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Pericentromeric regulation of chromosome segregation in mitosis

by

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DISSERTATION

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Pericentromeric regulation of chromosome segregation in mitosis

Heather D. Eshleman

ABSTRACT

Sister chromatid bi-orientation on the mitotic spindle is essential for proper chromosome segregation. Defects in bi-orientation are sensed and corrected to prevent chromosome mis-segregation and aneuploidy. This response depends on the adaptor protein Sgo1, which associates with pericentromeric chromatin in mitosis. The mechanisms underlying Sgo1 function and regulation are unclear. Here, we show that Sgo1 is an anaphase-promoting complex/cyclosome (APC/C) substrate in budding yeast (*Saccharomyces cerevisiae*), and that its mitotic destruction depends on an unusual D-box-related sequence motif near its C-terminus. We find that the removal of Sgo1 from chromosomes before anaphase is not dependent on its destruction, but rather on other mechanisms responsive to tension between sister chromatids. Additionally, we find that Sgo1 recruits the protein phosphatase 2A (PP2A) isoform containing Rts1 to the pericentromeric region prior to bi-orientation, and that artificial recruitment of Rts1 to this region of a single chromosome is sufficient to perform the function of Sgo1 on that chromosome. We conclude that in early mitosis, Sgo1 associates transiently with pericentromeric chromatin to promote bi-orientation, in large part by recruiting the Rts1 isoform of PP2A.
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Chapter 1

Introduction
The cell cycle and mitosis

Cell division is essential for the propagation of life, from single-celled organism such as yeast and bacteria to multicellular organisms, including humans. Central to the process of the cell division cycle is the accurate segregation of a duplicated genome. Significant errors in these processes can be disastrous, both for single cells and multicellular organisms. Potential consequences include cell death, cancer, genetic disorders, and infertility.

To ensure accurate chromosome segregation in eukaryotes, there are four stages to the eukaryotic cell cycle, with critical stages governed by checkpoints (Morgan, 2007). During S phase, chromosomes are duplicated into a pair of sister chromatids and are held together by a complex called cohesin. Following a gap phase (G2), cells enter mitosis, which prepares the duplicated chromosomes for separation and subsequent segregation by the mitotic spindle into two daughter cells.

Sister chromatid bi-orientation

It is essential that each daughter cell receives an equal copy of the duplicated chromosomes. To achieve this, each sister chromatid of a pair is attached to opposite poles of the mitotic spindle, a state referred to as bi-orientation (Foley and Kapoor, 2013). Individual pairs of sister chromatids are held in this attachment state until all pairs of sister chromatids have achieved bi-orientation. Driving cells through the cell cycle and into this state, called metaphase, is the activity of the cyclin-dependent kinase (CDK) (Morgan, 2007). The binding of various
cyclins directs CDK toward specific substrates, thus phosphorylating and modifying their functions at different stages of the cell cycle.

Once all pairs of sister chromatids have bi-oriented on the mitotic spindle, the spindle-assembly checkpoint (SAC) is satisfied and the anaphase-promoting complex (APC) is released from inhibition (Sullivan and Morgan, 2007). The APC is a complex E3 ubiquitin ligase that targets multiple mitotic regulators for ubiquitination and destruction by the proteasome. The essential substrates of the APC are the cyclins, destruction of which inhibits CDK activity, and securin (Thornton and Toczyski, 2003). Destruction of securin releases the protease separase to cleave the cohesin linking sister chromatids, releasing them to be pulled by the mitotic spindle into two daughter cells. It is essential that all pairs of sister chromatids are bi-oriented on the mitotic spindle before anaphase begins. Attachment errors such as syntely (both sister chromatids attached to the same pole) or monotely (attachment of only a single sister chromatid) will result in chromosome missegregation if left uncorrected (Musacchio and Salmon, 2007).

Attachment to the mitotic spindle is regulated primarily at the level of the kinetochore, a complex protein structure linked to each sister chromatid through specialized centromeric histones (Foley and Kapoor, 2013). Interactions between outer kinetochores proteins, particularly the Ndc80 complex and KNL1, and microtubules link the sister chromatids to the mitotic spindle. When both pairs of sister chromatids are attached to opposite spindle poles, the forces of the spindle stretch the pair of kinetochores in opposite directions, resulting in tension across the kinetochores and between sister chromatids.
When there are errors in sister chromatid attachment to the spindle, such as in the cases of syntely or monotely, tension between the sister chromatids is absent. In this state, the kinase Aurora B, a key member of the chromosomal passenger complex (CPC), is active towards substrates mediating the kinetochore-microtubule attachment, particularly Ndc80 and KNL1 (Kelly and Funabiki, 2009; Lampson and Cheeseman, 2011). This releases any existing attachment, allowing microtubules to re-attempt attachment. Additionally, lack of attachment of any sister chromatids signals to the spindle assembly checkpoint to inhibit the APC until all attachments have been stabilized. Once correct attachment is achieved, Aurora B is stretched away from its outer kinetochore substrates and the attachment is stabilized.

Additionally, the kinetochore-localized phosphatases PP2A and PP1 are required to modulate the phosphorylation levels of kinetochores to tune the responsiveness to destabilization signals (Funabiki and Wynne, 2013). Both phosphatases act on numerous substrates to regulate both mitosis and other cellular processes. While some substrates and phosphorylation sites targeted by these kinases have been identified, there are likely many more yet to be discovered. PP1 has been implicated in silencing the spindle checkpoint to allow for anaphase onset, and in reversing Aurora B-dependent phosphorylations. PP2A is composed of three subunits: a scaffold subunit (A), a regulatory subunit (B), and a catalytic subunit (C). In mitosis, the primary regulatory subunits are B55 and B56 (Cdc55 and Rts1 in the budding yeast *Saccharomyces cerevisiae*, respectively). Both regulatory subunits have functions in regulating mitosis, but the B56 subunit has specifically been implicated in the regulation of microtubule attachments (Foley et al., 2011; Suijkerbuijk et al., 2012). In these ways, Aurora B, PP2A, and PP1 create a dynamic system that monitors and adjusts attachment of chromosomes to the mitotic spindle both spatially...
and temporally, and only allows anaphase to occur when all pairs of sister chromatids are bi-oriented.

While Aurora B appears to be the primary kinase responsible for responding to errors in attachments, there are numerous other kinases that drive the cell cycle and make significant contributions to the function and regulation of the kinetochore and its attachment to microtubules (Funabiki and Wynne, 2013). These include Mps1, Polo, Bub1, and CDK. How exactly these kinases, along with the counter-activity of phosphatases, fine-tune the attachment of the chromosomes to the mitotic spindle remains poorly understood. It is important to sort out the functions of both specific phosphorylation events and the integrated effects of these events to determine how these work together to create a dynamic system that is responsive to attachment and ensure an accurate anaphase.

Shugoshins in mitosis

The conserved family of shugoshin proteins plays an essential role in promoting sister chromatid bi-orientation. Shugoshin is localized primarily at the centromere, and its localization is ensured by multiple inputs, including the activity of the Mps1 and Bub1 kinases (Fernius and Hardwick, 2007; Kawashima et al., 2010; Liu et al., 2012; London et al., 2012; Storchová et al., 2011; Tang et al., 2004; Yamagishi et al., 2010; 2008). Most organisms examined thus far express two isoforms of shugoshin, typically called Sgo1 and Sgo2 (Kitajima et al., 2004; Rabitsch et al., 2004), to complete two distinct functions: ensuring sister chromatid bi-orientation
and protecting cohesin from premature removal. Shugoshin appears to act as a scaffold to coordinate these functions.

**Shugoshins and cohesin protection**

Shugoshin was originally discovered as a protein essential for meiosis, a specialized process of cell division that creates four haploid nuclei from a single diploid nucleus (Morgan, 2007). To achieve this, one round of chromosome replication is followed by two rounds of chromosome segregation. In meiosis I, homologous chromosomes are segregated on the mitotic spindle. Cohesin is then cleaved in the second round of meiotic division, meiosis II, to finally allow the sister chromatids to be segregated into daughter cells. Cells lacking shugoshin randomly segregate their chromosomes at meiosis II, due to premature cleavage of cohesin in meiosis I (Katis et al., 2004; Kerrebrock et al., 1992; Kitajima et al., 2004; Marston et al., 2004). Further experiments showed that shugoshin recruits PP2A with the B56/Rts1 regulatory subunit to the pericentromere to dephosphorylate the Scc1 subunit of cohesin, thus preventing it from being cleaved by separase during meiosis I (Brar et al., 2006; Ishiguro et al., 2010; Katis et al., 2010; Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006; Tanno et al., 2010). At meiosis II, shugoshin is removed, and cohesin is again subject to cleavage by separase, allowing the sister chromatids to finally separate.

In metazoans, cohesin is also removed prior to anaphase and sister chromatid separation in a separase-independent process called the ‘prophase pathway’ (Waizenegger et al., 2000). This process depends on phosphorylation of sororin and the cohesin subunit SA2, which leaves
cohesin susceptible to destabilization by WAPL. As in meiosis, it is essential that cohesin surrounding the centromere remain intact to allow sister chromatids to remain attached until anaphase. To this end, Sgo1 recruits PP2A-B56 to dephosphorylate sororin and SA2, making it resistant to removal in prophase (Liu et al., 2012; McGuinness et al., 2005). This centromeric pool of cohesin is then cleaved in anaphase, releasing sister chromatids.

*Shugoshins and bi-orientation*

In addition to their function in protecting cohesin, shugoshins are also essential for ensuring sister chromatid bi-orientation (Huang et al., 2007; Indjeian et al., 2005; Kawashima et al., 2007; Kitajima et al., 2004; Vanoosthuyse et al., 2007). Sgo1 is required in yeast for sensing lack of tension between sister chromatids and signaling for a spindle checkpoint response. One important way shugoshins regulate this is through recruitment of the chromosomal passenger complex (CPC), which includes the Aurora B kinase (Peplowska et al., 2014; Tsukahara et al., 2010; Vanoosthuyse et al., 2007; Verzijlbergen et al., 2014; Yamagishi et al., 2010). However, how shugoshins sense lack of tension, how they relay this information to Aurora B, and whether other functions of shugoshin contribute to this response is unclear.

*Division of function among shugoshin homologs*

Most eukaryotic organisms studied thus far, including fission yeast, mice, and humans, express two homologs of shugoshin. The bi-orientation and cohesin-protection functions of shugoshin are divided between these homologs (Gutiérrez-Caballero et al., 2012). In the case of
fission yeast, these functions appear to be distinct: Sgo1 protects meiotic cohesin by recruiting PP2A, and Sgo2 is required for recruitment of the CPC (Kawashima et al., 2007). However, the specific functions of shugoshin homologs in other organisms are less distinct. In humans, for example, both hSgo1 and hSgo2 contribute to localization of Aurora B and PP2A (Gutiérrez-Caballero et al., 2012; Tanno et al., 2010; Tsukahara et al., 2010). Thus, dissecting the specific dependencies of bi-orientation and sister chromatid cohesion on shugoshin, Aurora B, and PP2A has been difficult.

The budding yeast Saccharomyces cerevisiae expresses a single shugoshin, Sgo1, which both recruits Aurora B in mitosis and PP2A-Rts1 in meiosis. It is not essential for unperturbed mitosis, but becomes crucial for accurate chromosome segregation when there are defects in spindle attachment at the metaphase-to-anaphase transition. Using this simplified budding yeast system has allowed us to gain insights into the significance of its interaction with PP2A in mitosis, and into the regulation of Sgo1 itself.
References


to sense lack of tension on mitotic chromosomes. Science 307, 130–133.


Peplowska, K., Wallek, A.U., and Storchová, Z. (2014). Sgo1 Regulates Both Condensin and
Ipl1/Aurora B to Promote Chromosome Biorientation. PLoS Genet 10, e1004411.


Chapter 2

Sgo1 recruits PP2A to chromosomes to ensure sister chromatid bi-orientation during mitosis
Accurate duplication and segregation of chromosomes during cell division is essential for faithful propagation of the genome, and errors in these processes can lead to cancer, genetic disorders, or infertility. To ensure accurate chromosome segregation, sister chromatid pairs are held together by cohesin and bi-oriented on the mitotic spindle, with each chromatid attached to opposite spindle poles. Following bi-orientation, sister chromatid separation and segregation are triggered by the anaphase-promoting complex or cyclosome (APC/C), a ubiquitin-protein ligase or E3 that initiates anaphase by promoting the proteasomal destruction of securin, cyclins, and other proteins (Foley and Kapoor, 2013; Morgan, 2007; Sullivan and Morgan, 2007).

The shugoshins are a conserved family of centromeric proteins that are necessary for proper bi-orientation and ultimately for accurate chromosome segregation. Most eukaryotes express two shugoshin family members, Sgo1 and Sgo2, whereas the budding yeast Saccharomyces cerevisiae has a single shugoshin protein, Sgo1 (Kitajima et al., 2004; Rabitsch et al., 2004). These proteins have functions in the control of both sister chromatid cohesion and bi-orientation. Shugoshin was first identified on the basis of its function during anaphase I of meiosis, when it is required to protect cohesin from cleavage by separase, thereby maintaining sister chromatid cohesion when the homologs are segregated (Katis et al., 2004; Kerrebrock et al., 1992; Kitajima et al., 2004; Marston et al., 2004). In metazoans, shugoshin also protects centromeric cohesin during mitosis from separase-independent removal by the ‘prophase pathway,’ which allows sister chromatid arms to condense and resolve while maintaining cohesion between the centromeric regions (Liu et al., 2012). Thus, depletion of shugoshin in
metazoans results in premature sister chromatid separation and an arrest in metaphase that results from activation of the spindle assembly checkpoint (Kitajima et al., 2005; McGuinness et al., 2005; Salic et al., 2004; Tang et al., 2004).

Shugoshins protect centromeric cohesin by recruiting protein phosphatase 2A (PP2A), which removes phosphates from cohesin and thereby inhibits its cleavage (Brar et al., 2006; Ishiguro et al., 2010; Katis et al., 2010; Kitajima et al., 2006; Riedel et al., 2006; Tang et al., 2006; Tanno et al., 2010). Similarly, during prophase, shugoshin recruits PP2A to dephosphorylate sororin, allowing it to block cohesin removal by Wapl (Liu et al., 2012; Nishiyama et al., 2010). PP2A is normally composed of three subunits: the scaffold (A), the regulatory subunit (B), and the catalytic subunit (C). Cells contain multiple distinct B subunits, and shugoshin has thus far been found associated with PP2A complexes containing the B’ subunit (B56 in humans, Rts1 in budding yeast). Structural analysis indicates that human Sgo1 interacts with PP2A through contacts between the Sgo1 N-terminal coiled-coil domain and both the B’ and C subunits of PP2A (Xu et al., 2009).

Shugoshins are also an important part of the kinetochore-based machinery that detects and corrects erroneous microtubule attachments, ensuring that sister chromatids achieve bi-orientation before anaphase onset (Huang et al., 2007; Indjeian et al., 2005; Kawashima et al., 2007; Vanoosthuyse et al., 2007). Deletion of Sgo1 in budding yeast renders cells unable to respond to a lack of tension between sister chromatids, a hallmark of mis-oriented chromatids (Indjeian et al., 2005; Kitajima et al., 2004; Rabitsch et al., 2004). In yeast and humans, shugoshin recruits the chromosomal passenger complex (CPC) containing the protein kinase
Aurora B (Peplowska et al., 2014; Tsukahara et al., 2010; Vanoosthuyse et al., 2007; Verzijlbergen et al., 2014; Yamagishi et al., 2010). In the absence of kinetochore tension, Aurora B phosphorylates multiple substrates at the kinetochore, thereby destabilizing microtubule attachments and signaling to the spindle assembly checkpoint to delay anaphase until the attachments have been corrected (Lampson and Cheeseman, 2011; Musacchio and Salmon, 2007). Aurora B is silenced, and attachments are thereby stabilized, when sister kinetochores are properly attached to microtubules from opposite spindle poles. The ability of shugoshin to recruit the CPC provides a logical explanation for its function in bi-orientation, but it is unclear whether budding yeast Sgo1 works through direct recruitment of the CPC in mitosis (Katis et al., 2004; Kerrebrock et al., 1992; Kitajima et al., 2004; Marston et al., 2004; Storchová et al., 2011; Verzijlbergen et al., 2014) or whether the bi-orientation function of Sgo1 depends in part on its association with PP2A. There is evidence both for and against a role for PP2A-Rts1 in budding yeast bi-orientation, and thus the importance of this phosphatase in bi-orientation remains unresolved (Nerusheva et al., 2014; Peplowska et al., 2014; Verzijlbergen et al., 2014).

Shugoshin function is regulated in part by its recruitment to pericentromeric regions, which is governed by kinetochore kinases. Phosphorylation of the outer kinetochore protein Spc105 by Mps1 recruits Bub1 to kinetochores, which in turn phosphorylates local histone H2A to create a binding site for shugoshin (Fernius and Hardwick, 2007; Kawashima et al., 2010; Liu et al., 2012; London et al., 2012; Tang et al., 2004). In fission yeast and humans, shugoshin also binds pericentromeric heterochromatin proteins to further refine its localization (Brar et al., 2006; Ishiguro et al., 2010; Katis et al., 2010; Kitajima et al., 2006; Riedel et al., 2006; Tang et
Shugoshin disappears from the centromere in mitosis and its total levels drop sharply, suggesting that its destruction may be important for its inactivation (Huang et al., 2007; Indjeian et al., 2005; Katis et al., 2004; Kawashima et al., 2007; Kitajima et al., 2004; Lianga et al., 2013; Marston et al., 2004; Vanoosthuyse et al., 2007). In human cells, Sgo1 has been shown to be an APC/C substrate, but its destruction is not necessary for anaphase progression, indicating that there may be other mechanisms for its inactivation (Karamysheva et al., 2009; Liu et al., 2012; Nishiyama et al., 2010). Recent work in budding yeast suggests that the recruitment of PP2A by Sgo1 could be contributing to its own release from centromeres by reversing phosphorylation of histones or other substrates in response to tension between sister chromatids (Nerusheva et al., 2014; Xu et al., 2009).

In this study, we examined the regulation and function of Sgo1 in the budding yeast Saccharomyces cerevisiae. We find that Sgo1 is an APC/C substrate in budding yeast, but that an additional mechanism ensures that Sgo1 is removed from centromeres once sister chromatids have properly attached to the spindle. We also find that Sgo1 recruits Rts1 to centromeres and that recruitment of Rts1 is sufficient for bi-orientation, supporting a function for PP2A-Rts1 in sensing and responding to tension between sister chromatids.
RESULTS

Sgo1 is an APC/C substrate in budding yeast

Sgo1 levels oscillate during the budding yeast cell cycle, accumulating at metaphase and declining near anaphase onset (Huang et al., 2007; Indjeian et al., 2005; Katis et al., 2004; Kawashima et al., 2007; Kitajima et al., 2004; Lianga et al., 2013; Marston et al., 2004; Vanoosthuyse et al., 2007). This pattern of protein levels is similar to that of known substrates of the APC/C, and vertebrate shugoshin is known to be a substrate for the APC/C (He et al., 2013; Karamysheva et al., 2009; Salic et al., 2004). Deletion of the APC/C in budding yeast results in Sgo1 stabilization (Lianga et al., 2013), suggesting that Sgo1 could also be a target of the APC/C in yeast. To test this possibility directly, we translated radiolabeled budding yeast Sgo1 in vitro and incubated it with purified APC/C components and ubiquitin. Sgo1 was extensively modified, suggesting that Sgo1 is ubiquitinated by the APC/C (Fig. 1A).

To assess the importance of Sgo1 ubiquitination at anaphase, we sought a mutant that would be resistant to ubiquitination by the APC/C. Ubiquitination of APC/C substrates typically depends on short sequence motifs or degrons, such as the “D-box” or “KEN box”, which interact with specific binding sites on the APC/C (Primorac and Musacchio, 2013). APC/C assays with a series of Sgo1 fragments suggested that Sgo1 ubiquitination requires amino acids 495-498 (Fig. 1B, C). Single point mutations in this region had little effect on Sgo1 ubiquitination, but short deletions of multiple amino acids revealed that removal of amino acids 494-498 (NKSEN) greatly reduced its ubiquitination by the APC/C in vitro (Fig. 1C). These and surrounding
residues are reminiscent of the human Sgo1 degrons and the high-affinity D-box of yeast Hsl1 (Burton and Solomon, 2001; Karamysheva et al., 2009) (Fig. 1D). Hereafter we refer to the Sgo1 mutant lacking the NKSEN sequence as the Sgo1-Δdb mutant.

The studies in Fig. 1 were performed using Cdh1 as APC/C activator, as this activator is more stable and reliable in vitro. In further studies, we also found that Sgo1 can be ubiquitinated in vitro, in a destruction-box-dependent manner, when Cdc20 is used as the activator (supplementary material Fig. S1).

We constructed a yeast strain expressing Sgo1-Δdb at the endogenous locus, and measured Sgo1 levels by western blotting of lysates of cells released from an alpha-factor-mediated G1 arrest. While wild-type Sgo1 levels rose after G1 and fell at anaphase, Sgo1-Δdb levels remained high throughout the cell cycle, indicating that it is resistant to the APC/C in vivo (Fig. 2A). The sgo1-Δdb strain did not display detectable defects in proliferation rate or sensitivity to the spindle poison benomyl, suggesting that Sgo1 destruction during mitosis is not required for normal cell function (Fig. 2B).

Wild-type Sgo1 levels declined earlier than those of the mitotic cyclin Clb2 (Fig. 2A), consistent with previous studies showing that Sgo1 levels decline at the same time as those of securin (Lianga et al., 2013). The timing of Sgo1 disappearance, together with evidence that APC/C^{Cdc20} ubiquitinates Sgo1 in vitro (supplementary material Fig. S1), suggests that Sgo1 is a target of the Cdc20-activated form of the APC/C.
To assess the effects of APC/C-dependent destruction on Sgo1 localization dynamics, we tagged Sgo1 with GFP at the endogenous SGO1 locus and analyzed its localization and levels by spinning-disk confocal fluorescence microscopy. We also tagged Spc42, a spindle pole body (SPB) component, with mCherry to mark the mitotic spindle poles. The initial separation of duplicated SPBs provides a useful indication of mitotic entry, whereas the initiation of spindle elongation marks the onset of anaphase (Lu et al., 2014; Pearson et al., 2001; Straight et al., 1997; Yaakov et al., 2012). Wild-type Sgo1-GFP appeared as a diffuse single or bi-lobed dot, first appearing at about the same time that the duplicated SPBs separated at the beginning of mitosis. Sgo1 remained localized within the region of the mitotic spindle, and then disappeared just before the spindle began to elongate at anaphase onset (Fig. 3A).

The fluorescence intensity of Sgo1-Δdb-GFP was significantly higher than that of wild-type Sgo1-GFP, presumably due to higher protein levels (Fig. 2A, 3A). Surprisingly, however, the stabilized Sgo1 mutant accumulated with normal timing as a diffuse focus between the spindle poles and then disappeared from the spindle at about the same time as the wild-type protein, while remaining concentrated in the nucleus. Thus, destruction via the APC/C is not required for Sgo1 removal from centromeres, indicating that other mechanisms promote Sgo1 delocalization.

Pericentromeric Sgo1 is removed when the spindle assembly checkpoint is satisfied

We carried out a more detailed analysis of the timing of Sgo1 disappearance, using spinning-disk confocal video microscopy and quantification of the intensity of the Sgo1-GFP
focus in single cells. We compared the timing and rate of Sgo1-GFP dot disappearance with the
timing of destruction of GFP-tagged Clb5 and securin (Pds1), two major APC/C substrates that
are destroyed in sequence during mitosis (Lu et al., 2014). We measured the intensity of the
brightest 25-pixel square in each of the strains, representing the highest concentration of GFP-
tagged protein.

In the Sgo1-GFP and Sgo1-Δdb-GFP strains, fluorescence was concentrated primarily in
the pericentromeric focus, and measurement of the brightest 25-pixel square enabled us to follow
pericentromeric Sgo1 levels over time. We found that both Sgo1-GFP and Sgo1-Δdb-GFP began
to disappear from the centromere 8-10 min before the onset of spindle elongation (Fig. 3B-D).
These results further indicate that destruction of Sgo1 via the APC/C does not make a significant
contribution to the timing of Sgo1 delocalization from centromeres prior to anaphase onset.

As expected, the fluorescence intensity of Sgo1-Δdb-GFP was higher than that of the
wild-type protein, and declined to a higher baseline in anaphase (supplementary material Fig.
S2). The intensity of the wild-type protein declined to very low levels as Sgo1 dissociated from
centromeres. These results, together with measurements of total Sgo1 levels by western blotting
(Fig. 2A), suggest that APC/C-mediated Sgo1 destruction occurs at about the same time or soon
after its dissociation from centromeres.

Further insights were gained by comparing the timing of Sgo1-GFP dot disappearance
with the timing of Clb5-GFP and securin-GFP destruction (Fig. 3B-D). For these proteins,
fluorescence intensity is distributed throughout the nucleus, and measurement of the brightest
25-pixel square provides the rate and timing of Clb5 or securin destruction (Lu et al., 2014). Our recent work indicates that Clb5 is destroyed immediately following spindle assembly checkpoint inactivation, while securin destruction begins about 6 min later, just before the onset of spindle elongation and anaphase (Lu et al., 2014). Both Sgo1-GFP and Sgo1-Δdb-GFP began to disappear from the centromere at about the same time as the beginning of Clb5 destruction; dot intensities then declined at a slower rate than the rate of Clb5 destruction (Fig. 3B, C).

The onset of Clb5 destruction is determined by the spindle assembly checkpoint: defects in the checkpoint result in premature Clb5 destruction but have no effect on the timing of securin destruction (Lu et al., 2014). The coincidence of Sgo1 delocalization and Clb5 destruction therefore suggested that Sgo1 delocalization occurs when sister chromatids achieve bi-orientation and the spindle assembly checkpoint is satisfied. To address this possibility further, we arrested cells in metaphase by shutting off expression of CDC20, which encodes the mitotic activator of the APC/C (Fig. 4). In this arrest, sister chromatids are bi-oriented and the spindle checkpoint is satisfied. We observed very few Sgo1-Δdb-GFP foci in this arrest, indicating that Sgo1 is not localized at centromeres when sister chromatids have bi-oriented. However, when we treated the arrested cells with nocodazole to promote spindle disassembly, Sgo1-Δdb-GFP formed chromosomal foci in most cells. Thus, as suggested by other recent studies, our results argue that Sgo1 is localized to pericentromeres specifically when sister chromatids are improperly attached to the spindle (Nerusheva et al., 2014).

**Rts1 localization requires Sgo1**
Budding yeast Sgo1 is known to interact with PP2A-Rts1 in meiotic cells (Riedel et al., 2006). In mitotic cells, Rts1 is found in foci that co-localize with kinetochores, raising the possibility that Sgo1 also recruits PP2A-Rts1 to pericentromeric regions in mitotic cells (Gentry and Hallberg, 2002; Peplowska et al., 2014). To test this possibility, we used fluorescence microscopy to determine whether Sgo1 is required for Rts1 localization in mitosis. We tagged Rts1 with GFP at the endogenous locus in a strain containing either wild-type or the stabilized SGO1-Δdb mutant. Interestingly, fluorescence intensity of Rts1-GFP was increased in the SGO1-Δdb mutant. Microscopy of this strain revealed that localization of Rts1-GFP is similar to that of Sgo1-Δdb-GFP: it appeared as a diffuse dot in the vicinity of the mitotic spindle during early mitosis, and was absent in anaphase cells (Fig. 5A) (Gentry and Hallberg, 2002).

Structural analysis has identified residues in the human Sgo1 coiled-coil domain that interact with the catalytic and regulatory subunits of PP2A-B56 (Xu et al., 2009). Mutations in residues of human Sgo1 that contact the catalytic subunit of PP2A abolish the interaction between Sgo1 and PP2A-B56. Mutating the analogous residues in budding yeast Sgo1 (sgo1-3A, Fig. 5B) causes defects both in meiotic cohesin protection and mitotic spindle assembly checkpoint activation (Xu et al., 2009). We found that the sgo1-3A mutant was also defective in recruiting Rts1 to the centromere in mitosis (Fig. 5A), consistent with a role for Sgo1 in recruitment of Rts1 to the centromere.

We next tested whether Sgo1 and Rts1 physically interact. We tagged Rts1 with an HA epitope in strains containing either Sgo1-9myc or Sgo1-3A-9myc. We immunoprecipitated the myc-tagged Sgo1 proteins and western blotted the precipitates with an anti-HA antibody. We
found that Sgo1-9myc co-immunoprecipitated with Rts1-6HA, and that this interaction was abolished in the sgo1-3A mutant (Fig. 5C). Together with the fluorescence microscopy data, these results suggest that Sgo1 directly recruits Rts1 to the centromere, consistent with recent studies (Peplowska et al., 2014; Verzijlbergen et al., 2014).

**The Sgo1-PP2A interaction is required for bi-orientation**

Sgo1 is known to be required for bi-orientation in cells released from a spindle assembly checkpoint arrest, but the mechanism underlying this function is unclear (Indjeian et al., 2005; Storchová et al., 2011). The sgo1-3A mutant is defective in both its response to spindle damage and in recruitment of Rts1, and so we tested whether Rts1 is required for Sgo1 to promote bi-orientation. To measure bi-orientation, we used an established assay with a strain in which the centromere of Chromosome V is marked with a fluorescent dot by integration of a LacO array and expression of GFP-tagged LacI (Biggins et al., 1999; Indjeian et al., 2005; Riedel et al., 2006; Storchová et al., 2011; Straight et al., 1997). We treated cells with benomyl to promote spindle disassembly and trigger a spindle assembly checkpoint arrest in mitosis. We then released the cells from benomyl and analyzed the fidelity of chromosome segregation 60 min after release (Fig. 6). Under these conditions, sgo1Δ cells cannot detect attachment errors that are made when the spindle reforms, and therefore the sister chromatids mis-orient and segregate randomly, resulting in co-segregation of the GFP dots in approximately 50% of the cells (Fig. 6A). We found that sgo1-3A cells also co-segregated the GFP dots approximately 50% of the time, suggesting that Sgo1 promotes bi-orientation through its interaction with PP2A-Rts1. Similar results have recently been reported by others (Peplowska et al., 2014). Interestingly,
deletion of Rts1 resulted in only a minor segregation defect, suggesting that Rts1 is not essential for bi-orientation. However, given that Sgo1 is known to interact directly with the PP2A catalytic subunit through the residues mutated in the Sgo1-3A mutant (Fig. 5B) (Xu et al., 2009), we suspect that the recruitment of PP2A, with or without the Rts1 subunit, promotes bi-orientation.

**Recruitment of Rts1 is sufficient for bi-orientation**

To further address the importance of PP2A-Rts1 in Sgo1 function in bi-orientation, we determined whether Rts1 recruitment to pericentromeres is sufficient to promote bi-orientation in the absence of Sgo1. We fused Rts1 to the C-terminus of GFP-LacI (GFP-LacI-Rts1) in the strain carrying a LacO array near the centromere of Chromosome V (Fig. 6B). Wild-type cells expressing GFP-LacI-Rts1 properly bi-oriented Chromosome V after release from benomyl. Most importantly, GFP-LacI-Rts1 rescued the bi-orientation defect of *sgo1Δ* cells, indicating that recruitment of Rts1 to the centromere is sufficient to restore Sgo1 function.

We also plated cells 90 min after benomyl release to assess their viability (Fig. 6C). As expected, *sgo1Δ* cells were unable to recover from the arrest, presumably due to massive chromosome mis-segregation. The growth defect of *sgo1Δ* cells was only slightly rescued by expressing GFP-LacI-Rts1, suggesting that the effect of recruiting Rts1 to the centromere is specific to Chromosome V, and that Rts1 promotes bi-orientation primarily on the chromosome to which it is recruited. These results also indicate that constitutive recruitment of Rts1 to the pericentromere, even after Sgo1 would normally be removed, does not result in significant growth defects.
As a control, we expressed GFP-LacI-Rts1 in a strain with Chromosome V marked with a TetR-GFP/TetO system, which does not recruit GFP-LacI-Rts1 (Fig. 6D). In this case, GFP-LacI-Rts1 did not rescue the bi-orientation defect of the sgo1Δ strain. Our results, like similar results published recently (Peplowska et al., 2014), indicate that the primary direct function of Sgo1 in bi-orientation is to recruit PP2A to the centromere.

**DISCUSSION**

Shugoshin proteins function by two distinct mechanisms to promote normal chromosome segregation: they protect centromeric cohesin from premature removal and they participate in sensing and correcting kinetochore attachment defects. The shugoshins carry out these functions in large part by serving as adaptor proteins that recruit two enzyme complexes to the centromere: PP2A-Rts1 and the CPC. Most eukaryotes have two homologs of shugoshin to complete these tasks. In the case of fission yeast, the two homologs are functionally distinct: spSgo1 recruits PP2A and spSgo2 recruits the CPC. Budding yeast has a single homolog, Sgo1, which recruits both PP2A and the CPC and fulfills both the cohesin protection and bi-orientation functions. While it is clear that Sgo1 recruits PP2A-Rts1 in meiosis to protect centromeric cohesin, there is no clear indication that Sgo1 serves a cohesin protection function in mitotic yeast cells, which do not employ a ‘prophase pathway’ to remove cohesin prior to metaphase and are therefore not expected to need centromeric cohesin protection. Instead, our work and other recent studies suggest that in budding yeast mitosis, Sgo1 recruits PP2A-Rts1 to promote bi-orientation, probably in collaboration with the CPC (Peplowska et al., 2014; Verzijlbergen et al., 2014).
found that PP2A-Rts1 is recruited by Sgo1 to the pericentromere during mitosis, and that defects in PP2A recruitment result in bi-orientation defects when cells recover from severe spindle damage.

We also found that recruitment of Rts1 to a single centromere is sufficient to allow bi-orientation at that centromere in the absence of Sgo1. To understand how Sgo1 and PP2A contribute to bi-orientation, it will be important to identify the key substrate(s) of PP2A. Studies in human cells showed that PP2A-B56 (Rts1) is important for stabilizing kinetochore-microtubule attachments and ensuring proper chromosome alignment on the mitotic spindle (Foley et al., 2011). Interestingly, phosphorylation of Dsn1 and Knl1 (both substrates of Aurora B) and BubR1 (a substrate of Plk1) increases in cells depleted of the B56 subunit, suggesting that these could be direct targets of PP2A (Foley et al., 2011; Suijkerbuijk et al., 2012). Another possibility is that PP2A-Rts1 regulates localization of Aurora B and the CPC to promote bi-orientation, as recent studies in budding yeast suggest that Sgo1 and Rts1 are required for maintenance of Aurora B (Ipl1) at the centromere (Peplowska et al., 2014; Verzijlbergen et al., 2014). Interestingly, a screen for Rts1 substrates identified two components of the CPC, Sli15 and Bir1, as being hyperphosphorylated in rts1Δ cells (Zapata et al., 2014).

In budding yeast, deletions of SGO1 or BUB1 also cause aberrations in mitotic chromosome architecture when cells are challenged with spindle poisons (Haase et al., 2012), suggesting that Sgo1 could be modulating the spatial conformation of sister chromatid attachments to promote bi-orientation. Sgo1 can recruit condensin to chromosomes and has the
capability to dephosphorylate cohesin, both of which contribute to chromosome structure and organization (Peplowska et al., 2014; Stephens et al., 2011; Verzijlbergen et al., 2014).

Interestingly, Rts1 itself is not strictly required for bi-orientation, as seen in our work and another recent study (Verzijlbergen et al., 2014). We suggest that Sgo1 is still able to recruit the catalytic subunit of PP2A even in the absence of Rts1. Binding studies with PP2A subunits in vitro reveal that mouse Sgo2 can associate with the C subunit of PP2A even in the absence of a B subunit (Xu et al., 2009). Thus, the B subunit might not be required for interaction of budding yeast Sgo1 with the C subunit. Additionally, mSgo2 can associate with three structurally distinct versions of human B subunits: B, B’, and B’’ (Xu et al., 2009). Further experiments will be required to determine whether Sgo1 can recruit active PP2A with the alternative regulatory subunit, Cdc55, or without any regulatory subunit at all.

Sgo1 and its associated PP2A-Rts1 dissociate from pericentromeres following bi-orientation, suggesting that their function is no longer required after satisfaction of the spindle assembly checkpoint. Interestingly, we found that constitutive recruitment of Rts1 to one pericentromere throughout mitosis did not cause significant mitotic defects, suggesting that removal of phosphatase activity is not essential after bi-orientation has been achieved.

We also find that Sgo1 is an APC/C substrate in budding yeast, but that it is removed from centromeres independent of its destruction once sister chromatid bi-orientation has been achieved. Consistent with this finding, studies in human cells demonstrate that localization of the PP2A-B56 subunit is also dependent on microtubule attachment (Foley et al., 2011). Mps1 and
Bub1 kinase activities are required for Sgo1 localization to the centromere, and phosphatases such as PP1 have been shown to counteract these kinases (London et al., 2012). It is likely that phosphatase activation following bi-orientation is required to silence the spindle checkpoint, and that displacement of Sgo1 is an important outcome of this silencing mechanism. Recent work suggests that Rts1 itself may contribute to release of Sgo1 following bi-orientation, though this is not the sole mechanism (Nerusheva et al., 2014). Together, these results demonstrate that Sgo1 recruits PP2A to centromeres in response to errors in sister chromatid attachment, and that centromeric PP2A promotes bi-orientation to ensure accurate chromosome segregation.
MATERIALS AND METHODS

Yeast strains and plasmids

Strains used in this study are listed in supplementary material Table S1. All yeast strains were derivatives of W303. C-terminal tagging of Sgo1, Rts1, and Spc42, and introduction of a GAL promoter upstream of CDC20, were performed using standard methods at the endogenous loci (Janke et al., 2004; Longtine et al., 1998). The SGO1 gene was subcloned from the budding yeast genome into pBS, pRS306, or pRS316 containing a 9-myc tag and the HIS3 marker. The 3A or Δdb mutations, as well as point mutants for ubiquitination assays, were made in these vectors using Quikchange site-directed mutagenesis (Agilent Technologies, La Jolla, CA). Lac or Tet operator arrays were integrated 1 kb from the centromere of Chromosome V using a two-step method (Rohner et al., 2008). pCUP1-GFP-LacI, pCUP1-GFP-LacI-RTS1, or pCUP1-GFP-TetR were cloned into pRS303 or single-copy integrating vectors and integrated at the corresponding locus (Chau et al., 2012; Yaakov et al., 2012; Zalatan et al., 2012).

Cells were grown in YPD, except where noted. To arrest cells in G1, cells were treated with 1 μg/ml alpha-factor for 3 h. To arrest cells in mitosis, cells were treated for 4 h at 30°C with 60 μg/ml benomyl or 2% Dextrose (in strains containing CDC20 under control of the GAL promoter). Cells in a GAL-CDC20 arrest were treated with 20 μg/ml nocodazole to promote spindle disassembly.

APC/C ubiquitination assays
E1 (Uba1), E2 (Ubc4), and APC/C (from a cdh1Δ strain) were purified and used in ubiquitination assays as previously described (Carroll and Morgan, 2002; Rodrigo-Brenni and Morgan, 2007). Cdh1 was translated using the TnT Quick Coupled Transcription/Translation systems (Promega, Madison, WI) and purified using a ZZ-tag and magnetic Dynabeads (Life Technologies, Carlsbad, CA) coupled to IgG. Cdh1 was cleaved from the beads using the TEV protease. PCR products of full length Sgo1 or truncations containing a T7 promoter were translated in vitro in the presence of 35S-methionine and subsequently treated with NEM to inhibit activities of E1 and E2 in the reticulocyte lysates. E1 and E2 were charged with methylated ubiquitin (Boston Biochem, Cambridge, MA) and added to reactions containing the purified Cdh1, APC/C, and Sgo1 translations to initiate ubiquitination of Sgo1. After 1 h, reaction products were separated by SDS-PAGE and visualized using a Typhoon Phosphorimager (GE Healthcare).

**Sgo1 immunoprecipitations**

50 ml cell cultures were grown to an A600 of ~1.0 and frozen as pellets in liquid nitrogen. Cell pellets were lysed by bead beating in IP lysis buffer (50 mM Hepes pH 8.0, 150 mM NaCl, 1% NP40, 50 mM beta-glycerophosphate, 50 mM NaF, 1 mM DTT, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotinin, 1 mM PMSF, 10% glycerol, 0.63 mg/ml benzamidine, 1 mM MgCl2, 50 U/ml Dnase I) and lysates were incubated with Protein G Dynabeads (Life Technologies, Carlsbad, CA) pre-incubated with an anti-myc antibody (9E10, Covance, Princeton, NJ). Beads were washed with lysis buffer and eluted from the beads in sample buffer. Elution products were separated by SDS-PAGE and analyzed by Western blot. Sgo1-9myc was
detected using an antibody against Sgo1 (gift of A. Rudner, University of Ottawa), Rts1-6HA was detected using an antibody against HA (12CA5, Roche), and Clb2 was detected using the sc-9071 antibody from Santa Cruz Biotechnology.

**Fluorescence microscopy and data processing**

Cells were grown in synthetic complete media containing 2% dextrose or galactose and raffinose to minimize background fluorescence. Cells were plated onto coverslips coated with ConA and imaged at the UCSF Nikon Imaging Center using a spinning disk confocal microscope (Nikon Ti-E inverted microscope with a Yokogawa CSU-22 scanner unit and a Photometrics Evolve EMCCD camera), 491 nm and 561 nm lasers, and Chroma ET525/50m and ET610/60m emission filters. Between 13 and 17 z slices (0.5 µm each) were taken for each image using a 60X/1.4 NA oil objective with a final pixel size of approximately 0.15 µm/pixel. Images for movies were acquired every minute for approximately 50 min. To obtain traces of GFP intensity relative to spindle elongation, images were processed as described (Lu et al., 2014).

**Bi-orientation assays**

Cells were arrested in YPD containing benomyl for 4 h and released into YPD without benomyl. After 60 min, cells were collected and fixed using 3.7% formaldehyde. After washing with PBS, cells were mixed with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 1µg/ml DAPI on microscope slides, and imaged using spinning disk confocal fluorescence microscopy.
Acknowledgements

We thank members of the Morgan lab for discussions; Dan Lu (UCSF) for reagents and assistance with image data processing; Scott Foster (UCSF) for assistance with ubiquitination assays; Norman Davey (UCSF) for assistance with D-box sequence analysis; Matilde Galli, Drew Thacker, and Dan Lu (UCSF) for comments on the manuscript; Kurt Thorn and the UCSF Nikon Imaging Center for assistance with microscopy; and Gilad Yaakov (UCSF), Adam Rudner (University of Ottawa, Canada), and Susan Gasser (Friedrich Miescher Institute, Basel, Switzerland) for reagents.
Figure 1

A

B

C

D

Yeast Sgo1
Human Sgo1 (C-term)
Human Sgo1 (N-term)
Yeast Hsl1

SSP
L
L
DITNKSEN
KK

HLS
KDN
S

KRMSKYKE

RAALS

SSPLL
DITNKSEN
KK

Sgo1-∆db

Sgo1-MeUb

SSPLL
DITNKSEN
KK

HLS
KDN
S

KRMSKYKE

RAALS

SSPLL
DITNKSEN
KK

Sgo1-∆db
Figure 1. Budding yeast Sgo1 is an APC/C substrate, and its destruction depends on a non-canonical D-box. (A) Radiolabeled Sgo1 was translated in rabbit reticulocyte lysates and incubated with purified APC/C, ubiquitin and Cdh1, an APC/C activator. Purified E1 and E2 were added to promote ubiquitination as indicated. Reaction products were analyzed by SDS-PAGE and autoradiography. Methylated ubiquitin was used to prevent polyubiquitin chain synthesis, which facilitates the clear detection of reaction products. The number of products generated in these reactions reflects the number of lysines that have been modified on the substrate, and modification of large numbers of lysines can lead to band heterogeneity and smearing. (B) Fragments of Sgo1 were used in APC/C ubiquitination assays to identify the Sgo1 D-box. Radiolabeled fragments were translated in vitro and assayed as in (A) using a higher percentage polyacrylamide gel to resolve smaller fragments. Yeast securin (Pds1) was used as a positive control in the reactions at far right. FL, full length Sgo1. (C) Various Sgo1 fragments and point mutants were used in APC/C assays, as in (A), to further define the D-box. In the top two panels, fragments containing the indicated residues were translated from truncated PCR products. In the bottom two panels, reactions were carried out with translated full-length Sgo1 carrying the indicated point mutations or short deletions. An asterisk (*) indicates the Sgo1-Δdb mutant. (D) Comparison of the budding yeast Sgo1 D-box to the Hsl1 D-box and the two APC/C degrons of human Sgo1 (Burton and Solomon, 2001; He et al., 2013; Karamysheva et al., 2009). Red text highlights similarities between all four sequences, while the green box highlights the similarities between C-terminal residues of the yeast Sgo1 D-box and the N-terminal degron of human Sgo1.
Figure 2
Figure 2. Deletion of the budding yeast Sgo1 D-box stabilizes Sgo1. (A) Wild-type (top panels) or SGO1-Δdb cells (bottom panels) were arrested in G1 with α-factor for 3 h and then released from the arrest. Samples were taken every 15 min, and lysates were analyzed by Western blotting with antibodies against Sgo1 or Clb2. Lysates of asynchronous cultures were also analyzed (asynch). (B) The indicated strains (sgo1Δ, or three isolates each of SGO1 or SGO1-Δdb) were plated as serial dilutions on YPD or YPD containing the indicated concentrations of benomyl.
Figure 3

A

B

C

D
Figure 3. Sgo1 and Sgo1-Δdb disappear from the pericentromere with similar timing. (A) Sgo1 or Sgo1-Δdb was tagged with GFP and imaged at one-minute intervals using spinning disk confocal microscopy. Spindle pole bodies were marked by fusing Spc42 to mCherry. An asterisk (*) denotes the onset of spindle elongation. Scale bar: 5 µm. (B) Quantification of GFP intensities relative to the onset of spindle elongation (Lu et al., 2014). The intensity of the brightest 5x5 pixel square in strains containing Sgo1-GFP (n=28), Sgo1-Δdb-GFP (n=53), Pds1-GFP (n=20), or Clb5-GFP (n=36) was measured as a function of time, smoothed, normalized to maximum intensity, and plotted relative to the onset of spindle elongation (dashed line). Data before normalization are found in supplementary material Fig. S2. (C) Averaged traces, where unsmoothed traces from all cells were first aligned to the same time reference point, averaged at each time point, and then normalized to maximum intensity (Lu et al., 2014). (D) To quantify and compare the timing of fluorescence changes for each protein, we determined the time point when 50% of the GFP intensity remained in each cell. This analysis was carried out with the subset of cells in which a clear fluorescence plateau was present before and after the decline. Each dot represents a single cell, the middle bar represents the median of each strain dataset, and the error bars represent the 25th and 75th percentiles.
Figure 4
Figure 4. Sgo1 is removed when the spindle assembly checkpoint is satisfied. Cells contained Sgo1-Δdb-GFP and Spc42-mCherry as in Fig. 3, as well as CDC20 under the control of the GAL promoter. In the presence of galactose (top panels), cells were distributed throughout the cell cycle. After shifting the cells into media containing dextrose for 3 h, Cdc20 synthesis was shut off and cells arrested in mitosis (middle panels). Nocodazole (20 µg/ml) was then added to the arrested cells (bottom panels) to promote spindle disassembly. In two independent experiments with over 50 cells each, 6.7% (+/- 1.4%; +/- s.d.) of cells displayed Sgo1-Δdb-GFP foci in the presence of dextrose, and 93% (+/- 2.2%; +/- s.d.) of cells displayed foci following addition of nocodazole. BF, bright-field image. Scale bar: 5 µm.
Figure 5

A

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B

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[Images and tables showing protein levels and interactions]
**Figure 5. Sgo1 recruits Rts1 to the mitotic spindle.** (A) The *sgo1-3A* mutant fails to recruit Rts1 to foci. Rts1-GFP was visualized in cells containing Sgo1-Δdb or Sgo1-3A-Δdb. Rts1-GFP foci were present in 29% of cells containing Sgo1-Δdb and in 1.6% of cells containing Sgo1-3A-Δdb (n=110 and 126, respectively). Sgo1-Δdb-GFP and Sgo1-3A-Δdb-GFP both localized normally. BF, bright-field image. Scale bar: 5 µm. Rts1-GFP fluorescence intensity was weak and difficult to quantify in a wild-type *SGO1* strain (not shown). (B) Diagram showing locations of the mutations in the *sgo1-3A* mutant relative to the structure of PP2A. The 3A mutations disrupt the interaction between Sgo1 and the catalytic subunit. (C) Sgo1 and Rts1 physically interact. Sgo1-9myc or Sgo1-3A-9myc were immunoprecipitated from lysates of strains containing Rts1-6HA, and Western blots of the IP and of total lysates were probed using anti-HA and anti-Sgo1 antibodies. Rts1-6HA was partly proteolyzed in the cell lysates, generating ~70 kDa fragments.
Figure 6

A

\[ \text{SGO1} \text{sgo1}^{\Delta} \text{SGO1} \text{sgo1}^{\Delta} +\text{GFP-LacI-Rts1} \]

\[ \% \text{Bi-oriented} \]

\[ 0 \mu g/ml \text{benomyl} \]

\[ 10 \mu g/ml \text{benomyl} \]

\[ \text{SGO1} \text{sgo1}^{\Delta} \text{SGO1} \text{sgo1}^{\Delta} \]

\[ \% \text{Bi-oriented} \]

\[ 0 \]

\[ 20 \]

\[ 40 \]

\[ 60 \]

\[ 80 \]

\[ 100\% \]

B

\[ \text{Rts1 fusion to GFP-LacI} \]

\[ \text{Bi-oriented} \]

\[ \text{Mis-oriented} \]

C

\[ \text{Pre-Arrest} \]

\[ sgo^{\Delta} \]

\[ 1.5 \text{ hours after release} \]

\[ sgo^{\Delta} \]

\[ \text{LacI} \]

\[ \text{LacI-Rts1} \]

\[ \text{LacI} \]

\[ \text{LacI-Rts1} \]

\[ \text{LacI} \]

\[ \text{LacI-Rts1} \]

\[ \text{LacI} \]

\[ \text{LacI-Rts1} \]

\[ \text{0 \mu g/ml benomyl} \]

\[ \text{10 \mu g/ml benomyl} \]

D

\[ \text{Rts1 fusion to GFP-LacI} \]

\[ \text{GFP-TetR} \]

\[ \text{Bi-oriented} \]

\[ \text{Mis-oriented} \]
Figure 6. Recruitment of Rts1 is sufficient for bi-orientation. Bi-orientation of sister chromatids after release from benomyl. (A) sgo1∆ or sgo1-3A mutants fail to properly bi-orient after release from benomyl. Cells containing a GFP-LacI/LacO dot near the centromere of Chromosome V were arrested in media containing benomyl to promote spindle disassembly. Cells were then released into media without benomyl to allow the spindle to reform. After 60 min, cells were fixed and stained with DAPI to visualize DNA. Only cells that had fully segregated their DNA were scored for localization of Chromosome V GFP dots (n ≥ 48 cells). Results are the mean of two experiments, with error bars representing the standard deviation. (B) A GFP-LacI-Rts1 fusion protein rescues the bi-orientation defect of sgo1∆ cells. The same bi-orientation assay as in (A) was performed using a GFP-LacI-Rts1 fusion in place of GFP-LacI. (C) GFP-LacI-Rts1 does not restore normal proliferation to cells lacking SGO1. Cells were arrested in benomyl and then released into media without benomyl. After 90 min, cells were plated in serial dilutions on media with or without benomyl as indicated. (D) The GFP-LacI-Rts1 fusion protein restores bi-orientation to sgo1∆ cells only when localized to the centromere. A GFP-LacI-Rts1 fusion protein was expressed as in (B), but in this case there were no LacO arrays on the chromosome. Chromosome segregation was instead monitored with a TetR-GFP/TetO dot on Chromosome V.
Figure S1

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<tr>
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[Image of gel electrophoresis with bands showing the differences between Sgo1 and Sgo1-Δdb with Cdc20 and Cdhl conditions.]
**Figure S1. Sgo1 is a substrate of Cdc20 and Cdh1 in vitro.** Radiolabeled Sgo1 or Sgo1-Δdb was translated *in vitro* and tested for APC/C-dependent ubiquitination as in Fig. 1A, using either purified Cdc20 or Cdh1 as the activator subunit. All reactions contained E1 and E2. Cdh1 is generally more stable and active than Cdc20 in these assays, resulting in higher activity.
Figure S2
Figure S2. Un-normalized traces of GFP-labeled proteins. (A) Smoothed fluorescence intensity measurements (arbitrary units) of Sgo1-GFP, Sgo1Δdb-GFP, Clb5-GFP, or Pds1-GFP before normalization for the plots in Fig. 2. (B) Average intensity of un-normalized traces.
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References


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Two Fission Yeast Homologs of Drosophila Mei-S332 Are Required for Chromosome Segregation during Meiosis I and II. Current Biology 14, 287–301.


Chapter 3

Conclusions
Precise regulation of kinetochore-microtubule attachments is essential for ensuring correct segregation of sister chromatids in anaphase. This regulation depends on the spatially and temporally coordinated activities of multiple kinases and phosphatases, but the specific contributions of these kinases and phosphatases are poorly understood.

Shugoshin is a crucial platform that coordinates the chromosomal passenger complex (CPC, including the Aurora B kinase) and the PP2A phosphatase to ensure accurate chromosome segregation in mitosis. As shugoshin has clear roles in regulating both the removal of cohesin and the stability of microtubule attachments, sorting out the potential contributions of Aurora B and PP2A to these apparently distinct pathways has been difficult. Adding to this complexity is the seemingly different ways that different organisms have co-opted isoforms of shugoshin to complete these tasks.

The research presented here provides evidence for a direct function for Sgo1-dependent recruitment of PP2A in ensuring sister chromatid bi-orientation in budding yeast mitosis. While Sgo1 likely also participates in direct recruitment of the CPC, PP2A is sufficient to serve the essential function of Sgo1. This finding also provides a precedent for studying a potential function of the Sgo1-PP2A complex in bi-orientation in other organisms. There is mounting evidence that PP2A plays a significant role at the kinetochore in mediating microtubule attachment, as the phosphorylation state of multiple kinetochore components is regulated by PP2A, either directly or indirectly (Foley et al., 2011; Suijkerbuijk et al., 2012). In humans, PP2A appears to be counteracting the activity of the Aurora B kinase and stabilizing microtubule attachments.
Because PP2A and Aurora B are likely both important for achieving sister chromatid bi-orientation but have opposing activities, their spatial and temporal regulation must be critical for their functions. The research presented here shows that the localization of Sgo1, and thus PP2A, is dynamically regulated in response to microtubule attachments. When sister chromatids are mis-oriented, Sgo1 is localized to the pericentromere. Once bi-orientation is achieved, Sgo1 is delocalized from chromosomes. Sgo1 localization to chromosomes is dependent on the Mps1 and Bub1 kinases, and its removal likely depends on the activation of phosphatases to counteract these kinases, though the precise mechanism remains unknown (Fernius and Hardwick, 2007; Kawashima et al., 2010; Liu et al., 2012; London et al., 2012; Storchová et al., 2011; Tang et al., 2004; Yamagishi et al., 2010; 2008). Whether Sgo1 is itself modified to regulate its localization or interaction with PP2A also remains unknown. This research shows that Sgo1 levels are ultimately regulated at the level of its destruction by the APC. Why there are multiple mechanisms to inactivate Sgo1 remains unclear. It will be important to first identify the Sgo1 delocalization pathway, and then inactivate this pathway in combination with preventing its destruction.

Many challenges remain for the future of this field, and sorting out the individual and collective contributions of single phosphorylation and dephosphorylation events to sister chromatid bi-orientation will be essential. An important step in this process will be to identify the protein sites that are modified and the specific kinases and phosphatases that act on them. Additionally, the precise timing of these modifications will also need to be characterized. It is possible that the timing of these events is dependent on site-specific localization of the kinases.
and phosphatases. For example, both BubR1 and Sgo1 can recruit PP2A in human cells, potentially directing it toward different substrates (Kitajima et al., 2006; Riedel et al., 2006; Suijkerbuijk et al., 2012). Thus, further understanding the regulation of Sgo1 will be important for understanding the function of PP2A in the dynamic regulation of kinetochore-microtubule attachments and its importance for accurate chromosome segregation during mitosis.
References


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