), analogous to the organisation of condensins on late prophase chromosomes in unperturbed cells (Fig 2F; Appendix Fig S2A). Quantitative immunofluorescence showed that siRNA-mediated knockdown reduced nuclear Sororin fluorescence to $16 \pm 5.8\%$ (mean $\pm$ SD) of the levels in control cells (Appendix Fig S2B and C), suggesting that low levels of residual cohesion between sister chromatids might prevent complete sister axis separation, potentially at centromere regions that are known to contain particularly high levels of cohesion. At intermediate levels of Sororin depletion, we observed local variations in the extent of cohesin axis splitting upon WAPL depletion (Appendix Fig S2D and E), consistent with regional fluctuations in the amount of cohesion between sister chromatid arms, although still more than 50% of cohesin axes were single axes even when Sororin levels were reduced by more than two-thirds compared with control cells (Appendix Fig S2D and E). Codepletion of WAPL in Sororin-depleted G2 cells also resulted in a small increase in sister chromatid resolution, reaching levels of separation observed in late prophase, with a separation score of 0.87 ± 0.09 in WAPL/Sororin-depleted cells, compared to 0.69 ± 0.11 (mean $\pm$ SD) in cells depleted of WAPL alone (Fig 2D–G). Thus, although chromosome arm cohesion prevents the separation of cohesin axes, it imposes only relatively small constraints on the resolution of sister chromatid DNA.

To investigate sister chromatid organisation in more detail relative to SMC protein axes, we analysed the distribution of DNA and SMC protein complexes along line profiles oriented perpendicular to the long axis of chromosomes. Measuring the distance between the peaks of F-ara-EdU-labelled and unlabelled DNA showed that the sister chromatids of WAPL depleted G2 cells were resolved almost as far apart as in unperturbed prophase or WAPL/Sororin-depleted G2 cells (Fig 3A–D), consistent with our separation score analysis. To assess the distribution of DNA relative to the SMC axes, we measured the distance between F-ara-EdU peaks and the closest SMC axis. This showed an outward-facing displacement of sister DNA relative to a single axis of cohesin in WAPL depleted G2 cells, in contrast to a symmetrical organisation of sister DNA around each of the

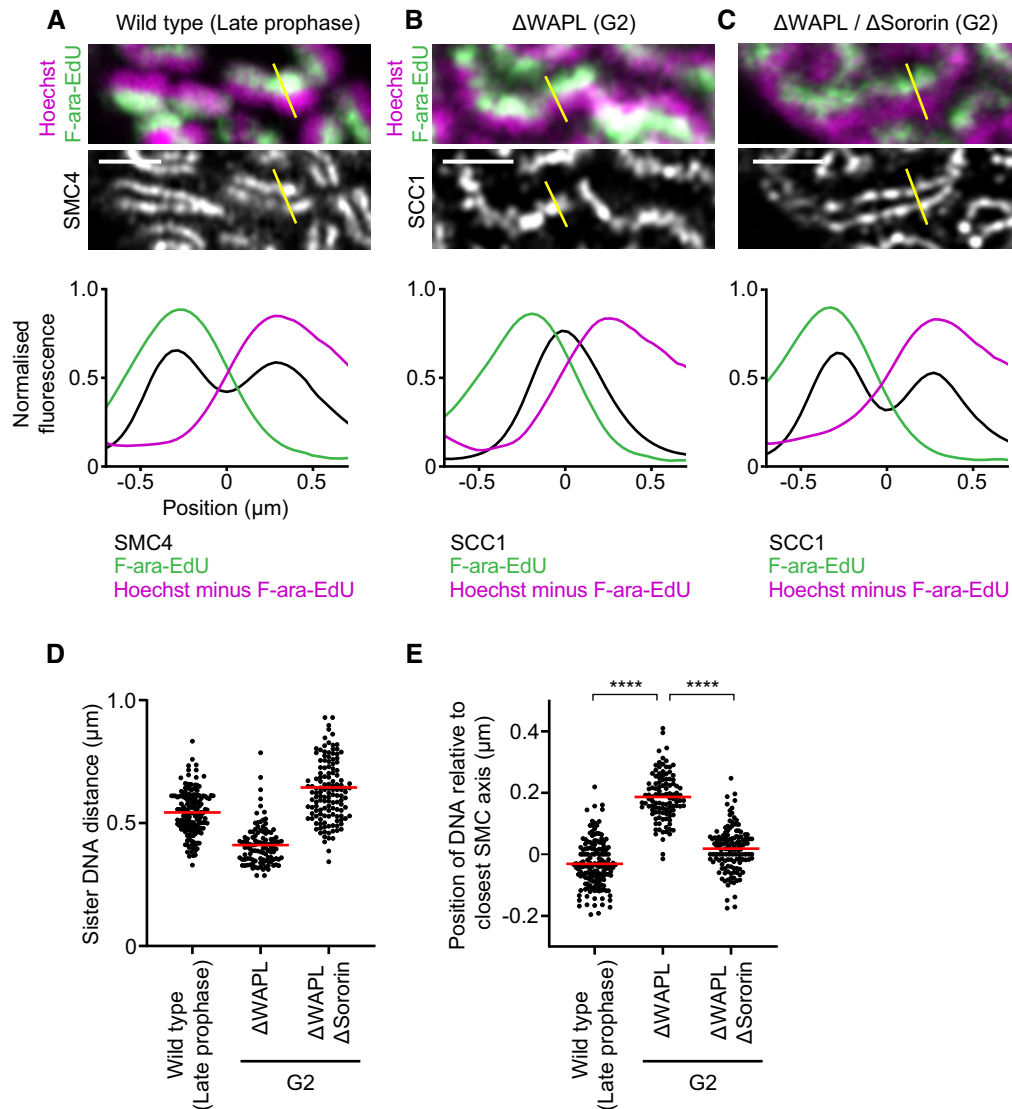


Figure 3. Organisation of sister chromatids around SMC protein axes.

A–C Analysis of sister DNA and SMC protein complex distribution in cross sections perpendicular to the long chromosome axis, as shown in Fig 2B, E and F. Density of Hoechst minus F-ara-EdU was calculated by subtracting F-ara-EdU fluorescence from Hoechst fluorescence. Line profiles indicate the mean curves of individual line profile measurements aligned to the midpoint between two SMC4 peaks (wild-type late prophase), the SCC1 peak (Δ WAPL G2 cells), or the midpoint between two SCC1 peaks (Δ WAPL Δ Sororin G2 cells). (A), Lines from wild-type late prophase ($n = 157$ lines from 31 cells). (B) Δ WAPL G2 cells ($n = 110$ lines from 18 cells). (C) Δ WAPL Δ Sororin G2 cells ($n = 138$ lines from 18 cells) were analysed.

D Measurement of the sister DNA peak-to-peak distance for line profiles as in (A–C). Dots represent individual distance measurements between chromatids; red bars indicate the mean.

E Measurement of the position of the F-ara-EdU labelled sister chromatid relative to the closest SMC peak for line profiles as in (A–C). A positive value indicates an outward displacement relative to the axis, a negative value an inward displacement. Dots represent single line profile measurements; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 5.64 \times 10^{-40}$ (late prophase); $P = 3.52 \times 10^{-34}$ (Δ WAPL Δ Sororin G2).

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological replicates: (A–E) Wild-type late prophase ($n = 4$), Δ WAPL G2 ($n = 3$), Δ WAPL Δ Sororin G2 ($n = 3$). Technical replicates: (A–E) Wild-type late prophase ($n = 7$), Δ WAPL G2 ($n = 6$), Δ WAPL Δ Sororin G2 ($n = 5$). Scale bars: 2 μ m.

Source data are available online for this figure.

split condensin or cohesin axes in unperturbed prophase cells or WAPL/Sororin-depleted G2 cells (Fig 3E). Thus, in the presence of arm cohesion, DNA loop extrusion promotes displacement of sister chromatid DNA away from a central cohesin axis, whereas under conditions of low arm cohesion, sister chromatids form two separate radially symmetrical bottlebrushes around split SMC axes.

Continuous loop extrusion is required to maintain resolved sister chromatids

Given the similarity of cohesin-organised sister chromatids in WAPL-depleted G2 cells to unperturbed mitotic prophase cells, we investigated to which extent cohesin might be able to compensate

for condensin depletion in mitotic cells. We therefore let cells progress through S and G2 phase in the absence of condensins, depleted WAPL in G2 to increase cohesin's looping processivity, before subsequently releasing cells from G2 into mitosis where they were arrested in prometaphase by STLC (Appendix Fig S3A). Under these conditions, sister chromatids still to a large extent formed chromatid threads, although their morphology was irregular with frequent kinks, and their separation score was lower than in prometaphase cells in which only WAPL was depleted (separation score of 0.94 ± 0.13 compared to 0.61 ± 0.12 (mean $\pm$ SD); Fig 4A and B). Consistent with our data from G2 cells, additionally reducing cohesin between sister chromatids by codepleting Sororin together with WAPL and condensin further increased sister chromatid resolution in prometaphase cells, with a mean separation score of 0.85 ± 0.14 , although chromosomes still had an irregular morphology (Fig 4A and B). Thus, WAPL depletion and the consequent retention of cohesin on chromosomes, can to some extent promote sister chromatid resolution in mitotic cells lacking condensin, although chromosomes do not resolve to the levels observed in unperturbed prometaphase cells and chromosome structure is perturbed.

That cohesin is unable to resolve sister chromatids to their full extent in prometaphase cells depleted of WAPL and SMC4 might be due to a lack of ongoing cohesin-mediated loop extrusion in mitotic cells. Indeed, NIPBL, which is an essential factor for cohesin-mediated loop extrusion (Schwarzer et al, 2017; Davidson et al, 2019; Kim et al, 2019; Mitter et al, 2020), dissociates from chromosomes during mitotic prophase, in wild-type and WAPL-depleted cells (Rhodes et al, 2017), raising the possibility that the cohesin retained on mitotic chromosomes no longer actively extrudes loops but instead maintains loops that preexisted before mitotic entry, and that continuous loop extrusion might be required not only to resolve sister chromatids but also to maintain them in the resolved state.

To address whether continuous loop extrusion is required for the maintenance of sister chromatid resolution in mitotic cells, we adapted our synchronisation protocol, first generating mitotic chromosomes with resolved sister chromatids and normal chromosome

morphology, before acutely depleting condensins once the cells were in prometaphase (Appendix Fig S3B). By imaging chromosomes at different time points after condensin degradation, we could therefore assess how sister chromatid resolution changed over time in cells in the absence of continuous loop extrusion (Fig 4C–E). Acute condensin depletion in prometaphase cells indeed resulted in reduced sister chromatid resolution and an increase in sister chromatid intermixing, as 120 min after inducing degradation the separation score decreased to 0.61 ± 0.21 , and further to 0.56 ± 0.23 at 240 min after induction of degradation (Fig 4E). Thus, active loop extrusion is not only required to establish sister chromatid resolution, but also to maintain it.

Genomic range of sister chromatid resolution

The segregation of entire sister chromatids during mitosis requires DNA resolution over very large genomic distances. Imaging-based approaches based on sister-chromatid-specific fluorescence labelling as described above do not inform on the genomic ranges of sister chromatid resolution, and previous visualisation of pairs of neighbouring genomic loci (Stanyte et al, 2018; Eykelenboom et al, 2019) also probe sister chromatid resolution only at a single local regime. To directly measure the genomic distance over which cohesin and condensin resolve sister chromatids, we therefore developed a new quantitative assay based on sister-chromatid-sensitive Hi-C (scsHi-C), a chromosome conformation technique that detects both intra- and inter-sister chromatid contacts based on labelling with the nucleotide analogue 4-thio-thymidine (Mitter et al, 2020, 2022).

In a closely juxtaposed and intertwined sister chromatid arrangement, the probability of a genomic locus contacting another genomic locus on the same DNA (cis sister contact) is expected to be as likely as contacting the sister DNA (trans sister contact), even over short genomic distances. With increasing degrees of sister chromatid resolution, cis sister contacts are expected to dominate trans sister contacts over increasing genomic intervals. On this basis, we calculated average cis/trans sister contact ratios over variable genomic distances and determined the interval at which cis sister contacts

Figure 4. Continuous loop extrusion is required to maintain resolved sister chromatids.

- A Representative images of one-sister labelled sister chromatids from Δ WAPL, Δ SMC4 Δ WAPL, or Δ SMC4 Δ WAPL Δ Sororin prometaphase cells, as indicated. A schematic of the synchronisation scheme used to generate Δ SMC4 Δ WAPL prometaphase cells can be found in Appendix Fig S3A. SMC4 was depleted in G1 through the addition of 5-Ph-IAA 1 h before the final release into S phase such that cells progressed through S and G2 phase in the absence of condensins. WAPL was depleted in G2 phase after completion of DNA replication to avoid potential effects of WAPL depletion on cohesin establishment.
- B Quantification of sister chromatid separation as in Fig 1D and E. Δ WAPL ($n = 21$ cells), Δ WAPL Δ SMC4 ($n = 25$ cells), and Δ WAPL Δ SMC4 Δ Sororin ($n = 28$ cells) prometaphase cells were analysed. Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 6.57 \times 10^{-8}$ (Δ WAPL); $P = 3.64 \times 10^{-7}$ (Δ WAPL Δ SMC4 Δ Sororin).
- C Representative images of control prometaphase cells labelled on one sister chromatid and depleted of SMC4 for different amounts of time as indicated. A schematic of the synchronisation scheme used can be found in Appendix Fig S3B. SMC4 was visualised by immunofluorescence using an anti-SMC4 antibody. Cells were synchronised to G2 phase by RO-3306, before being arrested in prometaphase by STLC for 60 min. SMC4 was then depleted through the addition of 1 μ M 5-Ph-IAA for 120 or 240 min, as indicated.
- D Quantification of mean SMC4 fluorescence within the chromatin mask for central Z-slices for the conditions shown in (C). Control prometaphase cells depleted of SMC4 for 0 min (Control, $n = 16$ cells), 120 min ($n = 15$ cells), or 240 min ($n = 20$ cells) were analysed. Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 2.32 \times 10^{-6}$ (SMC4 120 min); $P = 3.82 \times 10^{-7}$ (SMC4 240 min).
- E Quantification of sister chromatid separation as in Fig 1D and E. Control prometaphase cells depleted of SMC4 for 0 min (Control), 120 min, or 240 min were analysed. Sample numbers as in (D). Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 1.50 \times 10^{-5}$ (SMC4 120 min); $P = 6.28 \times 10^{-7}$ (SMC4 240 min).

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological replicates: (A, B) Δ WAPL prometaphase ($n = 3$), Δ WAPL Δ SMC4 prometaphase ($n = 3$), Δ WAPL Δ SMC4 Δ Sororin prometaphase ($n = 3$). Technical replicates: (C–E) Control ($n = 2$), SMC4 120 min ($n = 2$), SMC4 240 min ($n = 2$). All images show single Z-slices from 3D-stacks. Yellow boxes indicate inset regions. Scale bars large panels: 5 μ m, insets: 1 μ m.

Source data are available online for this figure.

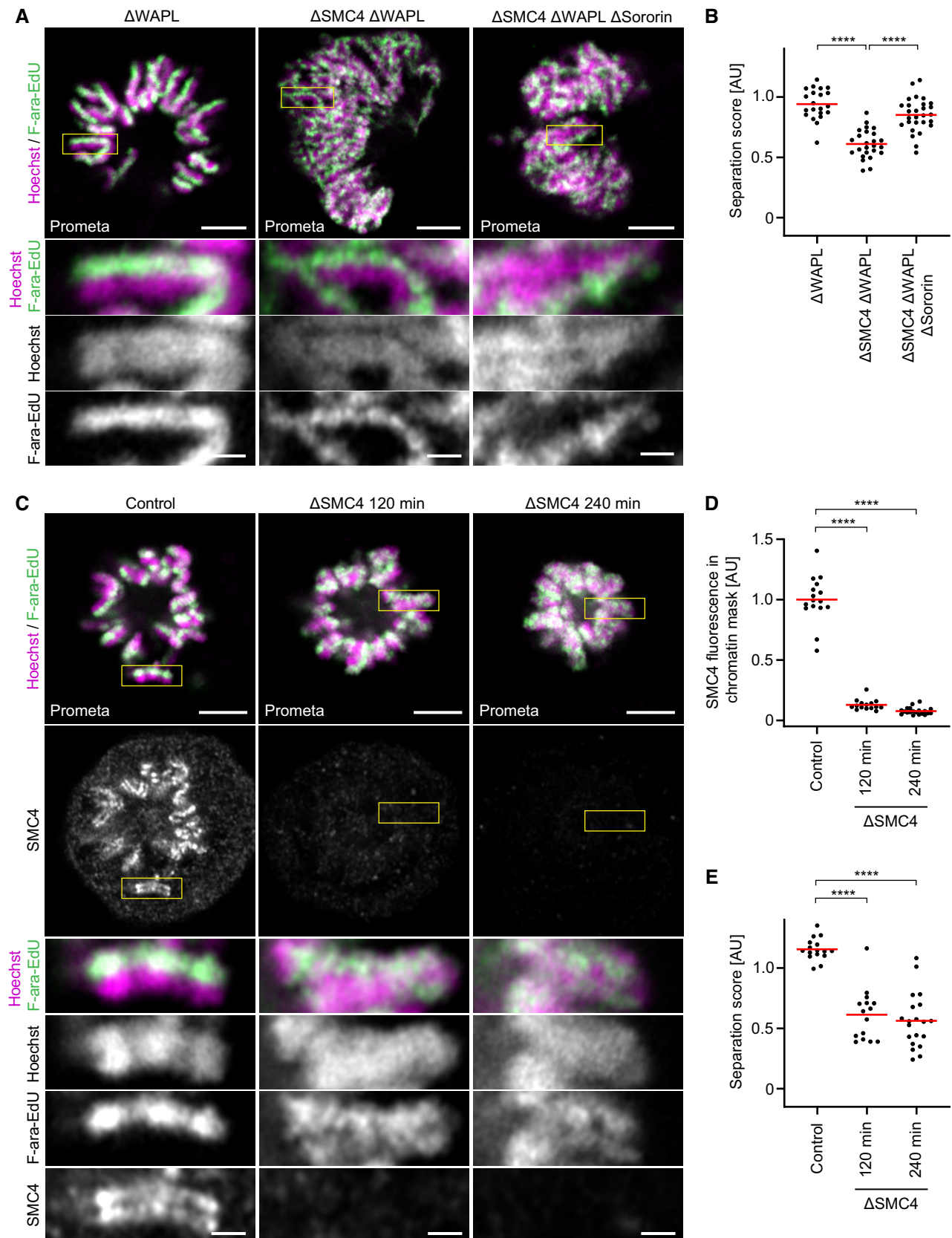


Figure 4.

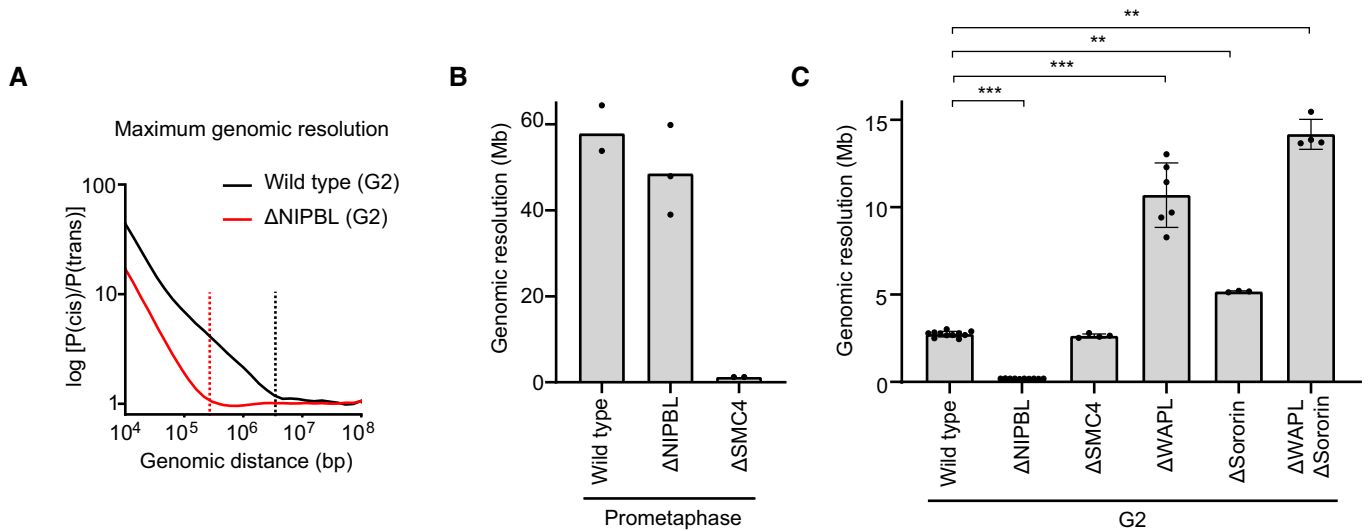


Figure 5. Genomic range of sister chromatid resolution.

A Analysis of sister chromatid resolution by sister-chromatid-sensitive Hi-C. Average contact probability curves over a range of genomic distances were calculated separately for cis and trans sister contacts (Fig EV6) to derive cis/trans sister contact ratio curves for wild-type (black) and ΔNIPBL (red) cells synchronised to G2. The genomic resolution intervals (dashed lines) were calculated by determining the genomic distance at which cis and trans sister contacts were equally abundant (at a threshold slightly above noise).

B Genomic resolution analysis for prometaphase cells. Wild-type, ΔNIPBL, and ΔSMC4 cells were analysed. Points indicate the values calculated for each replicate; bars indicate the mean.

C Genomic resolution analysis for G2 cells. Wild-type, ΔNIPBL, ΔSMC4, ΔWAPL, ΔSororin, and ΔWAPL ΔSororin cells were analysed. The wild-type and ΔSororin datasets were previously published in (Mitter et al 2020). Points indicate the values calculated for each replicate, bars indicate the mean and error bars indicate the standard deviation. Significance was tested using a two-tailed Mann-Whitney U test; $P = 1.24 \times 10^{-4}$ (ΔNIPBL G2); $P = 1.62 \times 10^{-4}$ (ΔWAPL G2); $P = 5.49 \times 10^{-3}$ (ΔSororin G2); $P = 1.47 \times 10^{-3}$ (ΔWAPL ΔSororin G2).

Data information: (***) $P < 0.001$; (**) $P > 0.01$; two-tailed Mann-Whitney U test. Biological replicates: (B) Wild-type prometaphase ($n = 2$), ΔNIPBL prometaphase ($n = 3$), ΔSMC4 prometaphase ($n = 2$). (C) Wild-type G2 ($n = 11$), ΔNIPBL G2 ($n = 10$), ΔSMC4 G2 ($n = 4$), ΔWAPL G2 ($n = 6$), ΔSororin G2 ($n = 3$), ΔWAPL ΔSororin G2 ($n = 4$).

Source data are available online for this figure.

became as likely as trans sister contacts (considering a threshold slightly above noise), to derive a genomic resolution score (Fig 5A).

With this genomic resolution assay, we analysed how cohesin and condensin contribute to sister chromatid resolution in interphase and mitosis (Fig EV6; Appendix Figs S4–S6). In wild-type prometaphase cells, sister chromatids were resolved on average to 56.9 Mb (Figs 5B and EV6A; Appendix Fig S4A). Mitotic sister chromatid resolution was almost completely suppressed by SMC4 depletion, whereas NIPBL depletion had little effect (Figs 5B and EV6B–D; Appendix Fig S4B–D), corroborating condensin's key function in promoting long-range sister chromatid resolution in mitosis.

To study genomic resolution of sister chromatids in G2, we first analysed published scsHi-C data from wild-type cells (Mitter et al, 2020), finding that their sister chromatids were resolved over 2.7 ± 0.2 Mb (Figs 5C and EV6E). We then performed scsHi-C in G2 synchronised cells depleted of NIPBL, detecting a more than 10-fold reduction in genomic resolution, whereas SMC4 depletion had no detectable effect (Figs 5C and EV6F–H; Appendix Fig S4E–H). These data confirm that the local resolution of sister chromatids in G2 depends on cohesin's loop-extruding activity but not that of condensin.

To investigate how hyperactivating cohesin's DNA loop-extrusion processivity affects sister chromatid resolution, we performed scsHi-C in G2 synchronised cells depleted of WAPL

(Fig EV6I; Appendix Fig S4I–O). WAPL depletion increased the genomic resolution almost fourfold over wild-type cells, to 10.7 ± 1.8 Mb, and resolution was further increased by codepleting Sororin (Fig 5C). However, depletion of Sororin alone had a smaller effect than WAPL depletion in the presence of Sororin, with an approximate twofold increase in maximum genomic resolution in Sororin-depleted cells compared with wild-type (Figs 5C and EV6J–L). Together, our results show that removing cohesive linkages alone promotes the local separation of sister chromatids but is not sufficient to resolve sister chromatids over large genomic distances. In contrast, increasing the DNA loop-extruding processivity of cohesin is sufficient to resolve sister chromatids over large genomic distances, even in the absence of mitotic activities, supporting that chromatin loop extrusion is a fundamental mechanism by which genomes are propagated to daughters.

Discussion

In our study, we have implemented novel assays for the detection of sister chromatid resolution based on microscopy and chromosome conformation capture. Our improved structural preservation and increased imaging resolution compared with previous work (Nagasaka et al, 2016) allowed us to study the fine structure of

sister chromatid resolution already in interphase cells, whereas our scsHi-C assays enabled direct measurement of the genomic distance over which sister chromatids resolve. In combination, these assays provide evidence for a previously underappreciated role of cohesin in sister chromatid organisation: cohesin not only holds sister chromatids together as previously known (Guacci *et al*, 1997; Michaelis *et al*, 1997; Losada *et al*, 1998; Gruber *et al*, 2003; Ivanov & Nasmyth, 2005; Watrin *et al*, 2006; Haering *et al*, 2008), but also moves them apart, most likely via its DNA loop-extruding activity, resulting in a balance between opposing forces (Fig 6A).

Our finding that increasing cohesin's looping capability by depleting WAPL induces prophase-like sister chromatid resolution is consistent with data from meiotic chromosomes where a meiosis specific variant of cohesin organises chromatids into threads (Klein *et al*, 1999; van Heemst *et al*, 1999; Zwettler *et al*, 2020; Ur & Corbett, 2021) and also with previous polymer simulations, which showed that a transition from a scarce regime with gaps between loops to a dense regime without gaps promotes efficient resolution of sister DNAs (Goloborodko *et al*, 2016a, 2016b). Counter to model predictions (Goloborodko *et al*, 2016a), however, our data show that sister chromatids can resolve their DNA over large genomic intervals even in the presence of abundant arm cohesion. In WAPL-depleted cells, sister DNAs resolve around a single cohesin axis that presumably contains both loop-forming and cohesive cohesin. Such resolution might be driven by steric repulsion between DNA loops (preprint: Polovnikov *et al*, 2022) when the directionality of loop extrusion is coordinated between neighbouring loops (Fig 6B). Whether loop-forming cohesins push and relocalise cohesive cohesins to new genomic positions, or if increasing cohesin looping promotes the removal of cohesive cohesin is an interesting open question, although our finding that cohesin is predominately organised into a single axis in the absence of WAPL argues against a strong reduction in arm cohesion.

Prior work suggested that sister chromatid resolution is impaired in WAPL-depleted cells (Gandhi *et al*, 2006; Kueng *et al*, 2006; Nagasaka *et al*, 2016; Eykelenboom *et al*, 2019). Our improved assays and time-controlled depletion, however, clarify that when WAPL is depleted after completion of DNA replication, it strongly

promotes sister DNA resolution rather than impairing it. As the sister DNAs organise around a single axis of cohesin, however, their resolution cannot be detected by conventional bulk DNA staining as in prior work (Gandhi *et al*, 2006; Kueng *et al*, 2006). Moreover, the phenotype analysis of RNAi-mediated WAPL depletion (Gandhi *et al*, 2006; Kueng *et al*, 2006; Nagasaka *et al*, 2016; Eykelenboom *et al*, 2019) is inherently limited by potential effects on both cohesin-mediated DNA looping and cohesion establishment, and therefore difficult to interpret.

We provide evidence that the resolution of sister chromatids into individual bodies does not require mitosis-specific activities, apart from increased DNA loop-extrusion processivity, while reduced cohesion between sister chromatids facilitates the additional separation of SMC-protein axes. Thus, while changes in histone posttranslational modifications (Cimini *et al*, 2003; Wilkins *et al*, 2014; Zhiteneva *et al*, 2017; Schneider *et al*, 2022) and the coating of chromosomes with a repulsive surface layer (Cuylen *et al*, 2016) are essential for proper interactions with spindle microtubules (Schneider *et al*, 2022) and nuclear assembly (Samwer *et al*, 2017; Cuylen-Haering *et al*, 2020), the crucial initial step of preseggregating sister chromatids can largely be mediated by increasing DNA loop extrusion processivity alone.

Although increasing cohesin-mediated looping processivity promotes high levels of sister chromatid resolution in G2 cells, cohesin is unable to resolve sister chromatids to their full extent in condensin-depleted mitotic chromosomes and chromosome structure is perturbed in mitotic chromosomes organised solely by cohesin, demonstrating that during mitosis condensins confer an essential functionality that cohesin cannot replace entirely. That sister chromatids become increasingly less resolved over time upon acute condensin depletion in prometaphase cells is consistent with continuous chromatin loop extrusion being necessary not only to resolve sister chromatids, but also to maintain them in the resolved state (Sen *et al*, 2016; Piskadlo *et al*, 2017).

In yeast, cohesion between sister chromatids brings sister DNAs into close spatial proximity that can result in topoisomerase II-mediated catenation (Farcas *et al*, 2011; Sen *et al*, 2016), with such catenations thought to resolve upon interaction of chromosomes

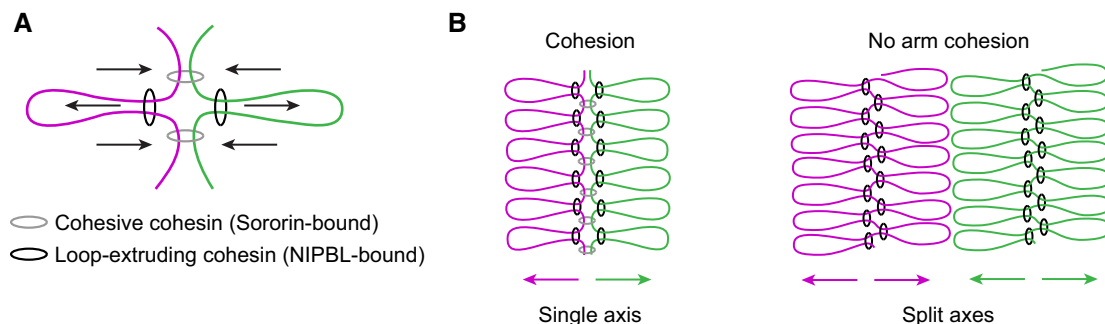


Figure 6. Models for sister chromatid organisation by cohesin.

- A Cohesin associated with NIPBL extrudes DNA loops to move sister chromatids apart, whereas cohesin associated with Sororin maintains linkages between sister chromatids.
- B Hyperactivating cohesin's loop extrusion processivity resolves sister chromatids in G2 despite the presence of arm cohesion, resulting in an asymmetrical distribution of adjacent DNA loops relative to a central cohesin axis. Additional removal of cohesion along chromosome arms promotes cohesin axis separation, resulting in a symmetrical organisation of DNA loops around two separate cohesin axes. Magenta and green indicate sister DNAs, grey and black rings indicate cohesive and loop-extruding cohesin, respectively.

with the mitotic spindle due to condensin-dependent overwinding of the DNA, that in turn promotes decatenation of sister DNAs by topoisomerase II (Sen *et al*, 2016). Similarly, in *Drosophila*, acute depletion of condensin from mitotic chromosomes results in rapid topoisomerase-dependent re-entanglement of sister chromatids (Piskadlo *et al*, 2017). Together, these results suggest that condensin-mediated changes in chromosome topology, presumably as a result of its loop extruding activity, bias topoisomerase II to productively decatenate sister chromatids, which might also play a role in the decatenation of sister DNAs upon increasing cohesin loop extruding processivity via WAPL depletion in G2 cells, despite abundant cohesion along chromosome arms.

NIPBL, which is essential for cohesin-mediated looping, dissociates from chromosomes during prophase (Rhodes *et al*, 2017), and thus the cohesin retained on prometaphase chromosomes following WAPL depletion is unlikely to be able to continue to actively extrude loops, and instead might only maintain the chromatin loops formed before mitotic entry. In prometaphase chromosomes depleted of both condensins and WAPL, it may therefore be that in the absence of continued extrusion the activity of topoisomerase is no longer biased towards decatenation, which could consequently result in increased entanglements between sister chromatids. The formation of a gapless array of consecutive loops by condensins is a prerequisite for efficient resolution in polymer simulations, and it could also be that in the absence of active loop extrusion, that gaps between loops begin to form that might also lead to reduced sister chromatid resolution.

Vertebrate cells strictly rely on condensin to resolve and segregate their sister chromatids in mitosis. Budding yeast on the other hand can to some extent resolve and segregate many chromosomal regions even in the absence of condensin, except for repetitive ribosomal DNA loci (Freeman *et al*, 2000; Schalbetter *et al*, 2017). This might be explained by the functional equivalence of cohesin- and condensin-mediated sister chromatid resolution by DNA loop extrusion, as demonstrated in our study, given that in budding yeast cohesin is not removed from chromosome arms during mitotic entry and has been shown to be necessary for chromosome compaction during G2 and mitosis (Guacci *et al*, 1997; Lazar-Stefanita *et al*, 2017; Schalbetter *et al*, 2017).

Structural maintenance of chromosomes protein complexes are also important organisers of chromosome architecture in prokaryotes. In *B. subtilis* and *C. crescentus*, the SMC-ScpAB complex facilitates the alignment and consequent individualisation of circular chromosome arms (Marbouty *et al*, 2015; Tran *et al*, 2017; Wang *et al*, 2017). This alignment depends on ParB-mediated loading of SMC complexes to ParS clusters near the replication origin, leading to the zipping up of chromosome arms that facilitates their individualisation prior to segregation. Chromosomes in *E. coli* do not undergo such zipping up but MukBEF, the *E. coli* SMC protein complex, is nonetheless essential for chromosome segregation (Hiraga *et al*, 1989). Modest overexpression of MukBEF results in the formation of a series of loops organised around an axial MukBEF core (Mäkelä & Sherratt, 2020), similar to the organisation of condensin on mitotic chromosomes, indicating that chromosomes in *E. coli* might be organised in a manner more similar to the organisation of eukaryotic mitotic chromosomes by condensins. SMC protein complexes are also known to regulate chromosome architecture in archaea (Palecek & Gruber, 2015; Takemata *et al*, 2019; Cockram

et al, 2021). Thus, DNA loop extrusion by SMC protein complexes might be an ancient and universal genome-segregating activity shared across kingdoms of life.

Materials and Methods

Cell culture

Each cell line used in this study has regularly tested negative for mycoplasma contamination. All cell lines in this study are derived from a parental HeLa cell line (Kyoto strain), which was validated using a Multiplex Human Cell line Authentication test (MCA) or from a HeLa Kyoto cell line previously described in (Schmitz *et al*, 2010). Cells were grown in Dulbecco's modified Eagle medium (DMEM, IMP/IMBA/GMI Molecular Biology Service/Media Kitchen), supplemented with 10% (v/v) fetal bovine serum (FBS): (Gibco, 10270-106, 2078432), 1% (v/v) penicillin-streptomycin (Pen/Strep): (Sigma Aldrich, P0781), GlutaMAX: (Invitrogen, 35050038), (here on referred to as wild-type medium), in combination with antibiotics, depending on the resistance of the respective cell line. To culture SMC4-AID, NIPBL-AID and WAPL-dTAG cell lines, cells were supplemented with blasticidin S ($6 \mu\text{g ml}^{-1}$, Thermo Fisher Scientific, A1113903). The WAPL-AID and Sororin-AID cell lines were supplemented with puromycin ($0.5 \mu\text{g ml}^{-1}$, Calbiochem, 540411). SMC4-AID_WAPL-dTAG cells were supplemented with blasticidin S ($6 \mu\text{g ml}^{-1}$, Thermo Fisher Scientific, A1113903) and hygromycin B (0.3 mg ml^{-1} , Roche, 10843555001). All cell lines were cultured in a humidified incubator at 37°C , with 5% CO_2 .

Genome editing using CRISPR/Cas9

The SMC4-AID cell line used in this study was published in (Schneider *et al* 2022). The Sororin-AID cell line used in this study was published in (Mitter *et al* 2020). The NIPBL-AID cell line was generated using a chimeric Cas9-human geminin fusion (Cas9-hGem), originally published in (Gutschner *et al*, 2016). A single guide RNA (sgRNA) (sequence: caccgtgtccccattactactcttg) was cloned into the Cas9-hGem expressing plasmid. To endogenously tag NIPBL at its N-terminus with a mini AID degron (mAID) (Kubota *et al*, 2013), a repair template was designed with homology arms of 682 and 685 bp flanking the insertion site 5' and 3', respectively. The repair template contained the following elements: 5' homology arm, mEGFP tag for visualisation, 2× Gly-Gly-Ser linker, mAID, Mattaj-Tandem linker, 3' homology arm. The protospacer adjacent motif (PAM) was also mutated to prevent re-editing of successfully edited clones. The repair template was produced as a gBlock® (IDT) and cloned into a donor vector (pCR2.1) for amplification. The sgRNA/Cas9 and repair template containing plasmids were cotransfected into cells using the Neon™ transfection system, using the standard manufacturer protocol for HeLa cells, as described previously (Schneider *et al*, 2022). Nine days after transfection, mEGFP-positive clones were sorted into 96-well plates. Homozygously edited clones were identified by genotyping as described previously (Samwer *et al*, 2017) using the following primers: gctagcagtgaa-gaaacaaact (forward), gctccaacagattactgaacatga (reverse). The OsTIR1^{F74G} ligase (Yesbolatova *et al*, 2020) was stably integrated into a homozygously edited clone at the “safe harbour” adeno-

associated virus integration (AAVS1) site as described previously (Li *et al*, 2019; Schneider *et al*, 2022). Clones that efficiently depleted NIPBL after integration of the ligase were identified by treating clones with 1 μ M 5-Ph-IAA (BioAcademia, 30-003) for 90 min, before analysis by flow cytometry using an iQue Screener Plus instrument. The WAPL-dTAG cell line was generated using a nickase Cas9 strategy originally described in (Ran *et al*, 2013). The guide RNA and repair template plasmids were as described previously (preprint: Nagasaka *et al*, 2022). In brief, two sgRNAs were cloned into separate nickase Cas9-expressing plasmids (guide 1 sequence: caccgctaagggtagctcggtt, guide 2 sequence: caccgtggga-gagaccacatta). To endogenously tag WAPL at its N-terminus, a repair template was designed with homology arms of 1,108 and 719 bp flanking the insertion site 5' and 3', respectively. The repair template contained the following elements: 5' homology arm, blasticidin resistance cassette, Gly-Ser-Gly linker, P2A sequence, 2 $\times$ HA tag, Gly-Ser-Gly linker, FKBP12^{F36V} (dTAG) sequence, 15 $\times$ Gly linker, 3' homology arm. The repair template was cloned into a donor plasmid (pBSKII) for amplification. The sgRNA/Cas9 and repair template containing plasmids were also cotransfected into cells using the NeonTM transfection system. Eight days after transfection antibiotic selection was performed using 6 μ g ml⁻¹ blasticidin S (Thermo Fisher Scientific, A1113903) to identify cells which had undergone productive editing. Homozygously edited clones were identified by genotyping using the following primers: gcacaagctctctggcggag (forward), gtcacagcgcaaatattacattacaccaag (reverse). Cells that efficiently depleted WAPL were identified using confocal microscopy and Western blotting after treatment of the cells with 1 μ M dTAG-7 (Tocris, 6912) for 3 h. To generate the SMC4-AID_WAPL-dTAG cell line, WAPL was endogenously tagged at its N-terminus with dTAG in the SMC4-AID cell line referenced above and published in (Schneider *et al* 2022). The same sgRNAs were used as described for the WAPL-dTAG cell line. A different repair template was used, with homology arms of 700 bp either side of the site of insertion. The repair template contained the following elements: 5' homology arm, hygromycin resistance cassette, Gly-Ser-Gly linker, P2A sequence, 2 $\times$ HA tag, 5 $\times$ Gly linker, FKBP12^{F36V}(dTAG), Mattaj Tandem linker, 3' homology arm. The plasmids were cotransfected into the cells using electroporation as described above. Eight days after transfection, productively edited cells were selected with 0.3 mg ml⁻¹ hygromycin B (Roche, 10843555001). Homozygously edited clones were identified using the same genotyping strategy as described above and cells that efficiently depleted WAPL were identified using confocal microscopy and Western blotting. The Halo-AID-WAPL_SCC1-mEGFP cell line was generated by first endogenously tagging SCC1 at its C-terminus with monomeric EGFP (L221K) using the same guides and genotyping primers described in Wutz *et al* (2017). WAPL was then N-terminally tagged with a mini-AID (amino acids (aa) 71–114) degon (Morawska & Ulrich, 2013), using the same gRNAs described above. The repair template contained homology arms of 1,108 and 719 bp 5' and 3' to the insertion site, respectively, and contained the following elements: 5' homology arm, HaloTag, Ala-Ser-Gly-Leu-Arg-Ser-Arg-Gly linker, mAID, 5 $\times$ Gly linker, 3' homology arm. Homozygously edited clones were identified using the following genotyping primers: tgattttcattccttaggccttg (forward), tacaagtgatactggcccaa (reverse). OsTIR1 was introduced into a

homozygously edited clone using lentivirus as described in (Wutz *et al* 2017) and cells were characterised as described above.

Sister chromatid labelling cell culture protocol

In all experiments, cells were grown on NuncTMLabTekTM 8-well chambered cover glass (8-well Labteks). The Labteks were treated with 0.01% Poly-L-Lysine (Sigma Aldrich, P8920) for >15 min and washed at least four times with dH₂O before seeding the cells. All cell lines were synchronised using a four block and release protocol, apart from NIPBL-AID cells, where a three block and release protocol was used instead. For a four block and release protocol, cells were first presynchronised to the G1/S boundary by incubation with 2 mM thymidine (Sigma Aldrich, T1895) in wild-type medium for 16–18 h. Cells were then washed twice with prewarmed PBS and released into fresh medium. 10–12 h after release, cells were blocked again using 3 μ g ml⁻¹ aphidicolin (Sigma Aldrich, A0781) for 16–18 h. To generate one-sister chromatid labelled cells, 10 μ M F-ara-EdU (Sigma Aldrich, T511293) was added to cells 15 h after the second release into S phase (after the first release for the NIPBL-AID cell line) to generate a pool of the compound within the cell, while the cells were in aphidicolin. The following day, cells were washed twice with prewarmed PBS and released into fresh medium containing 10 μ M F-ara-EdU. A final block with aphidicolin was then performed as described above. The following day, cells were washed twice with prewarmed PBS and released into fresh nucleotide-free medium, such that the cells became labelled on only one sister chromatid. To generate sister chromatids labelled on two chromatids, 10 μ M F-ara-EdU was added at the time of the final aphidicolin block. For the final S phase release, cells were then released into medium containing 10 μ M F-ara-EdU.

Sister chromatid labelling—G2 and mitotic arrests, and protein depletions

After labelling of one or two sister chromatids, cells were synchronised to G2 using the CDK1 inhibitor RO-3306 (Sigma Aldrich, SML0569). RO-3306 was added 5–6 h after the release from the final aphidicolin block. Protein depletions, siRNA transfections and the timing of fixation were performed as follows: Wild-type G2 samples were fixed 15 h after the release from the final aphidicolin block. To deplete SMC4 in the SMC4-AID cell line, 1 μ M 5-Ph-IAA (BioAcademia, 30-003) was added 1 h before the release from the final aphidicolin block, and samples were fixed 15 h after the release. For NIPBL-AID experiments, 1 μ M 5-Ph-IAA was added 10 h after the release from the final (third) aphidicolin block, and cells were fixed 15 h after release. To deplete WAPL in WAPL-dTAG or SMC4-AID_WAPL-dTAG cell lines, 1 μ M dTAG-7 (Tocris, 6912) was added 14 h after the release from the final aphidicolin block. To deplete SMC4 in the SMC4-AID_WAPL-dTAG cell line, 1 μ M 5-Ph-IAA (BioAcademia, 30-003) was added 1 h before the release from the final aphidicolin block. For experiments using the WAPL-dTAG or SMC4-AID_WAPL-dTAG cell lines, samples were fixed 24 h after the release from the final aphidicolin block, and siRNA transfections using XWNeg or Sororin siRNAs was performed as described below (section “siRNA transfection” of [Materials and Methods](#)). G2 samples fixed after 15 h were treated with 8 μ M RO-3306, while G2 cells fixed after 24 h were treated with 10 μ M RO-3306. To generate

prometaphase samples in the wild-type, SMC4-AID, and NIPBL-AID cell lines, 7 μ M RO-3306 was added to cells as described above. 15 h after release from the final aphidicolin block, cells were washed three times with prewarmed wild-type medium. After the final wash, cells were released into wild-type medium containing 5 μ M STLC (Enzo Life Sciences, ALX-105-011-M500), and fixed 30–60 min later. To generate prometaphase samples in the SMC4-AID_WAPL-dTAG cell line, 10 μ M RO-3306 was added to cells as described above. In Fig 4A and B, 24 h after the release from the final aphidicolin block, cells were washed three times with prewarmed wild-type medium and fixed 30–60 min later. In Fig 4C–E, cells were washed three times with prewarmed wild-type medium, before release into wild-type medium containing 5 μ M STLC for 60 min. After 60 min release, STLC-containing medium supplemented with 1 μ M 5-Ph-IAA was added to the cells to deplete SMC4. Cells were then fixed after either 120 or 240 min of treatment with 5-Ph-IAA. Control samples were fixed 60 min after washout into STLC-containing medium. For all samples where proteins were depleted, 5-Ph-IAA or dTAG-7 were added to all wash buffers and medium after they had been added to the samples.

Cell synchronisation—Measurement of Sororin fluorescence and extent of cohesin axis splitting in G2

To assess nuclear Sororin fluorescence in Sororin depleted, WAPL-depleted and WAPL/Sororin-depleted G2 cells, cells were first presynchronised to the G1/S boundary by incubation with 2 mM thymidine (Sigma Aldrich, T1895) in wild-type medium for 16–18 h. Cells were then washed twice with prewarmed PBS and released into fresh wild-type medium. 6 h after the first release, WAPL-AID cells were treated with Control (16 nM) or Sororin (0.75 or 16 nM, as specified) siRNAs as described below (section “siRNA transfection” of Materials and Methods). 10–12 h after the first release, cells were blocked for the second time using 3 μ g ml^{−1} aphidicolin (Sigma Aldrich, A0781) for 16–18 h. The following day, cells were washed twice with prewarmed PBS and released into fresh wild-type medium. 6 h after the second release, RO-3306 was added to a final concentration of 10 μ M to arrest cells in G2 phase. 14 h after the second release, 500 μ M auxin was added to the cells to deplete WAPL (WAPL-AID) or Sororin (Sororin-AID) respectively. After 10h depletion, cells were fixed and stained for immunofluorescence.

Immunofluorescence

Immunofluorescence was performed in parallel in all sister chromatid labelling experiments, before click chemistry was performed. In all experiments, cells were grown on Nunc™ LabTek™ 8-well chambered cover glass (8-well Labteks). At the time of fixation, cells were washed twice with PBS before fixation with PBS containing 4% methanol-free formaldehyde (Thermo Fisher Scientific, 28906) for 5 min. The fixative was quenched using 10 mM TRIS-HCl (Sigma Aldrich) pH 7.5 in PBS for 3 min. Cells were permeabilised by incubation with PBS containing 0.2% Triton-X-100 (Sigma Aldrich, X100-100 ml) for 5 min, washed once with PBS to remove residual detergent, and then blocked for 30 min with 0.45 μ m filtered 2% BSA (Sigma-Aldrich, A7030) in PBS (blocking buffer). Primary and secondary antibodies were diluted with blocking buffer. All primary antibody incubations were performed for >16 h at 4°C with gentle

shaking. Incubations with the anti-GFP nanobody were performed at room temperature for 2 h. After primary incubations, samples were washed 3 $\times$ 10 min with PBS, before incubation with secondary antibodies for 1 h at room temperature with gentle shaking. Samples were then washed 3 $\times$ 10 min with PBS. For samples labelled with F-ara-EdU, click chemistry staining was performed as described below (section “click chemistry” of Materials and Methods). Otherwise, Hoechst 33342 was added to a final concentration of 1.62 μ M in the wash buffer after secondary antibody incubation. Proteins were visualised using the following primary and secondary antibody combinations: Cyclin B1 was detected with a monoclonal rabbit antibody (Cell Signaling Technology, 12231S, 1:800) and visualised using a goat anti-rabbit Alexa Fluor 647 secondary antibody (Molecular Probes, A21244, 1:1,000) or detected with a monoclonal mouse antibody (SantaCruz, sc-245, 1:250) and visualised with a donkey anti-mouse Alexa Fluor 647 secondary antibody (Molecular Probes, A31571, 1:1,000). SCC1/RAD21 was detected with a monoclonal mouse antibody (Millipore, 05-908, 1:500) and visualised with a goat anti-mouse Alexa Fluor 568 secondary antibody (Molecular Probes, A11004, 1:1,000), a goat anti-mouse Alexa Fluor 488 secondary antibody (Molecular Probes, A11001: 1:1,000) or a donkey anti-mouse Alexa Fluor 647 secondary antibody (Molecular Probes, A31571, 1:1,000). SMC4 was detected using a polyclonal rabbit antibody (Abcam, ab229213, 1:250) and visualised with a goat anti-rabbit Alexa Fluor 568 secondary antibody (Molecular Probes, A11011, 1:1,000). HA-WAPL was detected using a monoclonal rabbit antibody against the HA-tag (Cell Signaling Technology, 3724S, 1:1,000) and visualised with a goat anti-rabbit Alexa Fluor 568 secondary antibody (Molecular Probes, A11011, 1:1,000). Phosphorylated Histone H3-Ser10 was detected using a monoclonal mouse antibody (Millipore, 05-806, 1:5,000) and visualised using a goat anti-mouse Alexa Fluor 568 secondary antibody (Molecular Probes, A11004, 1:1,000). Sororin was detected using a custom monoclonal mouse antibody (clone 3D5-G10, generated by the Max Perutz Labs Monoclonal Antibody Facility (E. Ogris and S. Schüchner, Medical University of Vienna), 1:50) and visualised using a goat anti-mouse Alexa Fluor 568 secondary antibody (Molecular Probes, A11004, 1:1,000). mEGFP-mAID-NIPBL and SCC1-mEGFP were detected and visualised with an anti-GFP nanobody booster (Chromotek, gba488-100, 1:250).

Click chemistry

All immunofluorescence staining was performed before the click chemistry reaction. Visualisation of F-ara-EdU labelled sister chromatids was performed using Molecular Probes Click iT Cell Reaction Buffer Kit (ThermoFisher, C10269), in combination with AF-488-Picoyl-Azide (Jena Bioscience, CLK-1276-1). 10x Click iT reaction buffer was diluted 1 in 10 with monoQ H₂O to make a 1 $\times$ solution freshly before use. A 1 $\times$ solution of Click iT buffer additive was prepared with the same diluent freshly before use. The click reaction cocktail was prepared by mixing the following components (in the same order as they are written here) according to the manufacturer's instructions: 1 $\times$ Click-iT reaction buffer, AF-488-Picoyl-Azide (5 μ M final concentration), copper sulphate (2 mM final), 1 $\times$ Click-iT buffer additive. The reaction cocktail was added to cells for 30 min with gentle rocking on a shaker, protected from light. The cocktail was removed, and the cells incubated for 5 min with 0.45 μ m filtered 2%

BSA in PBS. Cells were then washed twice with 1.62 μ M Hoechst 33342 for 3×10 min to stain DNA, and then stored at 4°C until imaging.

siRNA transfection

siRNA transfections using Sororin and XWNeg siRNAs were performed in 8-well Labteks. siRNAs were transfected using lipofectamine RNAiMax (Life Technologies, 13778150) according to the manufacturer's instructions. siRNAs were used in microscopy experiments at a concentration of 16 nM unless otherwise stated. Sororin was targeted using a custom silencer select siRNA originally published in (Schmitz *et al* 2007) (sense strand, GCCUAGGUGUC-CUUGAGCUtt, Ambion, including a 3' tt overhang for increased efficiency). XWNeg was used as a custom silence select siRNA (sense strand, UACGACCGGUCUAUCGUAGtt, Ambion, including a 3' tt overhang for increased efficiency). For sister chromatid labelling experiments, the transfection mix was added to cells 6 h after the third release (see above, sister chromatid labelling for details). The transfection mix was left on the cells for 16–18 h, before being washed out and replaced with wild-type medium. For scsHi-C experiments, siRNA transfections using the same siRNAs were performed as described, but in 25 cm² flasks, and using 32 nM siRNAs.

Labelling cells with HaloTag TMR Ligand

SMC4 and WAPL were stained using HaloTag TMR Ligand (Promega, G8251) in Δ SMC4 or Δ WAPL cells and control cells for 30 min (1:1,000 dilution in wild-type medium). Cells were then washed three times with fresh wild-type medium. The medium was exchanged once more, and cells left for 30 min in the absence of ligand before fixation as described above (section: Immunofluorescence).

Microscopy and image processing

All sister chromatid labelling experiments were imaged on a custom Zeiss LSM 980 microscope fitted with an additional Airyscan2 detector, using a 63 $\times$ NA 1.4 oil DIC Plan-Apochromat (Zeiss) objective and ZEN 3.3 Blue 2020 software. Experiments were performed with optimal sectioning (150 nm between each Z-slice) using the AiryScan SR (Super Resolution) mode. 3D Airyscan processing was performed for each acquired image using default parameters. Airyscan processed images were then registered to correct for chromatic shift from the microscope using either TetraSpeckTM Fluorescent Microspheres Size Kit (mounted on slide) (Thermo Fisher Scientific, T14792, 0.2 μ m bead size) or TetraSpeckTM Microspheres, 0.2 μ m (Thermo Fisher Scientific, T7280, prepared in-house and spread onto glass slides) in combination with a custom Fiji (Schindelin *et al*, 2012) script. A four colour Z-stack image of the bead slide was acquired using the same Z-section interval and zoom as the images to be registered, before running the script to register the cells in X, Y and Z dimensions. As the AiryScan processing procedure added an additional 10,000 grey pixel values to each processed image, 10,000 pixel values were subtracted from each registered image in Fiji using the 'Subtract' module before quantification. Experiments to measure protein depletions were performed on a custom Zeiss LSM 780 microscope using a 63 $\times$ NA 1.4 oil DIC Plan-Apochromat (Zeiss) objective and ZEN Black 2011 software.

Image analysis—Separation score

The separation score for each condition was calculated using a custom pipeline written in Python. In brief, the pipeline performed the following steps. For each processed Z-stack image, the central slice was calculated by determining the centre of mass of the chromatin (Hoechst) channel, using the scipy "ndimage" module (Virtanen *et al*, 2020). A subset of slices around the central slice (5 above, 5 below, 11 slices in total) was chosen for the F-ara-EdU and Hoechst channels for each image. For each chosen slice, the Hoechst channel was segmented using Otsu thresholding to generate a chromatin mask. By default, objects that touched the image border were excluded using the "clear_border" functionality from the skimage "segmentation" module (van der Walt *et al*, 2014). These cells were subsequently analysed separately with the "clear_border" parameter switched off. Small objects and cellular debris were excluded from the mask with a size filter using the "remove_small_objects" functionality from the skimage "morphology" module (van der Walt *et al*, 2014). The chromatin mask was applied to the F-ara-EdU and Hoechst channels, and all pixel values within the mask were extracted. The Spearman correlation coefficient (SCC) between the two channels was then calculated to give a single SCC value per slice. This operation was performed for each chosen slice, before subsequently calculating the mean SCC per cell. The SCC value per cell for each condition was then normalised relative to the mean SCC values of wild-type two (0 value) and one (1 value) sister labelled prometaphase cells, to generate the separation score metric.

Image analysis—Distribution of sister DNAs around SMC protein axes

Line profiles were drawn across single or split SMC protein axes in Fiji using the line profile tool with a line width of 5 pixels. All cells analysed were one-sister chromatid labelled. The line profiles were drawn across axes in a consistent way, moving always from the F-ara-EdU labelled chromatid to the unlabelled chromatid. The line profile coordinates and pixel intensities at each position were then extracted and saved as a csv file using a custom Fiji script. All line profile regions of interest (ROIs) from this analysis were also saved. These data were then further analysed using a custom Python script. The line profile distances and pixel intensities for the SCC1/SMC4, F-ara-EdU and Hoechst channels were extracted from the csv file. A min/max normalisation was performed on each channel to account for the differences in raw signal intensity. To overcome the limitation that only one sister chromatid was specifically labelled using F-ara-EdU in our protocol, the normalised F-ara-EdU signal was subtracted from the normalised Hoechst signal at positions where the normalised F-ara-EdU values were greater than the normalised Hoechst values. This subtraction operation generated a separate "Hoechst minus F-ara-EdU" plot profile. The Hoechst minus F-ara-EdU profile (here on referred to simply as Hoechst) was then again min/max normalised to rescale the data. A polynomial fit was then performed on the normalised SCC1/SMC4, F-ara-EdU and Hoechst profiles using the numpy "poly1d" operation. Peaks were then identified for the three profiles using the scipy 'signal' module (Virtanen *et al*, 2020). For WAPL-depleted cells, only profiles with a single peak for the SCC1, F-ara-EdU and Hoechst

channels were considered for the downstream analysis. For all other conditions, where split axes are expected, only profiles with two peaks for the SCC1/SMC4 channel and a single peak for the F-ara-EdU and Hoechst channels were considered. The amount of sister chromatid resolution around single or split SMC protein axes was calculated by measuring the peak-to-peak distance of the normalised F-ara-EdU and Hoechst profiles. To calculate the radial displacement of sister chromatids relative to a single axis, the distance between the SCC1 peak and the F-ara-EdU peak was calculated. To calculate the radial displacement of sister chromatids relative to split SMC protein axes, the distance between the F-ara-EdU peak and the closest SCC1/SMC4 axis was calculated. To plot the data, normalised Hoechst and F-ara-EdU mean curves were aligned relative to single or split SMC protein axes. For resolved sister chromatids around a single cohesin axis, for each set of profiles the peak of the normalised SCC1 channel was identified (as described above) and the normalised F-ara-EdU and Hoechst channels aligned relative to this. For cells with split axes, the two peaks for the normalised SCC1 or SMC4 profile were identified and the midpoint between the two peaks calculated. The normalised Hoechst and F-ara-EdU profiles were then aligned relative to this midpoint.

Image analysis—Measurement of mean pixel intensities, single cells

To measure mean intensities of single cells, a custom Python script was used. For mean intensity measurements, the central slice of each Z-stack was first determined as described above, by identifying the centre of mass of the chromatin (Hoechst) channel using the `scipy "ndimage"` module (Virtanen *et al*, 2020). Mean intensity measurements were then taken for each channel in this central slice. For all measurements, the Hoechst channel was segmented to create a mask. For mean intensity measurements, G2 and prophase cells were segmented using Li thresholding and prometaphase cells using Otsu thresholding. The mask was then applied to all channels of interest and mean fluorescence within the mask calculated. In Fig 4D, background fluorescence in the SMC4 channel was calculated by measuring fluorescence in noncell regions of four prometaphase cells and calculating the mean. These data were then normalised relative to the mean background fluorescence of the SMC4 channel (0 value) and mean SMC4 fluorescence of control (no 5-Ph-IAA treatment) prometaphase cells (1 value) for the conditions of interest. In Figs EV2B and 5D, background fluorescence in the phospho-H3-Ser10 channel was calculated by measuring fluorescence in nonchromatin regions of five prometaphase cells and calculating the mean. These data were then normalised relative to the mean background fluorescence of the phospho-H3-Ser10 channel (0 value) and mean phospho-H3-Ser10 fluorescence of prometaphase cells (1 value) for the conditions of interest. In Fig EV2D, the background fluorescence in the cyclin B1 channel was calculated by measuring fluorescence in non-cell regions of five G2 cells and calculating the mean. These data were normalised relative to the mean background fluorescence of the cyclin B1 channel (0 value) and the mean nuclear cyclin B1 fluorescence of prophase cells (1 value). In Appendix Fig S2E, the background fluorescence in the Sororin channel was calculated by measuring fluorescence in noncell regions of five Δ WAPL G2 cells and calculating the mean. These data were then normalised relative to the mean background fluorescence of

the Sororin channel (0 value) and the mean nuclear Sororin fluorescence of Δ WAPL G2 cells (1 value).

Image analysis—Measurement of mean pixel intensities, fields of cells

Fields of cells were analysed using a custom Python script. Before measuring mean fluorescence to assess protein depletion efficiency, the following criterion was pre-established: only interphase cells were considered for the analysis. A size filter was applied to the data to exclude mitotic cells, so that the range of measured values was not distorted by proteins which either dissociate from chromosomes during mitosis (e.g., NIPBL), or are enriched on chromosomes during mitosis (e.g., SMC4). A Gaussian blur was applied to the Hoechst channel ($\sigma = 1.0$) using the `skimage "filters"` module and the Hoechst channel was then segmented using Li thresholding. Cells which touched the border of the image were excluded using the "clear_border" functionality from the `skimage "segmentation"` module. Individual masks were labelled and applied to each cell in the field. Overlapping cells were distinguished using the "watershed" functionality of the `skimage "segmentation"` module. The area of the nuclear mask and mean fluorescence within the mask was then calculated. In Fig EV3D, Appendix Fig S1F and S4J, wild-type cells were stained with HaloTag TMR Ligand and the mean Halo-TMR fluorescence within the segmented nuclei then calculated. These data were then normalised relative to the mean nuclear Halo-TMR fluorescence of wild-type cells (0 value) and control cells (1 value). In Fig EV4D, Appendix Fig S1D and G, and S2C, background fluorescence in the channel of interest was calculated by measuring fluorescence in noncell areas of three fields and calculating the mean. These data were then normalised relative to the mean background fluorescence of the channel of interest (0 value) and the mean nuclear fluorescence of the protein of interest in control cells (1 value).

Image analysis—Calculation of percentage labelled sister chromatids per cell in prometaphase cells

For wild-type two and one-sister chromatid labelled prometaphase cells, sister chromatid pairs were identified using the Hoechst (to identify DNA) and SMC4 (to identify axes) channels. With only these two channels switched on, line profiles were drawn in Fiji (5-pixel line width) along the length of the chromatid pair. The length of each line for the Hoechst channel was then measured for each line in the cell, the ROIs saved, and the measurements saved as a csv file. For each measured line, the F-ara-EdU channel was then switched on, and the length of 0, 1 or 2 sister chromatid labelled fragments measured within the original measured line, and then saved as a csv file. A custom Python script was then used to sort and group the data such that the percentage of 0, 1 or 2-sister labelled fragments was calculated on a per cell basis.

Image analysis—Calculation of percentage split SMC axes per cell

Cohesin or condensin axes were identified using the SCC1/SMC4 channels. Line profiles were then drawn in Fiji (5-pixel line width) using the segmented line tool to trace along the length of the axes.

The length of each drawn axis was first measured along the entire length of the line for each line in the cell, the ROIs saved, and the measurements saved as a csv file. For each measured line, the length of segments with single (Δ WAPL) or split (Δ WAPL Δ Sororin, late prophase) axes was then measured, the ROIs saved, and the measurements saved as a csv file. A custom Python script was then used to group the data to determine the percentage of single or split SMC axes on a per cell basis for each condition.

Image analysis—Classification of late prophase cells

Cells were identified as late prophase through immunostaining to determine cyclin B1 and SMC4 mean intensity and localisation, in addition to staining with Hoechst 33342 to assess chromosome morphology. Mean nuclear cyclin B1 fluorescence was determined in a central Z-section as described above, and the cells classified into three classes (early, mid, late prophase) based on mean nuclear cyclin B1 intensity. After this stratification, cells were manually verified based on chromosome morphology, extent of SMC4 axis formation and localisation of cyclin B1.

Expression of full-length recombinant Sororin protein

Hi5 insect cells were used to overexpress full length Sororin protein (with a N-terminal His tag) from a pGB expression vector at 27°C. The cell pellet was harvested 3 days after proliferation arrest. Pellets from 2 l of culture were resuspended in 150 ml IMAC Buffer A (50 mM HEPES pH 7.5, 1,000 mM NaCl, 1 mM TCEP, 20 mM Imidazole) + 200 μ l benzonase, 0.15% NP-40 and 3 tablets of protease inhibitors (Roche, EDTA free). Lysate was cleared by centrifugation at 21,000 g for 30 min at 4°C. Cleared lysate (SN) was applied (1 ml/min) to a 5 ml HisTrap column, which had been equilibrated with IMAC buffer A. After loading, the column was washed with IMAC buffer A until UV returned to baseline; followed by 20 ml of 4% IMAC B buffer (50 mM HEPES pH 7.5, 500 mM NaCl, 1 mM TCEP, 500 mM Imidazole). The protein was eluted with 100% IMAC buffer B. The eluate from IMAC was treated with 3C protease in a molar ratio of 1:100 (protease: protein), left to incubate while dialysing against Dialysis buffer (PBS pH 7.4, 20 mM Imidazole, $\pm$ 1 mM) for 18 h in the cold room with constant mixing. The sample from the 3C treatment was reloaded (flow rate 1 ml/min) onto a 5 ml HisTrap FF column equilibrated with RI buffer A (PBS pH 7.4, 20 mM Imidazole, $\pm$ 1 mM) for reverse chromatography in which the Tag-free protein eluted in the flow through. The flow through from reverse IMAC was concentrated to 4 ml using a Vivaspin 20 concentrator (MWCO 10 kDa) and then loaded onto a HiLoad 16/60 Superdex 200 pg previously equilibrated with SEC buffer (PBS pH 7.4, 5% Glycerol) at a flow rate of 1 ml per minute. To increase the purity of the sample, the SEC fractions containing the Tag-free Sororin were pooled together, the salt concentration was adjusted, and then applied to a 1 ml Resource S column equilibrated with RS buffer A (50 mM Phosphate pH 7.4, 50 mM NaCl). The column was washed with 10 ml of RS buffer A and then eluted in a gradient to 50% of RS buffer B (50 mM Phosphate pH 7.4, 1 M NaCl) for 20 ml. The Sororin-containing fractions with the highest purity from IEXC were pooled together and concentrated to 2 ml using a Vivaspin 20 concentrator (MWCO 10 kDa) and then loaded onto a

HiLoad 16/60 Superdex 200 pg previously equilibrated with SEC buffer at a flow rate of 1 ml per minute.

Generation of monoclonal mouse anti-Sororin antibody

50 μ g of recombinant, full-length Sororin in Freund's adjuvant was subcutaneously injected into one mouse. After three immunisations, 30 μ g of Sororin (without adjuvant) was intravenously injected as a final boost. After 4 days, splenocytes were fused with the myeloma cell line X63-Ag8.653 using polyethylene glycol. The cells were seeded into 96-well plates, and fused hybridoma cells were selected in HAT-containing growth medium. Hybridoma mix clone supernatants were screened for Sororin-specific antibodies by ELISA and Western blot against full-length Sororin. Positive clone 3D5 was subcloned to give the single clone 3D5-G10.

scsHi-C cell culture

Cell culture for G2 scsHi-C was performed as described in (Mitter et al 2020, 2022). Wild-type medium without selection antibiotics was used to synchronise the cells, and all chemicals used were diluted in this medium to the specified concentration. All centrifugation steps before formaldehyde fixation were performed at 1,100 g, and all centrifugations after fixation at 2,500 g. In brief, asynchronous cells were synchronised to the G1/S boundary with 2 mM thymidine (Sigma Aldrich, T1895) for 16–18 h. The following day, cells were washed twice with prewarmed PBS, before the addition of fresh wild-type medium. 10–12 h postrelease, 3 μ g ml⁻¹ aphidicolin (Sigma Aldrich, A0781) was added to the cells to arrest them at the G1/S boundary. 2 mM 4-thiothymidine (4sT) (Carbosynth, NT06341) was also supplemented to the medium at this stage. 16–18 h later, cells were washed twice with prewarmed PBS before the addition of 2 mM 4sT. For all G2 samples shown in Fig 5, 10 μ M RO-3306 (Sigma Aldrich, SML0569) was added to the cells 5–6 h after the second release, and samples were harvested 24 h after the second release. Experiments with protein depletions were performed as follows: For SMC4-AID experiments, 5-Ph-IAA (BioAcademia, 30-003) was added 1 h before the second release to a final concentration of 1 μ M. For NIPBL-AID experiments, 5-Ph-IAA was added 8 h after the second release to a final concentration of 1 μ M. For WAPL-AID experiments, indole-3-acetic-acid (auxin) (Sigma Aldrich, I5148) was added to a final concentration of 500 μ M 14 h after the second release. For all samples where proteins were depleted, auxin or 5-Ph-IAA were added to all wash buffers and medium after they had been added to the samples. For WAPL-AID experiments, siRNA transfections using XWNeg or Sororin siRNAs was performed as described above (section “siRNA transfection” of [Materials and Methods](#)), 6–7 h after the release from the thymidine block. To harvest cells, cells were washed twice with PBS, with banging against a solid support each time to remove dead and mitotic cells. Cells were then trypsinised to detach them from the culture flask before quenching with wild-type medium. Collected cells were spun down and the pellet washed once with PBS. Cells were spun down, PBS removed, and the cells were fixed with PBS containing 1% methanol-free formaldehyde (Thermo Fisher Scientific, 28906) for 4 min with gentle rotation. Cells were spun down, the formaldehyde removed and 20 mM TRIS-HCl pH 7.5 in PBS added to quench residual formaldehyde. Samples were spun down once more, the

liquid removed, and samples stored at -20°C until further processing. To generate prometaphase samples, cells were treated as described above until the addition of RO-3306. Cells were also cultured in 75 cm^2 rather than 25 cm^2 flasks to generate sufficient cell numbers for the experiment. RO-3306 was added to a final concentration of $7\text{ }\mu\text{M}$ to cells 7 h after the second release. Before adding RO-3306, cells were washed twice with PBS and banged against a solid support to remove dead and mitotic cells. 14 h after the second release, cells were washed three times with wild-type medium. After the final wash, wild-type medium containing 200 ng ml^{-1} nocodazole (Sigma Aldrich, M1404) was added to the cells. 60 min later, mitotic cells were detached from the culture flask by banging the flask against a solid support. The medium was then collected, cells spun down and washed once with PBS, before fixation with 1% formaldehyde as described above.

Library prep and scsHi-C sample preparation

Hi-C sample preparation was performed as described in (Mitter *et al* 2020, 2022). All centrifugations were performed at $2,500\text{ g}$, apart from those in the ethanol precipitation step, which were performed at $21,000\text{ g}$. Frozen cell pellets were thawed and then lysed using ice cold Hi-C lysis buffer (10 mM TRIS-HCl pH 8 [Sigma], 10 mM NaCl [Sigma], 0.2% Nonidet P-40 substitute [Sigma], $1\times$ complete EDTA-free protease inhibitor [Roche] for 30 min at 4°C). The DNA was then digested using Dpn II (375 U DpnII [NEB, R0543 T/M/L] in $1\times$ Dpn II buffer [NEB, B0543S] for 16 h at 37°C with shaking at 800 rpm on a thermal block. Cells were centrifuged, the supernatant discarded, and fill-in mix added ($38\text{ }\mu\text{M}$ biotin-14-dATP [Thermo Fisher Scientific, 19524016], $38\text{ }\mu\text{M}$ dCTP [Thermo Fisher Scientific, R0152], dGTP [Thermo Fisher Scientific, R0162], dTTP [Thermo Fisher Scientific, R0172], 50 U Klenow Polymerase [NEB, M0210L], $1\times$ NEB 2 buffer, B7002S). Cells were then incubated for 1 h at 37°C with gentle rotation. Cells were then spun down, the supernatant discarded, ligation mix added ($1\times$ T4 DNA ligase buffer [Thermo Fisher Scientific, B69], 0.1% Triton X-100 [Sigma, X100-100 ML], $100\text{ }\mu\text{g ml}^{-1}$ BSA [Sigma, A7030-10G], 50 U T4 DNA ligase [Thermo Fisher Scientific, EL0011]) before incubation at room temperature for 4 h with gentle rotation. Cells were spun down, the supernatant discarded, and the pellet resuspended in $200\text{ }\mu\text{l}$ PBS. Genomic DNA (gDNA) was then purified using the DNeasy Blood and Tissue Kit (Qiagen, 69506) according to the manufacturer's instructions for tissue culture cells. During gDNA purification, after addition of lysis buffer, RNaseA (Qiagen, 19101) and proteinase K (Qiagen, 19131), samples were incubated at 65°C for 6 h to reverse crosslinks, before continuing with the rest of the protocol. The purified DNA was then sheared on a Covaris S2 instrument (duty cycle 10%, intensity 5.0, cycles/burst 200) for 25 s. The DNA was sheared in Covaris microTUBEs (Covaris, 520045). Size-selection was then performed to isolate fragments of the correct size using AMPure XP beads (Beckmann Coulter, A63881). A double size selection was performed according to the manufacturer's instructions. In brief, $0.8\times$ sample volume of beads was first added to the beads, the supernatant was then transferred to a new tube and $0.12\times$ sample volume beads was then added. The final size-selected DNA was resuspended in dH_2O . A biotin-pull down was then performed to select biotin-labelled DNA fragments using Dynabeads MyOne Streptavidin C1 beads (Thermo

Fisher Scientific, 65001), resuspended in biotin binding buffer (5 mM TRIS-HCl pH 7.5 [Sigma], 0.5 mM EDTA [AppliChem, 1103.1000], 1 M NaCl [Merck]). Beads were incubated with the samples for 1 h at room temperature with gentle rotation before two washes with Tween wash buffer (5 mM TRIS-HCl, pH 7.5 [Sigma], 0.5 mM EDTA [AppliChem, 1103.1000], 1 M NaCl [Merck], 0.05% Tween20 [Sigma, P1379-250ML]). A final wash with dH_2O was then performed to remove residual detergent before the library preparation. The beads were then resuspended in dH_2O , and library prep performed on the beads using the NEBNext Ultra II DNA library prep kit for Illumina (NEB, E7645L), according to the manufacturer instructions. The beads were then washed $4\times$ with Tween wash buffer. Elution of DNA from the beads was performed by incubation with 95% formamide (Sigma Aldrich, F9037-100ML), 10 mM EDTA (AppliChem) at 65°C for 2 min. Samples were then ethanol precipitated (80% EtOH (Sigma, 32221-1L/2.5L), 100 mM sodium acetate pH 5.2 (Sigma Aldrich, 71180-1 kg), incubation at -70°C for $>16\text{ h}$). $1\text{ }\mu\text{L}$ GlycoBlue (Thermo Fisher Scientific, AM9515) was also added during the precipitation to better visualise the pellet. Samples were spun down, the supernatant removed, washed once with 75% EtOH, spun down once more and then resuspended in dH_2O . 4sT was then converted to methyl-cytosine using $\text{OsO}_4/\text{NH}_4\text{Cl}$ as described in (Mitter *et al*, 2020, 2022). EtOH precipitation was then performed once more to the converted samples as described above. Finally, qPCR was performed to amplify libraries using the NEBUltra Ultra II DNA library prep kit for Illumina (NEB, E7645L). The final libraries were purified using AMP Pure XP beads (Beckmann Coulter, A63881) at $0.55\times$ sample volume, according to the manufacturer's instructions.

Western blotting

For scsHi-C experiments, 5–10% of the harvested sample was set aside for Western blot analysis. $2\times$ Laemmli buffer (Bio-Rad, 1610737), 10 mM DTT (Roche, 10708984001) was added to the samples, before incubation at 95°C for 10 min. For cells grown in 12-well or 24-well plates, Laemmli buffer was added directly to the well and the sample harvested, before incubation at 95°C for 10 min. Before loading, samples were spun down once at $21,000\text{ g}$ for 5 min. Samples were separated using Nu-Page 4–12% BisTris cassettes (Invitrogen, NP0326BOX) in $1\times$ MES running buffer (Invitrogen, NP0002) according to the manufacturer's instructions, apart from blots against NIPBL which were separated using 3–8% Tris-Acetate cassettes (Invitrogen, EA0375BOX) and $1\times$ Tris acetate running buffer (Invitrogen, LA0041) according to the manufacturer's instructions. Samples were then transferred to a $0.45\text{ }\mu\text{m}$ polyvinylidene (PVDF) membrane (activated using EtOH or MeOH) using a wet transfer ($1\times$ transfer buffer, IMP/IMBA/GMI Molecular Biology Service/Media Kitchen, 20% EtOH or MeOH, or $1\times$ NuPage™ transfer buffer, Thermo Fisher, NP0006-1, without methanol, for anti-NIPBL blots) for 1 h at room temperature. Membranes were blocked using 5% milk (w/v) (Maresi, “fixmilch” Instant milk powder), dissolved in PBS containing 0.05% Tween-20, for 30 min at room temperature. Primary and secondary antibodies were also diluted in 5% milk. Primary antibody incubations were carried out for $>16\text{ h}$ at 4°C , before $3\times 10\text{ min}$ washes with 5% milk 0.05% Tween-20 (Sigma, P1379-250ML). Secondary antibody incubations were carried out for 1 h at room temperature before $3\times$ washes with PBS

containing 0.05% Tween-20. Proteins were detected as follows: SMC4 was detected using a polyclonal rabbit antibody (Abcam, ab229213, 1:2,500). NIPBL was detected using a rat monoclonal antibody (Absea, 010702F01, 1:500). Sororin was detected using a custom rabbit antibody (1:500), kindly provided by J.-M. Peters and previously published in (Mitter *et al*, 2020). WAPL was detected using a custom rabbit antibody (Peters lab internal ID A961, 1:1,000), kindly provided by J.-M. Peters and previously published in (Tedeschi *et al*, 2013) or a mouse monoclonal antibody (Santa-Cruz Biotechnology, sc-365189, 1:1,000). GAPDH was detected using a rabbit polyclonal antibody (Abcam, ab9485, 1:2,500). Vinculin was detected using a recombinant monoclonal rabbit antibody (Abcam, ab129002, 1:15,000). The following horseradish peroxidase (HRP)-conjugated antibodies were used against antibodies raised in the requisite organism: goat anti-rat HRP (Amersham, NA935, 1:2,000), goat anti-mouse HRP (Bio-Rad, 1706516, 1:5,000), goat anti-rabbit HRP (Bio-Rad, 1706515, 1:5,000). Blots were incubated with Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, 32132) or Clarity Max Western ECL Substrate (BioRad, 1705062) and then visualised on a Bio-Rad ChemiDoc imager. Uncropped Western blots can be found in Appendix Fig S5A–N.

Flow cytometry—DNA Content analysis and cell cycle stage verification

Flow cytometry was performed to assess the cell cycle stage of all scsHi-C samples generated during this study as described in (Mitter *et al* 2020). All centrifugation steps were performed at 1,100 g for 1 min. In brief, when scsHi-C samples were harvested, 10% of the cells were set aside for flow cytometry analysis. Cells were transferred to 15-ml Falcon tubes, washed once with PBS, plus/minus auxin/arresting compounds as appropriate, spun down and then fixed with 70% EtOH (Sigma Aldrich, 32221-2.5L) at 4°C for >30 min. Samples were spun down and then permeabilised on ice for 15 min with 0.25% Triton-X-100 (Sigma Aldrich, X100-100 ML). After spinning down, samples were incubated with a mouse monoclonal anti-phospho-H3Ser10 antibody (Millipore, 05-806, 0.25 µg per sample) diluted in PBS containing 1% BSA for 1 h at room temperature. Cells were spun down, washed once with PBS containing 1% BSA, and then incubated with the secondary antibody, diluted as for the primary antibody (goat anti-mouse Alexa Fluor 488, Molecular Probes, A11001, 1:300) for 30 min at room temperature, protected from light. Cells were then stained with 200 µg ml⁻¹ RNaseA (Qiagen, 19101), 50 µg ml⁻¹ propidium iodide (Sigma Aldrich, 81845) for 30 min protected from light to determine the DNA content. Cells were analysed on either a FACS Canto II (BD Biosciences) or Penteon (Novacyte) instrument. Analysis was performed using FlowJo(v10). Cells were gated in the following way: Cells were gated by plotting FSC-A/SSC-A. Singlets were then gated by plotting FSC-A/FSC-H, and then PI-A/PI-H. Cell cycle stage was then determined by a scatter plot of FITC (H3S10P signal) and PI (DNA content). An example of the gating strategy can be found in Appendix Fig S6A–C.

Sequencing

All samples in this study were sequenced on an Illumina Novaseq 6000 instrument. Patterned SP flow cells were using with paired end sequencing (PE250 read mode).

Hi-C data preprocessing

Preprocessing of scsHi-C samples was performed using a custom Nextflow pipeline, as described in (Mitter *et al* 2020).

Scaling plot and cis/trans sister contact ratio analyses

Scaling plots for cis and trans sister chromatid contacts were generated as described in (Mitter *et al*, 2020). In Fig EV6, scaling plot analysis was performed for all G2 conditions simultaneously before visualisation of each condition independently. As multiple samples were compared at the same time, downsampling was performed using the ngs package (<https://github.com/gerlichlab/ngs>) such that the total number of cis sister and trans sister contacts separated by more than 1 Kb was equal across different samples. The different prometaphase conditions were analysed in the same way. The cis/trans ratio for each condition was calculated by dividing the average cis contact probability at a given genomic distance by the average trans contact probability at the same genomic distance. Cis/trans ratios were then plotted against genomic distance. To calculate the genomic resolution score, the cis/trans ratio for each condition was first interpolated using the “CubicSpline” operation from the scipy “interpolate” module (Virtanen *et al*, 2020). The interpolated data were then smoothed using a Savitzky Golay filter. A threshold of 1.25 (slightly above the noise of the data) was then applied to the cis/trans ratio data. The genomic resolution score was calculated by determining the first genomic distance at which the cis/trans ratio threshold was reached.

Data reporting

No statistical methods were used to predetermine sample size. The experiments were not randomised. The investigators were not blinded to allocation during experiments.

Sample number and statistical analyses

To test significance, the robust, nonparametric Mann–Whitney *U*-test was used. The statistical tests used, and exact *P*-values (wherever possible) are indicated in the figure legends. In some cases, the calculated *P*-value was beyond the precision limit of the module used. In these cases, it is noted in the figure legend and an upper bound given. For each condition analysed using microscopy in this study, the number of biological and technical replicates, and the number of cells analysed for each condition is indicated in the requisite figure legend. Technical replicates were performed in different wells of the same Labtek. For each condition analysed using scsHi-C in this study, at least two biological replicates were performed, and the number of replicates is indicated in the requisite figure legend. A summary of the cell cycle stage analysed by flow cytometry for each scsHi-C replicate in this study can be found in Appendix Tables S1 (G2 samples) and S2 (prometaphase samples). A detailed summary of the cell lines used in this study can be found in Appendix Table S3. A detailed summary of the read statistics for each scsHi-C replicate can be found in Appendix Tables S4–S10. All statistical tests were performed using scipy (Virtanen *et al*, 2020).

Published datasets

All published datasets used in this study can be found in Appendix Table S11.

Reagent availability

All plasmids generated in this study are available at Addgene. All other reagents generated in this study will be distributed upon request, in accordance with the Materials Transfer Agreement of IMBA.

Data availability

All scsHi-C datasets generated in this study have been made available via the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE227694>) under the series accession number GSE227694 and are also available from the authors upon request. The IPython notebooks used to perform analysis of microscopy and sequencing data generated within this work have been uploaded to GitHub (https://github.com/gerlichlab/Batty_et_al_EMBO.J_2023) along with a detailed description of each script and the figure panels they produce. The microscopy datasets generated in this manuscript have been made available via the Image Data Resource (IDR) (<https://idr.openmicroscopy.org>) under the accession number idr0149.

Expanded View for this article is available [online](#).

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Author contributions

Daniel W Gerlich: Conceptualization; supervision; funding acquisition; writing – original draft; project administration; writing – review and editing.

Paul Batty: Conceptualization; resources; software; formal analysis; validation; investigation; methodology; writing – original draft; writing – review and editing. **Christoph CH Langer:** Software; methodology.

Zsuzsanna Takács: Formal analysis; investigation. **Wen Tang:** Resources.

Claudia Blaukopf: Resources. **Jan-Michael Peters:** Resources; funding acquisition; writing – review and editing.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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Expanded View Figures

Figure EV1. Validation of sister chromatid resolution assay.

- A Schematic of experimental procedure for generation of two or one-sister labelled chromatids in wild-type cells. Cells were treated with the requisite compounds as indicated.
- B Bar plot indicating the percentage of chromosome segments with 0, 1 or 2 labelled sister chromatids for wild-type prometaphase cells, fixed during the first mitosis after labelling with F-ara-EdU. $n = 121$ sister chromatid pairs from 15 cells from seven replicates analysed. Bar indicates mean; error bars indicate standard deviation.
- C Bar plot indicating the percentage of chromosome segments with 0, 1 or 2 labelled sister chromatids for wild-type prometaphase cells, fixed during the second mitosis after labelling with F-ara-EdU. $n = 129$ sister chromatid pairs from 27 cells from seven replicates analysed. Bar indicates mean; error bars indicate standard deviation.
- D Schematic indicating the image analysis pipeline to calculate the sister chromatid separation score. Extract central Z-sections: The central slice of an input Z-stack is calculated, and the five slices above and below are chosen (11 slices in total). Segment chromatin, extract pixel intensities: The chromatin channel is segmented to generate a mask, which is applied to the Hoechst and F-ara-EdU channels. Pixel intensities within the mask are extracted. Pixel correlation analysis: The Spearman correlation coefficient (SCC) between Hoechst and F-ara-EdU pixel values in the mask is calculated first per slice, and then per cell. See [Materials and Methods](#) for details of the normalisation procedure to generate the separation score. Plot is an example scatter plot representation of pixel value intensities for Hoechst and F-ara-EdU fluorescence in a prometaphase cell labelled on one sister chromatid.
- E Scatter plot representation and SCC of pixel value intensities for Hoechst and F-ara-EdU fluorescence for a single slice of a wild-type prometaphase cell, fixed during the 1st mitosis after labelling with F-ara-EdU (two-sister labelled). Real data is shown.
- F Scatter plot representation and SCC of pixel value intensities for Hoechst and F-ara-EdU fluorescence for a single slice of a wild-type prometaphase cell, fixed during the 2nd mitosis after labelling with F-ara-EdU (one-sister labelled). Real data is shown.
- G Quantification of the Spearman correlation coefficient between Hoechst and F-ara-EdU for wild-type prometaphase chromosomes fixed during the 1st (two-sister labelled) or 2nd (one-sister labelled) mitosis after labelling with F-ara-EdU, as indicated. Individual data points represent the mean SCC per cell, red bars indicate the mean. $n = 59$ cells (1st mitosis), $n = 83$ cells (2nd mitosis). Significance was tested using a two-tailed Mann–Whitney U test; $P = 3.91 \times 10^{-24}$.
- H Quantification of sister chromatid separation as in Fig 1D and E, for wild-type prometaphase chromosomes fixed during the 1st (two-sister labelled) or 2nd (one-sister labelled) mitosis after labelling with F-ara-EdU. Dots represent individual cells; red bars indicate the mean. Sample numbers and statistics as in (G). Quantification for one-sister labelled cells is the same as in Fig 1D (wild-type) to allow side by side comparison with two-sister labelled prometaphase cells.
- I Quantification of sister chromatid separation as in Fig 1D and E. Two-sister labelled ($n = 44$ cells) and one-sister sister labelled ($n = 69$ cells) wild-type cells synchronised to G2 by RO-3306 were analysed. Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 4.06 \times 10^{-19}$. Quantification for one-sister labelled G2 cells is the same as in Fig 1E (wild-type) to allow side by side comparison with two-sister labelled G2 cells.

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological and technical replicates: (G–I) Two-sister and one-sister labelled wild-type prometaphase and G2 cells, as in Fig 1.

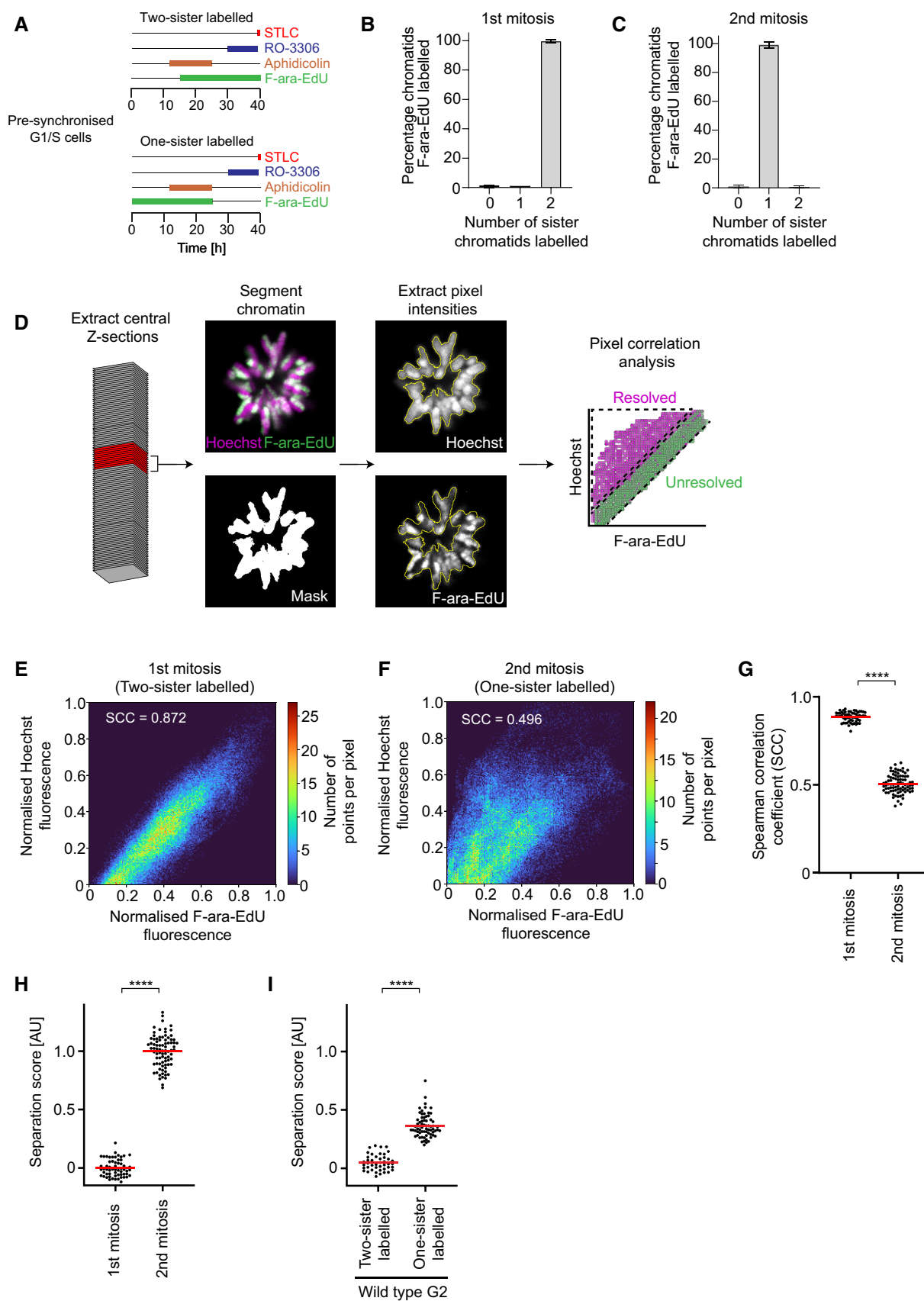


Figure EV1.

Figure EV2. Validation of cell cycle stage for sister chromatid labelling.

Cells were F-ara-EdU labelled on one sister chromatid and synchronised to different cell cycle stages as in Figs 1 and 2 to determine the abundance and localisation of the mitotic histone modification phospho-H3-Ser10 and cyclin B1 by immunofluorescence. Prometaphase and prophase cells were treated with STL, G2 cells were treated with RO-3306. Representative images for one-sister labelled sister chromatids in cells stained with an anti-phospho-H3-Ser10 antibody from wild-type prometaphase, prophase and G2 cells as indicated.

- B Quantification of mean phospho-H3-Ser10 fluorescence within the chromatin mask for central Z-slices for the conditions shown in (A). Prometaphase ($n = 48$ cells), prophase ($n = 32$ cells), and G2 ($n = 31$ cells) cells were analysed. Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 9.61 \times 10^{-12}$ (prophase), $P = 8.33 \times 10^{-14}$ (prometaphase).
- C Representative images for one-sister labelled sister chromatids in cells stained with an anti-cyclin B1 antibody, from wild-type prometaphase, prophase and G2 arrested cells as indicated.
- D Quantification of mean cyclin B1 fluorescence within the chromatin mask for central Z-slices for the conditions shown in (C). Calculation of mean fluorescence was performed as described in (B). Prometaphase ($n = 48$ cells), prophase ($n = 32$ cells), and G2 ($n = 69$ cells) cells were analysed. Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann–Whitney U test; $P = 7.95 \times 10^{-16}$ (prophase).
- E Representative images for one-sister labelled sister chromatids from G2 cells, treated with RO-3306 or untreated, as indicated.
- F Quantification of sister chromatid separation as in Fig 1D and E for one-sister labelled wild-type G2 cells, for the conditions shown in (E). RO-3306 treated ($n = 69$ cells) and untreated ($n = 16$ cells) cells were analysed. Dots represent individual cells; red bars indicate the mean. Quantification for RO-3306 treated cells is the same as in Fig 1E (wild-type) to allow side by side comparison with untreated cells. A different example RO-3306 treated cell is shown.

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological replicates: (A–F) One-sister labelled wild-type G2 (RO-3306 treated) as in Fig 1. Technical replicates: (A–D) Wild-type prometaphase ($n = 3$), wild-type prophase ($n = 4$). (E, F) Wild-type G2 (untreated) ($n = 2$). All images shown are single Z-slices from 3D-stacks. Scale bars: 5 μm .

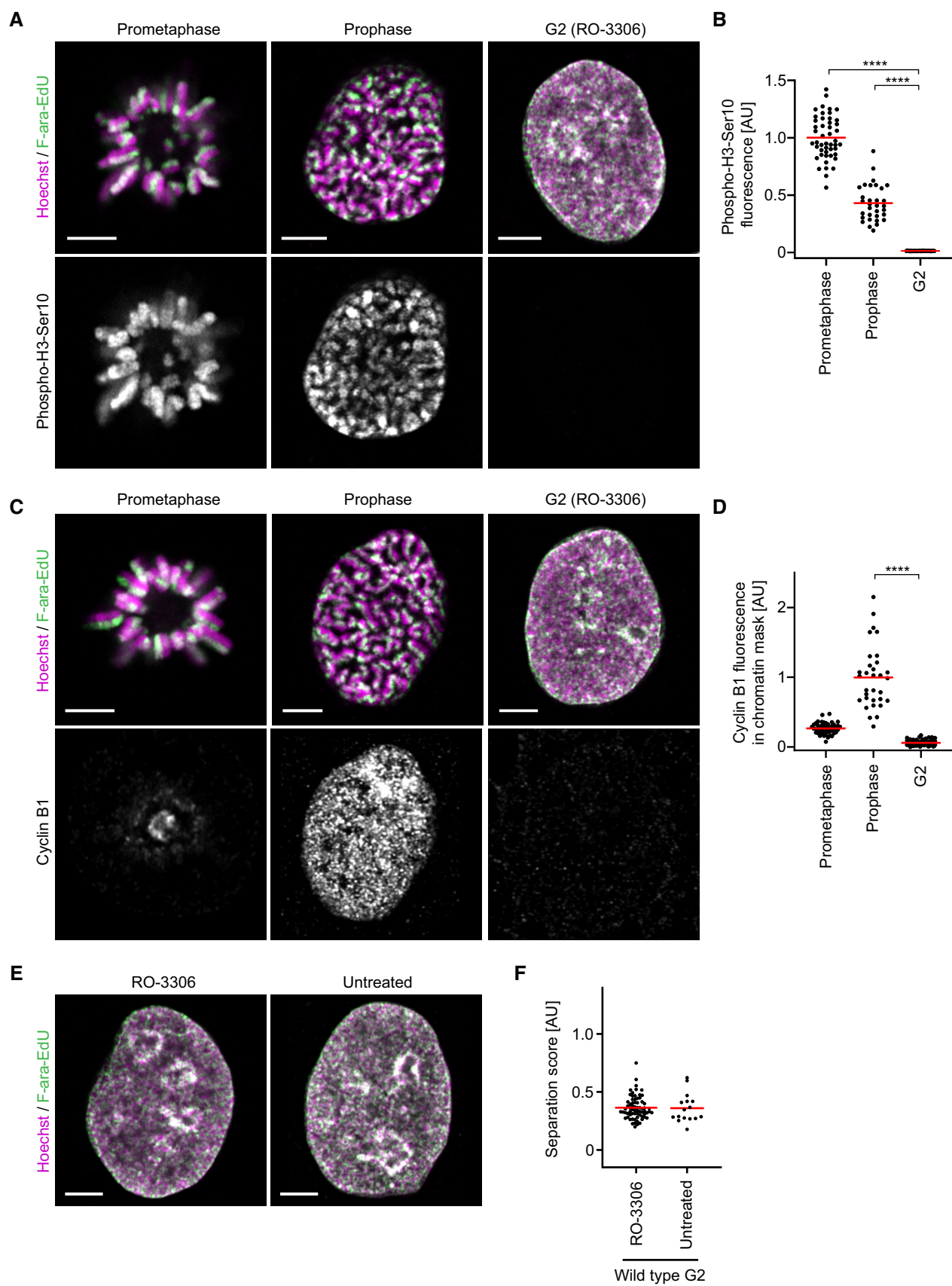


Figure EV2.

Figure EV3. Validation of protein depletion efficiency and sister labelling controls in SMC4-AID cells.

- A Schematic of experimental procedure for generation of one-sister labelled chromatids in Δ SMC4 G2 cells. Cells were treated with the requisite compounds as indicated; the end point indicates the time of fixation. Prometaphase samples were generated by washing out RO-3306 and releasing into medium containing STL. C.
- B Immunoblot analysis of SMC4 in wild-type (WT) cells, untreated SMC4-AID cells and SMC4-AID cells treated for either 1 or 3 h with 5-Ph-IAA. Representative example of $n = 3$ biological replicates.
- C Immunofluorescence analysis of HeLa cells homozygously tagged for SMC4-mAID-Halo and stably expressing OsTIR1^{F74G}. Cells were incubated for 3 h with (Δ SMC4) or without (Control) 1 μ M 5-Ph-IAA before subsequently staining of SMC4 with HaloTag TMR Ligand. DNA was stained with Hoechst 33342.
- D Quantification of mean nuclear SMC4 fluorescence per cell, as shown in (C). Dots represent individual cells; red bars indicate the mean. Wild-type cells were stained with HaloTag TMR Ligand and the mean Halo-TMR fluorescence within the segmented nuclei then calculated. Normalisation was performed relative to the mean nuclear Halo-TMR fluorescence of wild-type cells (0 value) and control SMC4-AID cells (1 value). For each condition two experimental replicates were performed. $n = 1,159$ cells analysed for control SMC4-AID cells, $n = 1,000$ cells analysed for Δ SMC4 cells. Significance was tested using a two-tailed Mann–Whitney U test; $P < 10^{-324}$ (precision limit of floating-point arithmetic).
- E Representative images from Δ SMC4 prometaphase cells labelled on one or two sister chromatids as indicated.
- F Representative images from Δ SMC4 G2 cells labelled on one or two sister chromatids as indicated.
- G Quantification of sister chromatid separation as in Fig 1D and E, for Δ SMC4 prometaphase cells labelled on one or two sister chromatids. Dots represent individual cells; red bars indicate the mean. Two-sister labelled ($n = 26$ cells) and one-sister labelled ($n = 75$ cells) cells were analysed. Quantification for one-sister labelled Δ SMC4 prometaphase cells is the same as in Fig 1D (Δ SMC4) to allow side by side comparison with two-sister labelled cells. A different example prometaphase cell labelled on one sister chromatid is shown.
- H Quantification of sister chromatid separation as in Fig 1D and E, for Δ SMC4 G2 cells labelled on one or two sister chromatids. Dots represent individual cells; red bars indicate the mean. Two-sister labelled ($n = 19$ cells) and one-sister labelled ($n = 50$ cells) cells were analysed. Quantification for one-sister labelled Δ SMC4 G2 cells is the same as in Fig 1E (Δ SMC4) to allow side by side comparison with two-sister labelled cells. A different example G2 cell labelled on one sister chromatid is shown.

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological replicates: B ($n = 3$), (E–H), Δ SMC4 two-sister labelled prometaphase ($n = 4$), Δ SMC4 two-sister labelled G2 ($n = 3$), Δ SMC4 one-sister labelled prometaphase and G2 as in Fig 1. Technical replicates: C, D ($n = 2$), (E–H) Δ SMC4 two-sister labelled prometaphase ($n = 9$), Δ SMC4 two-sister labelled G2 ($n = 5$), Δ SMC4 one-sister labelled prometaphase and G2 as in Fig 1. Images with fields of cells (panel C) are single Z-sections. Images with single cells (panels E, F) are single Z-slices from 3D-stacks. Scale bars fields: 20 μ m. Scale bars single cells, large panels: 5 μ m, insets: 1 μ m.

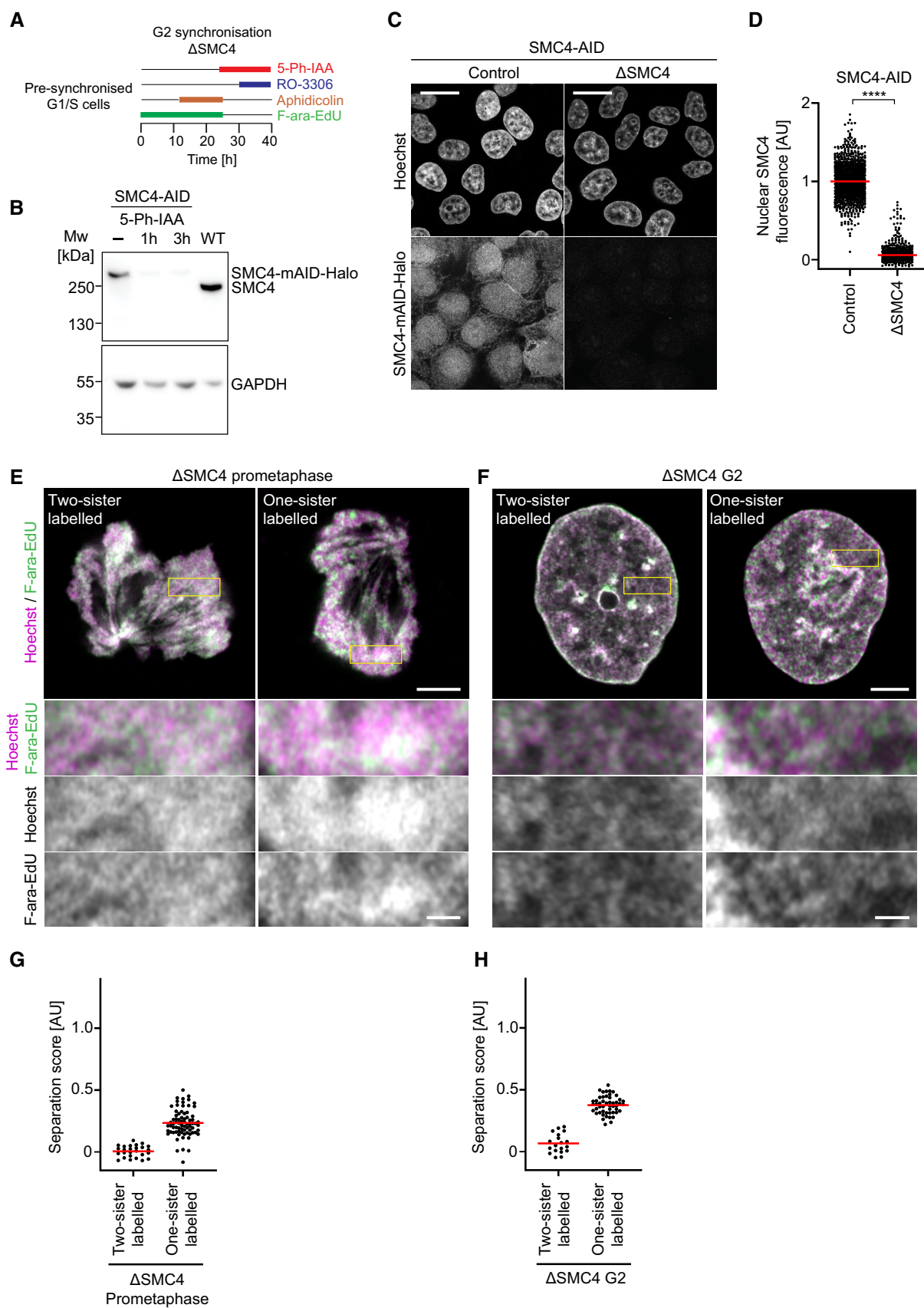


Figure EV3.

Figure EV4. Validation of protein depletion efficiency and sister labelling controls in NIPBL-AID cells.

- A Schematic of experimental procedure for generation of one-sister labelled chromatids in Δ NIPBL G2 cells. Cells were treated with the requisite compounds as indicated; the end point indicates the time of fixation. Prometaphase samples were generated by washing out RO-3306 and releasing into medium containing STLIC.
- B Immunoblot analysis of NIPBL in wild-type (WT) cells, untreated NIPBL-AID cells and NIPBL-AID cells treated for either 1 or 3 h with 5-Ph-IAA. Representative example of $n = 2$ biological replicates.
- C Immunofluorescence analysis of HeLa cells homozygously tagged for mEGFP-mAID-NIPBL and stably expressing OstIR1^{F74G}. Cells were incubated for 2 h with (Δ NIPBL) or without (Control) 1 μ M 5-Ph-IAA before subsequently fixing and staining for mEGFP with an anti-GFP nanobody. DNA was stained with Hoechst 33342.
- D Quantification of mean nuclear mEGFP-mAID-NIPBL fluorescence per cell, as shown in (C). Dots represent individual cells; red bars indicate the mean. For each condition three experimental replicates were performed. $n = 1,205$ cells analysed for control NIPBL-AID cells, $n = 1,223$ cells analysed for Δ NIPBL cells. Significance was tested using a two-tailed Mann–Whitney U test; $P < 10^{-324}$ (precision limit of floating-point arithmetic).
- E Representative images from Δ NIPBL prometaphase cells labelled on one or two sister chromatids as indicated.
- F Representative images from Δ NIPBL G2 cells labelled on one or two sister chromatids as indicated.
- G Quantification of sister chromatid separation as in Fig 1D and E, for Δ NIPBL prometaphase cells labelled on one or two sister chromatids. Dots represent individual cells; red bars indicate the mean. Two-sister labelled ($n = 24$ cells) and one-sister labelled ($n = 48$ cells) cells were analysed. Quantification for one-sister labelled Δ NIPBL prometaphase cells is the same as in Fig 1D (Δ NIPBL) to allow side-by-side comparison with two-sister labelled cells. A different example prometaphase cell labelled on one sister chromatid is shown.
- H Quantification of sister chromatid separation as in Fig 1D and E, for Δ NIPBL G2 cells labelled on one or two sister chromatids. Dots represent individual cells; red bars indicate the mean. Two-sister labelled ($n = 54$ cells) and one-sister labelled ($n = 70$ cells) cells were analysed. Quantification for one-sister labelled Δ NIPBL G2 cells is the same as in Fig 1E (Δ NIPBL) to allow side by side comparison with two-sister labelled cells. A different example G2 cell labelled on one sister chromatid is shown.

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological replicates: B ($n = 2$), C, D ($n = 2$), (E–H) Δ NIPBL two-sister labelled prometaphase ($n = 3$), Δ NIPBL two-sister labelled G2 ($n = 5$), Δ NIPBL one-sister labelled prometaphase and G2 as in Fig 1. Technical replicates: C, D ($n = 3$), (E–H) Δ NIPBL two-sister labelled prometaphase ($n = 5$), Δ NIPBL two-sister labelled G2 ($n = 8$), Δ NIPBL one-sister labelled prometaphase and G2 as in Fig 1. Images with fields of cells (panel C) are single Z-sections. Images with single cells (panels E, F) are single Z-slices from 3D-stacks. Scale bars fields: 20 μ m. Scale bars single cells, large panels: 5 μ m, insets: 1 μ m.

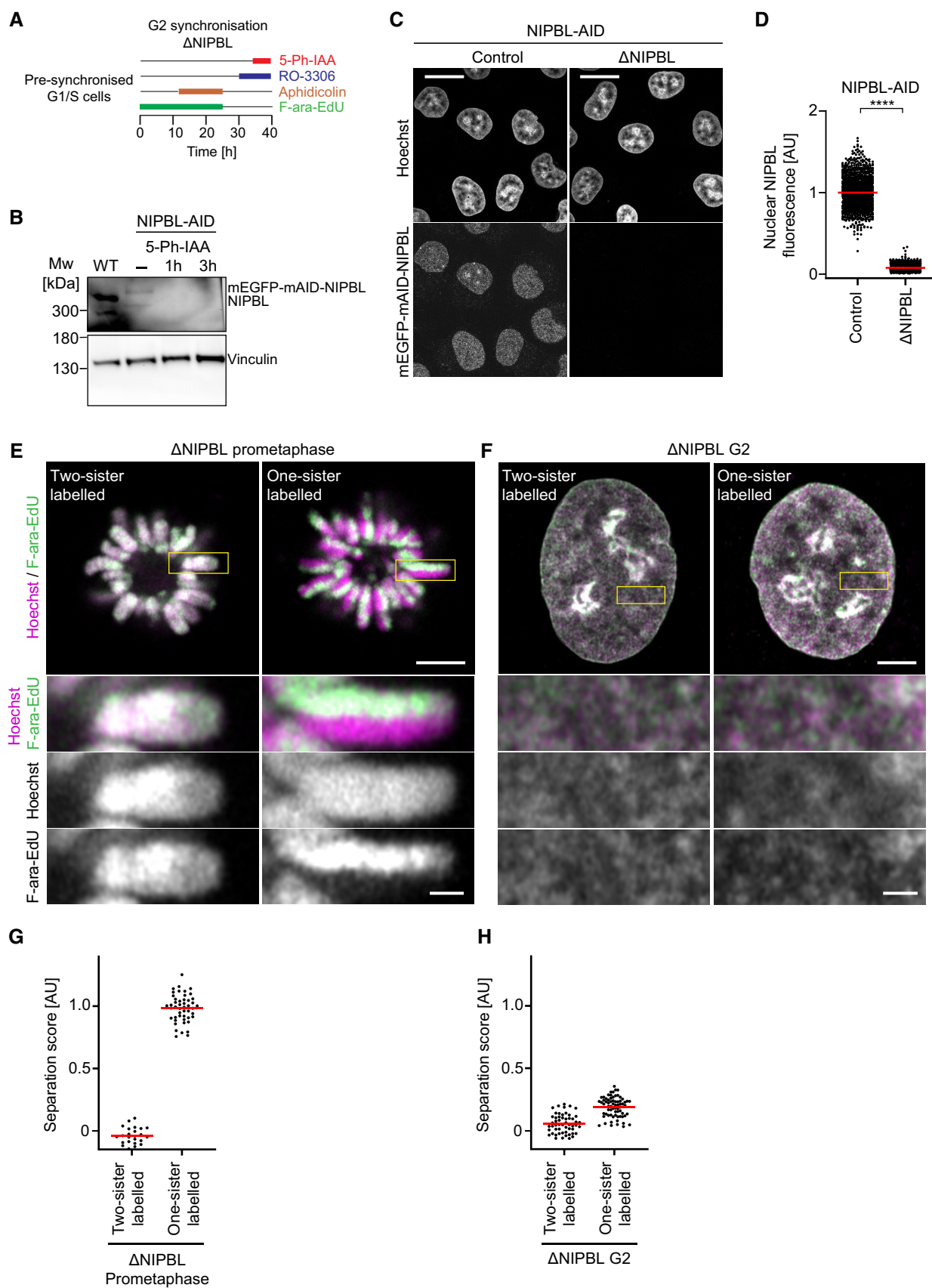


Figure EV4.

Figure EV5. Condensin is not required to resolve sister DNAs in WAPL depleted G2 cells.

- A Schematic of experimental procedure for generation of one-sister labelled chromatids in Δ SMC4 Δ WAPL G2 cells. Cells were treated with the requisite compounds as indicated; the end point indicates the time of fixation.
- B Representative images of one-sister labelled sister chromatids from Δ WAPL or Δ SMC4 Δ WAPL G2 cells, as indicated. Cells were labelled and fixed as in Fig 2B. SMC4 was depleted in G1 through the addition of 5-Ph-IAA 1 h before the final release into S phase such that cells progressed through S and G2 phase in the absence of condensins. WAPL was depleted in G2 phase after completion of DNA replication to avoid potential effects of WAPL depletion on cohesion establishment. SMC4 was visualised by staining with HaloTag TMR Ligand. Wild-type cells were stained with HaloTag TMR Ligand to determine the fluorescence background for background subtraction. The SMC4 channel is displayed after background subtraction.
- C Quantification of sister chromatid separation as in Fig 1D and E. Δ WAPL G2 ($n = 45$ cells) and Δ SMC4 Δ WAPL G2 cells ($n = 39$ cells) cells were analysed. Dots represent individual cells; red bars indicate the mean.
- D Validation of cell cycle stage in Δ WAPL cells by phospho-H3-Ser10 immunofluorescence in cells arrested in prometaphase (prometa) by STLC and cells arrested in G2 by RO-3306.
- E Quantification of mean phospho-H3-Ser10 fluorescence for central Z-stack slices for the conditions shown in (D). Dots represent individual cells; red bars indicate the mean. Δ WAPL prometaphase ($n = 19$ cells) and Δ WAPL G2 ($n = 33$ cells) cells were analysed. Significance was tested using a two-tailed Mann–Whitney U test; $P = 2.72 \times 10^{-9}$.
- F Immunoblot analysis of Sororin in WAPL-dTAG cells treated with either Control (Ctrl) or Sororin (Sor) siRNAs as indicated, as in Fig 2E. Cells were harvested 40 h after transfection. Representative example of $n = 2$ biological replicates.

Data information: (****) $P < 0.0001$; two-tailed Mann–Whitney U test. Biological replicates: (B, C) Δ WAPL G2 ($n = 3$), Δ WAPL Δ SMC4 G2 ($n = 3$). F ($n = 2$). Technical replicates: (B, C) Δ WAPL G2 ($n = 5$), Δ WAPL Δ SMC4 G2 ($n = 5$), D, E ($n = 3$). All microscopy images are single Z-slices from 3D-stacks. Yellow boxes indicate inset regions. Scale bars large panels: 5 μ m, insets: 2 μ m.

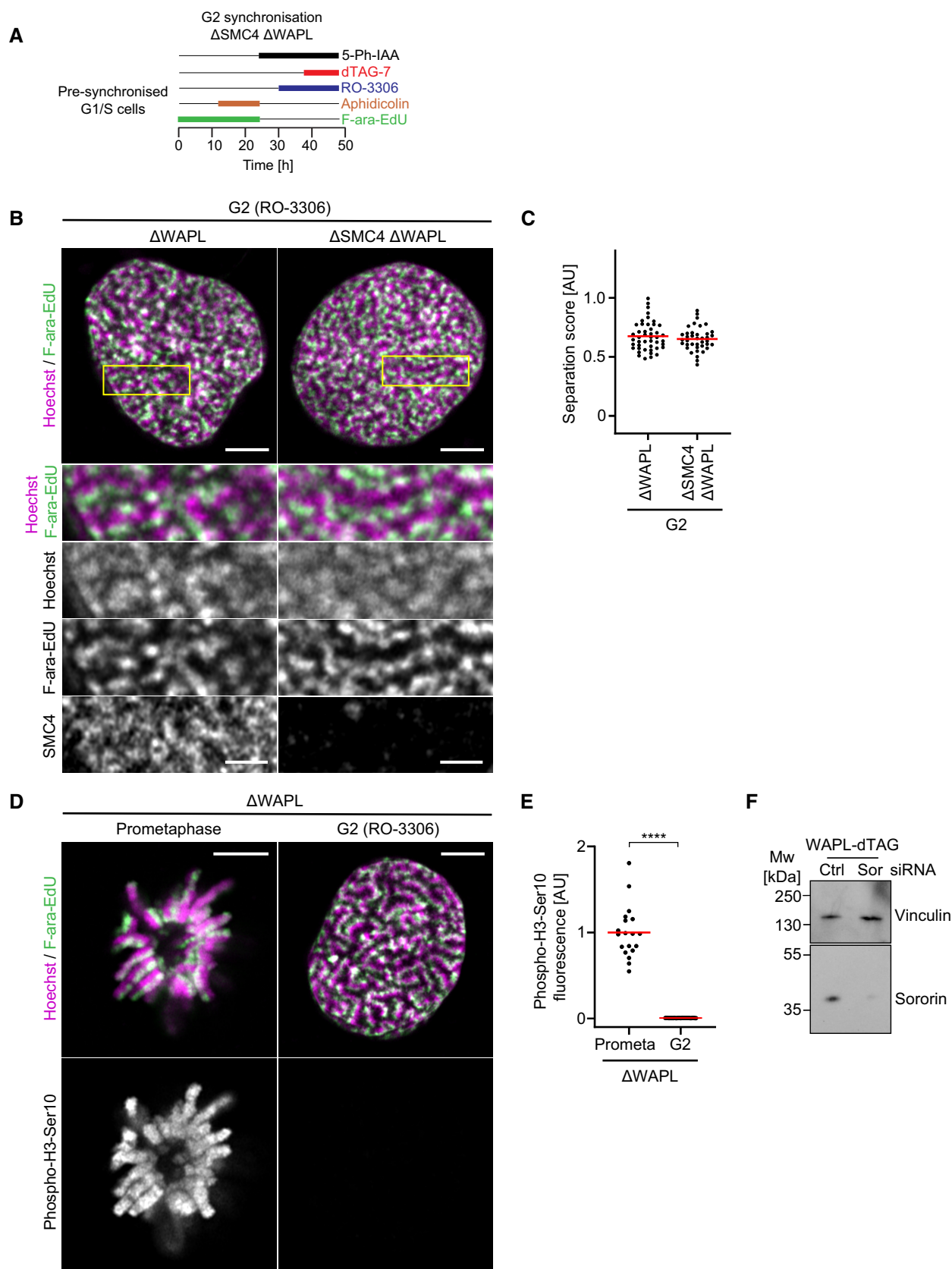


Figure EV5.

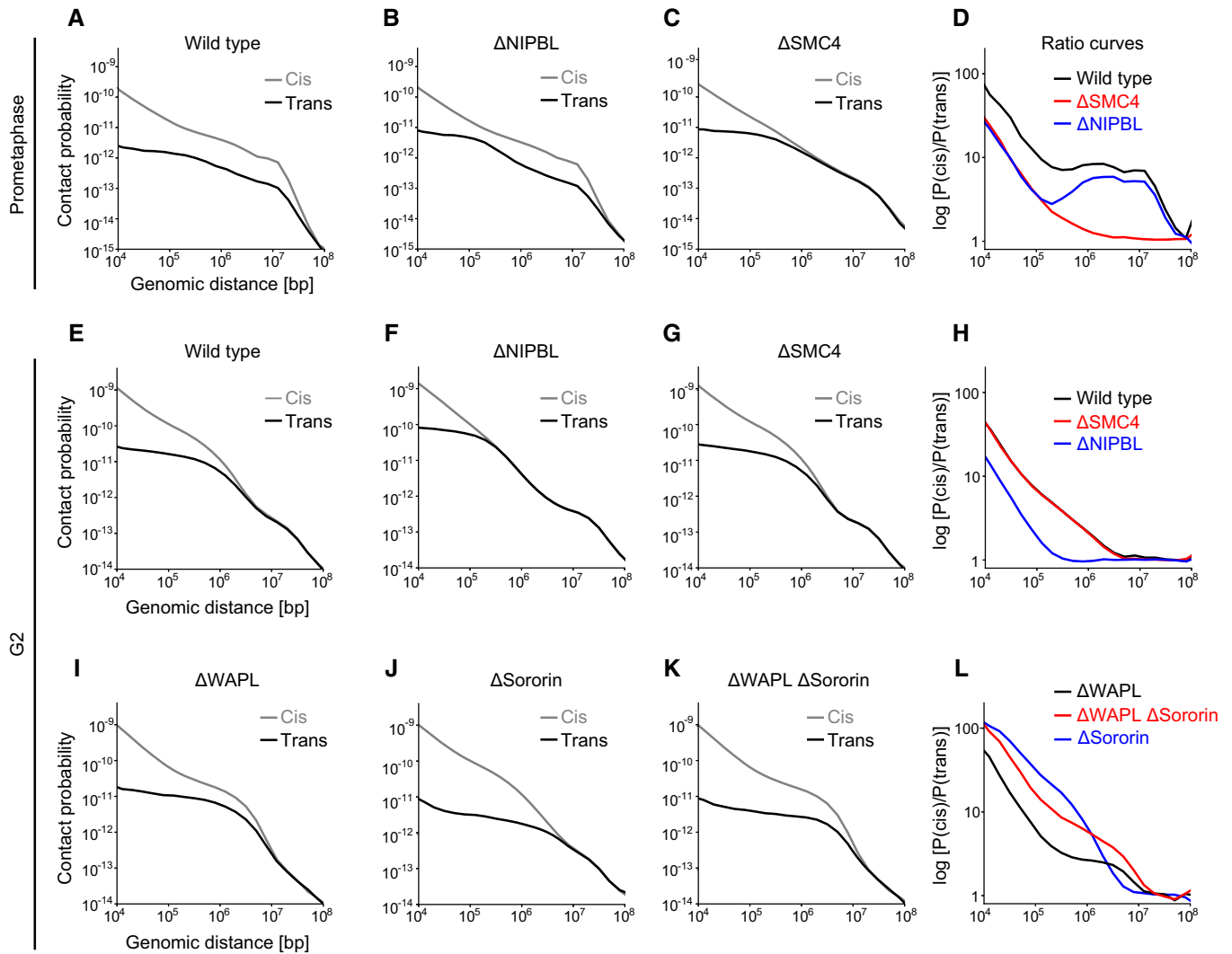


Figure EV6. scsHi-C contact probability curves.

A–L Sister-chromatid-sensitive Hi-C experiments were analysed by calculating the average contact probability for cis sister and trans sister contacts over variable genomic intervals. Merged curves of the individual replicates are shown for all panels. (A) Cis sister and trans sister contact probability curves for wild-type prometaphase cells. (B) Cis sister and trans sister contact probability curves for Δ NIPBL prometaphase cells. (C) Cis sister and trans sister contact probability curves for Δ SMC4 prometaphase cells. (D) Ratio curves plotting cis sister contact probability / trans sister contact probability against genomic distance for the conditions in (A–C). (E) Cis sister and trans sister contact probability curves for wild-type G2 cells. (F) Cis sister and trans sister contact probability curves for Δ NIPBL G2 cells. (G) Cis sister and trans sister contact probability curves for Δ SMC4 G2 cells. (H) Ratio curves plotting cis sister contact probability/trans sister contact probability against genomic distance for the conditions in (E–G). (I) Cis sister and trans sister contact probability curves for Δ WAPL G2 cells. (J) Cis sister and trans sister contact probability curves for Δ Sororin G2 cells. (K) Cis sister and trans sister contact probability curves for Δ WAPL Δ Sororin G2 cells. (L) Ratio curves plotting cis sister contact probability / trans contact probability against genomic distance for the conditions in (I–K). Biological replicates: Wild-type prometaphase ($n = 2$), Δ NIPBL prometaphase ($n = 3$), Δ SMC4 prometaphase ($n = 2$), wild-type G2 ($n = 11$), Δ NIPBL G2 ($n = 10$), Δ SMC4 G2 ($n = 4$), Δ WAPL G2 ($n = 6$), Δ Sororin G2 ($n = 3$), Δ WAPL Δ Sororin G2 ($n = 4$).

Appendix

Cohesin-mediated DNA loop extrusion resolves sister chromatids in G2 phase

Paul Batty^{1,2#}, Christoph C.H. Langer¹, Zsuzsanna Takács¹, Wen Tang³, Claudia Blaukopf¹, Jan-Michael Peters³, Daniel W. Gerlich^{1#}

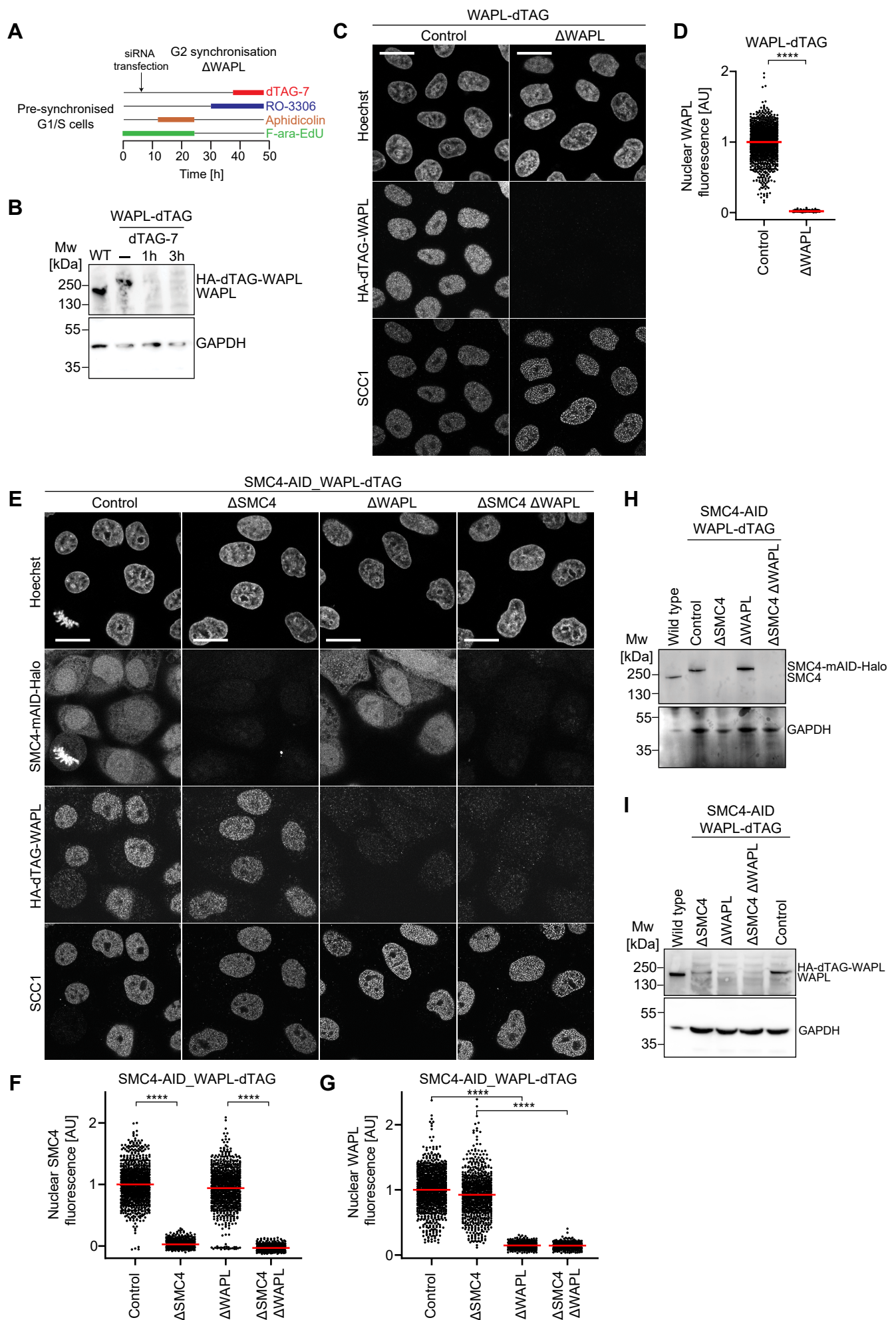
¹Institute of Molecular Biotechnology of the Austrian Academy of Sciences (IMBA), Vienna BioCenter (VBC), 1030 Vienna, Austria

²Vienna BioCenter PhD Program, Doctoral School of the University of Vienna and Medical University of Vienna, A-1030, Vienna, Austria

³Research Institute of Molecular Pathology (IMP), Vienna BioCenter (VBC), Vienna, 1030 Vienna, Austria

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Appendix Figure S1 | Validation of protein depletion efficiency in WAPL-dTAG and SMC4-AID_WAPL-dTAG cells.
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Appendix Figure S1 | Validation of protein depletion efficiency in WAPL-dTAG and SMC4-AID_WAPL-dTAG cells.

A, Schematic of experimental procedure for generation of one-sister labelled chromatids in Δ WAPL G2 cells. Cells were treated with the requisite compounds as indicated; the end point indicates the time of fixation. For conditions where WAPL was not depleted, dTAG-7 was not added to the cells.

B, Immunoblot analysis of WAPL in wild type (WT) cells, untreated WAPL-dTAG cells and WAPL-dTAG cells treated for either 1 h or 3 h with dTAG-7. Representative example of $n = 2$ biological replicates. For source gel data, see Appendix Fig. S5D.

C, Immunofluorescence analysis of HeLa cells homozygously tagged for HA-dTAG-WAPL. Cells were incubated for 3 h with (Δ WAPL) or without (Control) 1 μ M dTAG-7 before subsequently fixing and staining for HA-WAPL and SCC1, with anti-HA and anti-SCC1 antibodies respectively. DNA was stained with Hoechst 33342.

D, Quantification of mean nuclear HA-WAPL fluorescence per cell, as shown in C. Dots represent individual cells; red bars indicate the mean. For each condition two experimental replicates were performed. $n = 1466$ cells analysed for control WAPL-dTAG cells, $n = 1372$ cells analysed for Δ WAPL cells. Significance was tested using a two-tailed Mann Whitney U test; $P < 10^{-324}$ (precision limit of floating-point arithmetic).

E, Immunofluorescence analysis of HeLa cells homozygously tagged for SMC4-mAID-Halo and HA-dTAG-WAPL, and stably expressing OsTIR1F74G. Cells were incubated with 5-Ph-IAA or dTAG-7 in the following combinations: untreated (Control), 3 h 5-Ph-IAA (Δ SMC4), 3 h dTAG-7 (Δ WAPL), 3 h 5-Ph-IAA + dTAG-7 (Δ SMC4 Δ WAPL). SMC4 was stained with TMR-HaloTag ligand and HA-WAPL and SCC1 were stained using antibodies as in B. DNA was stained with Hoechst 33342.

F, Quantification of mean nuclear SMC4 fluorescence per cell, as shown in E. Dots represent individual cells; red bars indicate the mean. Wild type cells were stained with Halo-TMR and the mean Halo-TMR fluorescence within the segmented nuclei then calculated. Normalisation was performed relative to the mean nuclear Halo-TMR fluorescence of wild type cells (0 value), and control SMC4-AID_WAPL-dTAG cells (1 value). For each condition two experimental replicates were performed. $n = 1007$ cells analysed for control SMC4-AID_WAPL-dTAG cells, $n = 848$ cells analysed for Δ SMC4 cells, $n = 867$ cells analysed for Δ WAPL cells, $n = 717$ cells analysed for Δ SMC4 Δ WAPL cells. Significance was tested using a two-tailed Mann Whitney U test; $P = 1.07 \times 10^{-297}$ (Δ SMC4), $P = 3.81 \times 10^{-244}$ (Δ SMC4 Δ WAPL).

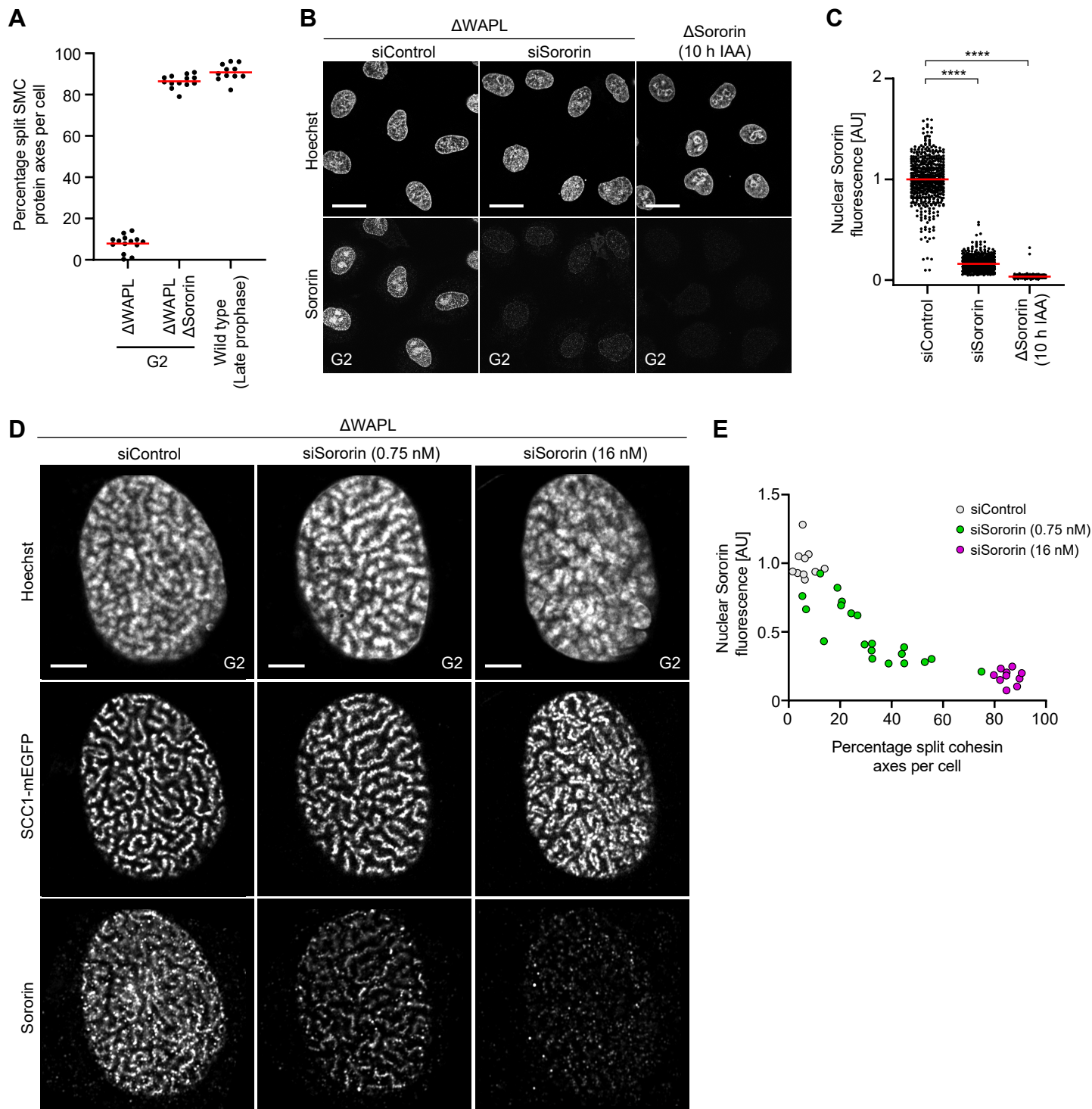
G, Quantification of mean nuclear HA-WAPL fluorescence per cell, as shown in E. Sample numbers as in F. Dots represent individual cells; red bars indicate the mean. Significance was tested using a two-tailed Mann Whitney U test; $P = 6.36 \times 10^{-305}$ (Δ WAPL), $P = 5.15 \times 10^{-253}$ (Δ SMC4 Δ WAPL).

H, Immunoblot analysis of SMC4 in wild type and SMC4-AID_WAPL-dTAG cells treated as follows: untreated (Control), 3 h 5-Ph-IAA (Δ SMC4), 3 h dTAG-7 (Δ WAPL), 3 h 5-Ph-IAA + dTAG-7 (Δ SMC4 Δ WAPL). Representative example of $n = 2$ biological replicates. For source gel data, see Appendix Fig. S5E, F.

I, Immunoblot analysis of WAPL in wild type and SMC4-AID_WAPL-dTAG cells treated as follows: untreated (Control), 3 h 5-Ph-IAA (Δ SMC4), 3 h dTAG-7 (Δ WAPL), 3 h 5-Ph-IAA + dTAG-7 (Δ SMC4 Δ WAPL). Representative example of $n = 2$ biological replicates. For source gel data, see Appendix Fig. S5G.

Data information: (****) $P < 0.0001$; two-tailed Mann Whitney U test. Biological replicates: **B** ($n = 2$), **H**, **I** ($n = 2$). Technical replicates:

C-G ($n = 2$). All microscopy images are single Z-sections. Scale bars: 20 μ m.



Appendix Figure S2 | Cohesin axes split upon WAPL depletion in Sororin depleted G2 cells.

A, Scatter plot of the percentage of split cohesin (SCC1) axes in ΔWAPL G2 and ΔWAPL ΔSororin G2 cells, and the percentage of split condensin (SMC4) axes in wild type late prophase cells, as indicated. Line profiles were drawn along cohesin or condensin axes and the percentage of split axis segments along the total axis length then calculated on a per cell basis. ΔWAPL G2 cells (n = 230 lines from 14 cells from 6 replicates), ΔWAPL ΔSororin G2 cells (n = 299 lines from 13 cells from 4 replicates), and wild type late prophase cells (n = 101 lines from 11 cells from 5 replicates) were analysed. Dots represent individual cells; red bars indicate the mean.

B, Immunofluorescence analysis of HeLa cells homozygously tagged for Halo-mAID-WAPL (ΔWAPL) or Sororin-mAID-mEGFP (ΔSororin) as indicated. Cells were synchronised to G2 phase by RO-3306. WAPL and Sororin were depleted through the addition of 500 μM auxin (IAA) for 10 h. ΔWAPL cells were treated with 16 nM Control (siControl) or Sororin (siSororin) siRNAs as indicated. Sororin was stained using an anti-Sororin antibody, DNA was stained using Hoechst 33342.

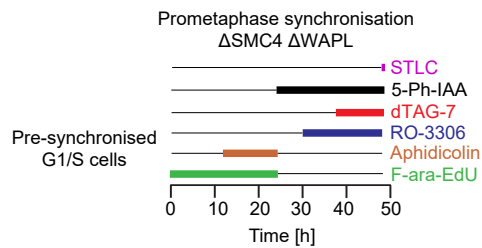
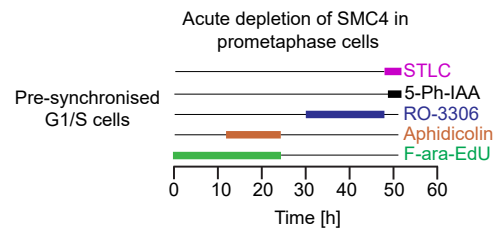
C, Quantification of mean nuclear Sororin fluorescence per cell, as shown in **B**. Dots represent individual cells; red bars indicate the mean. For each condition two experimental replicates were performed. n = 545 cells analysed for siControl, n = 990 cells analysed for siSororin, n = 971 cells analysed for ΔSororin. Significance was tested using a two-tailed Mann Whitney U test; P = 9.48 × 10⁻²²⁶ (siSororin), P = 3.77 × 10⁻²³¹ (ΔSororin).

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D, Immunofluorescence of Δ WAPL G2 cells to assess the localisation and abundance of Sororin and the extent of cohesin axis splitting upon siRNA-mediated depletion of Sororin. Cells were treated with Control (siControl) or Sororin (siSororin) siRNAs as indicated. Control siRNAs were used at 16 nM and Sororin siRNAs were used at 0.75 nM or 16 nM, as indicated. WAPL was depleted in G2 phase. SCC1 was homozygously tagged with mEGFP and was visualised using an anti-EGFP nanobody, Sororin was stained using an anti-Sororin antibody, DNA was stained using Hoechst 33342. To aid visualisation the SCC1-mEGFP channel is not contrast matched.

E, Scatter plot of the percentage of split cohesin (SCC1-mEGFP) axes against mean nuclear Sororin fluorescence on a per cell basis for Δ WAPL G2 cells treated with siRNAs as in **D**. Quantification of the percentage of split cohesin axes was performed as in Appendix Fig. S2A. Quantification of mean nuclear Sororin fluorescence was performed for central Z-stack slices. Dots represent individual cells. For each condition two experimental replicates were performed. $n = 135$ lines from 10 cells from 2 replicates analysed for Δ WAPL siControl, $n = 233$ lines from 10 cells from 2 replicates analysed for Δ WAPL siSororin (16 nM), $n = 441$ lines from 20 cells from 2 replicates analysed for Δ WAPL siSororin (0.75 nM).

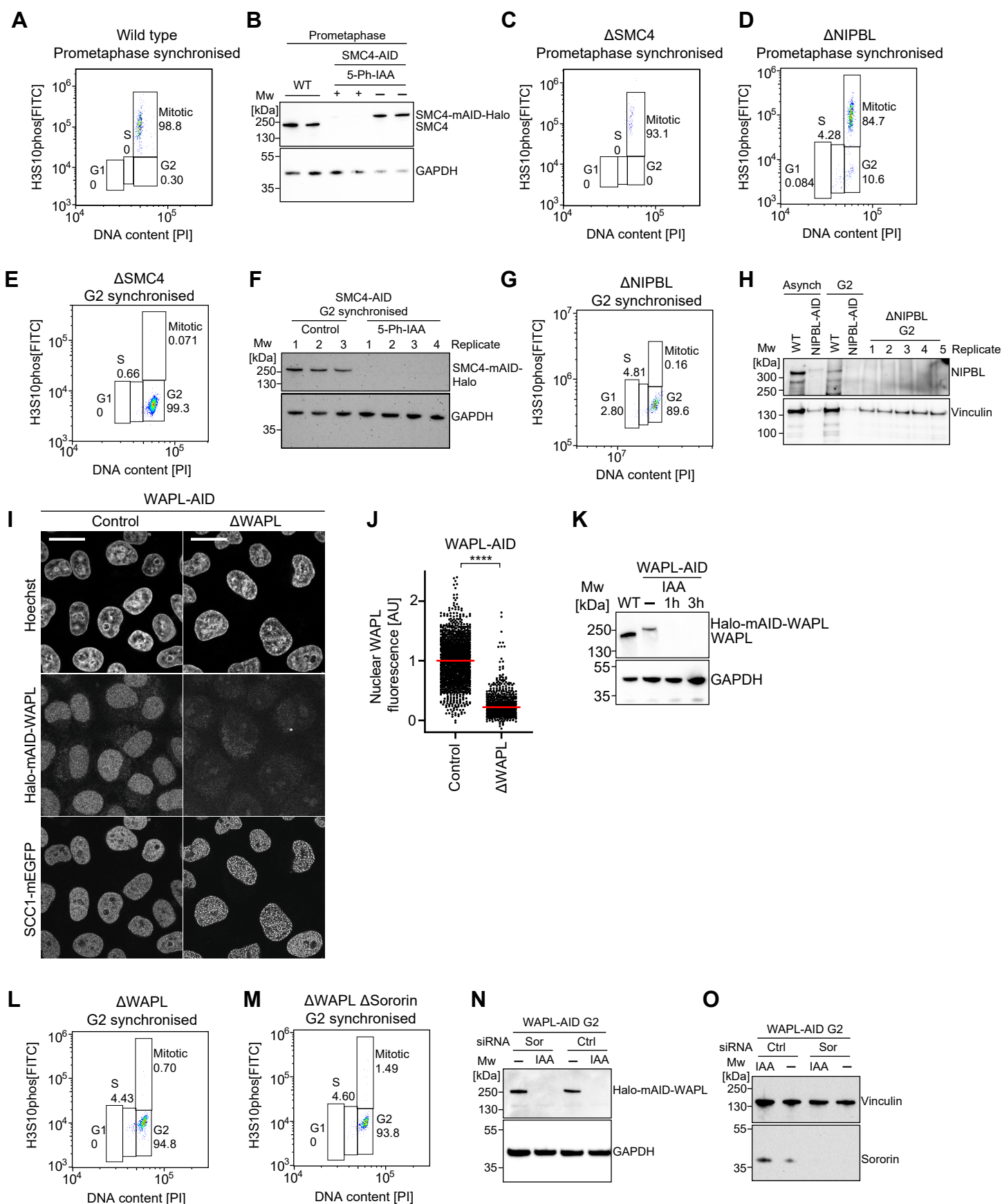
Data information: (****) $P < 0.0001$; two-tailed Mann Whitney U test. Biological replicates: **B-E** ($n = 2$). Images of fields are single Z-sections from Z-stack images. Images of single cells are single Z-slices from 3D-stacks. Scale bar fields: 30 μm , scale bars single cells: 5 μm .

A**B**

Appendix Figure S3 | Acute depletion of condensin and WAPL in prometaphase cells.

A, Schematic of experimental procedure for generation of one-sister labelled chromatids in Δ SMC4 Δ WAPL prometaphase cells, to test if cohesin is able to compensate for sister chromatid resolution defects resulting from condensin depletion. SMC4 was depleted in G1 through the addition of 5-Ph-IAA 1 h before the final release into S phase such that cells progressed through S and G2 phase in the absence of condensins. WAPL was depleted in G2 phase after completion of DNA replication to avoid potential effects of WAPL depletion on cohesion establishment. Cells were treated with the requisite compounds as indicated; the end point indicates the time of fixation.

B, Schematic of experimental procedure for generation of one-sister labelled chromatids in control prometaphase cells, before acute depletion of SMC4 through the addition of 5-Ph-IAA for 120 or 240 min. Schematic represents depletion of SMC4 for 240 min.



Appendix Figure S4 | Validation of cell cycle stage and protein depletion efficiency for scsHi-C.
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Appendix Figure S4 | Validation of cell cycle stage and protein depletion efficiency for scsHi-C.

A-M, Cell cycle analysis by flow cytometry (A, C, D, E, G, L, M). Cells were stained with propidium iodide to determine DNA content and an antibody against phospho-H3-Ser10 as a marker for mitotic cells. The gates indicate the cell cycle stage, and the numbers indicate the percentage of cells measured for a given cell cycle stage.

A, Representative flow cytometry plot for one of the wild type prometaphase samples (n = 2 biological replicates).

B, Immunoblot analysis of SMC4 in wild type (WT) and SMC4-AID prometaphase cells harvested for scsHi-C analysis, plus or minus 5-Ph-IAA as indicated. For source gel data, see Appendix Fig. S5I.

C, Representative flow cytometry plot for one of the Δ SMC4 prometaphase samples (n = 2 biological replicates).

D, Representative flow cytometry plot for one of the Δ NIPBL prometaphase samples (n = 3 biological replicates).

E, Representative flow cytometry plot for one of the Δ SMC4 G2 samples (n = 4 biological replicates).

F, Immunoblot analysis of G2 synchronised control SMC4-AID and Δ SMC4 cells harvested for scsHi-C analysis, plus or minus 5-Ph-IAA as indicated. For source gel data, see Appendix Fig. S5J.

G, Representative flow cytometry plot for one of the Δ NIPBL G2 samples (n = 10 biological replicates).

H, Immunoblot analysis of NIPBL in wild type (WT) and G2 synchronised NIPBL-AID cells harvested for downstream scsHi-C analysis. For source gel data, see Appendix Fig. S5K.

I, Immunofluorescence analysis of HeLa cells homozygously tagged for Halo-mAID-WAPL and stably expressing OsTIR1. Cells were incubated for 2 h with (Δ WAPL) or without (Control) 500 μ M auxin before subsequently staining of WAPL with TMR-HaloTag ligand. SCC1-mEGFP was stained with an anti-SCC1 antibody or an anti-mEGFP nanobody and DNA was stained with Hoechst 33342. Images show single Z-sections.

J, Quantification of mean nuclear WAPL fluorescence per cell, as shown in I. Wild type cells were stained with Halo-TMR and the mean Halo-TMR fluorescence within the segmented nuclei then calculated. Normalisation was performed relative to the mean nuclear Halo-TMR fluorescence of wild type cells (0 value) and control WAPL-AID cells (1 value). n = 1537 cells analysed for control WAPL-AID cells, n = 1199 cells analysed for Δ WAPL cells. Significance was tested using a two-tailed Mann Whitney U test; P = < 10⁻³²⁴ (precision limit of floating-point arithmetic).

K, Immunoblot analysis of WAPL in wild type cells, untreated WAPL-AID cells and WAPL-AID cells treated for either 1 h or 3 h with auxin (IAA). Representative example of n = 3 biological replicates. For source gel data, see Appendix Fig. S5L.

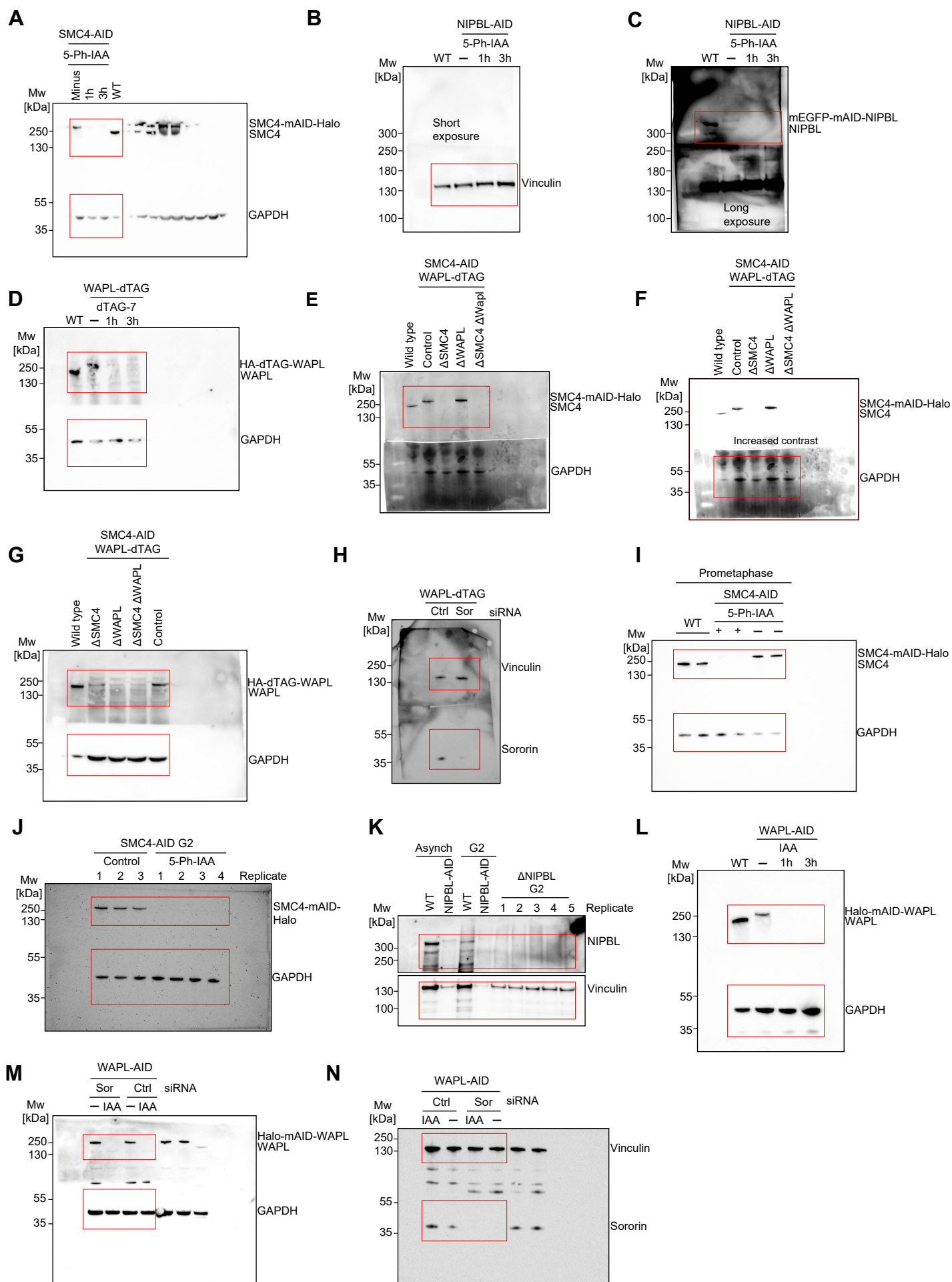
L, Representative flow cytometry plot for one of the Δ WAPL G2 samples (n = 6 biological replicates).

M, Representative flow cytometry plot for one of the Δ WAPL Δ Sororin G2 samples (n = 4 biological replicates).

N, Immunoblot analysis of WAPL in WAPL-AID cells harvested for scsHi-C analysis, treated with Control (Ctrl) or Sororin (Sor) siRNAs, plus or minus auxin (IAA), as indicated. For source gel data, see Appendix Fig. S5M.

O, Immunoblot analysis of Sororin in WAPL-AID cells harvested for scsHi-C analysis, treated with Control (Ctrl) or Sororin (Sor) siRNAs, plus or minus auxin (IAA) as indicated. For source gel data, see Appendix Fig. S5N.

Data information: (****) P < 0.0001; two-tailed Mann Whitney U test. Biological replicates: **A-H, K-O**: as in Fig. 5 and Fig. EV6, **I, J** (n = 2). Technical replicates: **I, J** (n = 3).



Appendix Figure S5 | Uncropped Western blot images.
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Appendix Figure S5 | Uncropped Western blot images.

Loading controls were run on the same gel as the target protein, and unless otherwise specified, the membrane was cut at 70 KDa after transfer to allow detection of different proteins. In all cases, the cropped images used in the requisite figure panels are indicated with red boxes.

A, Characterisation of SMC4-AID cells. Uncropped blot of wild type (WT) and SMC4-AID cells (untreated, 1 h 5-Ph-IAA, 3 h 5-Ph-IAA), as indicated, blotting against SMC4 and GAPDH. Cropped images shown in Fig. EV3B.

B, Characterisation of NIPBL-AID cells. Uncropped blot of wild type (WT) and NIPBL-AID cells (untreated, 1 h 5-Ph-IAA, 3 h 5-Ph-IAA, as indicated), short exposure time, showing the loading control (Vinculin). Cropped area shown in Fig. EV4B. Loading controls were run on the same gel and the membrane cut after transfer at 250 KDa to stain NIPBL and Vinculin with different antibodies

C, Uncropped blot as in **B**, long exposure time, blotting against NIPBL.

D, Characterisation of WAPL-dTAG cells. Uncropped blot of wild type (WT) and WAPL-dTAG cells (untreated, 1 h dTAG-7, 3 h dTAG-7, as indicated). Cropped area shown in Appendix Fig. S1B, blotting against WAPL and GAPDH.

E, Characterisation of SMC4-AID_WAPL-dTag cells. Uncropped blot of wild type and SMC4-AID_WAPL-dTAG cells (untreated, 3 h 5-Ph-IAA, 3 h dTAG-7, 3 h 5-Ph-IAA + dTAG-7, as indicated), showing blotting against SMC4. Cropped area shown in Appendix Fig. S1H, blotting against SMC4 and GAPDH.

F, Uncropped blot as in **E**, increased contrast, showing blotting against the loading control (GAPDH).

G, Characterisation of SMC4-AID_WAPL-dTAG cells. Same conditions as in **E**, **F**, blotting against WAPL and GAPDH. Cropped areas shown in Appendix Fig. S1I, blotting against WAPL and GAPDH.

H, Uncropped blot of WAPL-dTAG cells treated with Control (Ctrl) or Sororin (Sor) siRNAs, as indicated. Cropped areas shown in Fig. EV5F, blotting against Sororin and Vinculin.

I, Uncropped blot of prometaphase synchronised wild type (WT) and SMC4-AID cells harvested for scsHi-C analysis, plus or minus 5-Ph-IAA as indicated. Cropped areas shown in Appendix Fig. S4B, blotting against SMC4 and GAPDH.

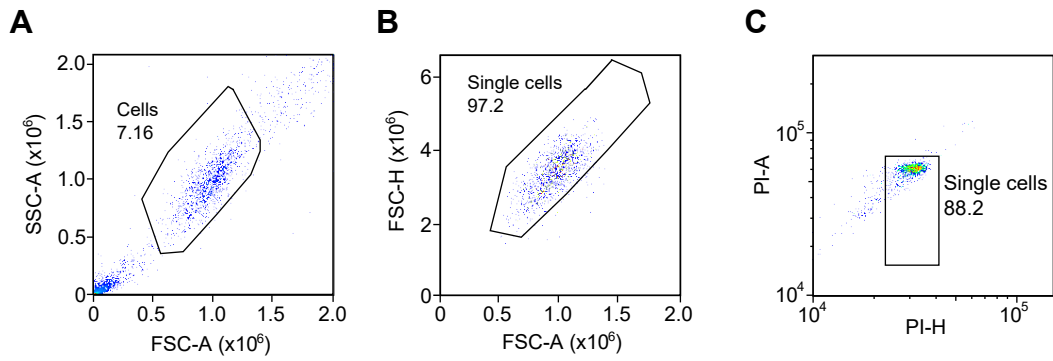
J, Uncropped blot of G2 synchronised control SMC4-AID and Δ SMC4 cells harvested for scsHi-C analysis, plus or minus 5-Ph-IAA as indicated. Cropped areas shown in Appendix Fig. S4F, blotting against SMC4 and GAPDH.

K, Uncropped blot of wild type (WT) and G2 synchronised NIPBL-AID cells harvested for scsHi-C analysis. Cropped areas shown in Appendix Fig. S4H, blotting against NIPBL and Vinculin.

L, Characterisation of WAPL-AID cell line. Uncropped blot of wild type (WT) and WAPL-AID cells (untreated, 1 h auxin (IAA), 3 h auxin (IAA), as indicated). Cropped area shown in Appendix Fig. S4K, blotting against WAPL and GAPDH.

M, Uncropped blot of G2 synchronised WAPL-AID cells harvested for scsHi-C analysis, treated with Control (Ctrl) or Sororin (Sor) siRNAs, plus or minus auxin (IAA), as indicated. Cropped areas shown in Appendix Fig. S4N, blotting against WAPL and GAPDH.

N, Uncropped blot of G2 synchronised WAPL-AID cells harvested for scsHi-C analysis, treated with Control (Ctrl) or Sororin (Sor) siRNAs, plus or minus auxin (IAA), as indicated. Cropped areas shown in Appendix Fig. S4O, blotting against Sororin and Vinculin.



Appendix Figure S6 | Flow cytometry gating strategy.

For all panels, numbers indicate the percentage of cells inside the gate. Cells were stained with propidium iodide to determine DNA content and an antibody against phospho-H3-Ser10 as a marker for mitotic cells.

A, FSC-A vs SSC-A gate to gate cells and remove debris, shown for one of the G2 synchronised Δ WAPL samples.

B, FSC-A vs FSC-H gate to identify single cells.

C, PI-H vs PI-A, second gate to identify single cells. Cells in this gate were subsequently used to analyse cell cycle stage in Appendix Fig. S4L. The same gating strategy was used for Appendix Fig. S4A, C, D, E, G, M.

Appendix Tables

Appendix Table S1: Flow cytometry sample statistics for G2 scsHi-C samples

Condition	Replicate	#G1	#S	#G2	#M	% G1	% S	% G2	%M
ΔWAPL G2	Replicate 1	87	57	1151	9	6.64	4.35	87.9	0.69
ΔWAPL G2	Replicate 2	4	5	260	1	1.48	1.85	95.9	0.37
ΔWAPL G2	Replicate 3	0	23	329	1	0	6.53	93.5	0.28
ΔWAPL G2	Replicate 4	0	51	1095	8	0	4.42	94.8	0.69
ΔWAPL G2	Replicate 5	1	107	1632	12	0	6.1	93	0.68
ΔWAPL G2	Replicate 6	0	53	613	9	0	7.83	90.5	1.33
ΔWAPL ΔSororin G2	Replicate 1	5	7	430	1	1.13	1.58	97.1	0.23
ΔWAPL ΔSororin G2	Replicate 2	0	74	541	4	0	11.9	87.1	0.64
ΔWAPL ΔSororin G2	Replicate 3	0	90	992	2	0	8.29	91.3	0.18
ΔWAPL ΔSororin G2	Replicate 4	0	91	594	8	0	13.1	85.3	1.15
ΔSMC4 G2	Replicate 1	0	14	1264	0	0	1.1	99.1	0
ΔSMC4 G2	Replicate 2	0	17	1317	0	0	1.28	98.9	0
ΔSMC4 G2	Replicate 3	0	7	1009	0	0	0.69	99.3	0
ΔSMC4 G2	Replicate 4	0	28	4195	3	0	0.66	99.3	0.071
ΔNIPBL G2	Replicate 1	18	31	577	1	2.8	4.81	89.6	0.16
ΔNIPBL G2	Replicate 2	17	56	661	2	2.25	7.41	87.4	0.26
ΔNIPBL G2	Replicate 3	18	50	518	1	3.01	8.35	86.5	0.17
ΔNIPBL G2	Replicate 4	20	67	665	1	2.6	8.7	86.4	0.13
ΔNIPBL G2	Replicate 5	18	65	816	2	1.97	7.12	89.4	0.22
ΔNIPBL G2	Replicate 6	52	112	1172	1	3.81	8.2	85.8	0.073
ΔNIPBL G2	Replicate 7	60	148	1466	0	3.5	8.64	85.6	0
ΔNIPBL G2	Replicate 8	43	83	874	0	4.2	8.1	85.3	0
ΔNIPBL G2	Replicate 9	36	87	889	0	3.44	8.32	85	0
ΔNIPBL G2	Replicate 10	96	136	1533	0	5.33	7.55	85.1	0

Appendix Table S2: Flow cytometry sample statistics for prometaphase scsHi-C samples

Condition	Replicate	#G1	#S	#G2	#M	% G1	% S	% G2	%M
WT prometaphase	Replicate 1	0	4	5	150	0	2.44	3.05	91.05
WT prometaphase	Replicate 2	0	0	2	666	0	0	0.3	98.8
ΔSMC4 prometaphase	Replicate 1	0	0	2	115	0	0	1.64	94.3
ΔSMC4 prometaphase	Replicate 2	0	0	0	147	0	0	0	93.1
ΔNIPBL prometaphase	Replicate 1	1	52	126	1010	0.084	4.36	10.6	84.7
ΔNIPBL prometaphase	Replicate 2	0	30	87	556	0	4.44	12.9	82.2
ΔNIPBL prometaphase	Replicate 3	0	52	82	707	0	6.09	10.3	82.8

Appendix Table S3: Cell lines used in this study

Name	Genotype	Plasmids used	Resistance marker	Internal Lab ID	Already published or generated for this study
HeLa Kyoto	Wild type	n/a	n/a	1	
HeLa Kyoto Sororin-AID	EGFP-AID-Sororin TIR1-3xMyc-T2A-Puro (Lentiviral integration)	EGFP-AID-Sororin repair template TIR1-3xMyc-T2A PuroLentivirus sgRNA expressing / Cas9 ^{D10A} expressing to tag Sororin	Puromycin	1712	Published in Mitter et al; 2020
HeLa Kyoto SMC4-AID	SMC4-mAID-Halo OsTIR1(F74G)-SNAP-IRES-Blast (integrated into AAVS1 safe harbour locus)	SMC4-mAID-Halo repair template OsTIR1 ^{F74G} -SNAP-IRES-Blast repair template sgRNA expressing/Cas9-human geminin fusion (Cas9-hGem) to tag SMC4 sgRNA / Cas9-human geminin fusion (Cas9-hGem) expressing to integrate OsTIR1 ^{F74G}	Blasticidin S Puromycin	2056	Published in Schneider et al; 2022
HeLa Kyoto NIPBL-AID	mEGFP-mAID-NIPBL OsTIR1(F74G)—SNAP-IRES-Blast (integrated into AAVS1 safe harbour locus)	mEGFP-mAID-NIPBL repair template OsTIR1 ^{F74G} -SNAP-IRES-Blast repair template sgRNA / Cas9-human geminin fusion (Cas9-hGem) expressing to tag NIPBL sgRNA / Cas9-human geminin fusion (Cas9-hGem) expressing to integrate OsTIR1 ^{F74G}	Blasticidin S	2045	Generated for this study
HeLa Kyoto WAPL-AID	Halo-mAID-WAPL SCC1-mEGFP TIR1-3xMyc-T2A-Puro (Lentiviral integration)	Halo-mAID-WAPL repair template SCC1-mEGFP repair template TIR1-3xMyc-T2A PuroLentivirus sgRNA expressing / Cas9 ^{D10A} expressing to tag WAPL	Puromycin	1802	Generated for this study
HeLa Kyoto WAPL-FKBP12 ^{F36V} (WAPL-dTAG)	Blasticidin-P2A-2xHA-FKBP12 ^{F36V} (dTAG)-WAPL	Blasticidin-P2A-2xHA-FKBP12 ^{F36V} repair template sgRNA / Cas9 ^{D10A} expressing to tag WAPL	Blasticidin S	2096	Generated for this study
HeLa Kyoto SMC4-AID_WAPL FKBP12 ^{F36V} (WAPL-dTAG)	SMC4-mAID-Halo Blasticidin-P2A-2xHA-FKBP12 ^{F36V} (dTAG)-WAPL OsTIR1 ^{F74G} -SNAP-IRES-Blast (integrated into AAVS1 safe harbour locus)	SMC4-mAID-Halo repair template HygromycinB-P2A-2xHA-FKBP12 ^{F36V} repair template OsTIR1 ^{F74G} -SNAP-IRES-Blast repair template sgRNA / Cas9-human geminin fusion (Cas9-hGem) expressing to tag SMC4 sgRNA / Cas9-human geminin fusion (Cas9-hGem) expressing to integrate OsTIR1 ^{F74G}	Blasticidin S Hygromycin B Puromycin	2108	Generated for this study, base cell line HeLa Kyoto SMC4-AID cell line mentioned above

Appendix Table S4: scsHi-C read statistics for ΔNIPBL G2 cells

Replicate	Total	Mapped	Unique	Cis	Trans Heterologue	Cis 1Kb+	Cis 10Kb+	Cis sister	Trans sister
Replicate 1	6.09E+07	5.00E+07	4.18E+07	3.41E+07	7.68E+06	2.57E+07	1.94E+07	2.08E+06	4.79E+05
Replicate 2	7.93E+07	6.30E+07	3.53E+07	2.85E+07	6.85E+06	2.13E+07	1.58E+07	1.51E+06	3.49E+05
Replicate 3	7.36E+07	5.98E+07	5.06E+07	4.14E+07	9.28E+06	3.08E+07	2.27E+07	2.47E+06	5.56E+05
Replicate 4	8.93E+07	7.22E+07	5.79E+07	4.74E+07	1.04E+07	3.53E+07	2.58E+07	2.56E+06	5.60E+05
Replicate 5	6.55E+07	5.37E+07	4.28E+07	3.49E+07	7.92E+06	2.61E+07	1.93E+07	2.00E+06	4.59E+05
Replicate 6	7.04E+07	5.74E+07	4.39E+07	3.59E+07	8.00E+06	2.60E+07	1.95E+07	9.43E+05	1.92E+05
Replicate 7	6.51E+07	5.19E+07	4.06E+07	3.27E+07	7.82E+06	2.35E+07	1.78E+07	6.74E+05	1.29E+05
Replicate 8	6.14E+07	5.01E+07	4.49E+07	3.60E+07	8.89E+06	2.62E+07	2.00E+07	1.28E+06	2.77E+05
Replicate 9	7.01E+07	5.57E+07	4.84E+07	3.81E+07	1.03E+07	2.76E+07	2.10E+07	1.39E+06	3.00E+05
Replicate 10	5.72E+07	4.61E+07	4.09E+07	3.35E+07	7.43E+06	2.23E+07	1.66E+07	7.86E+05	1.28E+05

Appendix Table S5: scsHi-C read statistics for ΔSMC4 G2 cells

Replicate	Total	Mapped	Unique	Cis	Trans Heterologue	Cis 1Kb+	Cis 10Kb+	Cis sister	Trans sister
Replicate 1	1.34E+08	1.02E+08	2.99E+07	2.60E+07	3.89E+06	1.38E+07	1.00E+07	1.88E+06	1.53E+05
Replicate 2	1.36E+08	1.10E+08	5.32E+07	1.10E+08	5.66E+06	2.52E+07	1.77E+07	3.83E+06	2.82E+05
Replicate 3	1.29E+08	1.01E+08	4.95E+07	4.35E+07	5.99E+06	2.36E+07	1.70E+07	4.34E+06	3.63E+05
Replicate 4	1.09E+08	7.26E+07	3.72E+07	2.52E+07	3.03E+06	1.54E+07	1.18E+07	3.32E+06	2.53E+05

Appendix Table S6: scsHi-C read statistics for ΔWAPL G2 cells

Replicate	Total	Mapped	Unique	Cis	Trans Heterologue	Cis 1Kb+	Cis 10Kb+	Cis sister	Trans sister
Replicate 1	3.50E+07	2.68E+07	1.64E+07	1.42E+07	2.25E+06	9.84E+06	7.64E+06	8.09E+05	1.06E+05
Replicate 2	4.64E+07	3.12E+07	9.40E+06	7.85E+06	1.55E+06	4.84E+06	3.80E+06	4.66E+05	5.27E+04
Replicate 3	5.62E+07	3.13E+07	1.15E+07	9.77E+06	1.70E+06	6.46E+06	5.02E+06	5.31E+05	6.16E+04
Replicate 4	7.99E+07	6.17E+07	1.02E+07	9.09E+06	1.11E+06	6.07E+06	4.54E+06	6.95E+05	9.36E+04
Replicate 5	1.00E+08	7.03E+07	1.92E+07	1.69E+07	2.32E+06	1.24E+07	9.86E+06	9.28E+05	1.53E+05
Replicate 6	9.30E+07	7.01E+07	8.50E+06	7.41E+06	1.09E+06	4.91E+06	3.78E+06	4.92E+05	6.75E+04

Appendix Table S7: scsHi-C read statistics for ΔWAPL ΔSororin G2 cells

Replicate	Total	Mapped	Unique	Cis	Trans Heterologue	Cis 1Kb+	Cis 10Kb+	Cis sister	Trans sister
Replicate 1	4.67E+07	3.52E+07	1.38E+07	1.17E+07	2.11E+06	7.45E+06	5.59E+06	7.30E+05	7.20E+04
Replicate 2	3.74E+07	2.77E+07	1.06E+07	8.99E+06	1.57E+06	6.33E+06	4.69E+06	4.08E+05	4.28E+04
Replicate 3	3.71E+07	2.73E+07	1.12E+07	9.14E+06	2.05E+06	6.27E+06	4.84E+06	5.50E+05	6.52E+04
Replicate 4	1.09E+08	7.26E+07	2.82E+07	2.52E+07	3.03E+06	1.54E+07	1.18E+07	1.95E+06	1.58E+05

Appendix Table S8: scsHi-C read statistics for wild type prometaphase cells

Replicate	Total	Mapped	Unique	Cis	Trans Heterologue	Cis 1Kb+	Cis 10Kb+	Cis sister	Trans sister
Replicate 1	7.88E+07	5.89E+07	3.12E+07	2.84E+07	2.87E+06	2.39E+07	2.09E+07	9.76E+05	8.35E+04
Replicate 2	6.53E+07	4.90E+07	2.22E+07	2.01E+07	2.08E+06	1.67E+07	1.44E+07	5.39E+05	5.17E+04

Appendix Table S9: scsHi-C read statistics for ΔNIPBL prometaphase cells

Replicate	Total	Mapped	Unique	Cis	Trans Heterologue	Cis 1Kb+	Cis 10Kb+	Cis sister	Trans sister
Replicate 1	3.65E+07	2.85E+07	7.66E+06	6.48E+06	1.18E+06	3.80E+06	3.15E+06	2.49E+05	2.47E+04
Replicate 2	4.20E+07	3.09E+07	1.45E+07	1.28E+07	1.76E+06	9.02E+06	7.69E+06	5.03E+05	4.62E+04
Replicate 3	2.78E+07	2.08E+07	6.71E+06	5.61E+06	1.10E+06	4.33E+06	3.74E+06	1.74E+05	2.11E+04

Appendix Table S10: scsHi-C read statistics for ΔSMC4 prometaphase cells

Replicate	Total	Mapped	Unique	Cis	Trans Heterologue	Cis 1Kb+	Cis 10Kb+	Cis sister	Trans sister
Replicate 1	7.56E+07	5.40E+07	3.54E+07	2.82E+07	7.24E+06	2.36E+07	2.00E+07	1.38E+06	4.72E+05
Replicate 2	7.38E+07	5.46E+07	4.34E+07	3.72E+07	6.14E+06	2.75E+07	2.18E+07	1.96E+06	3.79E+05

Appendix Table S11: Published datasets used in this study

Name	GEO ID	Description
Hi-C: G2	GSM4613674	Cooler files for wild type HeLa Kyoto cells, synchronised to G2 (All contacts, cis sister contacts, trans sister contacts)
Hi-C: Sororin-AID + auxin G2	GSM4613677	Cooler files for Sororin depleted HeLa Kyoto cells, synchronised to G2 (All contacts, cis sister contacts, trans sister contacts)