

sSgo1, a Major Splice Variant of Sgo1, Functions in Centriole Cohesion Where It Is Regulated by Plk1

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SUMMARY

Shugoshin 1 (Sgo1) functions as a protector of centromeric cohesion of sister chromatids in higher eukaryotes. Here, we provide evidence for a previously unrecognized role for Sgo1 in centriole cohesion. Sgo1 depletion via RNA interference induces the formation of multiple centrosome-like structures in mitotic cells that result from the separation of paired centrioles. *Sgo1*^{+/-} mitotic murine embryonic fibroblasts display split centrosomes. Localization study of two major endogenous splice variants of Sgo1 indicates that the smaller variant, sSgo1, is found at the centrosome in interphase and at spindle poles in mitosis. sSgo1 interacts with Plk1 and its spindle pole localization is Plk1 dependent. Centriole splitting induced by Sgo1 depletion or expression of a dominant negative mutant is suppressed by ectopic expression of sSgo1 or by Plk1 knockdown. Our studies strongly suggest that sSgo1 plays an essential role in protecting centriole cohesion, which is partly regulated by Plk1.

INTRODUCTION

Accurate chromosome segregation is critical for maintaining genomic stability during cell division. Spindle poles and kinetochores, two major microtubule organization centers (Luders and Stearns, 2007), play an essential role in coordinating chromosome segregation during mitosis. The centrosome duplicates once per cell cycle, which ensures the establishment of bipolar spindle poles during mitosis (Nigg, 2007). The two centrioles separate upon entry into G₁ (centriole disengagement) and this step is a prerequisite for centrosome duplication during S phase. As the cell cycle progresses through S and G₂, the duplicated centrosomes mature and become disconnected. Separated centrosomes are the structural basis of spindle poles from which bipolar mitotic spindles are formed that coordinate chromosome segregation during mitosis.

Centrosome (as well as centriole) cohesion and separation are tightly regulated during the cell cycle. Centrosome duplication and genome replication are initiated at the onset of S phase.

Both events are tightly coordinated and are triggered by the activity of Cdk2-cyclin E (Hinchcliffe and Sluder, 2002; Sauer and Lehner, 1995). Over the past few years, effort has been made to identify and characterize the molecular entities that both restrict centrosome duplication to once per cell cycle (Wong and Stearns, 2003; Doxsey et al., 2005) and control sister chromatid cohesion before anaphase entry (Uhlmann, 2004; Watanabe, 2005). Recent studies have shed light on the mechanism by which cells coordinate centrosome/centriole cohesion with sister chromatid cohesion during cell division. Tsou and Stearns (2006) have shown that separase, a caspase-related protease known to trigger sister chromatid separation by cleavage of cohesin, functions as a licensing factor that controls centriole disengagement at anaphase. Supporting this discovery, Thein et al. (2007) have recently demonstrated that depletion of astrin, a protein associated with both spindle poles and kinetochores during mitosis, causes premature sister chromatid separation as well as centriole disengagement, leading to the formation of multipolar spindles; moreover, separase is activated in cells that have been depleted of astrin, whereas separase depletion suppresses centriole disengagement and premature sister chromatid separation. These newly recognized functions of separase and astrin thus underscore the importance of a coordinated control of centrosome dynamics and the chromosome cycle in order to maintain genomic stability during cell division.

Separation of sister chromatids is achieved by sequential removal of the cohesin complex from chromosome arms by the prophase pathway and from centromeres by separase at the anaphase onset (Waizenegger et al., 2000). Work by several independent groups has revealed a new pathway by which centromeric cohesion of sister chromatids is protected in prophase by an evolutionarily conserved protein called Shugoshin (McGuinness et al., 2005; Kitajima et al., 2004; Salic et al., 2004; Tang et al., 2004). Mammalian cells contain two structurally related Shugoshin proteins, Sgo1 and Sgo2 (Kitajima et al., 2004; Wang and Dai, 2005). Alternatively, spliced forms of Sgo1 are present in human and mouse cells (McGuinness et al., 2005; Wang et al., 2006). In humans, two major species of Sgo1 mRNA encode a 527 amino acid isoform (Sgo1) and a 292 amino acid isoform (sSgo1), respectively. Sgo1 at centromeres is required for timely chromosomal separation at anaphase entry and is essential for mediating the function of Bub1 in fission yeast and humans (Kitajima et al., 2004, 2005; Tang et al., 2004). The shorter isoform, sSgo1, lacks 268 amino acids encoded by

exon 6 but contains 33 amino acids encoded by exon 9 (Wang et al., 2006). The unique structural features of sSgo1 suggest that it may have a distinct function in vivo.

The identification of a role for separase in centriole disengagement has prompted us to study whether other molecules critical for regulating centromeric cohesion of sister chromatids also play a role in the centrosome cycle during mitosis. We demonstrate that Sgo1 is not only essential for mediating sister chromatid cohesion but also involved in controlling centriole cohesion and that these bifurcate functions appear to be mediated by two splice variants of Sgo1. Moreover, we show that the polo-like kinase 1 (Plk1), known to be important for the centrosome cycle (Mayor et al., 1999), regulates subcellular localization and function of the centrosomal Sgo1 splice variant.

RESULTS

Sgo1 Depletion Results in the Formation of Extra Centrosomal Foci

Sgo1 is an evolutionarily conserved protein that functions as a protector of centromeric cohesion during mitosis. Inactivation of Sgo1 function or depletion of Sgo1 in mammalian cells causes missegregation of chromosomes (McGuinness et al., 2005; Tang et al., 2004; Kitajima et al., 2004; Yang et al., 2006). As both spindle poles and kinetochores are important in coordinating chromosome alignment and segregation during mitosis, we designed experiments in which spindle pole integrity as well as chromosome segregation could be monitored simultaneously after Sgo1 depletion via RNAi. Sgo1 was effectively depleted in rounded-up mitotic cells where neither the mitotic (p-Sgo1) nor the interphase form of Sgo1 was detected (Figure 1A). Evidence that the slower migrating band seen by western blotting in rounded-up cells after nocodazole treatment is the phosphorylated form is provided by the fact that it was converted to the interphase form after λ -phosphatase treatment (Figure S1A, see the Supplemental Data available with this article online).

Close examination of DAPI-stained HeLa cells by fluorescence microscopy revealed altered segregation of chromosomes in Sgo1-depleted cells (Figure 1B). The missegregated chromosomes exhibited several distinct patterns which we have defined as Type I, Type II, and Type III, in order to distinguish them from one another. Chromosome patterns included those with missegregated chromosomes which formed two clusters at the spindle pole regions in addition to those congregated at the midzone (Type I), triangular shaped chromosome clusters (Type II), and those in which chromosomes were loosely congregated at the metaphase plate (the midzone) with apparent lagging chromosomes (Type III; Figure 1B). The "Others" category includes chromosome patterns of relatively normal metaphase appearance or with polyploid contents.

It has been suggested that Sgo1 is involved in generating tension at the kinetochores and affecting microtubule dynamics (Salic et al., 2004). Therefore, Sgo1 depletion might be expected to have an effect on spindle pole integrity. In order to examine this possibility, we stained Sgo1-depleted HeLa cells with an antibody to γ -tubulin, a centrosome marker. Sgo1 depletion caused an increase in γ -tubulin-positive foci in mitotic, but not in interphase cells (data not shown), and these foci exhibited a few distinct patterns (Figure 1C). Whereas about half the cells

contained two apparently separated γ -tubulin foci formed around each spindle pole (Type 1), a significant number of cells contained three or four γ -tubulin foci, some of which were well separated from either spindle pole (Types 2 and 3). The Type 1 spindle pole pattern was tightly associated with Type I missegregated chromosomes. Type 2 spindle pole patterns were often associated with the tripolar arrangement of missegregated chromosomes (Type II). However, there was no apparent correlation between Type 3 spindle pole arrangements and chromosome segregation patterns. Time course studies indicated that the formation of extra γ -tubulin foci occurred within 12 hr posttransfection of Sgo1 siRNA, peaking around 24 hr (Figure 1D). These additional foci were likely to be functional for microtubule organization because they usually had associated microtubules (Figure 1E).

To exclude the possibility that Sgo1 depletion might affect the dynamics of γ -tubulin but not spindle pole integrity, we stained Sgo1-depleted cells with an antibody to NuMA, also a spindle pole protein (Dionne et al., 1999). Whereas NuMA was present as a distinct spot at each spindle pole in normal mitotic cells transfected with luciferase siRNA, in Sgo1-depleted cells NuMA formed additional foci. The extra NuMA foci overlapped with extra γ -tubulin foci (Figure 1F). Similar phenomena were also observed when other spindle pole markers including Plk1 and ninein were used (data not shown).

We further confirmed that the formation of extra centrosomal foci also occurred in normal fibroblasts (WI-38) after Sgo1 depletion (Figure S1B), indicating that the observed centrosomal abnormality in HeLa cells was not due to their transformed status. Moreover, individual Sgo1 siRNAs also efficiently induced the formation of extra γ -tubulin foci (Figure S1C). The formation of extra γ -tubulin foci in Sgo1-depleted cells did not seem to result from tension slack on the mitotic spindles because reducing tension by depolymerizing microtubules by nocodazole essentially eliminated, rather than increased, the number of extra γ -tubulin foci in these cells (Figures S2A and S2B).

Sgo1 Depletion-Induced Extra Centrosomal Foci Primarily Result from Centriole Splitting

The formation of additional γ -tubulin- or NuMA-positive foci could result from one of two possible mechanisms: centrosome amplification or centriole splitting. Due to the short time interval between Sgo1 depletion and the appearance of these foci (Figure 1D), we reasoned that they might be derived not from centrosome amplification but from centrosome splitting, perhaps through the separation of paired mother and daughter centrioles (referred to as "centriole splitting" hereafter). Consistent with this, the presence of more than four discrete γ -tubulin foci in one mitotic cell was infrequently observed (Figure S1C, "Others").

Centrin is a core centriole component, whose signal is confined to individual centrioles. To establish a system with which centrosome amplification or centriole splitting could be examined, we transiently transfected HeLa cells with a plasmid expressing GFP-centrin. Indeed, expressed GFP-centrin was not only localized at the centrosome, but also could discriminate two centrioles within a centrosome (Figure 2A, boxed areas enlarged). To exclude the possibility that the extra centrosomal foci in Sgo1-depleted mitotic cells were the result of centrosome

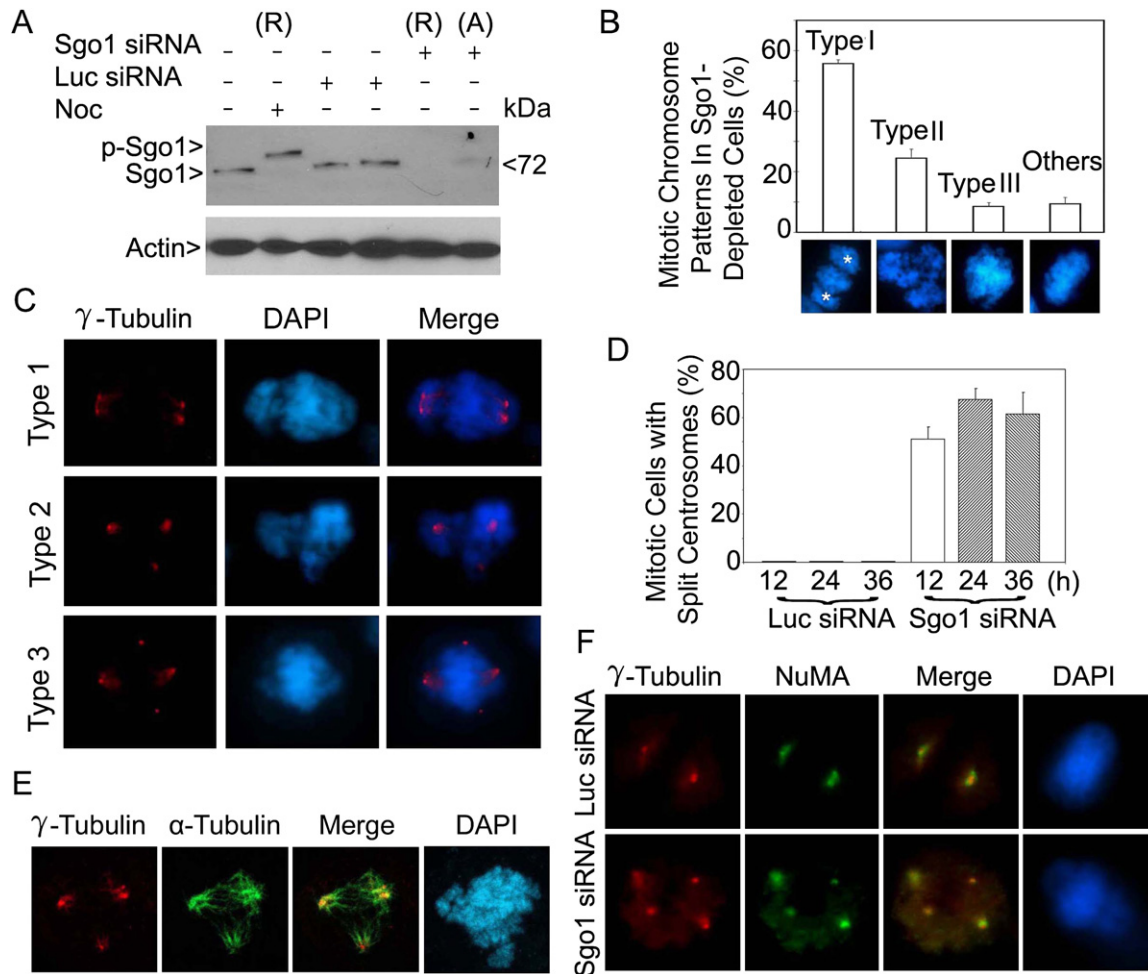


Figure 1. siRNA Depletion of Sgo1 Causes Formation of Extra γ -Tubulin Foci

(A) HeLa cells were transfected with Sgo1 or luciferase (Luc) siRNA for 24 hr. Rounded-up (R) and adherent (A) cells in Sgo1 siRNA transfected dishes were collected separately. An equal amount of each cell lysate was blotted for Sgo1 and β -actin. Nocodazole (Noc)-treated cell lysates were loaded as control. p-Sgo1 indicates phospho-Sgo1.

(B) Rounded-up cells induced as a result of Sgo1 depletion were examined for chromosome patterns after DAPI staining. The data were summarized from more than 300 mitotic cells depleted of Sgo1. The stars (*) denote spindle pole positions. The error bar represents the standard deviation.

(C) HeLa cells transfected with Sgo1 siRNA were stained with an antibody to γ -tubulin (red). DNA was stained with DAPI (blue). Representative images of cells with centrosome splitting are shown.

(D) The percentage of siRNA-transfected cells with extra centrosomal foci was summarized from 200 mitotic cells at each time point.

(E) A representative cell transfected with Sgo1 siRNA for 24 hr and stained with antibodies to γ -tubulin (red) or α -tubulin (green) is shown.

(F) HeLa cells transfected with Sgo1 or Luc siRNA were stained with antibodies to γ -tubulin (red) and NuMA (green). Representative images are shown.

amplification, we examined the centriole number (GFP-centrin foci) in interphase HeLa cells stably expressing GFP-centrin. We found that the number of interphase cells containing more than four centrioles after transfection with Sgo1 siRNAs for 24 hr was not increased compared with that of the control cells transfected with luciferase siRNA (Figure 2B), suggesting that centrosome amplification was not responsible for the increased centrosomal foci in mitotic cells. The mild increase in interphase cells with four centrioles (Figure 2B) may reflect reduction of G_1 cell population after Sgo1 depletion.

To further confirm the role of Sgo1 in mediating centriole cohesion, we looked at mitotic effects of Sgo1 depletion in HeLa cells stably expressing GFP-centrin. Sgo1 depletion resulted in the formation of extra GFP-centrin foci, which superimposed with

γ -tubulin foci (Figure 2C). A close examination revealed that each spindle pole consisted of a pair of GFP-centrin signals in luciferase siRNA-transfected cells, indicative of the presence of intact mother-daughter centriole pairs. In contrast, GFP-centrin foci detected in HeLa cells transfected with Sgo1 siRNA were discrete and showed no close association of companion centrioles (Figure 2C, boxed areas enlarged; Table S1).

Our further study of ninein, another centriole marker, confirms this effect of Sgo1 depletion on centriole splitting. Depletion of Sgo1 in HeLa cells via RNAi caused the formation of extra ninein foci as well as chromosomal missegregation. Unlike two spindle pole ninein foci in normal mitotic cells (Figure 2D, boxed areas enlarged), extra ninein foci in Sgo1-silenced cells were distinct and showed no companion centriole signals (Figure 2E, insets).

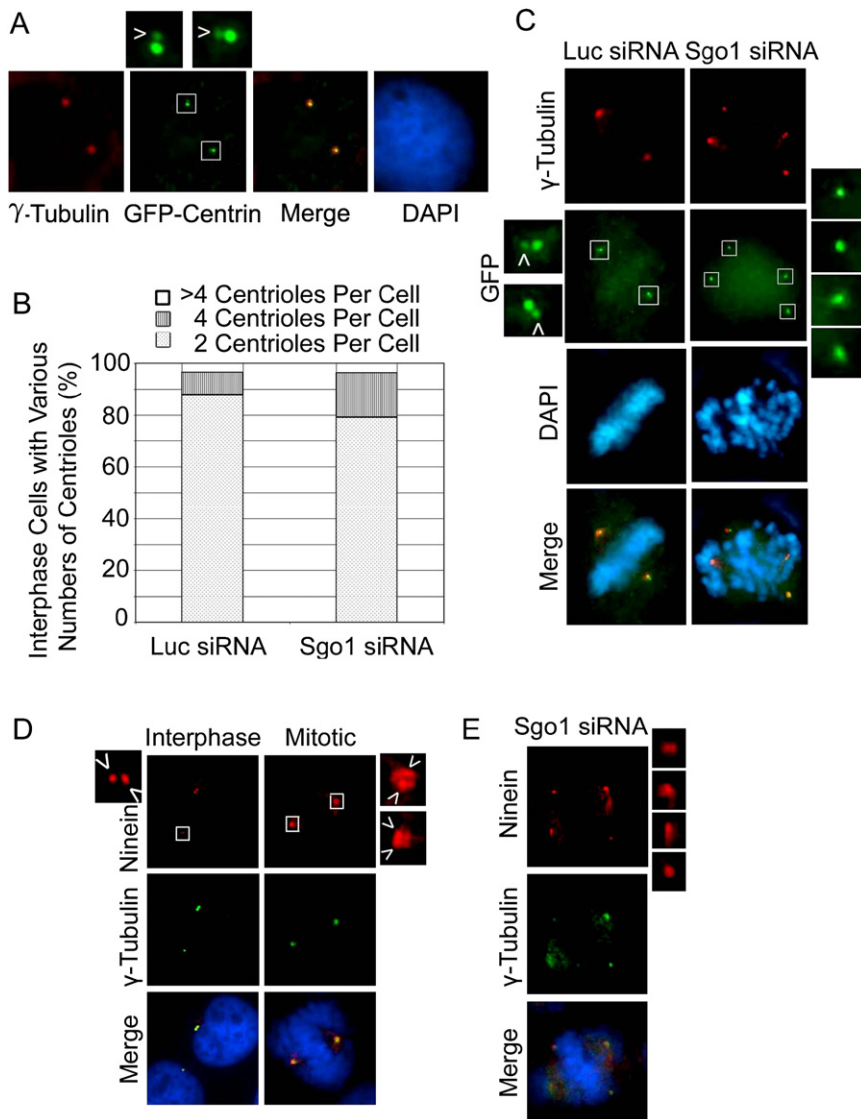


Figure 2. Sgo1 Depletion-Induced Formation of Extra Centrosomal Foci Results from Centriole Splitting

(A) HeLa cells transfected with a GFP-centrin expression plasmid for 24 hr were stained with an antibody to GFP (green) or γ -tubulin (red). The daughter centrioles are indicated by arrows.

(B) HeLa cells stably expressing GFP-centrin transfected with Sgo1 or Luc siRNA for 24 hr were fixed and stained with the antibody to GFP. The number of centrioles in individual interphase cells (100 cells/experiment) was scored under the microscope. The data were summarized from three independent experiments.

(C) GFP-centrin-expressing cells were transfected with Sgo1 or Luc siRNA for 24 hr. Cells were fixed and stained with antibody to GFP or γ -tubulin. Representative cells are shown. The boxed areas were enlarged. Daughter centrioles are indicated by arrows.

(D) HeLa cells were fixed and stained with the antibody to ninein (red) or γ -tubulin (green). Representative cells are shown. Paired centrioles are indicated by arrows.

(E) HeLa cells transfected with Sgo1 siRNA for 24 hr were stained with the antibody to ninein or γ -tubulin. The ninein foci were enlarged.

Taken together, these experiments provide strong support for the hypothesis that extra spindle pole-like foci induced by Sgo1 silencing results primarily from the separation of paired centrioles.

Sgo1 Has a Centrosomal Localization

The observation that Sgo1 depletion was linked to spindle pole integrity suggested its physical presence at the centrosome. To detect centrosome/spindle pole localization of Sgo1, we stained HeLa cells with antibodies to Sgo1 and γ -tubulin. Through rigorous extraction of the cells before fixation (Gregson et al., 2001), we were able to detect endogenous Sgo1 colocalized with γ -tubulin at the spindle poles in mitotic HeLa cells (Figure 3A, the area pointed by the upper right arrow enlarged).

Plk1 is localized to both spindle poles and kinetochores in mitotic cells (Arnaud et al., 1998). Similar to Plk1, endogenous Sgo1 signals were present both at the spindle poles and the kinetochores in mitotic U2OS cells (Figure 3B, upper panels, the area pointed by arrow enlarged). The spindle pole Sgo1 signal was

specific because transfection with Sgo1 siRNA abolished Sgo1 signals at both mitotic apparatus locations (Figures 3B and 3C). Endogenous Sgo1 also colocalized with γ -tubulin in interphase cells (Figure 3D), suggesting that it plays a role in centrosome dynamics throughout the cell cycle. Moreover, the localization of Sgo1 at the centrosome was independent of microtubules because brief treatment with nocodazole did not diminish centrosomal Sgo1 signals (Figure S2C).

GenBank databases contain numerous Sgo1 cDNA clones which correspond primarily to two major splice variants. One Sgo1 variant codes for the full-length protein detected as a 72 kDa protein by western blotting, while the other variant transcript appears to code for a much smaller protein that lacks the sequence encoded by exon 6 (Wang et al., 2006). In order to gain more understanding of the regulation of expression of these two variants and to explore the possibility that the two forms play different roles during cell division, we examined whether HeLa cells expressed both isoforms. Immunoblot analysis revealed that, in addition to the Sgo1 band migrating around 72 kDa as previously reported, HeLa cells also contained a protein of about 40 kDa that was immunoreactive to the Sgo1 antibody; this protein, as well as the 72 kDa one, was specifically depleted upon transfection with Sgo1 siRNA (Figure 3E). Expression of the short Sgo1 (termed "sSgo1" hereafter) was also detected in other cell lines tested including U2OS, A549, and MCF-7 cells (data not shown). To determine which form of Sgo1 was localized or enriched at the centrosome, we isolated the organelle from HeLa cells using

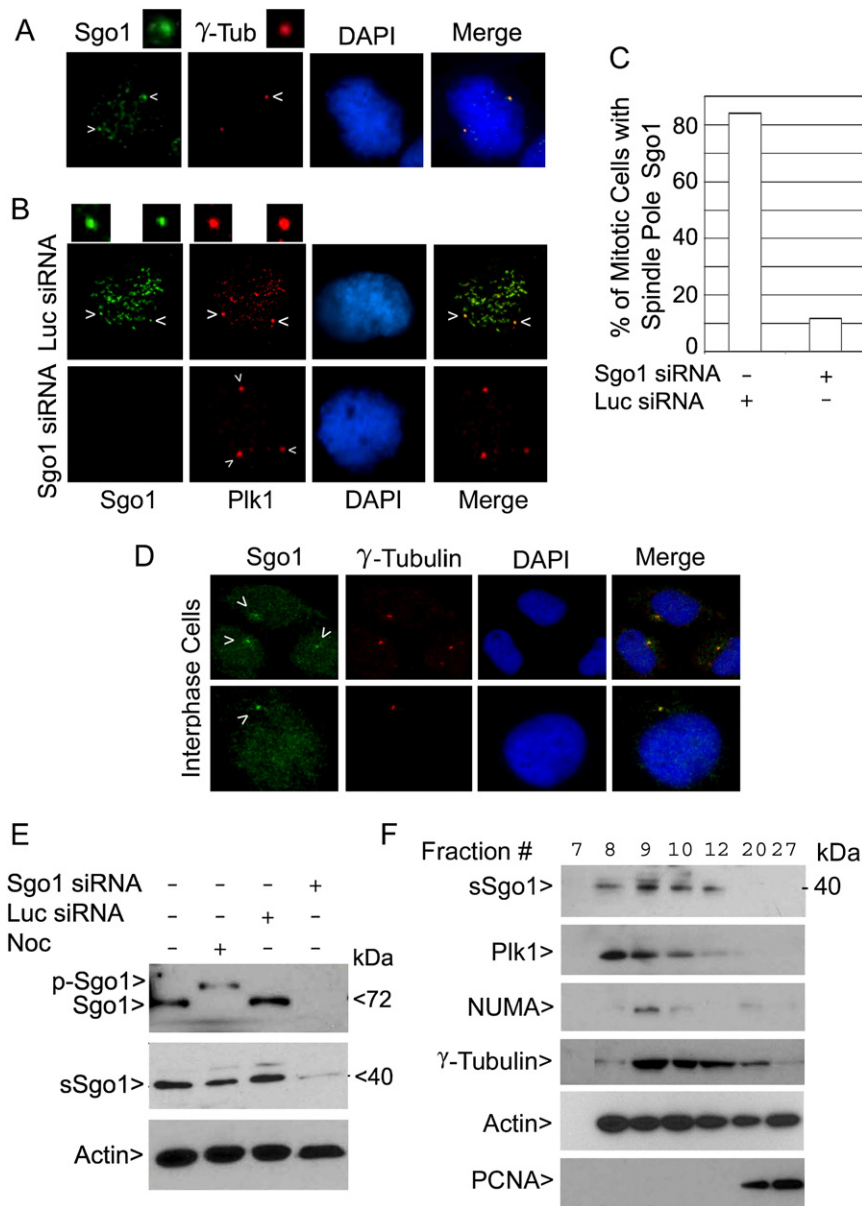


Figure 3. Sgo1 Is Localized to the Centrosomes/Spindle Poles

(A) HeLa cells were stained with antibody to Sgo1 (green) or γ -tubulin (red). Arrows indicate spindle pole signal. The area indicated by the upper right arrow was enlarged.

(B) U2OS cells transfected with Luc or Sgo1 siRNA for 24 hr were stained with the antibody to Sgo1 (green) or Plk1 (red). Arrows indicate spindle pole signal. The areas indicated by arrows were enlarged.

(C) Data were summarized from over 100 mitotic cells shown in (B) after transfection of Sgo1 or Luc siRNA.

(D) HeLa cells were stained with antibodies to Sgo1 γ -tubulin. Arrows indicate the centrosome positions.

(E) An equal amount of lysates from HeLa cells transfected with Sgo1 or Luc siRNA for 24 hr was blotted for Sgo1 and β -actin. Noc-treated cell lysates were loaded as controls.

(F) Centrosomes were isolated from HeLa cells. An equal volume of sample from selected centrosomal isolation fractions was blotted for sSgo1, Plk1, NuMA, γ -tubulin, β -actin, and PCNA.

among various mammals (Figure 4A), suggesting a conserved function. To test the possibility that exon 6 might dictate the subcellular localization of Sgo1, we expressed cDNAs coding for the two major splice variants as GFP-fusion proteins. Fluorescence microscopy revealed that whereas GFP-Sgo1 colocalized with CREST at the kinetochores during mitosis, GFP-sSgo1 localized to the spindle poles and the mitotic spindles but not at the kinetochores (Figure 4B). This supports the hypothesis that these two variants are differentially regulated in the cell.

As a first attempt to study the function of sSgo1 in regulating centrosome integrity, we made and expressed a deletion construct (Myc-Sgo1¹⁻¹⁹⁶) containing only

density gradient centrifugation. Representative centrosomal fractions were western blotted with antibodies to Sgo1 as well as to centrosomal markers. The fast migrating form, sSgo1, cofractionated with NuMA, Plk1, and γ -tubulin; in contrast, sSgo1 did not cofractionate with PCNA, a nuclear antigen (Figure 3F), suggesting its absence from kinetochores. The cytoplasm also contains a significant amount of γ -tubulin (Moudjou et al., 1996), which may account for its presence in many fractions of the samples (Figure 3F). The presence of intact centrosomes in fraction #9 was also confirmed by fluorescence microscopy. The centrosomes isolated from HeLa cells were positive for Sgo1, as well as for γ -tubulin (Figure S3A).

The full-length Sgo1 protein contains an exon 6-encoded region that is absent from sSgo1 (Wang et al., 2006). An analysis of intron and exon boundaries of Sgo1 cDNAs reveals that exon 6 (or its structural counterpart) exists as an independent unit

the N-terminal 196 amino acids of Sgo1. As it does not contain a region coded by a functional exon 6, Sgo1¹⁻¹⁹⁶ is similar in general sequence structure to sSgo1 (Figure S3B). When ectopically expressed, Myc-Sgo1¹⁻¹⁹⁶ exhibited both cytoplasmic and nuclear localizations during interphase (Figure 4C). A significant amount of ectopically expressed Myc-Sgo1¹⁻¹⁹⁶ colocalized with γ -tubulin during mitosis (Figure 4D). These cells frequently contained extra γ -tubulin foci (Figures 4D and 4E, arrows), reminiscent of those induced by transfection of Sgo1 siRNA. These studies indicate that Myc-Sgo1¹⁻¹⁹⁶ may exhibit a dominant negative function, perhaps by interfering with endogenous sSgo1 activity. Supporting this, when GFP-centrin-expressing cells were transfected with the Myc-Sgo1¹⁻¹⁹⁶ construct, this mutant Sgo1 protein also caused centriole splitting (Figure 4F, arrows). No kinetochore Myc-Sgo1¹⁻¹⁹⁶ was detected in mitotic cells when it was ectopically expressed (data not shown), suggesting that the

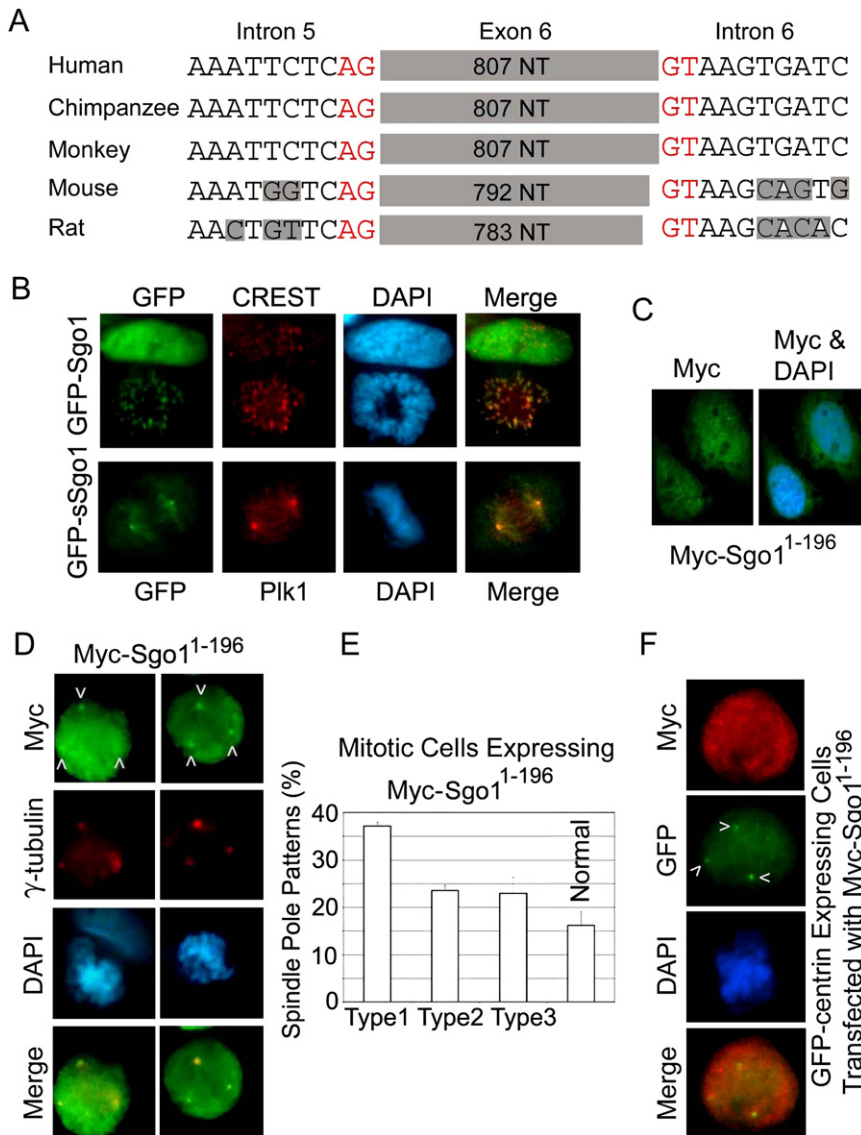


Figure 4. sSgo1 Functions in Regulating Spindle Pole Integrity

(A) The intronic sequences surrounding human exon 6 or its equivalent in other mammals were aligned. Red color marks the invariant splice donor and splice acceptor sequences. The shaded areas indicate nonconserved sequences in introns.

(B) HeLa cells transfected with GFP-Sgo1 or GFP-sSgo1 expression constructs for 24 hr were fixed and stained with antibodies to GFP (green), CREST (red, upper panel), or Plk1 (red, lower panel).

(C) HeLa cells transfected with Myc-Sgo1¹⁻¹⁹⁶ expression plasmid for 24 hr were stained with antibody to Myc.

(D and E) HeLa cells transfected with Myc-Sgo1¹⁻¹⁹⁶ plasmid for 24 hr were stained with antibodies to Myc (green) and γ -tubulin (red; [D]). Various abnormal spindle pole patterns were summarized from three independent experiments ([E]; 150 mitotic cells/experiment). The error bar represents the standard deviation.

(F) HeLa cells expressing GFP-centrin transfected with Myc-Sgo1¹⁻¹⁹⁶ plasmid for 24 hr were stained with antibodies to Myc (red) and GFP (green). Arrows indicate the position of split centrioles.

formation of extra γ -tubulin foci is primarily due to the disruption of sSgo1 function but not due to chromosome missegregation caused by Sgo1 depletion. In fact, Myc-Sgo1¹⁻¹⁹⁶ did not affect the localization of GFP-Sgo1 at kinetochores (Figure S3C).

As an additional approach to study sSgo1 function, we designed a Myc-sSgo1 expression construct and an siRNA duplex corresponding to the sequence of the 3'-untranslated region (3'UTR) of Sgo1 mRNA that was absent from Myc-sSgo1. HeLa cells were cotransfected with the Myc-sSgo1 expression construct and the Sgo1 3'UTR siRNA. Immunoblotting revealed that whereas endogenous sSgo1 was efficiently silenced by the 3'UTR siRNA, ectopically expressed Myc-sSgo1 remained in transfected cells (Figure 5A), indicating that Myc-sSgo1 mRNA was largely resistant to the silencing by the 3'UTR siRNA. Moreover, through examination of mitotic cells expressing transfected Myc-sSgo1, we observed that centriole splitting induced by Sgo1 silencing was significantly rescued (Figure 5B, Table S2), indicating a protective function of sSgo1 at the spindle poles. On the other hand, expression of Myc-sSgo1 did not suppress

the rate of chromosomal missegregation caused by depletion of endogenous Sgo1 (Figure 5C, Table S2), supporting the notion that sSgo1 may not have a direct function at kinetochores. Combined, these observations support the hypothesis that sSgo1 plays a primary role in protecting the integrity of the spindle poles through maintaining centriole cohesion.

We further examined the role of Sgo1 in protecting centriole cohesion using a genetic approach. Through gene-trapping and transgenic mouse genetics, as described in our previous study (Wang et al., 2004), we obtained *Sgo1* haploinsufficient mice (unpublished data), from which we derived *Sgo1*^{+/-} murine embryonic fibroblast (MEF) cells (Figure S4). Fluorescence microscopy revealed that a significantly higher fraction of *Sgo1*^{+/-} mitotic MEFs contained extra γ -tubulin foci than did wild-type mitotic MEFs (*Sgo1*^{+/+}; Figures 5D and 5E). Furthermore, expression of transfected Myc-sSgo1 significantly reduced the number of mitotic cells with extra γ -tubulin foci (Figure 5F). These studies thus provide a line of unequivocal genetic evidence indicating the involvement of Sgo1 in regulating centrosome function.

Plk1 Regulates sSgo1 Subcellular Localization and Function

In *Drosophila*, the Plk1 homolog POLO is involved in regulating Mei-S332, the homolog of mammalian Shugoshin (Clarke et al., 2005). In addition, Plk1 regulates centrosome dynamics during the cell cycle. Hence, we reasoned that Plk1 may regulate the function of sSgo1 during mitosis. To test this possibility, we examined the subcellular localization of Myc-sSgo1 in cells

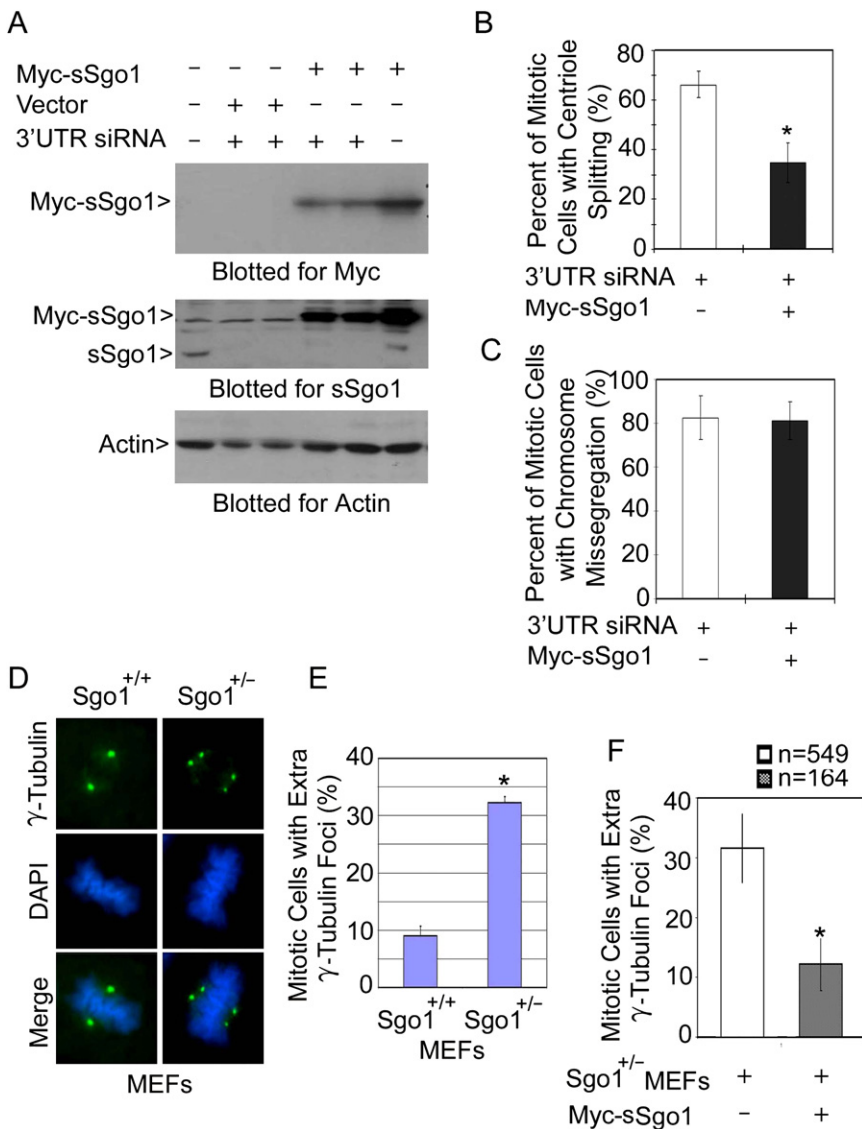


Figure 5. Ectopic Expression of sSgo1 Suppresses Centriole Splitting Induced by Sgo1 Depletion

(A) HeLa cells cotransfected with the Myc-sSgo1 expression construct and Sgo1 3'UTR siRNA for 48 hr were collected and an equal amount of cell lysate was blotted for Myc, endogenous sSgo1, and β-actin.

(B and C) HeLa cells transfected with the Myc-sSgo1 expression construct and Sgo1 3'UTR siRNA for about 32 hr were fixed and stained with antibodies to the Myc tag and γ-tubulin. The data were summarized from over 200 transfected mitotic cells. The error bars represent the standard deviation.

(D) Wild-type (Sgo1^{+/+}) and Sgo1^{+/-}-MEF cells were stained with antibody to γ-tubulin (green).

(E) The number of mitotic cells with extra γ-tubulin foci in Sgo1^{+/+}- and Sgo1^{+/-}- MEF cells was scored. The data were summarized from three independent experiments (100 mitotic cells/experiment).

(F) Sgo1^{+/-}-MEFs transfected with a Myc-sSgo1 expression construct for 24 hr were stained with antibodies to γ-tubulin and the Myc tag. The number (n) of mitotic cells with extra γ-tubulin foci was scored (two independent experiments). * denotes significant statistical difference (p < 0.01). The error bar represents the standard deviation.

(Oshimori et al., 2006). The physical interaction between Plk1 and sSgo1 suggests that Plk1 may regulate sSgo1 through phosphorylation. A survey of the sSgo1 amino acid sequence revealed four putative sites that conform to the Plk1 phosphorylation motif. One putative site (serine 260) falls within the region encoded by exon 9, which is spliced out in certain sSgo1 isoforms, suggesting that it may not have a conserved function. Therefore, we first focused on the other three motifs

transfected with Plk1 siRNA. Compared with control cells, Plk1 depletion abolished the localization of Myc-sSgo1 at the spindle poles and mitotic spindles (Figures 6A and 6B). Plk1 downregulation via RNAi also delocalized GFP-sSgo1 at the spindle poles (Figure S5A). Plk1 downregulation was efficient as confirmed by immunoblotting (Figure 6C).

To examine the possibility of a physical interaction between these two proteins, we performed coprecipitation assays using histidine-tagged sSgo1 (His₆-sSgo1). We observed that Ni-NTA resin precipitated not only His₆-sSgo1 but also Plk1 from HeLa cells transfected with His₆-sSgo1 plasmid, but not with the control plasmid (Figure 6D). To confirm the physical interaction between Sgo1 and Plk1, we performed coimmunoprecipitation experiments using anti-Sgo1 antibodies. The Sgo1 antibodies, but not the preimmune IgGs, were capable of precipitating a significant amount of Plk1 from mitotic HeLa cells (Figure 6E).

Plk1 has been shown to directly phosphorylate centrosomal proteins that are important for the regulation of the centrosome cycle or the maintenance of spindle pole integrity during mitosis

in mediating sSgo1 function. We made a series of mutants in which serines or threonines were replaced with alanines; mutant sSgo1 proteins, as well as the wild-type, were expressed as Myc-tagged fusion products. When ectopically expressed, Myc-sSgo1^{S129A} and the wild-type Myc-sSgo1 were largely confined to the spindle poles and the mitotic spindles; however, Myc-sSgo1^{S73A} and Myc-sSgo1^{T146A} mutant proteins did not properly localize to the spindle pole regions in a significant fraction of mitotic cells (Figures 7A and 7B), supporting the idea that phosphorylation by Plk1 is important for the localization and perhaps the function of sSgo1. Immunoblotting revealed that various sSgo1 mutant proteins were expressed at a similar level in HeLa cells (Figure S5B). We then analyzed if ectopic expression of these putative phosphorylation mutants affected spindle pole integrity. Mitotic cells expressing Myc-sSgo1^{S73A} and Myc-sSgo1^{T146A}, but not Myc-sSgo1^{S129A}, exhibited a significant increase in split spindle poles compared with those expressing wild-type Myc-sSgo1 (Figure 7C), suggesting that correct localization of sSgo1 is necessary for its spindle pole function during

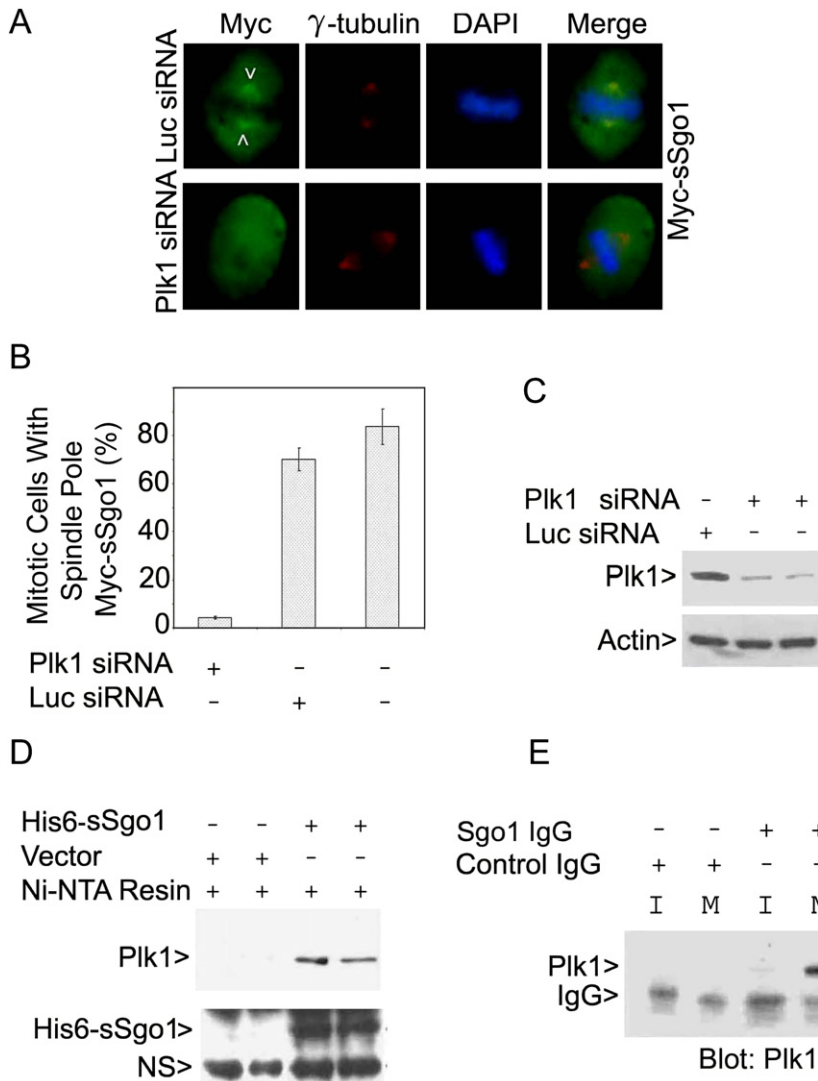


Figure 6. PIK1 Is Physically Associated with sSgo1

(A) HeLa cells cotransfected with Myc-sSgo1 plasmid and PIK1 or Luc siRNA for 24 hr were stained with antibodies to the Myc tag (green) and γ -tubulin (red).

(B) HeLa cells were cotransfected with Myc-sSgo1 expression plasmid and PIK1 or Luc siRNA. The data were summarized from three independent experiments (100 PIK1-depleted mitotic cells/experiment). The error bar represents the standard deviation.

(C) HeLa cells transfected with PIK1 or Luc siRNA for 24 hr were blotted for PIK1 and β -actin.

(D) Equal amounts of HeLa cell lysates after transfection with a His₆-sSgo1 plasmid or the vector alone for 48 hr were incubated with Ni-NTA resin. Proteins specifically bound to the resin were blotted for PIK1 or Sgo1. NS> denotes a nonspecific signal.

(E) An equal amount of interphase (I) or mitotic (M) HeLa cell lysate was immunoprecipitated with the anti-Sgo1 antibody or with a control IgG. The immunoprecipitates were blotted for PIK1.

strongly suggest that Sgo1 is involved in controlling spindle pole integrity and that its function is at least partially mediated through phosphorylation by PIK1.

DISCUSSION

This study reveals, to our knowledge, a new function of Sgo1 in the regulation of spindle pole integrity. Given the tight coordination of DNA replication and centrosome duplication during S phase, it is not surprising to observe crosstalk between chromosomes and spindle poles during mitosis. In fact, important regula-

tor molecules including PIK1, aurora kinases, and ERKs are present at both the kinetochores and the spindle poles. Recent studies indicate that key components, including PP2A and astrin, involved in mitotic control or sister chromatid cohesion are also present at both the spindle poles and kinetochores (Schlaitz et al., 2007; Thein et al., 2007). Moreover, during late mitosis, the mother centriole repositions itself by migrating to the intercellular bridge, apparently influencing cell cleavage at cytokinesis in certain cell types (Piel et al., 2001). It is conceivable that premature centriole splitting or maternal centriole migration before the completion of chromosome segregation would allow the formation of additional microtubule nucleating foci, a condition favoring chromosome missegregation and genomic instability.

Given the observation that separase, a key enzyme in controlling sister chromatid cohesion, also plays an essential role in centriole disengagement during M phase to facilitate their subsequent duplication (Tsou and Stearns, 2006), it is tempting to speculate that a mechanism similar to that of cohesin cleavage may exist at the centrosomes. Our current study supports the notion that separase may have a centrosomal target(s). The

mitosis. The relatively low percentage of split spindle poles in mitotic cells expressing either Myc-sSgo1^{S73A} or Myc-sSgo1^{T146A} suggests that phosphorylation of sSgo1 on multiple sites may be necessary for its full activity.

To further elucidate PIK1 function in regulating spindle pole integrity mediated by sSgo1, we asked whether PIK1 depletion could affect spindle pole abnormalities induced by the dominant negative protein Myc-Sgo1¹⁻¹⁹⁶. Fluorescence microscopy revealed that whereas expression of Myc-Sgo1¹⁻¹⁹⁶ alone induced centriole splitting in a majority of mitotic cells, PIK1 silencing by RNAi greatly suppressed Myc-Sgo1¹⁻¹⁹⁶-induced centriole splitting (Figures 7D and 7E). Transfection of PIK1 siRNA also suppressed chromosomal missegregation induced by Myc-Sgo1¹⁻¹⁹⁶ (Figure 7F). PIK1 depletion confirmed by immunoblotting did not affect the expression of transfected Myc-Sgo1¹⁻¹⁹⁶ in mitotic cells (Figure S5C). We also observed some structural alterations in spindle poles in PIK1-depleted cells; however, these changes, characterized mainly by diffused γ -tubulin signals along mitotic spindles (Figure S5D), were different from that induced by Sgo1-deficiency. Combined, these studies

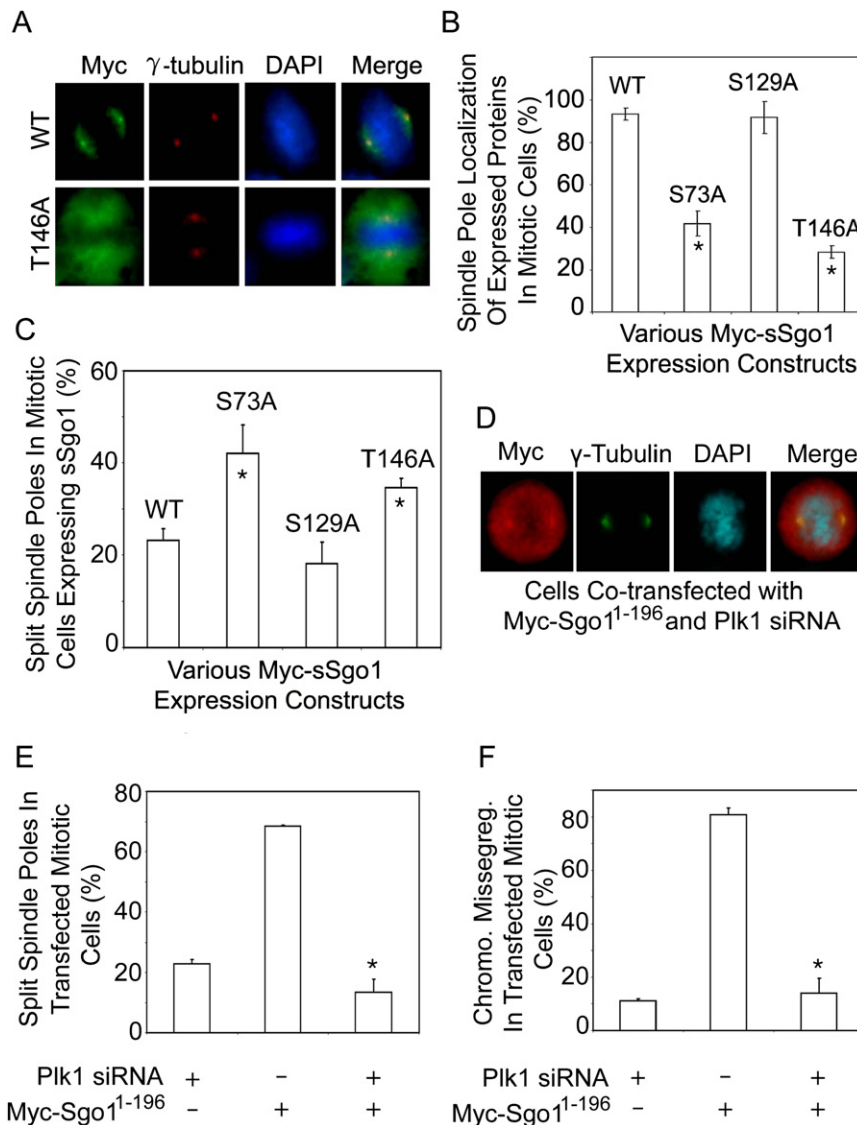


Figure 7. Plk1 Regulates Sgo1 Function

(A) HeLa cells transfected with Myc-sSgo1 or Myc-sSgo1^{T146A} expression plasmid for 24 hr were stained with antibodies to the Myc tag (green) and γ -tubulin (red).

(B) HeLa cells transfected with Myc-sSgo1 expression plasmid (WT) or with various Myc-tagged sSgo1 mutant plasmids as indicated for 24 hr were stained with antibody to the Myc tag. The data were summarized from three independent experiments (>100 transfected mitotic cells). * denotes significant statistical difference ($p < 0.01$). The error bar represents the standard deviation.

(C) HeLa cells transfected with Myc-tagged sSgo1 (WT) or with mutant expression plasmids for 24 hr were stained with antibodies to the Myc tag and γ -tubulin. The data were summarized from three independent experiments (>300 transfected mitotic cells). * denotes significant statistical difference ($p < 0.05$). The error bar represents the standard deviation.

(D) HeLa cells cotransfected with Myc-Sgo1¹⁻¹⁹⁶ expression plasmid and Plk1 siRNA for 24 hr were stained with antibodies to the Myc tag (red) and γ -tubulin (green).

(E and F) HeLa cells cotransfected with Myc-Sgo1¹⁻¹⁹⁶ expression plasmid and Plk1 siRNA for 24 hr were stained with antibodies to the Myc tag and γ -tubulin. The data were summarized from three independent experiments (>300 transfected mitotic cells). * denotes significant statistical difference ($p < 0.01$). The error bars represent the standard deviation.

nate efficiently with spindle poles and kinetochores to carry out nuclear and cytoplasmic division and suppress genomic instability during mitosis.

A straightforward explanation for Sgo1's role in mediating centriole cohesion would be its physical presence at the spindle poles. Indeed, our experiments

most straightforward model would be based on the concept that sSgo1 at the spindle poles may work in a fashion similar to that of Sgo1 at the kinetochores by protecting cohesin. In fact, Rad21, a cohesin subunit cleavable by separase, is transiently associated with the centrosomes during metaphase and anaphase in *Drosophila* (Warren et al., 2000). In mammalian cells, the cohesin complex also localizes to the spindle poles during mitosis where it interacts with NuMA, a protein associated with the spindle poles, to play an essential role in spindle pole organization (Gregson et al., 2001). Alternatively, sSgo1 can be a potential substrate of separase because its sequence contains a core separase cleavage consensus site at amino acids 128–131 [D/E XXR] (Sullivan et al., 2004). This would place sSgo1 downstream of separase. Supporting this notion, depletion of separase by RNAi did not significantly suppress massive centriole splitting induced by Sgo1 depletion (data not shown). The discovery that separase is involved in controlling centriole splitting (Tsou and Stearns, 2006) is significant because this would not only allow the centrosome to duplicate once per cell cycle, but also coordi-

confirm that centrosome/spindle pole sSgo1 signals are detected in interphase and mitotic cells and that these signals disappear upon transfection with Sgo1 siRNA, which depletes both Sgo1 and sSgo1. The failure to detect spindle pole Sgo1 (sSgo1) signals by others may be due partly to different in situ extraction conditions or the nature of the antibodies because sSgo1 contains no sequences encoded by exon 6. The modified protocol using a more stringent extraction process before staining allows us to detect Sgo1 signals at the centrosomes/spindle poles (which, we believe, were otherwise masked). Spindle pole localization of Sgo1 was also observed in a separate study in male meiotic cells in *Drosophila* (Lee et al., 2005).

Plk1 is a major protein kinase regulating centrosome maturation and spindle pole function during mitosis. Our current study indicates that Plk1 plays an important role in regulating centriole cohesion mediated by Sgo1. (1) It physically interacts with sSgo1, and the interaction between sSgo1 (and/or Sgo1) and Plk1 appears to be mitosis-specific. (2) Functional knockdown of Plk1 abolishes the subcellular localization of sSgo1 at mitotic

spindles and spindle poles. (3) Plk1 depletion greatly suppresses centriole splitting and chromosome missegregation induced by Myc-Sgo1¹⁻¹⁹⁶, a dominant negative mutant. (4) sSgo1 mutants (especially, serine^{S73A} and threonine^{T146A}) fail to localize properly to spindle poles due to lack of Plk1 phosphorylation sites, and this is correlated with an increase in split spindle poles. Serine 73 is localized in the region mediating microtubule binding (Salic et al., 2004); it is also in the coiled-coil structure, which is known to mediate protein-protein interactions. It is conceivable that phosphorylation of sSgo1 by Plk1 may directly regulate its interaction with microtubules and spindle pole components such as PP2A. In fact, the N-terminal region of Sgo1 interacts with PP2A (Tang et al., 2006). Structural alterations due to changes in phosphorylation status or due to truncation (Sgo1¹⁻¹⁹⁶) may disrupt proper interaction with PP2A, thus inducing centriole splitting. This is consistent with the observation that depletion of PP2A via RNAi also compromises the integrity of spindle poles (data not shown) and causes the formation of extra γ -tubulin foci (Kitajima et al., 2006).

The dominant negative protein Myc-Sgo1¹⁻¹⁹⁶ may interfere with the function of endogenous sSgo1 (and Sgo1, as well) by direct physical association because existing evidence indicates that Sgo1 is capable of forming a dimer (Tang et al., 1998). Plk1-mediated phosphorylation of Sgo1 may be required for efficient dimer formation in vivo. Therefore, in the absence of Plk1, the association between the mutant protein and cellular sSgo1 and Sgo1 can be conceivably compromised, resulting in significant functional rescue of defects in mitotic cells expressing transfected Myc-Sgo1¹⁻¹⁹⁶. It is not surprising to observe that Plk1 knockdown suppresses both spindle pole splitting and chromosome missegregation because Plk1 exhibits localization at both spindle poles and kinetochores (Arnaud et al., 1998).

Accurate chromosome segregation during mitosis is one of the most fundamental processes that allows cells to faithfully transmit their genetic information from one generation to another. Failures in the maintenance of chromosome stability during mitosis inevitably lead to either mitotic catastrophe or malignant transformation. Our current study indicates that Sgo1 mediates the integrity of both kinetochores and spindle poles, two major mitotic apparatuses, during cell division and the dysregulated function of Sgo1 may be one of the major underlying causes of chromosomal instability.

EXPERIMENTAL PROCEDURES

Cell Culture

HeLa, U2OS, and A549 cell lines were obtained from American Type Culture Collection (ATCC). These cells were cultured under 5% CO₂ in Dulbecco's Minimum Essential Medium (DMEM), 15% fetal bovine serum (FBS), and antibiotics (100 μ g/ml penicillin, 50 μ g/ml streptomycin sulfate). WI-38 cells from ATCC were cultured under 5% CO₂ in Minimum Essential Medium (MEM) with 10% FBS and 2 mM L-glutamine.

RNA Interference

Sgo1 small interfering RNAs (siRNAs; mixed pool) were obtained from Dharmacon which correspond to the following sequences: 5'CAUCUUAGCCUGAAGGAUUAU3' (designated as siRNA-1), 5'UGAAAGAAGCCCAAGAUUAU3' (siRNA-2), and 5'AAACGCAGGUCUUUAUUAUU3'. The 3'UTR sequence used for designing siRNA for silencing Sgo1 is 5'GAGGAUCUGUAAGAGUACACAUU3'. Plk1 siRNA corresponding sequence is 5'AAGGGCGGCUUUGCCAAGUGC3'. Sgo1 or Plk1 siRNA duplexes were transfected into HeLa or

U2OS cells with Lipofectamine2000 (Invitrogen). Briefly, cells seeded at 50% confluency in an antibiotic-free culture medium were transfected with siRNA duplexes at a final concentration of 100 nM for 24 hr (unless otherwise specified). Negative controls were cells transfected with 100 nM siRNA duplex targeting firefly (*Photinus pyralis*) luciferase (5'UUCCTACGCTGAGTACTTCCA3', GL-3 from Dharmacon). In some experiments, HeLa cells were cotransfected with Sgo1 siRNA and various Sgo1 or sSgo1 expression constructs for 24 or 48 hr.

Fluorescence Microscopy

Cells fixed in methanol or 4% paraformaldehyde (PFA) were treated with 0.1% Triton X-100 on ice and then washed three times with PBS. After blocking with 2.0% BSA in PBS for 15 min, cells were incubated for 1 hr with antibodies to Sgo1, γ -tubulin (Sigma), α -tubulin, NuMA, green fluorescence protein (GFP, Santa Cruz), Myc tag (Cell Signaling), Plk1 (Zymed), ninein (Abcam), NuMA, and CREST, washed with PBS, and then incubated with appropriate secondary antibodies conjugated with Rhodamine-Red-X or FITC (Jackson Immuno Research). Cells were finally stained with 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/ml, Fluka). For detecting Sgo1 signals at spindle poles/centrosomes, we followed the procedure as described (Gregson et al., 2001). Briefly, HeLa cells washed with a cytoskeleton (CSK) buffer (10 mM Pipes [pH 7.0], 100 mM NaCl, 300 mM sucrose, and 3 mM MgCl₂) were extracted by incubation in the CSK buffer containing 0.1% Triton X-100 at RT for 2 min. After brief washing with the CSK buffer, the extracted cells were fixed with methanol or PFA for 5 min at 4°C and treated with 0.1% Triton X-100 in PBS for 5 min on ice followed by blocking and antibody incubation as described above. Fluorescence microscopy was performed on a Nikon microscope, and images were captured using a digital camera (Optronics). For confocal imaging, Leica TCS SP5 and Bio-Rad MRC-1000 confocal microscopes (MRC-1000, Bio-Rad) were utilized.

Centrosome Isolation

Centrosomes were isolated from HeLa cells essentially as described (Blomberg-Wirschell and Doxsey, 1998). Briefly, five confluent 150 mm dishes of HeLa cells were treated with nocodazole (5 μ g/ml, Sigma) and cytochalasin B (10 μ g/ml, Sigma) for 90 min at 37°C. All subsequent steps were performed on wet ice or at 4°C using solutions that had been prechilled on ice. Cell lysates were collected into a 15 ml plastic conical tube and various components were supplemented to achieve a final concentration of 10 mM PIPES, 1 mM EDTA, and 1 μ g/ml protease inhibitors. Lysates were filtered through a 40 μ m nytex membrane and then gently laid on top of 20% (w/v) Ficoll (MW 400,000). The samples were then subjected to centrifugation at 25,000 g in a swinging bucket rotor for 20 min at 4°C. After centrifugation, centrosomes were layered about 2 mm on top of the Ficoll cushion. Samples (200 μ l per fraction) were collected from the bottom of the centrifuge tubes and placed on ice for subsequent western blot and fluorescence microscopic analyses.

Cell Synchronization

To obtain cells that were arrested at the G₁/S boundary, double thymidine block was carried out by first culturing HeLa cells in medium containing 2 mM thymidine overnight. After incubating in fresh medium for 8 hr, a second overnight incubation in 2 mM thymidine was performed prior to washing and releasing the block in medium for 8 hr. For mitotic arrest, cells were released into medium containing nocodazole (0.5 μ g/ml) for 16 hr. At the end of block or release, cells were collected for preparation of lysates. Double thymidine block was also employed to enrich mitotic cells expressing transfected Myc-sSgo1. Briefly, HeLa cells were cultured in medium containing 2 mM thymidine overnight. After washing, cells were cultured in fresh medium for 4 hr before cotransfection with the Myc-sSgo1 construct and 3'UTR Sgo1 siRNA. Four hours after transfection, cells were supplemented with medium containing 2 mM thymidine for 18 hr. Cells were then washed and cultured in fresh medium for 10 hr before being processed for fluorescent microscopy.

Western Blot

HeLa cells were transfected with Sgo1 or Plk1 siRNA for 24 hr or treated with nocodazole (0.5 μ g/ml) for 16 hr. Mitotic cells were collected by shake-off. The remaining cells were also harvested after trypsin treatment. An equal amount of proteins were subjected to immunoblotting with antibodies to Sgo1, Plk1

(Zymed), NuMA, PCNA (Sigma), γ -tubulin (Sigma), or β -actin (Sigma). Specific signals were detected with horseradish peroxidase-conjugated goat-anti-rabbit (or anti-mouse) secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

SUPPLEMENTAL DATA

Supplemental Data include five figures and two tables and can be found with this article online at <http://www.developmentalcell.com/cgi/content/full/14/3/331/DC1/>.

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