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Priority Report

# Shugoshin 1 Plays A Central Role in Kinetochore Assembly and is Required for Kinetochore Targeting of Plk1

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**KEY WORDS**

spindle checkpoint, tension sensing, Shugoshin, Plk1, aurora

**ABBREVIATIONS**

| | |
|---|---|
| Sgo1 | Shugoshin 1 |
| CPC | chromosomal passenger complex |
| Plk1 | Polo-like kinase 1 |
| AurB | Aurora B kinase |
| RT | room temperature |
| aa | amino acids |
| Plx1 | *Xenopus* Polo like kinase 1 |

**ACKNOWLEDGEMENTS**

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## ABSTRACT

Physical connection between the sister chromatids is mediated by the cohesin protein complex. During prophase, cohesin is removed from the chromosome arms while the centromeres remain united. Shugoshin1 (Sgo1) is required for maintenance of centromeric cohesion from prophase to the metaphase-anaphase transition. Furthermore, Sgo1 has been proposed to regulate kinetochore microtubule stability and sense interkinetochore tension, two tasks which are tightly coupled with the function of the chromosomal passenger complex (CPC) and Polo-like kinase 1 (Plk1). Here we show that depletion or chemical inhibition of Aurora B kinase (AurB), the catalytic subunit of the CPC, disrupts accumulation of Sgo1 on the kinetochores in HeLa cells and causes Sgo1 to localize on the chromosome arms. RNAi assays show that depletion of Sgo1 did not affect AurB localization but diminished Plk1 kinetochore binding. Furthermore, we demonstrate that vertebrate Sgo1 is phosphorylated by both AurB and Plk1 in vitro. The data presented here includes an extensive analysis of kinetochore targeting interdependencies of mitotic proteins that propose a novel branch in kinetochore assembly where Sgo1 and Plk1 have central roles. Furthermore our studies implicate Sgo1 in the tension sensing mechanism of the spindle checkpoint by regulating Plk1 kinetochore affinity.

## INTRODUCTION

Cohesion between the sister chromatids is established by a protein complex called cohesin. During prophase, the cohesin is removed from the chromosome arms while the sister centromere regions remain connected. Attachment of sister kinetochores to microtubules emanating from opposite spindle poles (termed chromosome bi-orientation) results in microtubule-mediated pulling forces with the purpose to separate the two copies of a chromosome. The cohesin complex resists these separating forces until the spindle checkpoint is satisfied by chromosome bi-orientation and congression to the metaphase plate. Inactivation of the spindle checkpoint leads to removal of the cohesin from the centromeres allowing the physical separation of sister chromatids.

MEI-S332 (the *Drosophila* Shugoshin 1 homolog) was first identified as a protector of cohesion at centromeres during anaphase I in *Drosophila* spermatocytes.[1,2] More recently, Shugoshin 1 (Sgo1) has also been shown to play a role during mitosis in both yeast and vertebrates; Sgo1 is required for maintenance of centromeric cohesion from prophase to the metaphase-anaphase transition,[3,4] tension sensing between sister chromatids,[5] and regulation of kinetochore microtubule stability.[4] Furthermore, human Sgo1 has been implicated in kinetochore driven formation of kinetochore microtubules.[6] The latter two tasks are tightly coupled with the function of the chromosomal passenger complex (CPC) and Polo-like kinase 1 (Plk1).[7-11]

Here we show that in HeLa cells kinetochore accumulation of Sgo1 is disrupted after RNAi or chemical inhibition of Aurora B kinase (AurB), the catalytic subunit of the CPC. Instead, Sgo1 accumulated along the chromosome arms. We also show that Sgo1 is not required for CPC localization at the inner centromeres. However, Plk1 kinetochore binding and accumulation of the 3F3/2 phospho-epitope, which is created by Plk1[7,9] at the kinetochores which are not under microtubule-mediated tension,[12-15] is diminished in the absence of Sgo1. This implicates Sgo1 in the tension sensing mechanism of the spindle checkpoint by regulating Plk1 kinetochore affinity. Moreover, we demonstrate that vertebrate Sgo1 is phosphorylated by both AurB and Plk1 in vitro. In addition to these experiments, we conducted extensive kinetochore interdependency RNAi assays that suggest a central role for Sgo1 and Plk1 in the hierarchical assembly of the kinetochore.

## MATERIAL AND METHODS

**Plasmids.** For localization studies Sgo1 was fused to the N-terminus of YFP (YFP-hSgo1). The Sgo1 coding sequence was amplified from Human Small Intestine QUICK-Clone cDNA (BD Biosciences) using primers that introduce specific restriction sites at the 5' and 3'-ends (XhoI and BamHI) and cloned into pEYFP-C1 (BD Biosciences).

**Transfections.** For plasmid transfections, HeLa cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers' protocol, with the exception that 20% of the recommended Lipofectamine 2000 was used. siRNA transfections of HeLa cells were performed using Oligofectamine (Invitrogen) according to the manufacturers' recommendations. The following siRNAs (Dharmacon) were used: 5'-AAGAAGCAGATTGAGCAGAAG-3' (INCENP[16]), 5'-AAGAUAUCAUCCUACAGCUGA-3' (Sgo1[4]), 5'-AAGATCACCCTCCTTAAATAT-3' (Plk1[17]), 5'-AAAUACCAC AAUGACCCAAGA-3' (Bub1) and 5'-AAACAACCUCCUUAAGA GUCA-3' (Cenp-F).

**Immunofluorescence.** After incubation for 40-48 h on coverslips under normal growth conditions, the cells were simultaneously fixed and extracted (3.5% paraformaldehyde/0.5% Triton X-100/PHEM for 15 min). Then, the cells were rinsed in 10 mM MOPS (pH 7.4), 150 mM NaCl, and 0.05% Tween-20 (MBST) and blocked for 1 hr in 20% boiled normal goat serum (BNGS) in MBST. Subsequently, the cells were incubated with the primary antibody diluted in MBST containing 5% BNGS for 1 hr at RT. The following primary antibodies were used at the dilutions indicated: mouse anti-Bub1 (1:125, Upstate), rabbit anti-INCENP (1:1000,[16] a kind gift from Dr. E. Nigg), rabbit anti-AurB (1:1000, Abcam), rabbit anti-Sgo1 (1:200,[18] a kind gift from Dr. W. Dai), rabbit anti-Plk1 (1:125, Abcam), mouse anti-Cenp-F (1:400, BD Biosciences), mouse anti-Hec1 (1:500, Abcam), and CREST auto-immune serum (1:200, Antibodies Inc.). Cells on coverslips were washed three times with MBST and then incubated for 1 h at RT with FITC- (1:600), CY3- (1:1000) or Cy5-conjugated (1:600) goat anti-rabbit, goat anti-mouse or donkey anti-human secondary antibodies (Jackson Immuno Research) in MBST containing 5% BNGS followed by three washes in MBST. For DNA staining the cells were incubated 5 min in 25 ng/ml DAPI and then washed three times with water before mounting in Vectashield (Vector Laboratories). Chromosome isolation and anti-3F3/2 immunofluorescence were performed as described previously.[7]

**Microscopy and quantification of fluorescence intensities.** Fixed cells were imaged using a Zeiss Axiovert microscope equipped with 63x (NA 1.4) and 100x (NA 1.4) objectives, a Hamamatsu Orca-ER camera (Hamamatsu Photonics) and Metamorph imaging software (Universal Imaging). For quantification of protein amounts, background corrected fluorescence intensities at the kinetochore were normalized against the anti-CREST signals of the same kinetochore.[7] For each experiment over fifty kinetochores were quantified at least in five cells. Statistical analysis was performed using student's t-test.

**Western blotting.** Control, Plk1 siRNA- or Cenp-F siRNA-transfected HeLa cells, all arrested with 3 μM nocodazole for 16 h, were harvested by mitotic shake-off and centrifugation followed by a wash in phosphate buffered saline (PBS) (pH 7.2). Cell pellets were flash frozen in liquid nitrogen and stored at -70°C. For preparation of extracts, cell pellets were thawed in 20 mM Tris (pH 7.7), 100 mM KCl, 50 mM sucrose, 0.1 mM CaCl2, 1 mM MgCl2, and 0.5% Triton X-100 (APC buffer), containing 5 μg/ml protease inhibitor cocktail. Cells were incubated on ice for 10 min with intermittent vortexing and extracts were cleared by centrifugation at 14,000 x g for 10 min. Proteins were separated by SDS-PAGE with 4%–12% gradient gel (Cambrex) and transferred onto nitrocellulose membrane for Western analysis. Membranes were blocked in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween-20 (TBST) containing 5% nonfat dry milk. The membrane was incubated in rabbit anti-Plk1, mouse anti-Cenp-F and mouse anti-AurB as a control (all 1 μg/ml). After washing three times with TBST the membrane was incubated in IRDye® Conjugated 680 Goat Anti-Mouse or anti-Rabbit IgG (Rockland) diluted 1:5000. Antibodies were diluted in TBST, and incubations were carried out for 1 hr at RT. The Odyssey Infrared Imaging System (LI-COR Biotechnology) was used for the detection of proteins.

**In vitro phosphorylation assay.** Four overlapping fragments of *Xenopus* Sgo1 (amino acids 1-200, 200-350, 350-500 and 500-664) were subcloned into pET-30 (Novagen) by PCR from the full-length Sgo1 cDNA.[4] The recombinant proteins were expressed, purified[19,20] and used in an in vitro kinase assays as described previously.[20]

## RESULTS AND DISCUSSION

**Sgo1 localization at the inner kinetochores is lost when sister chromatids align at the metaphase plate.** Previous studies have localized Sgo1 either at the inner centromere,[21,22] at the (inner and outer) kinetochore,[4] or both the inner centromere and kinetochore.[23] To resolve this discrepancy we expressed an YFP-hSgo1 fusion protein in HeLa cells and analyzed anti-Sgo1 antibody labeling. YFP-Sgo1 localization was compared to Hec1, Cenp-F, AurB, and CREST centromere marker at different phases of cell division (Fig. 1). Hec1 and Cenp-F are markers of the outer kinetochore[24,25] while AurB is present at the inner centromere.[26] From prophase until metaphase, YFP-hSgoI accumulated at the kinetochores in two dot-like structures proximal to the Hec1, Cenp-F (Figs. 1A-C), and CREST (data not shown) domains, and mostly distal to, but partially overlapping with the AurB signal (Fig. 1). The localization of endogenous Sgo1 detected by an anti-Sgo1 antibody[18] was similar to that of YFP-hSgo1 (Fig. 1D shows Sgo1 and Hec1 localization). These data demonstrate that hSgo1 localizes at the inner-kinetochore. Interestingly, less (YFP-)Sgo1 was present at the kinetochores of aligned chromosomes compared to unaligned chromosomes (Fig. 1), which is similar to the behavior of AurB (Fig. 1; Ref. 20). One possible explanation for the observed reduction of Sgo1 and AurB in metaphase chromosomes may be the presence of interkinetochore tension. This hypothesis is supported by the observations that in Plk1 silenced (Fig. 2) or monastrol treated (data not shown) HeLa cells, which both arrest in mitosis with monopolar spindles, Sgo1 is present in high quantities at the kinetochores. Occasionally, we observed bright (YFP-)Sgo1 signals at a few kinetochores at the metaphase plate (Fig. 2, arrows). This could mark merotelic attachments of the metaphase kinetochores that recently were shown to exhibit bright AurB signals.[27] Temporal changes in the interkinetochore tension in the metaphase chromosomes or erroneous microtubule attachments may lead to recruitment of Sgo1 to metaphase kinetochores through the action of other spindle checkpoint proteins such as the CPC subunits. We conclude that Sgo1 localizes to the inner kinetochore from prophase to early metaphase and that this affinity may be regulated by microtubule-mediated tension.

**Kinetochore localization of Sgo1 depends on AurB and Bub1.** To test the idea that certain spindle checkpoint proteins are required

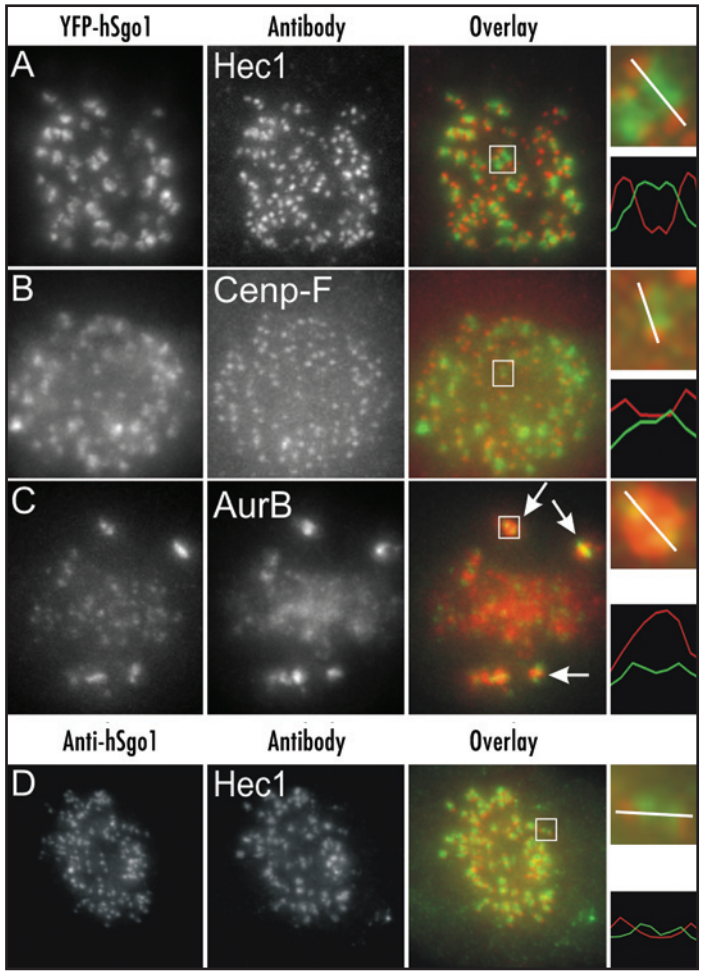


Figure 1. hSgo1 accumulates at the inner kinetochore during mitosis. (A–C) show prometaphase YFP-hSgo1 expressing HeLa cells stained with antibodies against Hec1 (A), Cenp-F (B), or AurB (C). (D) shows a prometaphase HeLa cell stained with antibodies against Sgo1 and Hec1. The overlays show YFP-hSgo1 (green) and antibody staining (red) (A–C), or anti-Sgo1 (green) and anti-Hec1 (red) antibody stainings (D). The right hand column shows enlargements of the sister chromatid pairs in the boxed region of the overlay picture and the corresponding line scans. Arrows indicate unaligned chromosomes. Sgo1 localizes at kinetochores in two distinct spots proximal to Hec1 and Cenp-F and distal to AurB, although some overlap with AurB was observed, indicative of accumulation at the inner kinetochore.

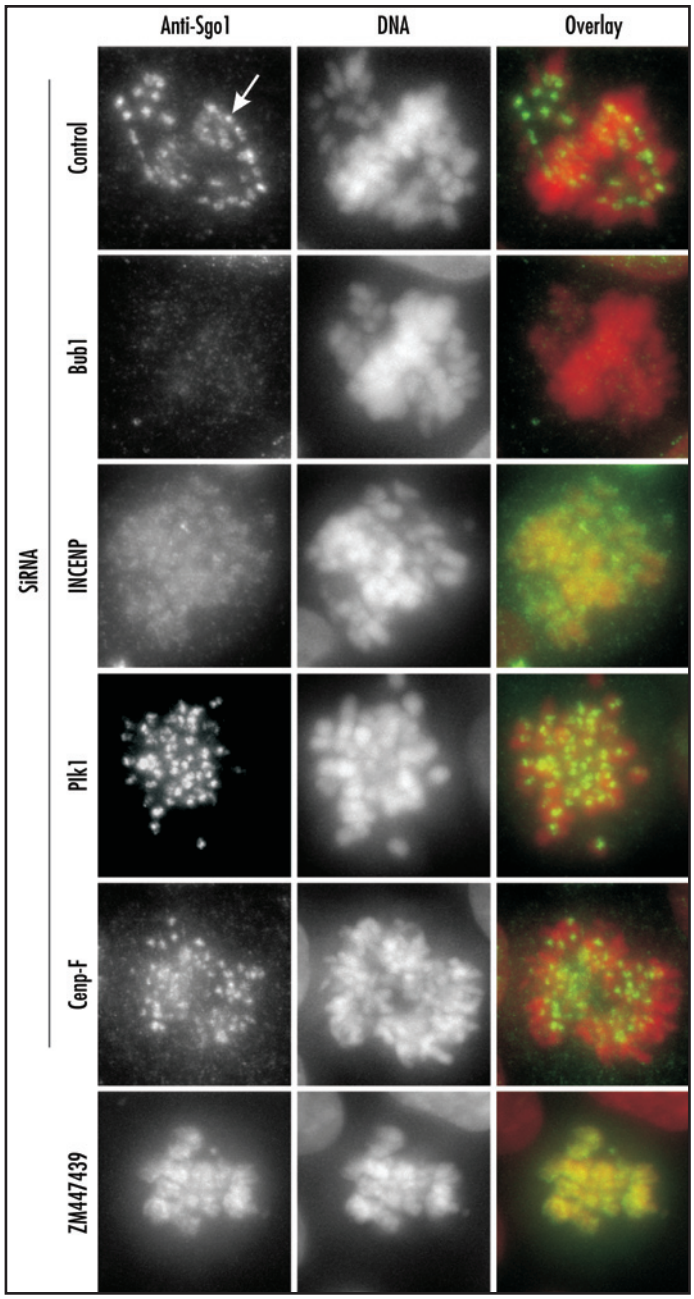


Figure 2. Kinetochore localization of Sgo1 depends on Bub1 and AurB kinase activity. All panels except the last (ZM447439) show HeLa cells that were transfected with the indicated siRNAs. The cells were fixed 40–48 h after transfection and stained with anti-Sgo1 antibody (green) and DAPI (red). The bottom panel (ZM) shows a HeLa cell expressing YFP-hSgo1 (green) treated with ZM447439, an Aurora kinase inhibitor, for 30 min before fixation and DNA staining (DAPI, red). The arrow indicates kinetochores at the metaphase plate with bright Sgo1 signals. Sgo1 accumulates on chromosome arms instead of exclusively at the kinetochores in the absence of AurB or when AurB kinase activity is chemically inhibited. Sgo1 is unable to target to the kinetochores in the absence of Bub1. Kinetochore targeting of Sgo1 does not depend on Plk1 and Cenp-F.

for Sgo1 kinetochore binding, we silenced Bub1, INCENP, Plk1 and Cenp-F in HeLa cells using RNAi and measured the Sgo1 protein levels at the kinetochores (Fig. 2). We compared integrated intensities of anti-Sgo1 antibody signals between silenced and nonsilenced cells after normalization against CREST signals (Table 1, $F_{siRNA}/F_{mock}$). Cenp-F and Plk1 were efficiently depleted as shown by Western blotting (Fig. S1). Importantly, levels of AurB in Cenp-F or Plk1 depleted cells were similar to controls. Western blotting could not be used to determine the depletion efficiencies of Bub1 and INCENP due to inability of the available antibodies to detect these proteins by Western blotting. However, recently it was shown that immunostaining of individual kinetochores is a better measure of protein depletion compared to blotting.[28,29] Immunofluorescent labeling showed that Bub1 and INCENP, as well as Cenp-F and Plk1, were successfully depleted in 60–80% of the cells. Antibody host species permitting, cells were stained for the depleted protein in addition to Sgo1 to confirm that only silenced cells were imaged and quantified. Plk1 silencing caused cells to accumulate in mitosis with monopolar spindles as reported before.[11,17] Plk1 and Cenp-F depleted mitotic cells exhibited similar Sgo1 levels at the prometaphase kinetochores

Table 1 **Centromere/kinetochore targeting interdependencies of mitotic proteins**

| Quantified Protein | Depleted Protein | | | | |
|---|---|---|---|---|---|
| | Bub1 | INCENP | Sgo1 | Plk1 | Cenp-F |
| Bub1 | | 0.80[28,41] | 4.02 | 0.94[41] | 0.78[47] |
| INCENP | 0.51 | | 0.91 | 1.29[10] | 0.86[47] |
| Sgo1 | 0.29[21,22] | ND* | | 0.88[21,39,40] | 1.09 |
| Plk1 | 0.30[41] | 0.43[8,41] | 0.26 | | 1.10 |
| Cenp-F | 0.14[48] | 0.17 | 0.45[4] | 0.17 | |

Centromere/kinetochore accumulation of various proteins was quantified in prometaphase HeLa cells after RNAi ($n \geq 50$). The numbers mark the ratio between the mean integrated fluorescence intensities corrected for background and normalized against CREST staining (F) in the silenced and nonsilenced control cells ($F_{siRNA}/F_{mock}$). Grey boxes show conditions where the difference between the protein levels at the kinetochores of silenced cells and nonsilenced cells is statistically highly significant ($p < 0.001$). The citations refer to articles in which similar results have been reported. *ND = not determined because Sgo1 is present on the chromosome arms masking the possible kinetochore localization.

compared to nonsilenced control cells (Fig. 2, Table 1). However, Sgo1 kinetochore binding is significantly reduced in Bub1 silenced HeLa cells compared to nonsilenced cells ($F_{siRNA}/F_{mock}$ = 0.29 ±0.05) (Fig. 2). This is in accordance with previous reports,[21,22] although we did not observe Sgo1 accumulation along the whole length of chromosome arm as reported by Kitajima et al.[22] As observed previously,[30-32] depletion of the CPC, through depletion of INCENP, changed the localization of Sgo1; instead of accumulating at the kinetochores, Sgo1 was present on the chromosome arms in the INCENP RNAi cells (Fig. 2). To determine if Aurora kinase activity is required for Sgo1 accumulation at kinetochores, YFP-Sgo1 expressing HeLa cells were treated with 20 μM ZM447439, an inhibitor of Aurora kinases,[33] for 30 min before sample fixation. As in the INCENP silenced cells, Sgo1 accumulated on the chromosome arms in ZM447439 treated cells (Fig. 2) indicating that Aurora kinase activity is required for Sgo1 localization at the kinetochores. ZM447439 has been shown to inhibit AurB more efficiently than AurA.[33,34] Moreover, the chromosome and spindle effects of ZM447439 phenocopy AurB RNAi but not AurA RNAi in human tissue culture cells[35,36] suggesting that AurB is the primary target of ZM447439. In conclusion, Bub1 and members of the CPC, as well as the kinase activity of AurB, regulate the kinetochore localization of Sgo1 in prometaphase cells.

**Kinetochore localization of Plk1 and Cenp-F depend on Sgo1.** To identify kinetochore/centromere proteins that depend on Sgo1 for their subcellular localization, we investigated kinetochore binding of Bub1, INCENP, Plk1, Cenp-F, and Hec1 in Sgo1 silenced cells (Fig. 3). Forty-eight hours after introduction of the Sgo1 siRNA HeLa cells were fixed and processed for immunofluorescence. The anti-Sgo1 antibody does not detect Sgo1 by Western blotting but analysis of anti-Sgo1 antibody labeling by immunofluorescence allowed us to identify cells with silenced Sgo1. Analysis of individual Sgo1 silenced cells demonstrated a ≥90% reduction in the average integrated Sgo1 signal intensity at the prometaphase kinetochores compared to controls. Sgo1 silenced cells accumulated at a pseudo-prometaphase with many unaligned chromosomes (Fig. 3), as reported previously.[4,23] We quantified Bub1, INCENP, Plk1, Cenp-F, and Hec1 protein levels at the kinetochores of Sgo1 silenced and nonsilenced cells, and normalized the values against CREST signals. Comparison of Sgo1 silenced cells and nonsilenced prometaphase cells revealed a statistically highly significant ($p < 0.001$) reduction in the amount of kinetochore bound Plk1 ($F_{siRNA}/F_{mock}$ = 0.26 ±0.04) and Cenp-F ($F_{siRNA}/F_{mock}$ = 0.45 ±0.10) in the absence of Sgo1 (Table 1, Fig. 3). In contrast, the mean intensities of Hec1 ($F_{siRNA}/F_{mock}$ = 0.86 ±0.10, p = 0.12) and INCENP ($F_{siRNA}/F_{mock}$ = 0.91 ±0.24, p = 0.17) signals at the kinetochores/inner centromeres of Sgo1 silenced cells were at the similar levels as in control cells (Table 1, Fig 3). Importantly, the spindle pole accumulation of Plk1 was unchanged in the absence of Sgo1 (Fig. 3, arrows). Interestingly, we observed that Bub1 levels were significantly higher on the Sgo1 depleted kinetochores compared to nonsilenced cells ($F_{siRNA}/F_{mock}$ = 4.02 ±0.47). To test if the observed enrichment of Bub1 at the kinetochores of Sgo1 silenced cells could be due to absence of tension or stable microtubule-kinetochore attachments, we compared intensities of Bub1 signals between nontreated, nocodazole treated (3 μM for 16 h) and taxol treated (0.6 μM for 16 h) cells. Nocodazole depolymerizes microtubules and induces unattached chromosomes while taxol treatment causes hyperstabilization of microtubules and results in chromosomes with attached microtubules but reduced interkinetochore tension.[37] In the nocodazole treated cells, the signal intensity of Bub1 labeling at the kinetochores was approximately two times higher compared to nontreated cells ($F_{siRNA}/F_{mock}$ = 2.27 ±0.69, p < 0.001) while in taxol treated cells no enhancement of Bub1 signals was observed ($F_{siRNA}/F_{mock}$ = 0.78 ±0.37, p = 0.015). We cannot exclude the possibility that it is the microtubule attachment that reduces Bub1 staining due to epitope masking. This is, however, unlikely since Bub1 was the only molecule of the several above mentioned proteins tested that showed increased signal intensity after addition of nocodazole. Importantly, depletion of Sgo1 did not significantly increase Bub1 levels in nocodazole treated cells ($F_{siRNA}/F_{mock}$ = 0.91 ±0.05, p = 0.18). We conclude that the kinetochore localization of Plk1 and Cenp-F is Sgo1 dependent while the inner centromere/kinetochore localization of INCENP and Hec1 is not. Furthermore, our data suggests that Bub1 is enriched at the kinetochores that lack microtubule attachments and thus strengthen the notion that Sgo1 modulates microtubule-kinetochore interactions.[4]

**Sgo1 is linked to the tension sensing mechanism of the spindle checkpoint.** Plk1 is a key mitotic kinase that regulates centrosome maturation and duplication, mitotic entry, bipolar spindle assembly, removal of cohesin from chromosome arms, and cytokinesis (reviewed in ref. 38). The observation that Sgo1 is required for kinetochore localization of Plk1 (Fig. 3) implicates Sgo1 in the tension-sensing mechanism of the spindle checkpoint. Plk1 creates the tension-sensing 3F3/2 phosphoepitope[7,9] at the kinetochores in the absence of interkinetochore tension.[12-15] Importantly, the 3F3/2 epitope is reduced at the kinetochores of Sgo1 depleted cells (Fig. 3) suggesting that either the 3F3/2 protein is lost from the kinetochores in the Sgo1 silenced cells or that the epitope is dephosphorylated in the absence of Sgo1. The latter is a more likely reason since the Sgo1 depleted cells have lost kinetochore bound Plk1 (Fig. 3). Previously, it was shown that Plk1 binds and phosphorylates MEI-S332 (the *Drosophila* Sgo1 homologue) in vitro[39] and that Plk1 activity is required to remove MEI-S332 and hSgo1 from centromeres at the metaphase-anaphase transition.[39,40] Mutations of Plk1 binding sites in MEI-S332 prevent its removal from the centromeres. Together with our findings, these published results suggest a model where Plk1 is targeted to the kinetochores after recognition of a priming

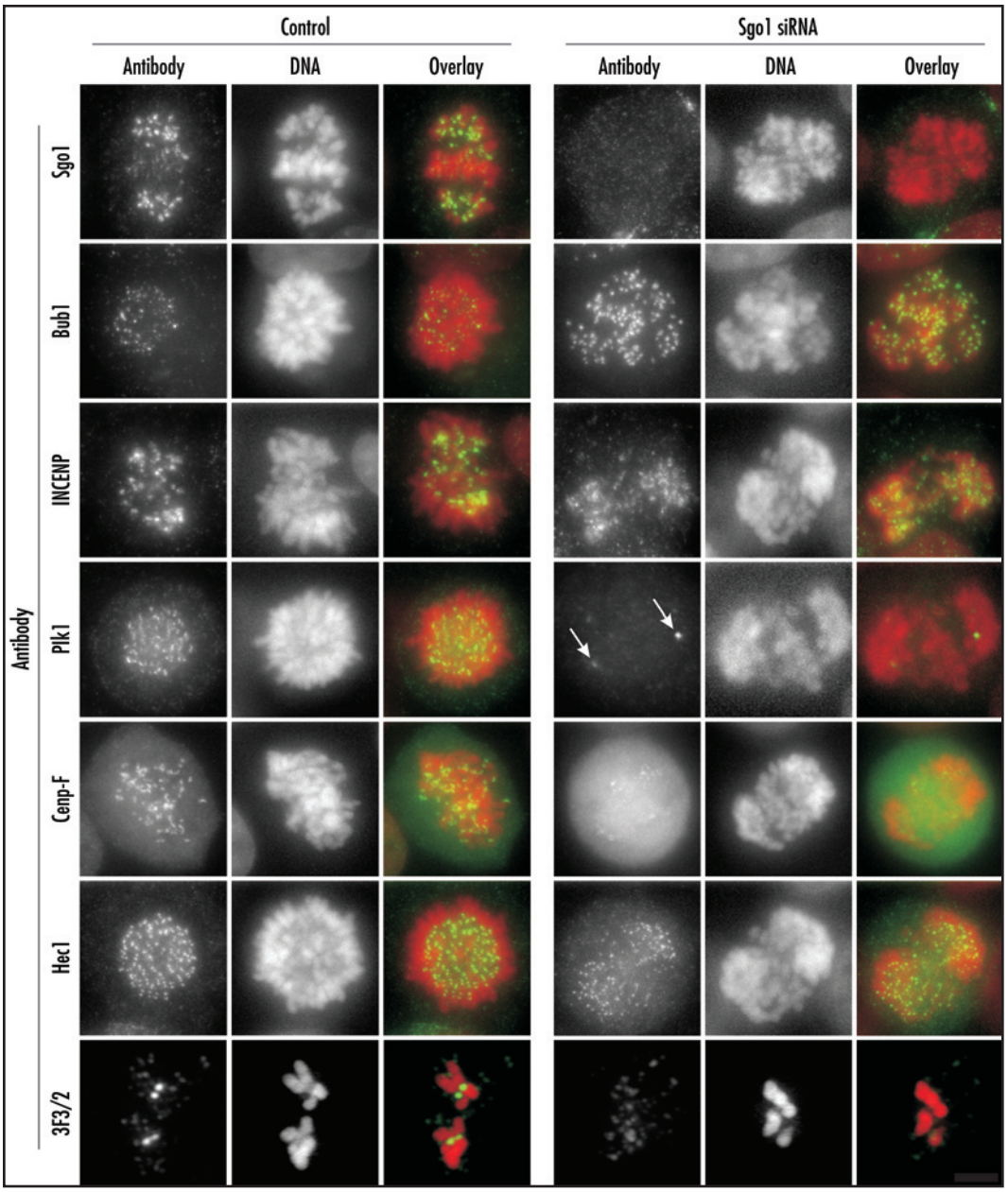


Figure 3. Kinetochore localization of Plk1, Cenp-F, and the 3F3/2 phosphoepitope depends on Sgo1. All panels except the last (3F3/2) show nonsilenced (left) and Sgo1 siRNA transfected HeLa cells fixed and immunolabeled with antibodies against the indicated proteins (green) and stained for DNA (DAPI, red). Arrows indicate spindle poles positive for Plk1. The lower panel shows isolated chromosomes from nocodazole arrested nonsilenced HeLa cells (left) and from nocodazole treated Sgo1 silenced cells stained with the anti-3F3/2 antibody (green) and DAPI (red). Kinetochore targeting of Plk1, Cenp-F, and 3F3/2 depend on Sgo1 while Hec1, Bub1, and INCENP accumulate normally at kinetochores in the absence of Sgo1.

phosphorylation in Sgo1 or in another kinetochore protein that depends on Sgo1 for its kinetochore localization. Qi et al.[41] showed that Plk1 phosphorylates Bub1, which was suggested to directly target Plk1 to the kinetochore. The observations that Sgo1 localization at the kinetochore depends on Bub1 and that Sgo1 depletion removes Plk1 but enhances Bub1 at the kinetochores provide an alternative model where Sgo1 initially recruits Plk1 to the kinetochore where it phosphorylates Bub1 and other targets. Furthermore, we suggest that INCENP is not the Plk1 kinetochore anchor, as proposed by Goto et al.[8] as in the Sgo1 RNAi cells Plk1 diminishes from the kinetochores despite normal INCENP levels. In summary, our data shows that Sgo1 is required for accumulation of Plk1 and the tension-sensing 3F3/2 phosphoepitope at the kinetochore, suggesting that Sgo1 participates in the tension-sensing mechanism of the spindle assembly checkpoint.

**Sgo1 is an in vitro substrate for both AurB and Plk1.** The disappearance of AurB and Bub1 from the kinetochores of aligned chromosomes[20,42] coincides with loss of Sgo1. Moreover, AurB activity is required for proper Sgo1 localization in prometaphase cells. These two observations point to the possibility that Sgo1 is a substrate for AurB. To test this, four overlapping recombinant fragments covering the entire *Xenopus* Sgo1 protein were purified from *E. coli* and used as substrates in an in vitro kinase assay with recombinant *Xenopus* AurB/INCENP (Fig. 4). Full length Sgo1 and the fragment containing amino acids (aa) 1–200 were not soluble and therefore were not included in the assay. As a positive control *Xenopus* MCAK, a bone fide AurB substrate,[20] was used. This experiment showed that the conserved basic C-terminal region of Sgo1 (aa 500–664) was efficiently phosphorylated by AurB (Fig. 4). The fragment containing aa 200-350, which is not conserved between *Xenopus* and human, was also phosphorylated by AurB although to a lesser extend than the C-terminal fragment (Fig. 4). Together with the partial colocalization of AurB and Sgo1 (Fig. 1) and the requirement of AurB activity for kinetochore localization of Sgo1 (Fig. 2), these data suggest that Sgo1 phosphorylation by AurB may lead to recruitment of Sgo1 to the kinetochores. The presence of Sgo1 along the chromosome arms in AurB inhibited/depleted cells might explain the role for AurB in removing chromosome arm cohesion during prophase and prometaphase.[43,44] Mapping of the AurB phosphorylation sites on Sgo1 combined with directed mutagenesis assays could provide further insights into the temporal and spatial control of Sgo1 during mitosis. A recent paper demonstrates that *Drosophila* AurB phosphorylates MEI-S332 in vitro and that the CPC is involved in regulating MEI-S332 localization and therefore the control of sister chromatid cohesion in meiosis.[45] However, MEI-S332 was absent from kinetochores as well as from chromosome arms in INCENP

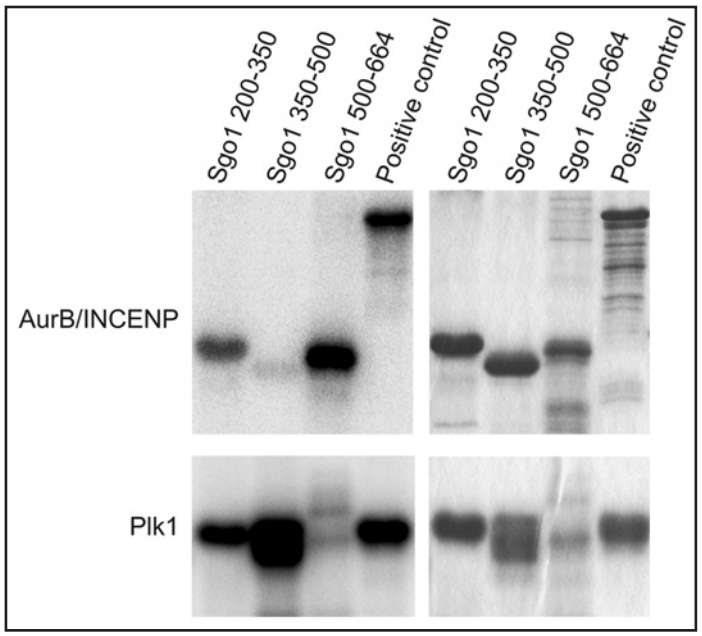


Figure 4. In vitro phosphorylation of xSgo1 fragments by xAurB and Plx1. Recombinant xAurB (upper panel) and Plx1 were used in an in vitro kinase assay with the indicated xSgo1 fragments as substrates. Both the autoradiogram (left) and a corresponding coommassie staining are shown. xAurB phosphorylated the basic C-terminal region of xSgo1 (amino acids 500–664) and the region spanning amino acids 200–350. Plx1 phosphorylated the Sgo1 regions spanning amino acids 200–350 and 350–500. Positive controls were *Xenopus* MCAK (upper panel) and casein.

depleted cells which is in contradiction to the observed chromosome arm localization of Sgo1 (data presented here and refs. 30–32). This discrepancy might be caused by differences between mitotic and meiotic systems, or between *Drosophila* and vertebrate cells. Nevertheless, the combined data strongly suggest a functional link between the CPC and Sgo1, which appears to be conserved in evolution and to be in operation in both mitosis and meiosis.

*Drosophila* Polo has been shown to bind and phosphorylate MEI-S332 in vitro.[39] To investigate if Plk1 targets Sgo1 in vertebrates, we used the above mentioned Sgo1 fragments as substrates for recombinant *Xenopus* Plk1 (Plx1) in an in vitro kinase assay (Fig. 4). Casein, a known Plk1 substrate,[46] was used as a positive control. This experiment showed that Plx1 very efficiently phosphorylates the Sgo1 fragment containing aa 350–500 and, to a lesser extend, the fragment consisting of aa 200–350. The aa 350–500 fragment shows a considerable mobility shift compared to the AurB/INCENP treated fragment, suggesting that phosphorylation by Plx1 occurs on multiple serine/threonine residues. In summary, our findings indicate that Sgo1 is an in vitro substrate for two major mitotic kinases AurB and Plk1.

**Sgo1 and Plk1 play a central role in kinetochore assembly.** To further clarify the role of Sgo1 in kinetochore assembly and the spindle checkpoint machinery we determined interdependencies between Bub1, INCENP, Plk1, and Cenp-F (Table 1), which are in agreement with recent publications (see references in Table 1). The results revealed a hierarchical kinetochore binding for these molecules (Fig. 5). In summary, our results, together with the published data discussed above, suggest a novel branch in kinetochore assembly with central roles for Sgo1 and Plk1 (Fig. 5). Presence of Bub1 and phosphorylation of Sgo1 by AurB are independent

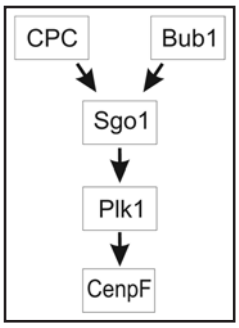


Figure 5. Model for hierarchical assembly of mitotic proteins onto kinetochores. Arrows indicate the direction of regulation. At the top of the hierarchy are Bub1 and the CPC which are independently required for the kinetochore accumulation of Sgo1. Sgo1 is required for kinetochore binding of Plk1, which subsequently attracts Cenp-F to the kinetochores.

requirements for Sgo1 kinetochore affinity. We propose that Sgo1 attracts Plk1 to the kinetochore, which in turn is required for kinetochore recruitment of Cenp-F. After interkinetochore tension is established, Plk1 phosphorylates Sgo1 which leads to removal of Sgo1 from the kinetochores. This is followed by loss of Cenp-F and Plk1 itself from the kinetochore. Since Plk1 is known to be required for the kinetochore recruitment of several key proteins involved in mitotic signaling,[7,9] these events possibly contribute to the eventual inactivation of the spindle checkpoint in metaphase cells. What events lead to phosphorylation of Sgo1 by Plk1 after establishment of tension remain to be discovered. Knowledge of these reactions would help to understand how the spindle checkpoint senses interkinetochore tension/microtubule attachments and how its activity is controlled.

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**Note**

Supplemental information can be found at:
www.landesbioscience.com/supplement/pouwelsCC6-13-sup.pdf

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