A highly conserved pocket on PP2A-B56 is required for hSgo1 binding and cohesion protection during mitosis

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Abstract

The shugoshin proteins are universal protectors of centromeric cohesion during mitosis and meiosis. The binding of human hSgo1 to the PP2A-B56 phosphatase through a coiled-coil (CC) region mediates cohesion protection during mitosis. Here we undertook a structure function analysis of the PP2A-B56-hSgo1 complex, revealing unanticipated aspects of complex formation and function. We establish that a highly conserved pocket on the B56 regulatory subunit is required for hSgo1 binding and cohesion protection during mitosis in human somatic cells. Consistent with this, we show that hSgo1 blocks the binding of PP2A-B56 substrates containing a canonical B56 binding motif. We find that PP2A-B56 bound to hSgo1 dephosphorylates Cdk1 sites on hSgo1 itself to modulate cohesion interactions. Collectively our work provides important insight into cohesion protection during mitosis.

Keywords cohesion; mitosis; phosphatase; PP2A-B56; shugoshin

Introduction

The shugoshin proteins (hSgo1 (Sgo11) and hSgo2 (Sgo12) in humans) are conserved protectors of centromeric cohesion by preventing premature release of the cohesion complex (Marston, 2015). The first shugoshin protein was discovered in Drosophila melanogaster through the isolation of a mutant, MEI-S332, that lost cohesion prematurely during meiosis (Kerrebrouck et al, 1992; Kerrebrouck et al, 1995; Tang et al, 1998). Subsequent genetic screens identified the shugoshin proteins in yeast (Katis et al, 2004; Kitajima et al, 2004; Marston et al, 2004). Common to these proteins is the presence of an N-terminal coiled coil (CC) region that binds to B56 regulatory subunits, hereby localizing PP2A-B56 to centromeres (Kitajima et al, 2006; Riedel et al, 2006; Tang et al, 2006; Xu et al, 2009). The function of the yeast PP2A-B56-Sgo1 complex during meiosis is to dephosphorylate Rec8, hereby preventing Separate cleavage of cohesion (Brar et al, 2006; Kitajima et al, 2006; Riedel et al, 2006; Ishiguro et al, 2010; Katis et al, 2010).

The PP2A-B56 protein phosphatase is a Ser/Thr phosphatase that dephosphorylates numerous substrates to regulate mitosis (Nilsson, 2019; Garvanska & Nilsson, 2020). PP2A-B56 is a trimeric holoenzyme composed of a scaffold subunit (PP2A-A) that connects the B56 subunit with the catalytic subunit (PP2A-C) (Fig 1A) (Xu et al, 2006; Cho & Xu, 2007). The B56 subunit of the holoenzyme carries substrate specificity by binding to interactors that target the phosphatase to its substrates. Most B56 interactors bind via a conserved LxxIxE peptide motif that engages a highly conserved pocket on B56 present in all five B56 isoforms (Hertz et al, 2016; Wang et al, 2016; Wu et al, 2017; Wang et al, 2020). A number of important mitotic regulators such as BubR1, Kif4A, and RacGAP1 bind to PP2A-B56 through a LxxIxE motif to regulate specific dephosphorylation events. There are five isoforms of B56 (B56α, β, γ, δ, and ε) that display distinct localization patterns during mitosis (Foley et al, 2011; Bastos et al, 2014; Vallardi et al, 2019).

In human somatic cells, hSgo1 recruits PP2A-B56α/ε and to a lesser extent the other PP2A-B56 isoforms, to the centromere (Meppelink et al, 2015; Vallardi et al, 2019). This protects cohesion complexes from the mitotic prophase pathway by locally antagonizing mitotic kinase activity and thus WAPL mediated removal of cohesion (Salic et al, 2004; Kitajima et al, 2005; McGuinness et al, 2005). Although hSgo2 has been reported to recruit the bulk of PP2A-B56α to centromeres, hSgo2 is not needed for cohesion protection during mitosis (Kitajima et al, 2006; Tang et al, 2006; Orth et al, 2011; Vallardi et al, 2019). Instead, hSgo2 protects Rec8 from cleavage by separase during meiosis (Lee et al, 2008). In contrast, depleting hSgo1 prevents cohesion protection despite having limited...
effect on PP2A-B56 centromeric levels (Kitajima et al, 2006; Tang et al, 2006; Vallardi et al, 2019). hSgo1 performs cohesion protection through a conserved cohesin binding motif that is absent from hSgo2 (Liu et al, 2013; Nishiyama et al, 2013; Hara et al, 2014). This hSgo1 cohesin binding motif is phosphorylated by Cdk1 during mitosis on Thr346 to promote cohesin binding (Liu et al, 2013). hSgo1 furthermore competes directly with the cohesin release factor WAPL for cohesin binding to prevent WAPL activity (Hara et al,

Figure 1. hSgo1 and LxxIxE motifs compete for binding to PP2A-B56.
A Structure of the PP2A-B56–hSgo1 complex (adapted from Xu et al, PDB: 3FGA). The hSgo1 coiled-coil homodimer interacts with both PP2A catalytic and B56 regulatory subunits. The model shows a LxxIxE peptide bound to B56 at its conserved binding pocket.
B YFP pull down from cells stably expressing YFP (control) or YFP-B56x enriches the entire PP2A-B56x holoenzyme on the beads. PP2A-A, scaffold subunit; PP2A-C, PP2A catalytic subunit.
C Coomassie-stained SDS–PAGE of the purified hSgo1 full length (FL) and hSgo11–155.
D Competition assay with the purified hSgo1 proteins shown in (C). Binding of YFP-B56x to indicated proteins was determined. Representative of 3 independent experiments.
E Peptide competition assay with a WT LxxIxE peptide or a mutated variant that does not bind B56 (LxxAxA). Binding of YFP-B56x to indicated proteins was determined and quantified by LI-COR.
F A PP2A-B56x–BubR11516–715 complex was reconstituted (blue box) and peak fractions pooled and incubated with hSgo11–155. Following incubation, this complex was analyzed by size exclusion chromatography and fractions analyzed by SDS–PAGE (black box).
G YFP-B56x pull down from cells stably expressing the indicated LxxIxE binding pocket variants of B56x and subsequent immunoblotting of indicated proteins. Representative of 4 independent experiments.

Source data are available online for this figure.
Figure 2.
Two proteins, Sororin and the cohesin subunit SA2, have been shown to be dephosphorylated by PP2A-B56-hSgo1 to protect cohesin (Hauf et al., 2005; Liu et al., 2013; Nishiyama et al., 2013). Indeed, expressing variants of Sororin and SA2 that cannot be phosphorylated bypass the need for hSgo1 (Liu et al., 2013; Nishiyama et al., 2013). Whether PP2A-B56 bound to hSgo1 dephosphorylates other substrates is unclear.

In addition to recruiting PP2A-B56, the shugoshin proteins also recruit the chromosomal passenger complex (CPC) to centromeres through their CC region (Kawashima et al., 2007; Vanoosthuyse et al., 2007; Tsukahara et al., 2010). The shugoshin-dependent localization of CPC to the centromere could also contribute to cohesion protection (Hengeveld et al., 2017). Although the interplay between shugoshin recruitment of PP2A-B56 and the CPC to centromeres is not fully established, recent work suggests that the ability of hSgo1/2 to recruit the CPC and PP2A-B56 are distinct activities (Bonner et al., 2020).

Crystallographic studies have determined the human PP2A-B56 in complex with a fragment of hSgo1 comprising residues 51–96, which represents most, but not the entire N-terminal CC domain (Xu et al., 2009). This hSgo1 fragment displays less affinity to PP2A-B56 than longer N-terminal fragments of hSgo1, but sufficient affinity to efficiently bind to PP2A-B56 under crystallization conditions using high protein concentrations. The structure revealed that the hSgo1 fragment forms a dimer which engages several residues of the last C-terminal HEAT repeat of B56γ and makes contacts to the PP2A catalytic subunit (Fig 1A). Although the crystal asymmetric unit shows a 1:1 interaction between hSgo1 peptide strands and PP2A holoenzymes, the hSgo1 peptide strands are arranged into a parallel CC homodimer, where one fragment is related to the other by a twofold crystallographic symmetry axis (depicted as chain A and Asym in Fig 1A). This arrangement allows them to interact symmetrically with PP2A enzymes on both sides. Thus, one PP2A-B56γ holoenzyme displays interactions with residues from both of the two alpha helices forming one hSgo1 CC region in the crystal, which is again consistent with biochemical experiments showing that dimerization of hSgo1 is required for binding to PP2A-B56γ (Tang et al., 1998; Xu et al., 2009). In the PP2A-B56γ-hSgo1 structure, the LxxlxE binding pocket of B56γ is fully exposed and indeed the N-terminal region of hSgo1 does not appear to contain any recognizable LxxlxE motif.

These observations raise the possibility that the PP2A-B56-hSgo1 complex can make higher order complexes with LxxlxE containing proteins, which could be important for mitotic cohesion protection. We explored this possibility, which revealed unanticipated aspects of the PP2A-B56-hSgo1 complex important for understanding cohesion protection during mitosis.

Results and Discussion

hSgo1 and LxxlxE motifs compete for binding to PP2A-B56

We first determined whether hSgo1 can bind to PP2A-B56 in complex with LxxlxE containing proteins. We generated stable inducible HeLa cell lines that express YFP-tagged B56α (stable inducible HeLa cell lines used throughout unless indicated) and arrested cells in prometaphase using nocodazole. Mitotic cells were collected by mitotic fixation, and hSgo1 and BubR1 binding was analyzed. Interestingly, all B56α variants unable to bind BubR1 failed to co-purify with LxxlxE-containing proteins. We generated stable inducible HeLa cell lines expressing hSgo1 variants fused to YFP and the Cenp B centromere-targeting domain (CB). Representative images of chromosome spreads are shown (CB targets all the rescue constructs [green] to the centromere. Scale bar, 5 μm. (E) Quantification of (D). The distance between the two peak intensities of YFP was measured for 5 kinetochore pairs and averaged for a single cell and plotted. The data are from independent experiments and the mean and SD are indicated.

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Source data are available online for this figure.
Figure 3.
hSgo1 (Fig 1G). The reason why the LxxIxE peptide does not fully displace hSgo1, in contrast to the B56 mutants, could reflect that the PP2A-B56-hSgo1 complex is very stable once formed. Collectively, these results indicate that LxxIxE motif-containing proteins and hSgo1 compete for a common binding surface on PP2A-B56α. As hSgo1 does not contain any recognizable LxxIxE motif in its CC region, it likely has a binding site that overlaps with the LxxIxE binding pocket of B56 subunits.

The LxxIxE binding pocket of PP2A-B56 is required for cohesion protection

The involvement of the B56α LxxIxE binding pocket in hSgo1 binding was surprising, given that hSgo1 binds the less conserved C-terminal HEAT repeat of B5γ in the reported structure of the PP2A-B56γ-hSgo1 complex (Fig 2A). To further analyze this, we investigated the B56α R222E LxxIxE pocket mutant in depth for hSgo1 binding and cohesion protection. We compared this to a B56α mutant (B56α 5A), in which all residues at the reported structural interface with hSgo1 were mutated (B56α 5A:Y365A, H377A, Y381A, L384A, M388A) (Fig 2A). First, we compared the binding of PP2A-B56α to hSgo1 and LxxIxE containing mitotic regulators. Consistent with the reported structure of the PP2A-B56γ-hSgo1 complex, we found that YFP-B56α 5A bound less hSgo1 while maintaining its interactions with BubR1 and Ki67A (Fig 2B). In contrast, B56α R222E (mutation in the LxxIxE binding pocket) lost both binding to hSgo1 and LxxIxE containing proteins. In a reciprocal experiment, cells stably expressing FLAG-tagged B56α variants were transfected with YFP-hSgo1, and then, YFP-hSgo1 was affinity-purified from mitotic cells. Again, we observed impaired binding to both B56α R222E and 5A, with the latter mutant retaining more binding to hSgo1 (Fig 2C). A similar result was obtained using YFP-hSgo2 (Fig EV1A). These experiments strengthen the conclusion that the LxxIxE binding pocket of B56 is an important binding determinant for the human shugoshin proteins.

We next analyzed the ability of the B56α mutants to support cohesion protection. All B56 isoforms were depleted by RNAi and cells were induced to express RNAi-resistant YFP-B56α variants at endogenous levels (Fig EV1B). This in our hands did not affect hSgo1 or hSgo2 localization to centromeres although we note that PP2A has been found to affect hSgo1 localization in another study (Tang et al, 2006) (Fig EV1C and D). Cells were synchronized in prometaphase using nocodazole and chromosome spreads were stained with CREST and DAPI to analyze cohesion integrity. The distance between the two peak intensities of CREST was measured, as premature cohesion removal results in longer distances. Indeed, depleting all B56 subunits increased the distance between centromeres, which was rescued by expressing B56α wild type (WT) (Fig 2D and E). As anticipated from the interaction studies, B56α R222E did not support cohesion protection at all while B56α 5A surprisingly did (Fig 2D and E). To further substantiate this result, we performed live cell imaging of the same conditions. Removing hSgo1 and consequently centromeric cohesion results in prolonged mitotic arrest because of activation of the spindle assembly checkpoint. Similarly, depleting all B56 isoforms resulted in a prolonged arrest which was rescued by YFP-B56α WT and 5A but not the R222E mutant, thus paralleling the chromosome spread results (Fig EV1E–G). Consistent with our binding experiments (Fig 2B and C), only YFP-B56α WT displayed clear localization to chromosomes as observed by live cell imaging (Fig EV1F and Movies EV1–EV3). We analyzed the YFP-B56α 5A phenotype over a range of expression levels and even low levels of expression rescued the B56 RNAi. These results show that mutating the LxxIxE binding pocket of B56α abolishes cohesion protection while the reported interface for binding the hSgo1 CC appears less critical for this.

To establish that B56α R222E can assemble an active PP2A holoenzyme capable of cohesion protection, we artificially recruited the B56α mutants to the centromere by fusing them to the centromere-targeting domain of Cenp B (CB). We then asked if in the absence of hSgo1, these B56α mutants supported cohesion protection (Fig EV2A and B for hSgo1 depletion). We performed chromosome spreads and measured the distance between CREST peak intensities. All variants of CB-B56α rescued the cohesion defect when hSgo1 was depleted, arguing that they form functional PP2A complexes (Fig 2F and G).

Collectively, our analysis of B56α R222E shows that this mutant is defective in cohesion protection most likely due to a defect in hSgo1 binding.

hSgo1 mutations affecting PP2A-B56 binding

We were surprised by the fact that the B56α 5A mutant fully supported cohesion protection despite showing a clear reduction in hSgo1 binding. To explore this further, we investigated the...
Figure 4.
Figure 4. Cohesion protection in hSgo1 mutants.

A Representative images of chromosome spreads from hSgo1 RNAi treated cells stably expressing the indicated YFP-hSgo1 variants. All the YFP-hSgo1 rescue constructs (green) localize to the centromeres (CREST, red). Scale bar, 5 μm.

B The distance between the two peak intensities of CREST was measured for 5 kinetochore pairs from the chromosome spreads in (A) and averaged for a single cell. The data are from 3 independent experiments and the mean and SD are indicated.

C IP of YFP-hSgo1 from cells stably expressing hSgo1 WT, 3A, and 4A followed by immunoblotting of cohesin components, Smc1 and Smc3. Representative blots are shown (top). Signals were quantification by LiCOR, normalized to YFP and plotted (bottom). Error bars represent SD (n = 4 for Smc1 and n = 3 for Smc3).

D Table summarizing the log2 differences between hSgo1 WT, 3A and 4A from quantitative MS analysis.

E Experimental protocol of the live cell imaging shown in (F).

F hSgo1 RNAi and rescue with the indicated hsgo1 RNAi-resistant constructs was performed. Representative still images captured during the live cell imaging showing DIC and YFP-hSgo1 WT, 3A, and 4A localization during mitosis. Time (min) from nuclear envelop breakdown (NEBD) is indicated. Scale bar, 15 μm.

G The time from NEBD to anaphase was measured from 3 independent live cell imaging experiments. Each circle represents an individual cell, and the median is indicated. Note that the WAPL KD condition is incorporated from the experiment which is shown in full in Fig EV4C for clarity.

Source data are available online for this figure.

Consequence of mutating the residues in hSgo1 involved in binding the C-terminal region of B56. We generated a hSgo1 mutant (hSgo1 4A) where the four residues (L83, K87, Y90, and C94) contacting B56γ in the reported structure were mutated to alanine residues (Figs 3A and EV2C). As a comparison, we used a previously reported hSgo1 3A mutant (Y57, N60, and K62 to alanine) which contains three mutated residues at the interface with the PP2A catalytic subunit (Xu et al., 2009). The reported interface with the PP2A catalytic subunit involves residues from both alpha helices of the hSgo1 CC region (Fig 3A). Stably expressed YFP-hSgo1 variants were purified from mitotic cells at two salt concentrations (50 and 150 mM NaCl), and binding to PP2A-B56x was determined (Fig 3B and C). Both hSgo1 4A and 3A showed a strong reduction in binding to PP2A-B56x components, and only hSgo1 4A maintained some residual binding at the low salt concentration (Fig 3B).

To analyze PP2A-B56-hSgo1 complex formation in cells, we took three separate approaches. Firstly, we analyzed centromeric B56x levels by immunofluorescence in cells depleted of hSgo1 and complemented with RNAi-resistant YFP-hSgo1 variants. By normalizing the B56x signal to YFP, it was clear that both hSgo1 3A and hSgo1 4A localized B56x less efficiently to centromeres (Fig 3D and E). However, it was evident that hSgo1 3A was more compromised in localizing B56x than hSgo1 4A, consistent with the binding assays. Secondly, we employed an assay where LacI-GFP fusions of hSgo1 variants are localized to a LacO array on chromosome 1 in budding yeast (Verzijlbergen et al., 2014). Consistent with these results, it has been reported that hSgo1 3A is less efficient in localizing Ipl1 (Aurora B) in budding yeast (Verzijlbergen et al., 2014).

Collectively, we characterize two hSgo1 mutants showing a reduction in PP2A-B56 binding.

Cohesin binding and protection in hSgo1 mutants

To determine the ability of hSgo1 mutants to support cohesion protection, we depleted endogenous hSgo1 and expressed RNAi-resistant hSgo1 mutants. Cells were arrested in mitosis; chromosome spreads were prepared and distances between kinetochore pairs were measured (Fig 4A and B). hSgo1 depletion resulted in complete loss of cohesion, which was rescued by hSgo1 WT and hSgo1 4A but not by hSgo1 3A. The lack of cohesion protection in hSgo1 3A is consistent with data from budding yeast meiosis (Xu et al., 2009). This was not due to a defect in cohesin binding, as both hSgo1 3A and hSgo1 4A enriched more cohesin components in YFP purifications as determined by immunoblotting and quantitative mass spectrometry analysis (Fig 4C and D and Dataset EV2). In particular, the hSgo1 3A mutant bound more Smc1 and Smc3 while hSgo1 4A only showed a slight increase in binding. We observed by mass spectrometry that phosphorylated hSgo1 Thr346 levels were increased in hSgo1 3A, paralleling the increase in cohesin binding (Fig 4D). This argues that PP2A-B56 bound to hSgo1 dephosphorylates Thr346 to negatively regulate cohesin binding. To further analyze the hSgo1 3A and 4A phenotypes, we performed a live cell analysis of cells complemented with the different hSgo1 variants and monitored mitotic progression (Fig 4E–G). hSgo1 depletion induced a strong mitotic arrest which was rescued by hSgo1 WT and hSgo1 4A but not by hSgo1 3A, in agreement with the chromosome spread results. From the live cell analysis, it was obvious that...

8 of 13 EMBO reports 22: e52295 | 2021 © 2021 The Authors
hSgo1 3A expressing cells did not align chromosomes, likely due to loss of cohesion protection (Fig 4F). Consistent with a specific defect in cohesion protection from the prophase pathway, the depletion of WAPL fully suppressed the hSgo1 3A phenotype (Figs 4G and EV4C). We analyzed all hSgo1 variants at a similar fluorescent intensity and over a range of fluorescent intensities, and this revealed that even low levels of hSgo1 4A expression were sufficient to support hSgo1 function. Further reduction in B56 levels by RNAi did not cause a mitotic phenotype in hSgo1 4A (Fig EV4D–F). This suggests that a substantial reduction in PP2A-B56-hSgo1 complex formation is required to prevent cohesion protection.

An important discovery from our work is that the highly conserved LxxIxE binding pocket of B56 subunits is required for hSgo1 and hSgo2 binding and cohesion protection during mitosis in somatic cells. Indeed, our results argue that the interaction between hSgo1 and the LxxIxE binding pocket of B56 is more critical than the reported interaction with the C-terminal HEAT repeats. Whether these observations extend to meiosis and other model organisms is unclear. This was surprising based on the reported structure of the human PP2A-B56γ-hSgo1 complex and the fact that the B56 binding region of hSgo1 and hSgo2 lacks a recognizable LxxIxE motif. One possibility is that the solved structure, which used only a short fragment of hSgo1, does not fully recapitulate the PP2A-B56-hSgo1 complex and crucial aspects of the structure are yet to be uncovered.

We anticipate that full-length hSgo1 binds in a manner that overlaps with the LxxIxE binding pocket of B56 and the C-terminal HEAT repeat as reported in the structure. Consistent with this, in vivo cross-linking mass spectrometry identified peptides of hSgo1 cross-linked to residues in close proximity to the LxxIxE binding pocket on B56 (Herzog et al., 2012). Given the strong conservation of the B56 LxxIxE binding pocket, our results explain why hSgo1 co-purifies with all isoforms of B56 (Kitajima et al., 2019). In addition to this, specific sequence elements present in B56 might further favor hSgo1/2 binding (Valdardi et al., 2019). An implication from our results is that hSgo1 and LxxIxE motifs compete for binding to PP2A-B56, which could regulate dephosphorylation at centromeres during mitosis.

Furthermore, our analysis shows that a substantial reduction in PP2A-B56-hSgo1 complex formation can be tolerated without affecting cohesin protection. Only when complex formation is strongly preserved, such as with the hSgo1 3A and B56 R222E mutants, is cohesin protection defective. This might not be surprising given that PP2A-B56 is very efficient in dephosphorylating substrates. Our analysis of the phosphorylation patterns of hSgo1 3A also reveals that one target of PP2A-B56 is Cdk1 phosphorylation sites on hSgo1 itself. Indeed, we find that Thr346 is dephosphorylated by PP2A-B56 and that this regulates hSgo1 association with cohesin during mitosis.

Whether dynamic phosphorylation-dephosphorylation of Thr346 is required for cohesin protection during mitosis will be important to investigate in future experiments.

Materials and Methods

Antibodies and RNAi oligos

Antibodies used in this study were as follows: Rabbit anti-hSgo1 (gift from Dr. Hongtao Yu, 1:200 IF), rabbit anti-hSgo1 (generated in-house, 1:2,000 WB), mouse anti-B56α (BD Biosciences 610615, 1:2,000 WB and 1:200 IF), rabbit anti-GFP (generated-in-house, 1:10,000 WB and 1:500 IF), mouse anti-GFP (Roche #11814460001, 1:2,000 WB and 1:200 IF), mouse anti-BubR1 (generated-in-house, 1:1,000 WB), rabbit anti-Kif4α (Bethyl Laboratories A301-074A, 1:3,000 WT), mouse anti-PP2A-C (Millipore clone D6 05-421, 1:1,000 WB and IF), mouse anti-FLAG M2 (Sigma F3165, 1:10,000 WT), human anti-CREST (Antibodies Inc, 1:500 IF), mouse anti-Aurora B (BD Transductions 611083, 1:1,000 IF), rabbit anti-Borealin (gift from Dr. Sally Wheatley), Smc1 (Bethyl Laboratories A300-055A, 1:2,000 WB), Smc3 (Bethyl Laboratories A300-060A, 1:2,000 WB), and GFP-Booster Att0488 (Chromotek gba488-100, 1:1,000 IF).

RNAi oligos used in this study were as follows: B56α (Dharmacon 5525), B56γ (Dharmacon 5527), B56δ (Dharmacon 5528), B56ε (Dharmacon 5529), hSgo1 (Silencer Select siRNA s45600, Thermo Fischer Scientific), and WAPL (Silencer Select siRNA s22948, Thermo Fischer Scientific).

Cloning

Standard cloning methods were used throughout the study. pcDNA5/FRT/TO vector was used unless otherwise stated. B56α variants were generated in our previous study (Hertz et al., 2016). B56α 5A, hSgo1 3A, and hSgo1 4A mutant constructs were synthesized by GeneArt (Thermo Fischer Scientific). BamHI and NotI were used to subclone B56α and hSgo1 constructs with various tags (YFP, FLAG, or TurboID). Full-length hSgo2 was amplified by PCR and inserted in pcDNA5/FRT/TO-YFP vector. For YFP-CenpB-B56α constructs, CenpB domain was amplified by PCR and inserted into pcDNA5/FRT/TO-YFP vector by standard restriction cloning, followed by subcloning of B56α variants into the vector using BamHI/NotI.

Cell culture

HeLa FRT/T-REx cells (gift from S. Taylor) were used throughout the study, unless otherwise stated. Stable cell lines were generated using the T-REx doxycycline Flip-In system (Invitrogen). For synchronization, 2.5 mM thymidine and 200 ng/µl nocodazole were used.

Expression and purification of recombinant hSgo1

BL21 (DE3) Gold E. coli cells expressing hSgo1 FL and truncations (hSgo11-155) were grown at 37°C/200 rpm to an optical density of 1.5 (OD600) and induced overnight at 18°C with 0.35 mM IPTG. Cells were resuspended in lysis buffer containing 20 mM Tris, 500 mM NaCl, 1 mM EDTA and supplemented with complete EDTA-free cocktail tablets (1 tablet/50 ml cells; Roche) and 0.01 mg/ml DNase (Sigma) and 1 mM PMSF. The lysate was sonicated at 60% amplitude for 8 min (2s on, 2s off) and centrifuged at approx. 58,000 x g for 50 min at 4°C, and the protein was batch purified using chitin beads (NEB). Post lysis and high salt chaperone wash, the chitin beads were washed with 3 CV of 20 mM Tris–HCl, 500 mM NaCl, 50 mM DTT and injected at RT overnight. The next day, the protein was eluted with the lysis buffer without DTT. The elutions were analyzed for protein quality on an SDS–PAGE, and the elutions containing hSgo1 were pooled and dialyzed in 20 mM Tris–HCl pH 8, 125 mM NaCl, 4 mM DTT overnight at 4°C. The
next day, the dialyzed sample was loaded onto a HiTrap Q HP (GE Healthcare) anionic exchange column. The excess DNA contamination was separated from hSgo1 by providing a 50% salt gradient over 20CV in an ÄKTA start system (GE Healthcare). The samples containing hSgo1 were pooled and concentrated, and the pure protein was finally obtained by a final size exclusion chromatography step with the column equilibrated with 20 mM Tris-HCl, 200 mM salt and 5 mM DTT (Superdex 200 Increase 10/300, GE Healthcare). PP2A(B56γ) and BubR1316–715 were produced and purified as previously described (Cho & Xu, 2007; Kruse et al, 2013).

Interaction analysis by size exclusion chromatography

The contribution of the PP2A(B56γ) Interaction analysis by size exclusion chromatography (SEC) using a Superdex 200 Increase 10/300 column (GE Healthcare). The column was previously equilibrated with 25 mM HEPES pH 7.5, 200 mM NaCl, 5% glycerol, and 2 mM DTT, and the SEC runs were done with a flow rate of 0.5 ml/min at 4°C. Purified PP2A(B56γ) and BubR1316–715 were mixed in a 1:5 molar ratio, respectively, and incubated for 30 min at 4°C prior to SEC analysis. Fractions containing the PP2A(B56γ)-BubR1316–715 complex were pooled and concentrated using a 30 kDa Vivavis® 500 centrifugal unit. The PP2A(B56γ)-BubR1316–715 complex was then mixed with a five-times molar excess of hSgo11–155, incubated for 30 min at 4°C and analyzed by SEC. Recombinant BubR1316–715 was run separately as a control. SEC fractions were analyzed by SDS–PAGE.

Immunoprecipitation and competition assays

Inducible, stable cell lines expressing indicated YFP-tagged bait were lysed in a low salt lysis buffer (50 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% NP-40, protease- and phosphatase inhibitors), unless otherwise stated. In some experiments, the same lysis buffer with 150 mM NaCl was used. Lysates were immunoprecipitated with GFP-trap beads (ChromoTek) according to the manufacturer’s recommendation. The beads were washed three times with wash buffer (50 mM Tris–HCl pH 7.5, 1 mg/ml BSA, 20% glycerol, and 1 mM DTT) and eluted in 2× sample buffer. For the peptide competition assays, a peptide containing LxxlxE motif (LPRSSLPTIHHEEELSLC) or a control mutant peptide that was unable to bind B56 (LPRSSLPTAHHEEELSLC) was used. For the competition assay with hSgo1 proteins, purified full-length hSgo1 or hSgo11–155 described above were used. The peptides/proteins were incubated with cell lysates 30 min prior to the addition of GFP-trap beads.

IP samples were resolved with 4–12% Bis-Tris gels (Thermo Fischer Scientific) and transferred to PVDF membranes. LI-COR Odyssey imaging system was used for visualization, and signals were quantified using Image Studio software (LI-COR).

Chromosome spreads

Indicated cells were seeded in a 6-well plate, RNAi knockdown was performed, and cells were synchronized using thymidine followed by nocodazole treatment. 48 h after RNAi transfection, mitotic cells were collected by shake-off. After hypotonic treatment with KCl, cells were spun onto microscopy slides with a Shandon Cytospin centrifuge (Thermo Fischer), fixed with 4% paraformaldehyde, and then immunocytochemistry was performed. Representative images were taken with a 100× objective on a DeltaVision fluorescent microscope under the same condition. The distance between the two peak intensities of CREST or YFP-CB was measured for 5 kinetochore pairs using ImageJ and averaged for a single cell. At least 45 cells from minimum of 3 independent experiments were analyzed.

Live cell imaging

Live cell imaging was performed using a DeltaVision fluorescent microscope. Cells were seeded in a 6-well ibidi dish (ibidi) a day before filming, the media was changed to Leibovitz’s L-15 (Life Technologies) immediately before the filming. Indicated channels were recorded at 7- to 8-min intervals, and data were analyzed using SoftWoRx (GE Healthcare). The time from nuclear envelope breakdown (NEBD) to anaphase was measured in single cells.

Immunofluorescence

Cells were seeded in a 6-well plate and treated as indicated. A day before fixing, cells were transferred in an 8-well ibidi dish. Cells were fixed in 4% PFA for 10 min at room temperature, and standard immunocytochemical methods were used. Fluorescent microscopy was performed using a DeltaVision fluorescent microscope. To ensure quantitative image quality, the imaging parameters were kept constant for a given experiment.

LacO-LacI assay

hSgo1-LacI-GFP constructs were cloned in a pAceBac1-CMV background (Hadders et al, 2020). Bacmids were generated using the Bac-to-Bac system in conjunction with EMBacY cells (Berger et al, 2004; Bieniossek et al, 2012). Baculovirus was then produced by transfection of bacmids into Sf9 cells using standard procedures. P2 viruses were harvested after 5 days, filtered (0.2 µm), and stored at 4°C till use. The lacO-LacI assays were performed as previously described in (Hadders et al, 2020). Briefly, U-2 OS LacO Haspin CM (CRISPR Mutant) cells were seeded on glass coverslips followed directly by addition of recombinant baculovirus encoding the hSgo1-LacI-GFP variants or LacI-GFP as a control. After 3–4 h, S-trityl-L-cysteine (STLC; 20 µM) was added overnight to block cells in mitosis. The next morning cells were fixed in 4% PFA (v/v) in PHEM buffer (60 mM HEPES KOH, 20 mM PIPES KOH, pH 6.8, 5 mM EGTA, and 1 mM MgCl2) for 10–15 min followed by permeabilization in ice-cold methanol for a minimum of 1 h.

For immunofluorescence, cells were washed with PBS with 0.01% Tween-20 (PBST), followed by blocking with 3% BSA in PBST for ≥ 30 min. Cells were then incubated with primary antibodies diluted in 3% BSA in PBST for 2 h followed by washing three times, again with PBST. Cells were then incubated with secondary antibodies, GFP-Booster, and DAPI (500 ng/µl) in PBST + 3% BSA for 1 h. Coverslips were washed again, twice with PBST, followed by a final wash with PBS, before mounting onto glass slides using Prolong Diamond Antifade Mountant (Thermo Fisher Scientific).

Fluorescence images were acquired on a DeltaVision imaging system (GE Healthcare), upgraded with a seven-color InsightSSi Module & TruLight Illumination System Module using a UPlanSApo
TurboID proximity labeling and label-free LC-MS/MS analysis

TurboID proximity labeling assay was performed as described previously (Branon et al., 2018). Doxycycline-inducible TurboID-hSgo1 WT, 3A, and 4A stable cell lines were generated in HeLa cells, and 50 µM biotin was added to the media 1 h prior to the harvest. Cells were collected by mitotic shake-off, lysed with RIPA buffer, and immunoprecipitation was performed using high-capacity Streptavidin agarose beads (Thermo Scientific). The beads were washed once with RIPA buffer, twice with 2% SDS, then again once with RIPA buffer and eluted in 2× sample buffer. For YFP-Sgo1 WT, 3A, 4A analysis, YFP purifications were performed as described above. The elutes were then run on SDS gels and sliced for MS analysis. Pull-downs were analyzed on a Q-Exactive Plus quadrupole, Fusion Orbitrap, or Fusion Orbitrap Lumos mass spectrometer (Thermo Scientific) equipped with Easy-nLC 1000 or Easy-nLC 1200 (Thermo Scientific) and nanospray source (Thermo Scientific). Peptides were resuspended in 5% methanol / 1% formic acid and analyzed as previously described (Kruse et al., 2020). Raw data were searched using COMET (release version 4.0.1.0) in high-resolution mode (Eng et al., 2013) against a target-decoy (reversed) (Elias & Gygi, 2007) version of the human proteome sequence database (UniProt; downloaded 2/2020, 40704 entries of forward and reverse protein sequences) with a precursor mass tolerance of ± 1 Da and a fragment ion mass tolerance of ± 0.02 Da, and requiring fully tryptic peptides (K, R; not preceding P) with up to three mis-cleavages.

Static modifications included carbamidomethylcysteine and variable modifications included oxidized methionine, and phosphorylated-edgeserines, threonines, and tyrosines. Searches were filtered using orthogonal measures including mass measurement accuracy (± 3 ppm), Xcorr for charges from +2 through +4, and ΔCn targeting a < 1% FDR at the peptide level. Quantification of LC-MS/MS spectra was performed using BasicQuan or MassChroQ (Valot et al., 2011) and the iBAQ method (Schwanhausser et al., 2011). Missing values were imputed from a normal distribution in Perseus to enable statistical analysis and visualization by volcano plot (Tyanova et al., 2016). For phosphorylation site analysis, retention time alignment, and smart quantification was performed. For further analysis, proteins had to be identified in the hSgo1 +biotin or hSgo1 WT samples with more than 1 total peptide and quantified in 2 or more replicates. For further analysis, phosphorylation sites had to identify in at least 5 out 9 samples. hSgo1 amounts were normalized to be even across WT, 3A, and 4A samples. Statistical analysis was carried out in Perseus by two-tailed Student’s t-test.
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