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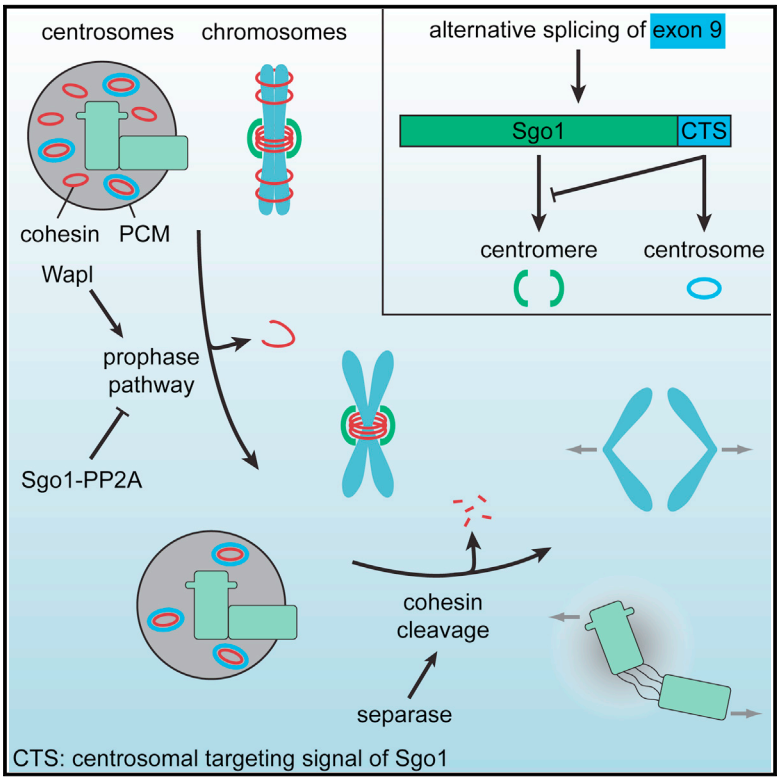
An Alternatively Spliced Bifunctional Localization Signal Reprograms Human Shugoshin 1 to Protect Centrosomal Instead of Centromeric Cohesin

Graphical Abstract



Highlights

- Alternatively spliced Sgo1 isoforms localize to either centromeres or centrosomes
- *SGO1* exon 9 encodes an anti-centromeric, pro-centrosomal targeting signal
- Sgo1 preserves centriole engagement by recruiting protein phosphatase 2A (PP2A)
- Sgo1-PP2A protects centrosomal cohesin from the prophase pathway

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In Brief

Mohr et al. demonstrate that alternatively spliced Sgo1 isoforms specifically localize and function at either centromeres or centrosomes. *SGO1* exon 9 encodes an anti-centromeric, pro-centrosomal targeting signal, which redirects Sgo1-PP2A to shield centrosomal cohesin from the prophase pathway, thereby protecting centriole engagement.

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An Alternatively Spliced Bifunctional Localization Signal Reprograms Human Shugoshin 1 to Protect Centrosomal Instead of Centromeric Cohesin

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SUMMARY

Separation of human sister chromatids involves the removal of DNA embracing cohesin ring complexes. Ring opening occurs by prophase-pathway-dependent phosphorylation and separase-mediated cleavage, with the former being antagonized at centromeres by Sgo1-dependent PP2A recruitment. Intriguingly, prophase pathway signaling and separase's proteolytic activity also bring about centriole disengagement, whereas Sgo1 is again counteracting this licensing step of later centrosome duplication. Here, we demonstrate that alternative splice variants of human Sgo1 specifically and exclusively localize and function either at centromeres or centrosomes. A small C-terminal peptide encoded by exon 9 of *SGO1* (CTS for centrosomal targeting signal of human Sgo1) is necessary and sufficient to drive centrosomal localization and simultaneously abrogate centromeric association of corresponding Sgo1 isoforms. Cohesin is shown to be a target of the prophase pathway at centrosomes and protected by Sgo1-PP2A. Accordingly, premature centriole disengagement in response to Sgo1 depletion is suppressed by blocking ring opening of an engineered cohesin.

INTRODUCTION

Error-free segregation of chromatids into newly forming daughter cells is one of the most critical steps of mitosis, as mistakes lead to aneuploidy.In order to segregate sister chromatids properly, the kinetochores of each chromosome are attached to microtubules emanating from opposite poles of the mitotic spindle. In most eukaryotic cells, each spindle pole harbors one centrosome, the major microtubule-organizing center (MTOC) of the cell. Like chromosomes, centrosomes also have to be duplicated and segregated in each cell cycle. This centrosome cycle has to be coordinated with the chromosome cycle to ensure bipolarity of the mitotic spindle and hence faithful chromosome segregation.

Sister chromatids are held together from the time of their synthesis in S phase until their separation at the metaphase to anaphase transition. This cohesion is mediated by the multi-subunit complex cohesin, a tripartite ring structure composed of Smc1 (structural maintenance of cohesion), Smc3, and Scc1 (sister chromatid cohesion) plus associated proteins like SA1/2 and Pds5A/B. The latter serves as a binding-platform for either Wapl or sororin in a mutually exclusive manner (Nishiyama et al., 2010). The complex topologically entraps both sister chromatids within its ring structure (Gruber et al., 2003; Haering et al., 2002). During vertebrate mitosis, cohesin is removed from chromatin in two waves. The bulk of cohesin, located on chromosome arms, is removed in a non-proteolytic manner in early mitosis by prophase pathway signaling (Waizenegger et al., 2000), while centromere-associated complexes remain protected by shugoshin 1 (Sgo1) until the metaphase to anaphase transition, when Scc1 is cleaved by the cysteine protease separase (Uhlmann et al., 2000). The prophase pathway depends on the phosphorylation of SA2 by Plk1 and sororin by aurora B and Cdk1 (Hauf et al., 2005; Nishiyama et al., 2013). This destabilizes the interaction of Pds5 with the cohesion-establishment factor sororin, upon which the latter is replaced by Wapl (Nishiyama et al., 2013). Wapl then drives opening of the cohesin ring at the Smc3-Scc1 interaction site (the so-called exit gate), leading to the release of cohesin from chromosome arms (Buheitel and Stemmann, 2013; Chan et al., 2012; Eichinger et al., 2013). At the centromere, Sgo1 in complex with protein phosphatase 2A (PP2A) is initially recruited to phosphorylated histone 2A (Kawashima et al., 2010). Upon Cdk1-dependent phosphorylation of Sgo1 at T346, the complex is then handed over to cohesin, where PP2A dephosphorylates SA2 and sororin, thus antagonizing mitotic phosphorylations and, by extension, the prophase pathway (Kitajima et al., 2006; Liu et al., 2013b; Riedel et al., 2006). This Sgo1-PP2A-dependent protection of centromeric cohesin renders final sister chromatid separation dependent on proteolytic cleavage by separase, which is tightly kept in check by its mutually exclusive inhibitors securin and cyclin B1-Cdk1 until the metaphase to anaphase transition (Gorr et al., 2005; Hellmuth et al., 2015; Stemmann et al., 2001).

At the beginning of G1 phase, each cell has one centrosome consisting of two centrioles and the surrounding pericentriolar material (PCM). When the cell enters S phase, the centrioles are duplicated as daughter centrioles are newly assembled

orthogonally to each of the existing mother centrioles (Kuriyama and Borisy, 1981). Mother and daughter centrioles are closely linked to each other, a state referred to as "engaged" (Kuriyama and Borisy, 1981). In G2 phase, the centrosomes mature and ultimately separate in order to form the spindle poles as the cell enters mitosis. After sister chromatid separation at the end of mitosis, the tight association of mother and daughter centriole is lost, while they remain loosely tethered by proteinaceous fibers (Bahe et al., 2005). This process, known as centriole disengagement, serves as a licensing step for later centriole duplication (Tsou and Stearns, 2006). Centriole disengagement has been shown to be dependent on combined Plk1 and separase activities (Schöckel et al., 2011; Tsou and Stearns, 2006; Tsou et al., 2009). For separase, two different targets at the centrosome have been described whose cleavage leads to disengagement of centrioles. We have previously shown that overexpression of a non-cleavable Scc1 cohesin subunit prevents centriole disengagement while ectopic cleavage of an engineered variant promotes it (Schöckel et al., 2011). Since other cohesin subunits (including Smc1 and -3) were also reported to localize to the centrosome (Beauchene et al., 2010; Gregson et al., 2001; Guan et al., 2008; Kong et al., 2009; Wong and Blobel, 2008), it is tempting to speculate that the whole cohesin ring might contribute to the cohesion between mother and daughter centriole. Apart from this, it was recently reported that separase-mediated cleavage of kendrin/pericentrin B (PCNT), a giant and highly abundant scaffold protein of the PCM, is also necessary and sufficient to trigger centriole disengagement (Lee and Rhee, 2012; Matsuo et al., 2012). The relative contributions of separase-dependent cleavage of cohesin and PCNT to centriole disengagement remain enigmatic.

The chromosome and centrosome cycles show striking parallels: (1) duplication of chromatids and centrosomes is limited to only once per cell cycle, (2) duplicated chromatids and centrosomes are evenly distributed to the newly forming daughter cells in mitosis, and (3) the regulation of both processes is marked by the dual use of several cell-cycle-coordinated key factors like Cdk1, Plk1, separase, and cohesin. Notably, Sgo1 has also been found to be involved in both processes: a knockdown of endogenous Sgo1 leads not only to premature loss of sister chromatid cohesion (due to abrogated cohesin-protection from the prophase pathway; McGuinness et al., 2005; Tang et al., 2004) but also to premature centriole disengagement (Schöckel et al., 2011; Wang et al., 2008; Yamada et al., 2012). It was reported that a smaller isoform of Sgo1 (sSgo1) localizes and functions at centrosomes rather than centromeres (Wang et al., 2006, 2008). In fact, there are 13 different mature transcripts of the Sgo1 gene derived from alternative splicing (ENSEMBL: ENSG00000129810). Of these, only 11 can theoretically be translated to a maximum of 7 different proteins (some mRNAs differ only in the length of their UTRs), of which 6 retain the two structural hallmarks of shugoshins, i.e. the N-terminal coiled-coil region and the conserved C-terminal Sgo C-box (UniProt: Q5FBB7). Sgo1 dimerizes via the N terminus and thereby forms an interaction site for PP2A (Xu et al., 2009). The Sgo C-box mediates binding to histone 2A, which needs to be pre-phosphorylated by the kinetochore-associated kinase Bub1 (Kawashima et al., 2010). So far, three Sgo1 isoforms have been investigated: the well-characterized, centromeric Sgo1 A1, whose mRNA contains exon 6 but misses exon 9; the centrosomal Sgo1 C2 (sSgo1) (Wang et al., 2006, 2008), with its mRNA missing exon 6 but containing exon 9; and Sgo1 B1, whose mRNA contains only part of exon 6 and lacks exon 9. Sgo1 B1 is overexpressed in certain cancer cells, localizes to the centromere, and has a dominant-negative effect on cohesion (Matsuura et al., 2013).

It is not yet understood how Sgo1 C2 is targeted to centrosomes instead of centromeres and how it protects centrosomes from premature disengagement. It has been proposed that the absence of the peptide encoded by exon 6 is the denominator for Sgo1 C2's centrosomal localization (Wang et al., 2008). Instead, we identify here the peptide encoded by exon 9 (consisting of only 40 amino acids) as the centrosomal targeting signal of human Sgo1 (CTS). The CTS not only is necessary and sufficient to direct Sgo1 and the fluorescent protein mCherry to centrosomes but also prevents targeting of Sgo1 to centromeres. Moreover, we demonstrate that centrosomal Sgo1 isoforms shield centriole engagement by PP2A-dependent protection of centrosomal cohesin from prophase pathway signaling.

RESULTS

Localization of Sgo1 Isoforms to the Centrosome Depends on the Presence of Exon 9

Alternative splicing gives rise to several isoforms of mammalian Sgo1. For human Sgo1, different isoforms have been described (Matsuura et al., 2013; Wang et al., 2006, 2008), which mostly differ in the presence or absence of amino acids encoded by the large exon 6 and the small exon 9 (Figure 1A). While the canonical Sgo1 A1 localizes to centromeres and protects sister chromatid cohesion, the short Sgo1 C2 (also called sSgo1) was reported to localize to centrosomes (Tang et al., 2004; Wang et al., 2006, 2008). Whether other isoforms have specific localizations and functions has not yet been studied. We generated stable transgenic Hek293 cell lines inducibly expressing Myc-tagged variants of Sgo1 A1, A2, C1, and C2 from small interfering RNA (siRNA)-resistant transgenes. Following their induction with doxycycline, the localization of these four Sgo1 isoforms relative to centromeres (as marked by CREST staining) and centrosomes (as marked by centrin 2 staining) was analyzed by immunofluorescence microscopy (IFM) using anti-Myc antibodies (Figure 1B). Sgo1 A1 localized to centromeres and Sgo1 C2 to centrosomes, as previously described, while Sgo1 A2 localized to centrosomes but not centromeres. This was surprising, since Sgo1 A2 is identical to A1 except for an additional 40 amino acids at its C terminus, which are encoded by exon 9. In contrast, Sgo1 C1, which represents C2 minus the 40 C-terminal amino acids encoded by exon 9, was found at centromeres and not centrosomes. These observations suggested that not the lack of exon 6 but rather the presence of the tiny exon 9 in the mRNA might dictate a centrosomal localization of Sgo1 protein isoforms. For these reasons, the peptide encoded by exon 9 will henceforth be referred to as "centrosomal targeting signal of human Sgo1" (CTS).

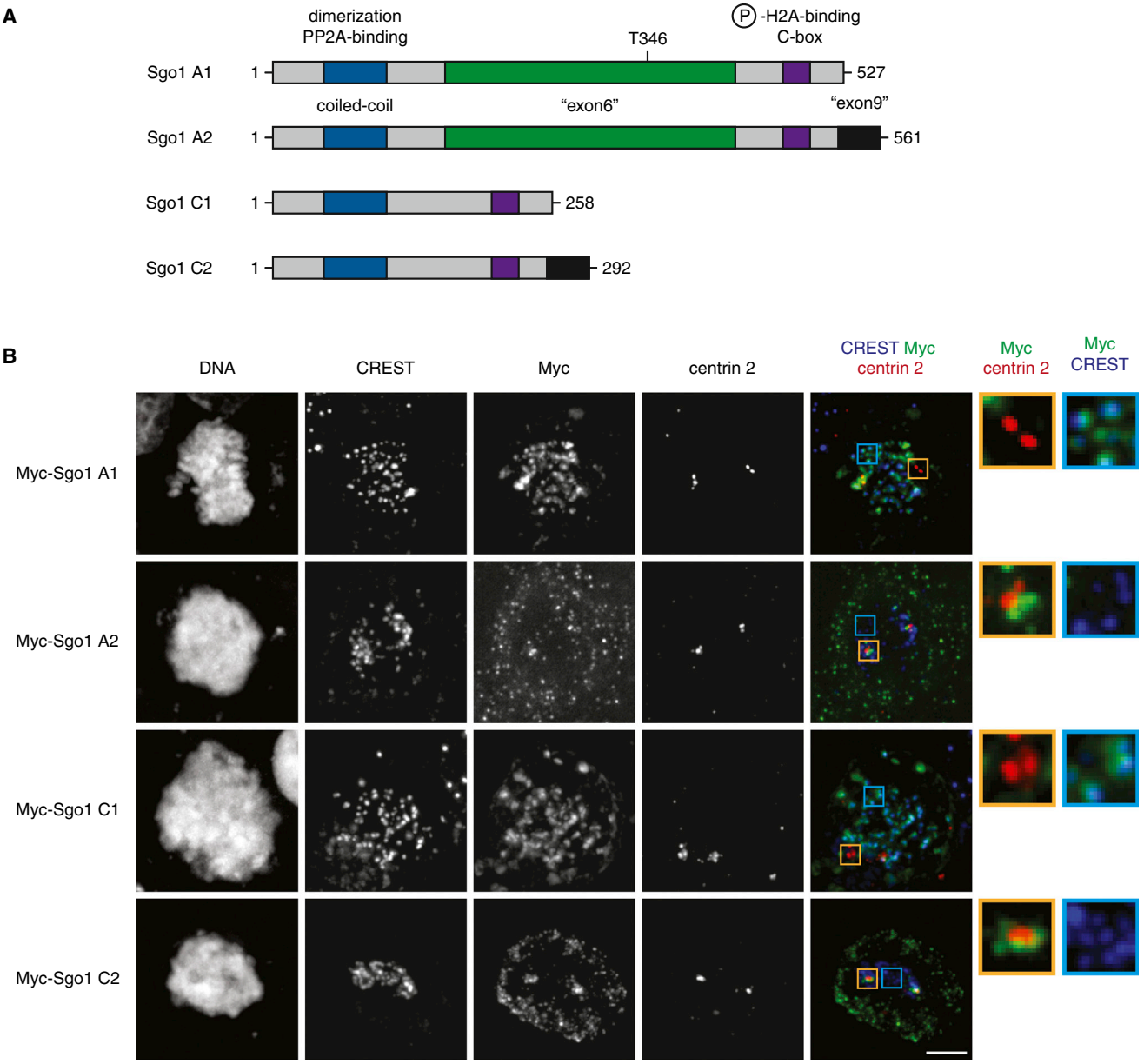

Figure 1. Localization of Sgo1 Isoforms

(A) Schematic view of Sgo1 isoforms drawn to scale.

(B) Sgo1 isoforms containing the peptide encoded by exon 9 localize to the centrosome. Expression of Myc-Sgo1 A1, A2, C1, or C2 was induced with doxycyline for 48 hr in stable Hek293 Flp-In T-REx cells. 24 hr before fixation, cells were transfected with *SGO1* siRNA. Cells were preextracted prior to fixation and CREST (centromere marker), centrin 2 (centrosomal marker), Myc (Sgo1 isoforms), and DNA (Hoechst 33342) were visualized by IFM. On the right, centrosomes (orange frame) and centromeres (blue frame) are shown at 4-fold magnification. Scale bar, 5 μm.

Site-Specific Rescue of Sgo1 Depletion Phenotypes by Specific Sgo1 Isoforms

It had been previously described that RNAi-mediated knockdown of Sgo1 leads to premature loss of sister chromatid cohesion as well as precocious centriole disengagement (Schöckel et al., 2011; Tang et al., 2004; Wang et al., 2008). We recapitulated this experiment and confirmed the reported Sgo1-depletion phenotype on sister chromatid cohesion in prometaphase-arrested cells as judged by spread chromosomes (Figures S1A and S1B). To quantify centriole (dis-) engagement, we immunofluorescently stained the distal centriole marker centrin 2 and the proximal marker C-Nap1 in fixed cells (Figure S1C) and on isolated centrosomes (Figure S1D). With both methods, we could equally verify that depletion of Sgo1 results in precocious centriole disengagement in addition to premature sister chromatid separation. To examine

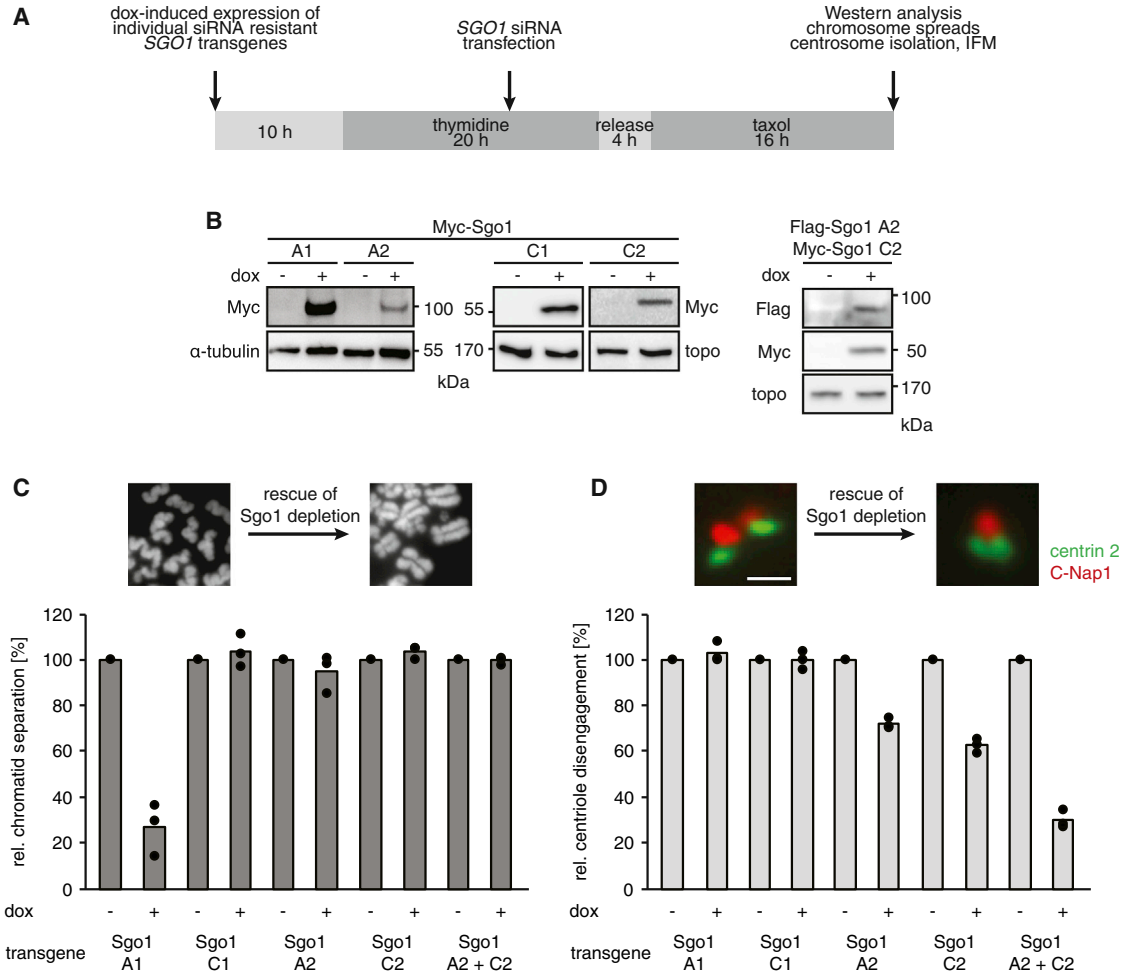

Figure 2. Site-Specific Rescue of Sgo1-Depletion Phenotypes by Specific Sgo1 Isoforms

(A) Experimental setup of Sgo1 knockdown-rescue experiments. At the indicated times, stable cell lines were induced by addition of doxycyline (dox) to express *SGO1* transgenes and transfected with *SGO1* siRNA to deplete all endogenous forms of Sgo1. Cells were synchronized in prometaphase by Taxol addition prior to analysis by western blotting, spreading of chromosomes, isolation of centrosomes, and IFM.

(B) Transgenic cell lines inducibly expressing siRNA-resistant Myc-Sgo1 A1, A2, C1, or C2 or both siRNA-resistant Flag-Sgo1 A2 and Myc-Sgo1 C2. The corresponding Hek293 Flp-In T-REx cell lines were treated as described in (A) and analyzed by Myc and Flag immunoblots for transgene expression. Immunodetection of α-tubulin or topoisomerase II α (topo) served as loading controls.

(C) Premature loss of sister chromatid cohesion in absence of endogenous Sgo1 is only suppressed by siRNA-resistant Sgo1 A1. Analysis of chromosome spreads.

(D) Only expression of (siRNA-resistant) Sgo1 A2 and/or C2 prevents premature centriole disengagement caused by depletion of endogenous Sgo1. Centrosomes were isolated and visualized by IFM using centrin 2 and C-Nap1 antibodies. Scale bar, 1 μm.

(C and D) The stable Hek293 Flp-In T-REx cell lines were treated as described in (A). Each column represents averages of three independent experiments (dots, 100 cells or centrosomes each). The amount of chromatid separation and centriole disengagement of + dox cells was normalized to the corresponding − dox samples (set to 100%).

See also Figures S1 and S2.

the function of specific Sgo1 isoforms, we capitalized on our stable Hek293 cell lines and induced the individual expression of transgenic Sgo1 A1, A2, C1, or C2 while simultaneously depleting all endogenous Sgo1 isoforms by RNAi. Following presynchronization in early S phase, cells were arrested in prometaphase and then analyzed (Figure 2A). Transgene expression was documented by immunoblotting (Figure 2B), the status of sister chromatid cohesion was assessed by chromosome spreading (Figure 2C), and centriole (dis-)engagement was examined by IFM on isolated centrosomes staining centrin 2 and C-Nap1 (Figure 2D). On the chromosomal level, only the canonical Sgo1 A1 was able to reduce the premature loss of sister chromatid cohesion by 73%, while the centrosomal isoforms Sgo1 A2 and C2 had no such effect (Figure 2C). Likewise, Sgo1 C1, despite localizing to centromeres, did not rescue the Sgo1 depletion phenotype at the chromosomes. Instead, cells transiently overexpressing Myc-Sgo1 C1 suffered from premature loss of sister chromatid cohesion and accumulated in

G2/M, indicating a dominant-negative effect (Figure S2). Similar observations were reported for cells overexpressing Sgo1 B1, a cancer-associated isoform lacking most of the peptide encoded by exon 6 (Matsuura et al., 2013), thereby making it very similar to C1 (Figure S2E). Premature centriole disengagement in Sgo1-less prometaphase cells could not be rescued by the expression of Sgo1 A1 and C1 but was partially rescued by the expression of Sgo1 A2 or C2. More specifically, the two centrosomal isoforms suppressed premature centriole disengagement by 28% and 37%, respectively (Figure 2D). Since these effects seemed rather small compared to the effect of Sgo1 A1 expression on the chromosomal phenotype (73% rescue), we asked if both isoforms might jointly be needed at centrosomes. To address this issue, we generated a doubly transgenic stable cell line that expressed Myc-tagged Sgo1 C2 and Flag-tagged Sgo1 A2 upon doxycycline addition (Figure 2B). Indeed, simultaneous expression of both centrosomal isoforms suppressed the centriole disengagement phenotype resulting from depletion of endogenous Sgo1 by 70% (Figure 2D). Thus, while centromeric Sgo1 A1 shields sister chromatid cohesion, centrosomal Sgo1 A2 and C2 protect centriole engagement.

Changing the C Terminus Reprograms Centrosomal Sgo1 A2 to Localize and Function at the Centromere

The localization of Sgo1 isoforms seems to depend on the presence of the CTS. But what are the minimum requirements for the localization and function of centrosomal Sgo1? The CTS consists of only 40 amino acids at the very C terminus of Sgo1 A2 and C2 and is conserved only in humans and higher primates (Figure S3A). The last seven amino acids, which are absent in orangutans, are also dispensable in humans, since Sgo1 C2 with the corresponding deletion still localized to the centrosome (data not shown). Remarkably, replacing the three conserved, consecutive amino acids ILY with alanines (Figure 3A) not only abolished centrosomal localization but also redirected the corresponding Sgo1 $A2^{AAA}$ to centromeres (Figure 3B). Crucially, and in line with its altered localization, this variant now rescued sister chromatid cohesion instead of centriole engagement in the absence of endogenous Sgo1 (Figure 3C). Thus, changing only three amino acids within the CTS is sufficient to reprogram Sgo1 A2 to mimic A1's localization and function.

The 40 Amino Acids Encoded by Exon 9 of Human Sgo1 Constitute a Transferrable Centrosomal Targeting Signal

In order to test whether the CTS of Sgo1 A2 or C2 might be sufficient for centrosomal localization, we expressed it C-terminally fused to mCherry. In both HeLa K and Hek293T cells, this mCherry-CTS localized to centrosomes. Changing the conserved ILY motif to AAA abrogated centrosomal localization without influencing the expression level of the fusion protein (Figures 4A and S3B). Moreover, the CTS-mediated centrosomal recruitment is independent of the presence of microtubules as mCherry-CTS still localized to the centrosome upon nocodazole treatment (Figure S3B). Although the murine *SGO1* gene lacks exon 9, the mechanism, which allows the CTS to mediate centrosomal recruitment, seems to be conserved, since mCherry-CTS expressed in mouse NIH 3T3 fibroblasts readily localized to the centrosome, while the corresponding ILY to AAA variant again failed to do so (Figure 4D). Therefore, even if Sgo1's centrosomal targeting signal is not conserved between human and mouse, the interaction partner of the human CTS at the centrosome certainly is. It has been reported that $Sgo1^{+/-}$ mouse embryonic fibroblasts (MEFs) suffer from premature centriole disengagement (Wang et al., 2008). To further investigate a potential role of Sgo1 at murine centrosomes, we expressed Myc-tagged mouse Sgo1 in NIH 3T3 cells, where it localized to both centromeres and centrosomes (Figure S3C). RNAi-mediated depletion of murine Sgo1 expectedly caused premature loss of sister chromatid cohesion in NIH 3T3 cells (Figure 4E). Consistently, it also resulted in premature centriole disengagement as judged by the increased distance of centrin 2 signals in situ (Figure 4F). Thus, mice seem to utilize a single Sgo1 isoform to fulfill both centromeric and centrosomal functions of shugoshin 1.

In a previous study, the absence of the peptide encoded by exon 6 was considered to be responsible for centrosomal localization, since a Myc-tagged N-terminal part of Sgo1 (amino acids 1–196) localized to centrosomes in HeLa cells (Wang et al., 2008). Trying to recapitulate these results, we expressed the same construct in Hek293T cells, in which it did not localize to the centrosomes (Figure S3D). In HeLa K cells, however, we were able to detect localization at the centrosomes as well as the spindle. Strikingly, the centrosomal staining was lost upon depletion of endogenous Sgo1 (Figure S3D), which leads us to conclude that centrosomal recruitment of this N-terminal fragment most likely depends on dimerization with endogenous Sgo1 via the coiled-coil domain. The binding to the spindle seems to be independent of dimerization and also specific for HeLa cells. Given that the CTS-dependent recruitment of mCherry to the centrosome occurred in Hek293T, HeLa K and even murine NIH 3T3 cells, we propose the CTS-mediated mechanism of centrosomal Sgo1-recruitment to be of more general importance. These experiments already strongly suggest that the CTS is recruited to the centrosome by direct binding to an as-yet-unknown centrosomal protein. If this was true, one would expect heavy overexpression of the CTS to outcompete endogenous Sgo1 A2 and C2 for binding to centrosomes and thereby phenocopy a Sgo1 depletion. Indeed, when transiently overexpressed in fusion with an FKBP (FK506 binding protein 12) tag, wild-type (WT) CTS, but not the ILY to AAA variant, triggered premature centriole disengagement in Hek293T cells (Figures 4B and 4C).

Recruitment of PP2A by Sgo1 Is Essential for Maintenance of Centriole Engagement

At centromeres Sgo1 protects cohesin by recruiting the B′ (B56) isoform of PP2A, thereby antagonizing the phosphorylation-dependent prophase pathway (Kitajima et al., 2006; Nishiyama et al., 2013; Riedel et al., 2006; Xu et al., 2009). We speculated that the phosphatase might have a similar function for the protection of centriole engagement. Accordingly, using an antibody against the catalytical PP2A-C subunit in IFM, we could show that PP2A also localized to the centrosome and that this localization depended on Sgo1, as the PP2A signal is lost upon Sgo1 depletion (Figure 5A). To test whether PP2A

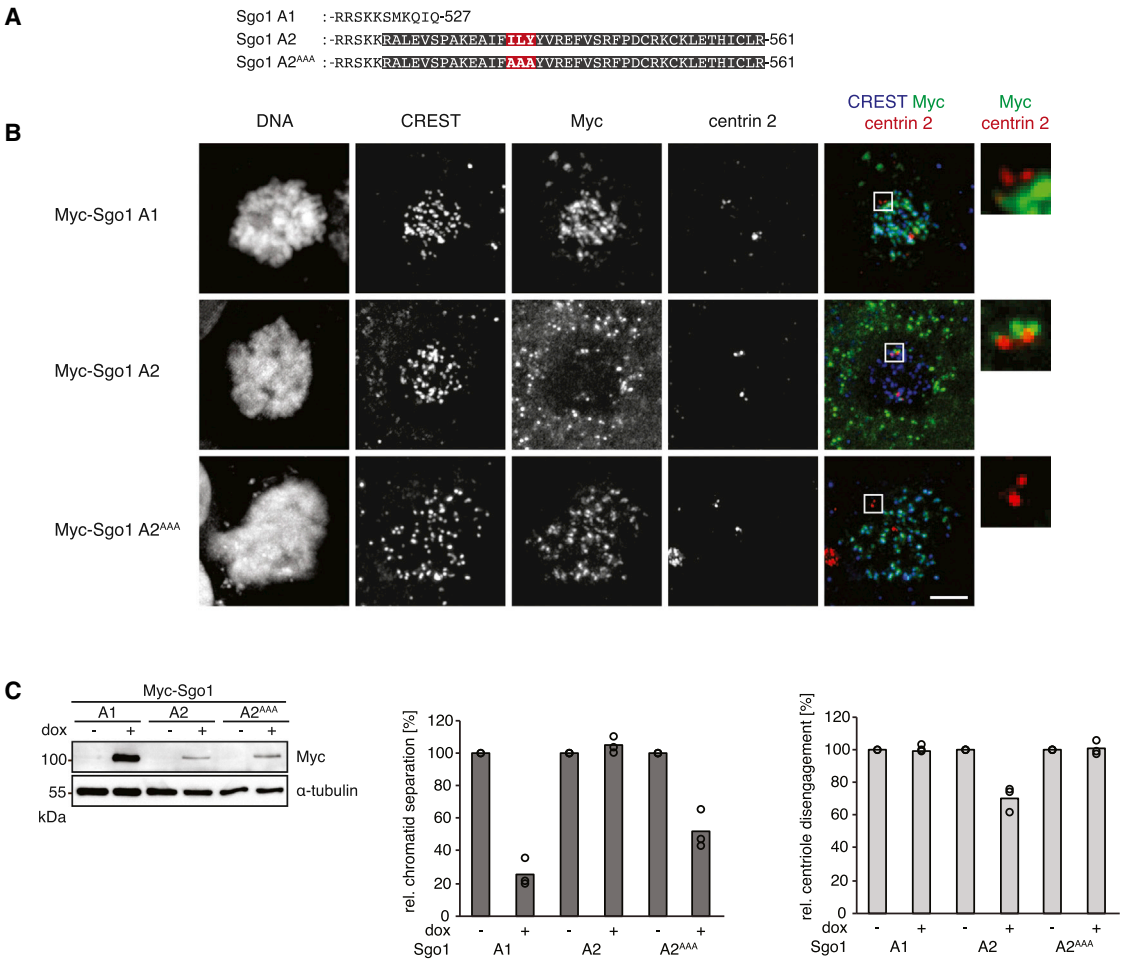

Figure 3. Changing the C Terminus Reprograms Centrosomal Sgo1 to Localize and Function at the Centromere

(A) Sequence alignment of the C termini of Sgo1 A1 and A2. Amino acids encoded by exon 9 are highlighted in black. The three consecutive amino acids important for centrosomal localization are colored red and mutated to alanines in the Sgo1 A2^{AAA} variant.

(B) Sgo1 A2^{AAA} localizes not to the centrosome but to the centromere. Expression of Myc-Sgo1 A1, A2 and A2^{AAA} was induced with dox for 48 hr in stable Hek293 Flp-In T-REx cells. 24 hr before fixation, cells were transfected with *SGO1* siRNA. Cells were preextracted prior to fixation and CREST, centrin 2, Myc (Sgo1 isoforms), and DNA (Hoechst 33342) were visualized by IFM. On the right, centrosomes are shown at 4-fold magnification. Scale bar, 5 μm.

(C) Expression of Sgo1 A2^{AAA} cannot prevent premature centriole disengagement but can prevent loss of sister chromatid cohesion caused by Sgo1 depletion. The transgenic cell lines inducibly expressing siRNA resistant Myc-Sgo1 A1, A2, or A2^{AAA} were treated as described in Figure 2A in the presence or absence of dox before being analyzed for transgene expression, sister chromatid cohesion, and centriole engagement status. Each column represents averages of three independent experiments (dots, 100 cells or centrosomes each). The amount of chromatid separation and centriole disengagement of + dox cells was normalized to the respective − dox cells (set to 100%).

recruitment is required for the Sgo1-mediated protection of centriole engagement, we introduced previously described compromising mutations (N61I and Y57A, K62A) into the PP2A binding site of Sgo1 A2 and C2 (Xu et al., 2009). Co-immunoprecipitation experiments confirmed that both variants indeed exhibited greatly reduced PP2A binding in comparison to WT Sgo1 A2 and C2 (Figure 5B). Additionally, while the mutants still localized to centrosomes (Figures S4A and S4C), they were not able to recruit PP2A to the centrosomes (Figures S4B and S4D). Most importantly however, PP2A-binding deficiency correlated with the inability of the variants to prevent premature centriole disengagement in the absence of endogenous Sgo1 (Figure 5C). These results strongly suggest that Sgo1's function as a recruitment factor for PP2A is conserved between centromeres and centrosomes. If this notion is true, then artificially tethering PP2A to centrosomes should bypass the need for Sgo1 to protect centriole engagement. To test this prediction, we expressed PP2A-B′α in fusion with an extended version of the CTS in Hek293T cells (Figure 5D). This fusion protein readily localized to the centrosomes in an Sgo1-depletion background, while the ILY to AAA variant and WT PP2A-B′α did not (Figures S4E and S4F). Crucially, assessment of centriole engagement status revealed that PP2A-B′α-CTS indeed suppressed premature centriole disengagement by 45%, while neither the corresponding ILY to AAA variant nor WT PP2A-B′α could rescue the Sgo1 depletion phenotype (Figure 5E). Similar

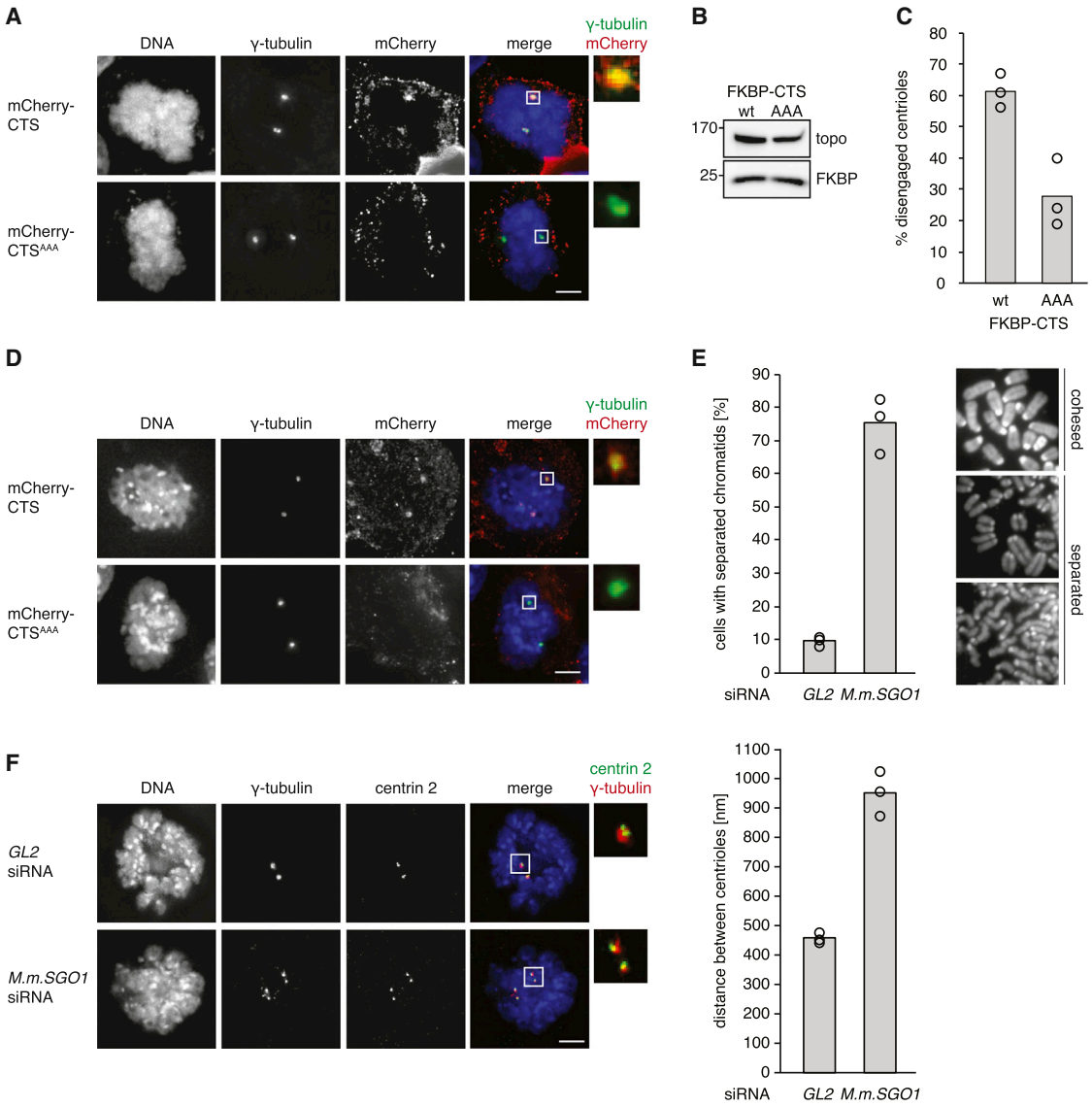

Figure 4. The 40 Amino Acids Encoded by Exon 9 Constitute a Transferrable Centrosomal Targeting Signal

(A) Fusion to the CTS (centrosomal targeting signal of Sgo1), but not the ILY to AAA variant thereof, directs mCherry to centrosomes. Wild-type and the AAA variant of mCherry-CTS were transiently expressed in Hek293T cells for 48 hr. To enrich for mitotic cells, presynchronized cells were released from a G1/S arrest 10 hr prior to preextraction, fixation, and staining for γ-tubulin, mCherry, and DNA (Hoechst 33342). On the right, centrosomes are shown at 4-fold magnification. Scale bar, 5 μm.

(B) Overexpression of the CTS induces premature centriole disengagement. Hek293T cells were transfected with plasmids encoding FKBP-CTS (WT or AAA) 24 hr prior to addition of thymidine. Cells were then treated as described in Figure 2A. Expression of transgenes was analyzed by western blot.

(C) Centrosomes from (B) were isolated and visualized by IFM using centrin 2 and C-Nap1 antibodies. Each column represents averages of three independent experiments (dots, 100 centrosomes each).

(D) mCherry-CTS localizes to the centrosomes in mouse cells. mCherry-CTS and mCherry-CTS^{AAA} were transiently expressed in NIH 3T3 cells for 36 hr. Cells were fixed and stained for γ-tubulin, mCherry, and DNA (Hoechst 33342). On the right, centrosomes are shown at 4-fold magnification. Scale bar, 5 μm.

(E) Knockdown of murine Sgo1 causes premature sister chromatid separation and centriole disengagement. NIH 3T3 cells were thymidine-arrested in early S phase and transfected with *M.m. SGO1* siRNA. After release, cells were synchronized in prometaphase with Taxol. Status of chromatid cohesion was analyzed by spreading of chromosomes. Each column represents averages of three independent experiments (dots, 100 centrosomes each).

(F) Aliquots of cells from (E) were fixed and stained for γ-tubulin, centrin 2, and DNA (Hoechst 33342). On the right, centrosomes are shown at 4-fold magnification. Scale bar, 5 μm. To discriminate between engaged and disengaged centrioles (in the absence of a working antibody against murine C-Nap1), the distance between the two centrin 2 dots, representing the centrioles of one centrosome, was measured. Each column represents average distances from three independent experiments (dots, 100 centrosomes each).

See also Figure S3.

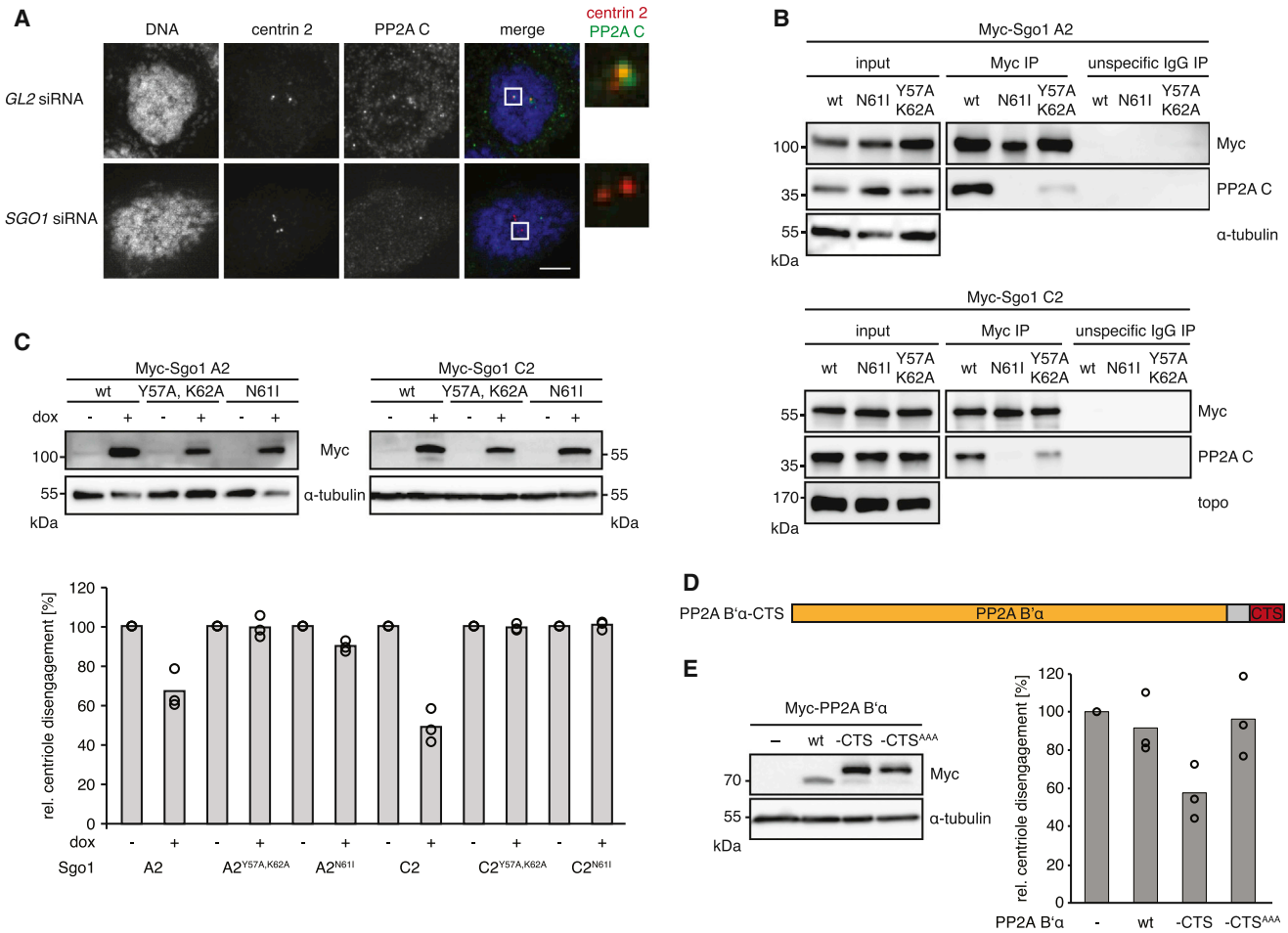

Figure 5. Recruitment of PP2A by Sgo1 Is Essential for Maintenance of Centriole Engagement

(A) Localization of PP2A to centrosomes is lost upon Sgo1 depletion. 24 hr before fixation, Hek293T cells were transfected with *GL2* or *SGO1* siRNA. Cells were preextracted prior to fixation and stained for centrin 2, PP2A-C, and DNA (Hoechst 33342). On the right, centrosomes are shown at 4-fold magnification. Scale bar, 5 μm.

(B) Variants of Sgo1 A2 and C2 bearing Y57A, K62A, or N61I mutations in the N-terminal coiled-coil domain can no longer bind to PP2A. Myc-tagged variants of A2 or C2 were transiently expressed in Hek293T cells for 36 hr. Cell lysates were subjected to immunoprecipitation (IP) with anti-Myc or unspecific immunoglobulin G. Inputs and eluates were finally analyzed by western blot using the indicated antibodies.

(C) Expression of PP2A binding-deficient variants of Sgo1 A2 and C2 does not rescue the premature centriole disengagement caused by Sgo1 depletion. Transgenic Hek293 cell lines inducibly expressing siRNA-resistant wild-type (WT) Myc-Sgo1 A2/C2 or PP2A binding-deficient variants thereof (Y57A, K62A or N61I) were treated as described in Figure 2 A before being analyzed by immunoblotting and centrosomes isolation followed by IFM using centrin 2 and C-Nap1 antibodies. Each column represents averages of three independent experiments (dots, 100 centrosomes each). The amount of centriole disengagement of + dox cells was normalized to – dox cells (set to 100%).

(D) PP2A-B′α can artificially be recruited to the centrosome by fusion to Sgo1's CTS. Schematic view of chimeric protein consisting of PP2A-B′α and the C terminus of Sgo1 A2 (aa 493–561) drawn to scale.

(E) CTS-mediated recruitment of PP2A to the centrosome prevents premature centriole disengagement caused by Sgo1 depletion. Hek293T were transfected with plasmids encoding Myc-tagged PP2A-B′α, PP2A-B′α-CTS, or PP2A-B′α-CTS^{AAA} and treated as described in Figure 2A before being analyzed by immunoblotting and IFM on isolated centrosomes. Each column represents averages of three independent experiments (dots, 100 centrosomes each).

See also Figure S4.

to mCherry-CTS (see Figure S3B), CTS-mediated recruitment of PP2A-B′α also does not depend on the presence of microtubules (IFM data not shown), as its ability to rescue the Sgo1 depletion phenotype is not abrogated upon nocodazole treatment (Figures S4G and S4H). Thus, Sgo1's centrosomal function lies in its ability to recruit PP2A, which then acts as the actual effector for the protection of centriole engagement.

Sgo1 Protects Centrosomal Cohesin from Prophase Pathway Signaling

Prophase pathway signaling causes phosphorylation-dependent cohesin opening at the Smc3-Scc1 interface (Buheitel and Stemmann, 2013; Eichinger et al., 2013) and is counteracted at centromeres by Sgo1-PP2A. Therefore, abrogating the prophase pathway by depletion of its key factor, Wapl, abolishes

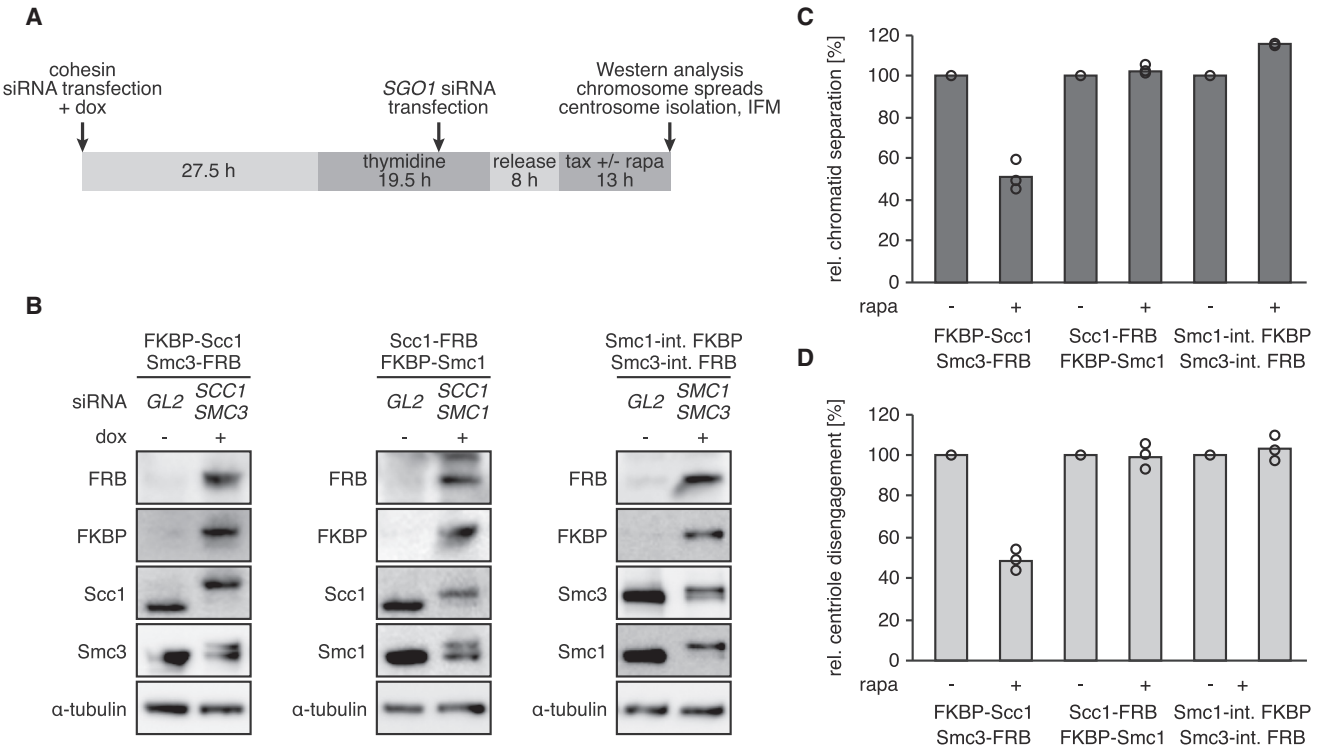

Figure 6. Sgo1 Protects Centrosomal Cohesin from Prophase Pathway Signaling

(A) Experimental setup of Sgo1 knockdown rescue. Expression of transgenes was induced in doubly stable cell lines transfected with cohesin and *SGO1* siRNAs as indicated. Cells were synchronized in prometaphase, supplemented with rapamycin (rapa) to close individual cohesion gates, and finally analyzed by western blotting, spreading of chromosomes, and IFM on isolated centrosomes.

(B) Three doubly transgenic cell lines inducibly co-expressing FKBP-Scc1 and Smc3-FRB, Scc1-FRB and FKBP-Smc1, or Smc1-int. FKBP and Smc3-int. FRB were transfected with *GL2*- or cohesin-directed siRNAs and incubated for 3 days in the absence (for *GL2* RNAi) or presence (for cohesin RNAi) of dox. Note that Smc3-FRB, Scc1-FRB, and Smc3-int. FRB migrate only slightly above the untagged proteins and, thus, are difficult to discern from the endogenous subunits in the mock-depleted samples. Note also that the western signals for Scc1-FRB and Smc3-FRB do not accurately reflect their expression levels because the corresponding antibodies display a greatly reduced sensitivity when their antigens are C-terminally tagged.

(C) Locking of the Scc1-Smc3 gate rescues premature loss of sister chromatid cohesion caused by Sgo1 RNAi. Analysis of chromosome spreads.

(D) Locking of the Scc1-Smc3 gate suppresses premature centriole disengagement caused by Sgo1 RNAi. Centrosomes were isolated and visualized by IFM using centrin 2 and C-Nap1 antibodies.

(C and D) Each column represents averages of three independent experiments (dots, 100 cells or centrosomes each). The amounts of chromatid separation and centriole disengagement of + rapa cells were normalized to − rapa cells (set to 100%).

See also Figure S5.

the need for Sgo1-mediated protection and rescues premature loss of sister chromatid cohesion usually caused by Sgo1 knockdown (Gandhi et al., 2006). To explore the possibility that Sgo1-PP2A's function might be conserved on centrosomes, we tested, whether a Wapl knockdown was able to also alleviate premature centriole disengagement associated with Sgo1 depletion. This was indeed the case, thus arguing for conservation of Sgo1-PP2A's role as a cohesion protector between chromo- and centrosomes (Figures S5A–S5C). If the prophase pathway was acting on cohesin also at centrosomes, then artificially locking the Smc3-Scc1 gate might prevent premature centriole disengagement caused by Sgo1 depletion. We capitalized on previously generated doubly transgenic Hek293 cell lines, in which each of the three cohesin gates (Smc1-Smc3, Smc3-Scc1, or Scc1-Smc1) is tagged with FKBP and FRB (FKBP-rapamycin binding domain of mTOR) in such a way that they can individually be locked by rapamycin-induced FKBP-FRB heterodimerization (Buheitel and Stemmann, 2013). To guarantee efficient replacement of endogenous cohesin by engineered ring complexes, the induced expression of each pair of FKBP/FRB-tagged variants was combined with simultaneous depletion of the corresponding endogenous subunits by RNAi. Two days later, the cells were synchronized in early S phase. During this arrest, the cells were depleted of Sgo1 by siRNA transfection and later released into early G2 phase. Then, taxol and rapamycin (or DMSO as control) were added, respectively, to arrest cells in prometaphase of the following mitosis and lock each of the cohesin gates in the corresponding cell line (Figure 6A). Finally, the expression of the transgenes, the efficiency of the cohesin knockdowns, and the degree of sister chromatid separation and centriole disengagement were analyzed as before (Figures 6B–6D). Consistent with our previous finding (Buheitel and Stemmann, 2013), the loss of sister chromatid cohesion in Sgo1-depleted cells could be

mitigated by closure of the Smc3-Scc1, but not by locking of the Smc1-Smc3 or Scc1-Smc1 gate (Figure 6C). Interestingly, the same effect could be observed at the centrosomal level: centriole disengagement in response to Sgo1 depletion was alleviated by blocking the Smc3-Scc1 gate but not by keeping the other gates closed (Figure 6D). Note that the absence of a phenotype for the Smc1-Smc3 and the Scc1-Smc1 cell lines is not due to non-functionality of FRB/rapamycin/FKBP-mediated closure of the corresponding gates (Buheitel and Stemmann, 2013; Figures S5D and S5E). Thus, Sgo1-PP2A is antagonizing the prophase pathway by preventing premature opening of cohesin's exit gate not only at centromeres but also at centrosomes.

Dissociation of Cohesin from Centrosomes in Late Mitosis Requires Separase Activity

While we do believe that a certain pool of cohesin is removed from centrosomes during prophase, removal of Sgo1-PP2A-protected cohesin and, thus, ultimate centriole disengagement still depends on the action of separase (Schöckel et al., 2011; Tsou and Stearns, 2006). To further corroborate this notion, we inactivated the prophase pathway by RNAi-mediated knockdown of its key player, Wapl, in transgenic HeLa cells expressing the separase inhibitor securin either in its WT form or as a non-degradable variant (KEN and D-box mutated = KD_{mut} (Hellmuth et al., 2014). The cells were presynchronized with thymidine and then released into a taxol-mediated prometaphase-arrest. Addition of the aurora B kinase inhibitor ZM447439 (ZM) was used to release the cells from the arrest and synchronously drive them through late mitosis into G1 phase (Figure 7A). Cells were harvested 30 min after the release and analyzed by immunoblotting (Figure 7B) and by IFM using γ-tubulin as a centrosomal and Smc1 as a cohesin marker (Figures 7C and 7D). In parallel, centrosomes were isolated and assessed for their centriole engagement status (Figure 7E). Quantification of cells displaying centrosomal Smc1 signals (Figure 7C) revealed that inactivation of the prophase pathway alone does not abrogate the dissociation of cohesin from centrosomes during transition from prometaphase into late mitosis, which is hallmarked by separase auto-cleavage, cyclin B1 degradation and histone 3 serine 10-dephosphorylation (Figure 7B). At the same time, centriole engagement was lost (Figure 7E). In contrast, overexpression of non-degradable securin incapacitated separase, as exemplified by lack of auto-cleavage, and resulted in continued association of cohesin with centrosomes (Figure 7D) and engagement of centrioles (Figure 7E). This phenotype was not due to a failure to resume cycling because cyclin B1 degradation and histone 3 serine10-dephosphorylation occurred on schedule (Figure 7B). To rule out unspecific binding of the Smc1 antibody to centrosomes, we performed RNAi-mediated knockdown of endogenous Smc1 for 2 days. The resulting weakened Smc1 band in western analysis correlated well with reduced signals on both chromatin and centrosomes in IFM, thus confirming the specificity of the antibody (Figure S6). Together, these results corroborate the importance of separase-mediated proteolysis for removal of cohesin from centrosomes at a time when they are scheduled to undergo centriole disengagement.

DISCUSSION

Extending a previous study (Wang et al., 2006, 2008), we demonstrate here that various splice variants of human Sgo1 exclusively localize to either centromeres or centrosomes. Whereas Sgo1 A1 binds only to centromeres, Sgo1 A2 and C2 are exclusively found at centrosomes. How Sgo1 A1 is targeted to centromeres has been extensively studied in the past. It binds via its Sgo-C box to Bub1-phosphorylated, centromeric histone 2A from where it is handed over to cohesin when Cdk1 phosphorylates T346 encoded by exon 6 (Liu et al., 2013a, 2013b). Here, we now identify the 40 C-terminal amino acids encoded by exon 9 of the A2 and C2 isoforms as the centrosomal targeting signal of human Sgo1 (CTS). Sgo1 A1 lacks the CTS, which readily explains why it is not found at centrosomes. Conversely, centrosomal Sgo1 A2 and C2 still contain the major centromeric targeting signal, the Sgo-C box, which strongly implies that the short CTS fulfills a dual function of mediating centrosomal targeting while simultaneously abolishing centromeric localization. The underlying mechanisms, however, remain unclear. The CTS has no homologies to the centrosome-localizing PACT domain of pericentrin and AKAP450 (Gillingham and Munro, 2000). With its 40 residues (of which we even know the last 7 to be dispensable), it is also much shorter than the 90-amino-acid-long PACT domain. Given that the CTS represents a transferrable centrosomal localization signal, which functions even in murine cells, it is conceivable that it binds to a conserved, yet hitherto unknown centrosomal component. The dominant anti-centromeric effect of the CTS might be explained by various models. One might envision that the CTS binds and thereby masks the Sgo-C box, although we could not detect such an interaction by genetic or biochemical assays (data not shown). Alternatively, the CTS might serve as a nuclear export sequence (NES), thereby excluding those isoforms from the nucleus and preventing binding to the centromere. In fact, while Sgo1 A1 localizes to the nucleoplasm in interphase, the CTS containing Sgo1 A2 and C2 are retained within the cytoplasm (Kang et al., 2011; data not shown). Furthermore, the CTS contains three sequence stretches weakly resembling a Crm1/exportin1-specific NES (Güttler et al., 2010). However, inhibition of Crm1-dependent nuclear export with leptomycin B did not result in an altered localization of the centrosomal isoforms (data not shown). Therefore, one might speculate that the CTS either alters Sgo1's three-dimensional structure in such a way that it is no longer accessible for binding to the centromere and/or that the affinity of the CTS for the centrosome exceeds that of the Sgo C-box to phosphorylated histone 2A.

Intriguingly, differential localization of human Sgo1 isoforms correlates with differential functions in that A1 protects centromeric sister chromatid cohesion whereas A2 and C2 prevent premature centriole disengagement. Sgo1 at centromeres mediates the PP2A-dependent protection of cohesin ring complexes from prophase pathway signaling, which would otherwise result in premature opening of the Smc3-Scc1 gate (Kitajima et al., 2006; Riedel et al., 2006). However, the centrosomal target of Sgo1-protection has remained enigmatic. Here, we now demonstrate that blocking the opening of the Smc3-Scc1 gate either indirectly via Wapl depletion or directly via rapamycin-mediated

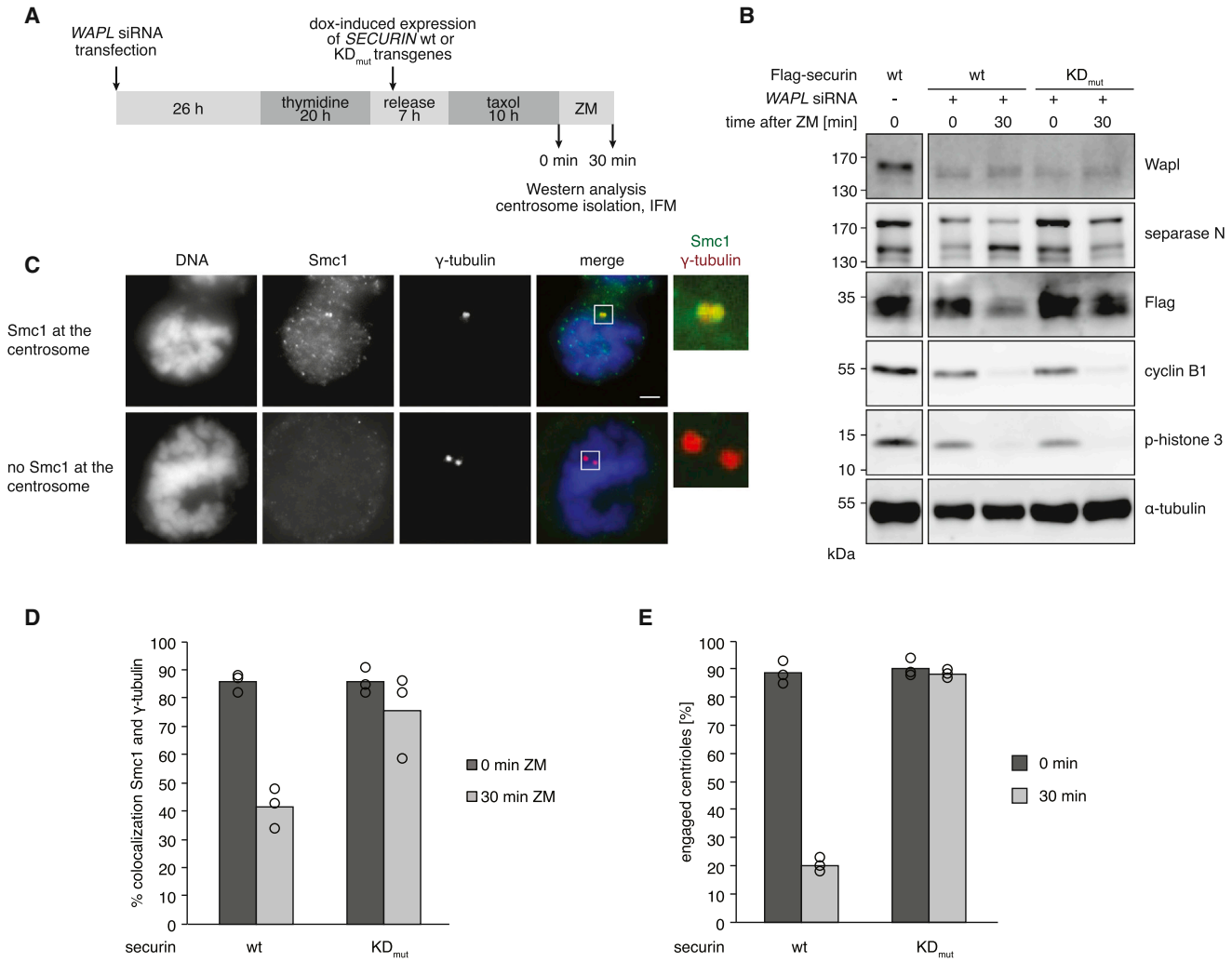

Figure 7. Dissociation of Cohesin from Centrosomes and Centriole Disengagement in Late Mitosis Require Separase Activity

(A) Experimental setup of override of Taxol arrest by ZM447439 (ZM). Transgenic HeLa cell lines inducibly expressing Flag-tagged versions of wild-type (WT) or non-degradable (KD_{mut}) securin were depleted of Wapl by RNAi prior to synchronization in early S phase by addition of thymidine. Cells were released into fresh medium, induced to express the *SECURIN* transgenes, and then arrested with Taxol 10 hr prior to addition of ZM to override the prometaphase arrest. Directly before (0 min) and 30 min after ZM addition, samples were taken for western blotting and IFM.

(B) HeLa cell lines treated as described in (A) were analyzed in immunoblots for transgene expression (anti-Flag), separase activation (auto-cleavage; anti-separase antibody raised against the N terminus), degradation of cyclin B1, dephosphorylation of histone 3 S10, and Wapl depletion efficiency. Anti-α-tubulin staining served as the loading control.

(C) Cells treated as described in (A) were preextracted, fixed, and stained for Smc1, γ-tubulin, and DNA (Hoechst 33342).

(D) Co-localization of Smc1 and γ-tubulin as shown in (C) was quantified. Each column represents averages of three independent experiments (dots, 100 cells each). Scale bar, 5 μm.

(E) Cells treated as described in (A) were analyzed by IFM on isolated centrosomes. Each column represents averages of three independent experiments (dots, 100 centrosomes each).

See also Figure S6.

heterodimerization of FRB/FKBP-tagged cohesin subunits (partially) rescues the precocious loss of centriole engagement in Sgo1-depleted, prometaphase-arrested cells. Thus, Sgo1's centrosomal function consists at least partly, if not exclusively (see below), in protection of cohesin from the prophase pathway. Extending the parallels to the situation on chromosomes, the role of centrosomal Sgo1 also lies in the recruitment of PP2A, as exemplified by the inability of PP2A-binding-deficient A2 variants to functionally replace endogenous Sgo1 at centrosomes. Strikingly, despite the fact that it lacks T346, which is of crucial importance for the Sgo1 A1-dependent protection of chromosomal cohesin, centrosomal Sgo1 C2 is still active in shielding centriole engagement. This might imply that Sgo1 does not need to bind centrosomal cohesin directly and that the tethering of PP2A in its proximity is sufficient to counteract phosphorylation-dependent opening of the ring. Consistently, expression of PP2A-B′α

in fusion with the CTS partially suppresses centriole disengagement in response to Sgo1 depletion. Alternatively, centrosomal cohesin might be guarded only by (T346-containing) Sgo1 A2-PP2A, in which case Sgo1 C2-PP2A could have a so far unidentified, different substrate. The additive rescue effect of the simultaneous expression of both A2 and C2 in Sgo1-depleted cells would be consistent with this scenario. An attractive yet highly speculative possibility is that this putative second substrate of centrosomal Sgo1 might be PCNT, which next to cohesin represents the other known centriole engagement factor and separase substrate. It should be emphasized that we assay premature centriole disengagement in prometaphase-arrested, Sgo1-depleted cells, in which the prophase pathway is active but separase is not. Therefore, a corollary of this model would be that PCNT represents a hitherto-unappreciated second substrate of the prophase pathway.

In both human and murine cells, Sgo1 localizes to centrosomes and protects centriole engagement. Despite this conservation, the CTS specifies the localization of primate Sgo1, whereas mouse Sgo1 is targeted to centrosomes by other means. CTS's absence from murine Sgo1 might be explained by the high evolutionary plasticity of alternative splicing with only 28% of exons present in minor splice forms (<50% of transcripts) being conserved between human and mouse (Harr and Turner, 2010). But why do primates employ different variants to fulfill the centromeric and centrosomal functions of Sgo1? The chromosome and centrosome cycles are usually strictly synchronized with each other, but this rule is violated on rare occasions as, for example, male meiosis (Cunha-Ferreira et al., 2009). Here, centrioles disengage and centrosomes duplicate between MI and MII, while DNA replication must not occur. Thus, the functional specialization of alternatively spliced Sgo1 variants might facilitate uncoupling of the centrosome cycle from the chromosome cycle in human spermatocytes. Naturally, this division of labor requires exquisite regulation, as imbalanced expression ratios of centromeric versus centrosomal Sgo1 isoforms could result in abnormal numbers of chromosomes and/or centrosomes, both of which have been associated with the formation of cancer (Chan, 2011; Hanahan and Weinberg, 2011). It will therefore be interesting to address in future studies how human cells usually ensure homeostasis of relative Sgo1 variant levels and whether imbalanced expression ratios of Sgo1 isoforms might be associated with cellular transformation.

EXPERIMENTAL PROCEDURES

Cultivation of Cells, RNAi, and Transfection

All cell lines were cultured in DMEM (PAA) supplemented with 10% fetal calf serum (FCS) (Sigma) at 37°C and 5% CO_2. siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) for HeLa, U2OS, and NIH 3T3 and the calcium-phosphate-based method for Hek293T and Hek293 Flp-In T-REx. The following siRNAs were used for the knockdown of endogenous proteins: *SGO1*: 5′-GAUAGCUGUUGCAGAAGUA-3′ (*SGO1*_5′UTR) and 5′-CAGUAGAACCUGCUCAGAA-3′ (*SGO1*_ORF1) or 5′-GAUGACAGCUCCAGAAAUU-3′ (*SGO1*_ORF2); murine Sgo1: 5′-GCUACACUACUGAGAUAUU-3′ (*M.m.SGO1*_ORF1) and 5′-GCAUUGAAAGAGAAGCUAA-3′ (*M.m.SGO1*_ORF2); Wapl: 5′-CGGACUACCCUUAGCACAA-3′ (Wapl1) and 5′-GGUUAAGUGUUCCUCUUAU-3′ (Wapl2); Scc1: 5′-ACUCAGACUUCAGUGUAUA-3′ (Scc1-1), and 5′- AGGACAGACUGAUGGGAAA-3′ (Scc1-2); Smc1: 5′-GGAAGAAAGUAGAGACAGA-3′; Smc3: 5′-UGGGAGAUGUAUAUAGUAA-3′ (Smc3-1) and 5′-UGUCAUGUUUGUACUGAUA-3′ (Smc3-2); Luciferase: 5′-CGUACGCGGAAUACUUCGAUU-3′ (GL2).

Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) for HeLa and NIH 3T3 cells and the calcium-phosphate-based method for Hek293T and Hek293 Flp-In cells.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.045.

AUTHOR CONTRIBUTIONS

L.M., J.B., L.S., and D.K. conducted the experiments. L.M. and J.B. wrote the manuscript. B.M. initiated the project. O.S. designed the experiments and contributed to writing of the manuscript.

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