The kinetochore encodes a mechanical switch to disrupt spindle assembly checkpoint signalling

Pavithra Aravamudhan¹, Alan A. Goldfarb² and Ajit P. Joglekar¹,²,³

The spindle assembly checkpoint (SAC) is a unique signalling mechanism that responds to the state of attachment of the kinetochore to spindle microtubules. SAC signalling is activated by unattached kinetochores, and it is silenced after these kinetochores form end-on microtubule attachments. Although the biochemical cascade of SAC signalling is well understood, how kinetochore–microtubule attachment disrupts it remained unknown. Here we show that, in budding yeast, end-on microtubule attachment to the kinetochore physically separates the Mps1 kinase, which probably binds to the calponin homology domain of Ndc80, from the kinetochore substrate of Mps1, Spc105 (KNL1 orthologue). This attachment-mediated separation disrupts the phosphorylation of Spc105, and enables SAC silencing. Additionally, the Dam1 complex may act as a barrier that shields Spc105 from Mps1. Together these data suggest that the protein architecture of the kinetochore encodes a mechanical switch. End-on microtubule attachment to the kinetochore turns this switch off to silence the SAC.

The attachment of sister kinetochores to microtubules from opposite spindle poles is necessary for accurate chromosome segregation during cell division. Unattached kinetochores activate the cell-cycle control known as the spindle assembly checkpoint¹² (SAC), which arrests the cell cycle until these kinetochores form stable attachment. The SAC thus ensures accurate segregation of chromosomes into daughter cells.

The kinetochore-based biochemical cascade that generates the SAC signal is well understood. Within unattached kinetochores, the highly conserved Mps1 kinase phosphorylates kinetochore proteins, and enables the sequential recruitment of SAC proteins³ (Fig. 1a). This cascade ultimately generates the ‘wait-anaphase’ signal and stalls the cell cycle. The formation of end-on kinetochore–microtubule attachment disrupts this cascade, presumably by interfering with one or more of its steps. Early cell biological observations of SAC silencing led to the hypothesis that a mechanical change within the kinetochore induced by end-on microtubule attachment silences the SAC (ref. 4). Concurrent changes in the state of SAC signalling and the nanoscale separations between various kinetochore proteins support this hypothesis⁵–⁷. However, the causative link between specific changes in kinetochore architecture induced by microtubule attachment and the disruption of specific steps in SAC signalling is missing. This is mainly because the kinetochore is a highly complex machine containing multiple copies of more than 60 different proteins⁸. A change in the structure, conformation and/or architecture of any of these proteins induced by microtubule attachment may affect SAC signalling. Consequently, the molecular basis for the mechanosensitivity of SAC signalling is unknown.

Here we investigate how the architecture of the kinetochore–microtubule attachment in the budding yeast Saccharomyces cerevisiae disrupts SAC signalling. We find that the phosphorylation of Spc105 by Mps1 is both necessary and sufficient to initiate the SAC cascade. End-on kinetochore–microtubule attachment restricts Mps1 kinase activity to the outer kinetochore and maintains the phosphodomain of Spc105 in the inner kinetochore to disrupt this crucial first step in the SAC cascade to silence the SAC.

RESULTS

Mps1, when artificially localized to the kinetochore, phosphorylates Spc105 and activates the SAC

Microtubule attachment to the kinetochore may silence the SAC by promoting the dissociation of SAC proteins from the kinetochore (Fig. 1a). If this is true, then persistent localization of key SAC proteins at the kinetochore should constitutively activate the SAC. To test this hypothesis, we used rapamycin-induced dimerization of 2xFkbp12 and Frb to artificially localize or ‘anchor’ key phosphoregulators and SAC proteins⁵: Mps1, Ipl1 (Aurora B), Glc7 (PP1) or Mad1 within the kinetochore (Fig. 1b). In the absence of rapamycin,
that the arrest was mediated by the SAC (Fig. 1d). These observations of large-budded cells that were arrested in metaphase (Fig. 1d,e). The addition of rapamycin to the culture media rapidly anchored it to the kinetochore subunit tagged with 2xFkbp12 (Fig. 1c and Supplementary Fig. 1b). The phosphorylation of the 

Kinetochore complex

Ndc80

Bub1

Bub3

Mad1

Mad2

Mitotic checkpoint complex

Figure 1 Cell-cycle effects of anchoring Mps1 to the kinetochore using rapamycin-induced dimerization. (a) The steps in the kinetochore-based signalling cascade of the SAC (magenta Ps indicate Mps1-mediated phosphorylation) that may be disrupted by microtubule attachment. (b) Top: Protein architecture of the metaphase kinetochore–microtubule attachment. (c) Top: Micrographs show the anchoring of Mps1–Frb–GFP at Mtw1-C (time after rapamycin addition indicated; scale bars, ~3 μm). The stereotypical distribution of kinetochores in between the spindle pole bodies in metaphase visualized with Mtw1–GFP and Spc97–mCherry is shown on the right. Schematic underneath depicts the metaphase spindle morphology. Bottom: Representative transmitted-light images of yeast cells before and 1 h after the addition of rapamycin to anchor Mps1 at Mtw1-C. Right: Localization of Bub1–GFP and Mad1–GFP, and kinetochores (visualized by Spc24–mCherry and Spc105–GFP) in untreated cells (control) and in cells that have Mps1 anchored at Mtw1-C (RAP). Scale bar, ~3 μm. (e) Top: Domain organization of Spc105. The end-to-end length of the unstructured domain of Spc105 (amino acids 1–455) is predicted to be 11.7 ± 5 nm (mean ± s.d. using the worm-like chain model). The maximum length of its α-helical region (amino acids 455–709) is 38 nm (3.6 amino acids per turn with a 0.54 nm pitch). The predicted kinetochore-binding domain (RWD) is ~6 nm long.

Figure 1 Cell-cycle effects of anchoring Mps1 to the kinetochore using rapamycin-induced dimerization. (a) The steps in the kinetochore-based signalling cascade of the SAC (magenta Ps indicate Mps1-mediated phosphorylation) that may be disrupted by microtubule attachment. (b) Top: Protein architecture of the metaphase kinetochore–microtubule attachment. (c) Top: Micrographs show the anchoring of Mps1–Frb–GFP at Mtw1-C (time after rapamycin addition indicated; scale bars, ~3 μm). The stereotypical distribution of kinetochores in between the spindle pole bodies in metaphase visualized with Mtw1–GFP and Spc97–mCherry is shown on the right. Schematic underneath depicts the metaphase spindle morphology. Bottom: Representative transmitted-light images of yeast cells before and 1 h after the addition of rapamycin to anchor Mps1 at Mtw1-C. Right: Localization of Bub1–GFP and Mad1–GFP, and kinetochores (visualized by Spc24–mCherry) in untreated cells (control) and in cells that have Mps1 anchored at Mtw1-C (RAP). Scale bar, ~3 μm. (e) Top: Domain organization of Spc105. The end-to-end length of the unstructured domain of Spc105 (amino acids 1–455) is predicted to be 11.7 ± 5 nm (mean ± s.d. using the worm-like chain model). The maximum length of its α-helical region (amino acids 455–709) is 38 nm (3.6 amino acids per turn with a 0.54 nm pitch). The predicted kinetochore-binding domain (RWD) is ~6 nm long.

Other than these estimated dimensions, the structure and organization of Spc105 is unknown. Therefore, the depiction is not drawn to scale. The six Mps1 phosphorylation sites (consensus sequence ‘MELT’) are depicted as bars. Bottom: Cell-cycle progression of asynchronous cells with the indicated genotypes observed on anchoring Mps1 at Mtw1-C. Accumulation of large-budded cells indicates mitotic arrest. Plotted points represent the average values calculated from 2 independent experiments. More than 50 cells were scored for each time point. The source data are shown in Supplementary Table 3.
the SAC indirectly by disrupting either microtubule attachment or force generation\(^\text{13}\) (Supplementary Fig. 1c,d). This observation is consistent with data from other organisms and with the dispensability of Ipl1 for SAC signalling in budding yeast\(^\text{14,15}\). Thus, anchoring Mps1 to the kinetochore is sufficient for inducing constitutive SAC signalling.

**SAC proteins that act downstream from Mps1 can function within attached kinetochores**

The above experiments were performed in asynchronous yeast cultures. Consequently, we could not ascertain whether the anchored Mps1 activated the SAC mostly in prometaphase, before all kinetochores attach to microtubules, or if Mps1 can reactivate the SAC when anchored within stably attached kinetochores. To test this, we repressed CDC20, the gene that encodes the activating subunit of the anaphase-promoting complex (APC), to prevent yeast cells from entering anaphase even after all of the kinetochores were attached and the SAC was satisfied\(^\text{16}\). We anchored Mps1 at Mtw1-C in such cells, released them from the arrest by inducing CDC20 expression, and then monitored cell-cycle progression (Fig. 2a). We found that cells that had Mps1 anchored at Mtw1-C underwent a persistent cell-cycle arrest, whereas control cells completed anaphase within 20 min (Fig. 2a). Thus, Mps1 can reactivate the SAC, when it is anchored to kinetochores with stable microtubule attachments.
The ability of Mps1 to activate the SAC depends on its position in the kinetochore. (a) Fluorescence recovery after photobleaching of Mps1–frb–GFP anchored at Ndc80-C (red circles), and loss of anchored protein from the unbleached cluster (green squares). Blue dashed line shows the expected rate of photobleaching as a result of imaging determined in cells expressing Ndc80–GFP (mean ± s.e.m. from n = 8 and 11 clusters for bleached and unbleached clusters, respectively; data pooled from 2 independent experiments). Scale bar, ∼3 µm. (b) Top: Structure of the Ndc80 complex and the positions of fluorescent tags used for FRET. Scatter plot: Proximity ratio, which is directly proportional to the FRET efficiency, for FRET between Spc25–mCherry or Nuf2–mCherry and Mad1–Frb–GFP anchored to Spc24-C (mean ± 95% confidence interval from n = 35, 33 and 44 kinetochore clusters analysed, from left to right; the bars show mean values calculated using data pooled from 2 independent experiments). The proximity ratio is defined as the acceptor fluorescence resulting from FRET normalized by the sum of mCherry cross-excitation and GFP emission bleed-through into the mCherry imaging channel. FRET between the anchored donor, Mad1–Frb–GFP, and the acceptor, Spc25–mCherry, was readily detected, but it was absent when the mCherry was fused to Nuf2-C. Spc25-C is <3 nm away from Spc24-C, where the donor is anchored, whereas Nuf2-C is >10 nm away. We used Mad1, rather than Mps1, in this experiment to ensure that the number of donors is equal to the number of acceptor molecules (either Spc25–mCherry or Nuf2–mCherry) for accurate FRET quantification. (c) Number of protein molecules anchored at Ndc80-C, measured 30 min after rapamycin addition (mean ± s.d. from n = 25, 33, 29, 20, 41 and 30 kinetochore clusters from left to right; the experiment was performed once). Scale bar, ∼3 µm. (d) Top: The organization of yeast kinetochore proteins along the microtubule axis. The N-terminal half of Spc105 is not drawn to scale. Bottom: Bar graph shows the number of colonies formed on rapamycin-containing plates relative to control plates. The experiment was repeated at least twice and the cumulative number of colonies scored is shown below the graph. Right: Representative photographs of plates for three strains.

These results also show that SAC proteins downstream from Mps1 can bind to and function from attached kinetochores. It is possible that the anchored Mps1 facilitates SAC protein binding by changing the overall organization of the kinetochore. However, we did not detect significant changes when we compared the nanoscale separation between key kinetochore domains in metaphase and rapamycin-treated cells using high-resolution co-localization (Fig. 2b). Even if architectural changes that facilitate SAC protein binding do occur, they can do so when the kinetochore is attached. On the basis of these data, we concluded that microtubule attachment to the kinetochore must hamper either Mps1 localization to the kinetochore or its kinase activity to silence the SAC.
Figure 4 The Dam1 complex defines a boundary for SAC signalling by anchored Mps1. (a) Schematic: Position of the Dam1 complex relative to the Ndc80 complex and subunit organization within the Dam1 complex. EMD1372 was used to infer the dimensions of the Dam1 complex. (b) Colony growth (also see Supplementary Fig. 4a) on control (Ctrl) and rapamycin (+Rap) plates. The number of days after plating is indicated at the top; the anchoring subunit is indicated on the left. (c) Cell-cycle progression when Mps1 is anchored to a Dam1 subunit (indicated on the left) in cells released from an experimentally imposed S-phase arrest. S-phase synchronization was used to ensure that the kinetochores formed end-on attachments and loaded the Dam1 complex before Mps1 was anchored. Plotted points represent the average values calculated from 2 independent experiments. The source data are shown in Supplementary Table 3. (d) Normalized distribution of Dad4–mCherry on the spindle when Mps1 was anchored to the indicated positions for 1 h (mean ± s.e.m. n = 43, 34, 28, 36, 73 and 38 cells for Dad1, Dad3, Ask1, Ctrl, Spc34 and Dad2, respectively). Control data are from untreated metaphase cells. Micrographs on the right show the localization of Dad4–mCherry relative to that of Mps1–frb–gfp anchored to the indicated subunits (scale bars, ∼3 µm). (e) The separation between kinetochore clusters in the cells in d, measured as the separation between maximum-intensity pixels in the two Dad4–mCherry puncta in each cell; mean ± 95% confidence interval, n = 43, 16, 16, 25, 72 and 38 cells for Dad1, Dad3, Ask1, Ctrl, Spc34 and Dad2, respectively. Although there is a small decrease in kinetochore cluster separation when Mps1 is anchored at Dad3-C, cell-cycle progression is unaffected as seen in c. (f) Left: Classification of Dam1 complex subunits inferred from the Mps1 anchoring experiments. Right: Activity map of the anchored Mps1 along the length of the kinetochore–microtubule attachment. Arrows from the Dam1 complex depict the proposed orientation of the C termini of subunits used as anchors. Possible variations in the conformation of the unstructured phosphodomain of Spc105 are also depicted.

**Endogenous Mps1 binds to attached kinetochores**

Microtubule attachment may inhibit Mps1 function by simply promoting its dissociation from the kinetochore. Indeed, Mps1 gradually disappears from the kinetochore clusters as yeast cells progress from prometaphase to metaphase (Supplementary Fig. 1e). However, Mps1 is targeted for degradation by the APC (ref. 17). This
process may contribute to the disappearance of Mps1 either directly or indirectly. Consistent with this hypothesis, when we inactivated the APC using CDC20 repression, Mps1–Frb–GFP autonomously localized to attached kinetochores (Fig. 2c, left). Importantly, this autonomously localized Mps1 did not activate the SAC, because both Bub3 and Mad1 were absent from the kinetochores (Fig. 2c, right). Furthermore, these cells entered anaphase without any detectable delay following release from the metaphase block (Fig. 2a, dotted grey line). Finally, Mps1 was present at the kinetochore even as these cells entered anaphase (Supplementary Fig. 1f). Thus, the removal of Mps1 from the kinetochore is not necessary for either SAC silencing or anaphase onset.

It is notable that the Mps1 molecules that autonomously localize to attached kinetochores do not activate the SAC, but a similar number of Mps1 molecules anchored at Mtw1-C activate it constitutively (Supplementary Fig. 1e). The inability of Mps1 to activate the SAC could be due to: its inability to reach and phosphorylate Spc105 from its endogenous binding position (Fig. 3a–c). First, we found that the anchoring was stable, as indicated by negligible turnover of Mps1–Frb–GFP anchored at Ndc80-C (Fig. 3a). Although this high stability is ideal for studying position-specific activity, it is likely to be non-physiological. Second, Förster resonance energy transfer (FRET) measurements suggested that the anchored protein is probably confined within a 10 nm region around the anchoring point (Fig. 3b). Finally, the total number of molecules anchored within the kinetochore was determined by the abundance of

The ability of Mps1 to activate the SAC depends on its position within the kinetochore

We first tested whether the binding position of Mps1 within the kinetochore can affect its ability to phosphorylate Spc105 and initiate SAC signalling. In metaphase, the budding yeast kinetochore spans ∼80 nm along its longitudinal axis, from the amino terminus of Ndc80 to the centromeric nucleosome. It contains ∼8 copies of the Ndc80 complex and Spc105 molecules distributed with an average inter-molecular spacing of ∼8 nm around the microtubule circumference, and with little inter-molecular staggering along the length of the microtubule. This architecture suggests that the proximity of Mps1 to Spc105 along the longitudinal axis of the kinetochore can affect its ability to phosphorylate Spc105.

To reveal the position-specific activity of Mps1, rapamycin-induced dimerization must stably anchor and confine it at specific kinetochore positions. We determined this to be the case using three measurements (Fig. 3a–c). First, we found that the anchoring was stable, as indicated by negligible turn-over of Mps1–Frb–GFP anchored at Ndc80-C (Fig. 3a). Although this high stability is ideal for studying position-specific activity, it is likely to be non-physiological. Second, Förster resonance energy transfer (FRET) measurements suggested that the anchored protein is probably confined within a 10 nm region around the anchoring point (Fig. 3b). Finally, the total number of molecules anchored within the kinetochore was determined by the abundance of

See Table 3. Asterisk: known Mad1 localization at the nuclear envelope. Scale bar, ∼3 μm.

| Time after rapamycin addition (min) | Percentage of large-budded cells |
|------------------------------------|----------------------------------|
| 0                                 | 0%                               |
| 60                                | 25%                              |
| 120                               | 50%                              |
| 180                               | 75%                              |
| 240                               | 100%                             |

Figure 5 Phosphorylation of the Spc105 phosphodomain by Mps1 is sufficient to activate the SAC. (a) Schematic of Spc105\textsuperscript{120,329}, the minimal Spc105 phosphodomain. NLS: nuclear localization signal used to send Spc105\textsuperscript{120,329} to the nucleus. (b) Cell-cycle kinetics following rapamycin addition to anchor the phosphorylatable (solid black line) or non-phosphorylatable Spc105\textsuperscript{120,329} (solid grey line) to Mps1-C. The dashed black line shows the cell-cycle progression of the mad2A strain after anchoring Spc105\textsuperscript{120,329} to Mps1. Plotted points represent the average values calculated from 2 independent experiments. More than 50 cells scored for each time point in each trial. The source data are shown in Supplementary Table 3. (c) Localization of Spc105\textsuperscript{120,329} or Spc105\textsuperscript{120,329,6A} when anchored to Mps1. Scale bars, ∼3 μm. (d) Strategy to anchor Spc105\textsuperscript{120,329} at N-Ndc80, and the localization of Spc105\textsuperscript{120,329} at the indicated times after rapamycin addition. Scale bar, ∼3 μm. (e) Recruitment of Mad1 to the kinetochore clusters when Spc105\textsuperscript{120,329} (top) or Spc105\textsuperscript{120,329,6A} (bottom) is anchored at N-Ndc80. Bars represent mean data pooled from 2 independent experiments. At least 45 cells were analysed for each sample in each trial. The source data are shown in Supplementary Table 3. Asterisk: known Mad1 localization at the nuclear envelope. Scale bar, ∼3 μm.
Figure 6 Spc105120:329 activates the SAC only when it is anchored in the outer kinetochore. (a) Representative micrographs of asynchronously dividing cells showing the localization of Spc105120:329 and cell-cycle progression as a function of the anchoring position (indicated at the top; scale bar, ∼3 µm). Large-budded cells with <2 µm separation between kinetochore clusters were characterized as metaphase-arrested cells. (b) Accumulation of metaphase-arrested cells after rapamycin addition, when either Spc105120:329 (solid lines) or its non-phosphorylatable version, Spc105120:329:6A (dashed lines), was anchored at the indicated positions. The experiment was performed once, and more than 70 cells were scored for each time point (source data are shown in Supplementary Table 3). (c) Mad1–mCherry localization after anchoring Spc105120:329 at the indicated positions for 1 h (scale bar, ∼3 µm). The bar graph shows the fraction of metaphase cells that recruit Mad1 to the kinetochores in each case. Bars present average values from 2 independent experiments. Total number of cells analysed in each case is indicated on top of the bars. (d) Top: Cell-cycle progression as in a when a modified version of the Spc105 phosphodomain that includes the Glc7-recruitment motif (Spc1052:329, solid lines) or its non-phosphorylatable version (Spc1052:329:6A, dashed line) was anchored at the indicated kinetochore positions. The experiment was performed once. More than 50 cells were scored for each time point and the source data are shown in Supplementary Table 3. Bottom: Micrographs (scale bar, ∼3 µm) and quantification of kinetochore-localized Bub3–mCherry 45 min after either Spc105120:329 or Spc1052:329 was anchored at Ask1-C in cells arrested in metaphase using CDC20 repression (mean ± 95% confidence interval from n = 102 and 100 kinetochore clusters analysed for Spc105120:329 and Spc1052:329 anchoring, respectively). P values computed using Mann–Whitney test. (e) Map of the SAC activity of the anchored Spc105120:329.

the anchored protein 25 and also its kinetochore anchor 21 (Fig. 3c). As a result, the entire nuclear pool of low-abundance proteins such as Mps1 and Ipl1 was anchored at the selected kinetochore position.

We constitutively anchored Mps1 at six distinct positions selected to sample the 80 nm length of the kinetochore–microtubule attachment (Fig. 3d, top). To assess the effects of anchoring Mps1 on
Spc105 to activate the SAC, the observed phenotypes probably reflect can affect its ability to activate the SAC. As Mps1 must phosphorylate (Supplementary Fig. 3d–g). Mad1 at the same positions did not result in the same phenotypes observed phenotypes (Supplementary Fig. 3c). Finally, the observed phenotypes (Supplementary Fig. 3b). Reducing the outer kinetochore, these differences did not strictly correlate with 50% higher than the number of Mps1 molecules anchored in the inner kinetochore positions was 30– no effect on colony growth (Fig. 3d). Although the number of Mps1 molecules anchored in the outer kinetochore, N-Ndc80 and Ask1-C (a Dam1 complex subunit), had Interestingly, Mps1 anchored at two positions located in the outer kinetochore, N-Ndc80N-Nuf2 N-Ndc80 N-Nuf2 deletion characterized the effects of anchoring Mps1 to the carboxy termini of seven other subunits of the heterodecameric Dam1 complex (Fig. 4a). In addition to Ask1, Mps1 anchored to three other Dam1 subunits did not affect the colony growth (Fig. 4b and Supplementary Fig. 4a). Slow colony growth was probably due to a transient SAC-mediated delay in the cell cycle (Fig. 4c, also Supplementary Fig. 2c). As before, reduced length of the flexible linker fusing the Mps1 kinase domain to Frb did not affect the observed cell-cycle delay (Supplementary Fig. 4b).

Mps1 anchored in the outer kinetochore does not activate the SAC
To confirm that the inability of Mps1 to activate the SAC from the outer kinetochore is only because it cannot phosphorylate Spc105, we characterized the effects of anchoring Mps1 to the carboxy termini of seven other subunits of the heterodecameric Dam1 complex (Fig. 4a). In addition to Ask1, Mps1 anchored to three other Dam1 subunits did not affect the colony growth (Fig. 4b and Supplementary Fig. 4a). Surprisingly, Mps1 anchored to four other subunits delayed colony formation, but did not seem to affect the number of colonies formed (Fig. 4b and Supplementary Fig. 4a). Slow colony growth was probably due to a transient SAC-mediated delay in the cell cycle (Fig. 4c, also Supplementary Fig. 2c). As before, reduced length of the flexible linker fusing the Mps1 kinase domain to Frb did not affect the observed cell-cycle delay (Supplementary Fig. 4b). We also investigated whether the anchored Mps1 in these experiments perturbed Dam1 complex localization and function, because Dam1 subunits are known Mps1 substrates. We quantified the distribution of Dad4 over the mitotic spindle after anchoring Mps1 to other Dam1 subunits (Fig. 4d). Dad4–mCherry co-localized with the anchored Mps1–Frb–GFP in every case, and its distribution was indistinguishable from Dad4 distribution in untreated cells.

the cell cycle, we plated an equal number of cells on control plates and on plates containing rapamycin, and compared the number of colonies formed in each case (Fig. 3d, right). We performed these experiments in heterozygous diploids that also expressed wild-type Mps1, because Mps1 activity is also essential for other cellular functions. Even though the wild-type, diffusible Mps1 provides these essential functions, it is not required for SAC activation (Supplementary Fig. 2a,b). Furthermore, haploids expressing only Mps1–Frb also exhibited identical SAC activation phenotypes (see below and Supplementary Fig. 2c).

When Mps1 was constitutively anchored at four different locations within the inner kinetochore, ranging from Ndc80-C to Ctf19-C, it completely inhibited colony growth (Fig. 3d). MAD2 deletion restored colony growth, indicating that the lack of growth was due to constitutive SAC activation (Supplementary Fig. 3a and Fig. 3d). Interestingly, Mps1 anchored at two positions located in the outer kinetochore, N-Ndc80 and Ask1-C (a Dam1 complex subunit), had no effect on colony growth (Fig. 3d). Although the number of Mps1 molecules anchored in the inner kinetochore positions was 30–50% higher than the number of Mps1 molecules anchored in the outer kinetochore, these differences did not strictly correlate with SAC activation phenotypes (Supplementary Fig. 3b). Reducing the length of the linker between Mps1 and Frb–GFP did not affect the observed phenotypes (Supplementary Fig. 3c). Finally, the observed effects were specific to Mps1: constitutive anchoring of Ip11 or Mad1 at the same positions did not result in the same phenotypes (Supplementary Fig. 3d–g).

These data show that the position of Mps1 within the kinetochore can affect its ability to activate the SAC. As Mps1 must phosphorylate Spc105 to activate the SAC, the observed phenotypes probably reflect whether or not the anchored Mps1 can access the phosphodomain of Spc105. It is also notable that Mps1 activates the SAC from different locations over a 30 nm span (the metaphase separation between Ndc80-C and Ctf19-C), even though its kinase activity is spatially confined to individual anchoring locations. To encounter the confined kinase activity over this wide span, the long and unstructured phosphodomain of Spc105 probably assumes variable conformations.

Figure 7 The proximity between the CH domains of Ndc80 and the phosphodomain of Spc105 within the kinetochore controls SAC signalling. (a) Scatter plot: Proximity ratio measurements for FRET between mCherry–Nuf2 or mCherry–Ndc80 and GFP–Spc105 in attached (metaphase) and unattached (nocodazole-treated) kinetochores. It should be noted that a 113-amino-acid-long unstructured tail connects the N-Ndc80 to the CH domain. Data pooled from 3 independent experiments, horizontal bars represent mean ± 95% confidence interval computed from n = 121, 37, 101 and 49 clusters (left to right). P values were computed using Mann–Whitney test. (b) Cell-cycle kinetics after anchoring Spc105[20,32,33] at the indicated positions in strains expressing spc105-6A. Plotted points represent average values calculated from 2 independent trials. More than 70 cells scored in each trial and the source data are shown in Supplementary Table 3. Scale bar, 3 µm.
Thus, the association of the Dam1 complex with the kinetochore remained unaffected. The separation between kinetochore clusters in rapamycin-treated cells was also indistinguishable from the corresponding length in untreated cells (Fig. 4e). This indicates that force generation at the kinetochore, a process in which the Dam1 complex is the dominant contributor, was not affected. Thus, the anchored Mps1 does not perturb Dam1 complex function, and the observed phenotypes reflect whether or not the anchored Mps1 can phosphorylate Spc105.

The strikingly different phenotypes induced by Mps1 anchored to Dam1 subunits are surprising. This is because dimensions of the Dam1 complex and its narrow distribution along the length of the kinetochore–microtubule attachment suggest that all of the anchoring points are confined within a ~10-nm-wide zone. Although the structure of the Dam1 complex is unknown, it is conceivable that the C termini of Dam1 subunits face towards or away from the centromere (Fig. 4f, arrows). This orientation may in turn constrain the orientation of the anchored Mps1, and determine whether or not it can phosphorylate Spc105 to activate the SAC.

Phosphorylation of Spc105 by Mps1 is sufficient to initiate SAC signalling

Our data show that the physical proximity between the Mps1 kinase and the phosphodomain of Spc105 can control the state of the SAC. Therefore, we investigated whether a forced interaction between the two outside the kinetochore is sufficient to activate the SAC. We engineered a minimal, anchorable phosphodomain comprising residues 120–329 of Spc105 (referred to as Spc105:120–329, Fig. 5a). It contains all 6 MELT motifs, but no known kinetochore-binding activity. When we anchored Spc105:120–329 to Mps1–Fkbp12 in asynchronously dividing cells, the cells arrested in metaphase (Fig. 5b). Spc105:120–329 also localized to kinetochore clusters under these conditions and recruited Mad1 (Fig. 5c and Supplementary Fig. 5a). The kinetochore localization of Mad1 and Spc105:120–329, when the latter anchored to Mps1, is probably mediated by Mps1 binding to the kinetochores. MAD2 deletion abolished the cell-cycle arrest indicating that the arrest resulted from SAC activation (Fig. 5b, dashed line). When Spc105:120–329:6A, the non-phosphorylatable version of Spc105:120–329, was anchored to Mps1, it did not activate the SAC (Fig. 5b,c). Thus, the phosphorylation of MELT motifs in Spc105:120–329 by Mps1 is necessary for the observed cell-cycle arrest.

To examine whether kinetochores contributed to the SAC signalling observed in the above experiment, we used cells carrying ndc10-1, a temperature-sensitive allele of the gene encoding the centromeric protein Ndc10 (ref. 30). At the restrictive temperature, these cells cannot assemble functional kinetochores, and are thus unable to activate the SAC. However, when Spc105:120–329 was anchored to Mps1 at the restrictive temperature, ndc10-1 cells experienced a cell-cycle delay similar to the delay seen in NDC10 cells under the same conditions (Supplementary Fig. 5b). Thus, the SAC signalling induced by the forced interaction between Spc105:120–329 and Mps1 does not require functional kinetochores. Together with our earlier results, these data demonstrate that the interaction between Mps1 and the phosphodomain of Spc105 is both necessary and sufficient to activate the SAC. The kinetochore may primarily serve as the scaffold that makes this interaction sensitive to microtubule attachment.

Spc105:120–329 activates the SAC when anchored in the outer kinetochore, but not the inner kinetochore

Our data reveal a potential organization of Mps1 and Spc105 relative to one another that can make their interaction sensitive to the attachment state of the kinetochore. When Mps1 is anchored in the inner kinetochore, proximal to the phosphodomain of Spc105, it activates the SAC constitutively even from attached kinetochores. In contrast, if it is anchored in the outer kinetochore, distal from the phosphodomain of Spc105, it activates the SAC conditionally, only from unattached kinetochores (Supplementary Fig. 6). Therefore, to implement attachment-sensitive SAC signalling, endogenous Mps1 should bind to a site within the outer kinetochore. Consistent with this expectation, Mps1 physically interacts with the CH domain of Ndc80, which is located in the outer kinetochore.

To investigate whether endogenous Mps1 binds within the outer kinetochore, we anchored Spc105:120–329 at N-Ndc80, proximal to the CH domain (Fig. 5d, top). In metaphase cells, the anchored Spc105:120–329 exhibited the stereotypical, metaphase kinetochore distribution: two distinct puncta separated by ~1 μm. It also recruited Mad1, and the cells remained arrested for a prolonged period (Fig. 5d,e). The cell-cycle arrest was absent when Spc105:120–329:6A was anchored to N-Ndc80, revealing that the phosphorylation of the MELT motifs in Spc105:120–329 by kinetochore-localized Mps1 is required for SAC activation. These results demonstrate that catalytically active Mps1 binds to the outer kinetochore even after stable microtubule attachments form.

We next probed the entire kinetochore for additional Mps1-binding sites (Fig. 6a). When we anchored Spc105:120–329 to Dam1 subunits expected to face towards the outer kinetochore (Ask1-C, Dam1-C, or Dad1-C, see Fig. 4f), the kinetochores recruited Mad1, and the
cells arrested in mitosis (Fig. 6b top and Fig. 6c). Strikingly, when Spc105Δ120-329 was anchored to positions in the inner kinetochore, including the Dam1 subunit termini predicted to face towards the centromere (Dad4-C, Spc34-C and Spc19-C), it had no effect on the cell cycle (Fig. 6b bottom and Fig. 6c). As expected, Spc105Δ120-329:6A did not affect the cell cycle when anchored at any of the positions (dashed lines in Fig. 6b). These results demonstrate that catalytically active Mps1 is absent from the inner kinetochore.

The N terminus of Spc105 localizes to the inner kinetochore and contains a Glc7-binding motif, which is not present in Spc105Δ120-329. Therefore, the lack of Glc7 activity in the outer kinetochore, rather than localized Mps1 activity, could also produce the observed SAC activation phenotypes. To determine whether this is the case, we constructed a phosphodomain that contains the Glc7-binding motif (Spc105Δ1, Fig. 6d). Spc105Δ2-329 anchored at N-Ndc80 or at Ndc80-C produced the same phenotypes as Spc105Δ120-329 (Fig. 6d, top). To determine whether Spc105Δ2-329 recruits Glc7 activity, we anchored either Spc105Δ2-329 or Spc105Δ120-329 to Ask1-C, and quantified the kinetochore-recruitment of Bub3 in each case (Fig. 6d). As Bub3 specifically binds to phosphorylated MELT motifs, significantly reduced Bub3 recruitment in the former case confirmed that Spc105Δ2-329 recruits Glc7 activity (Fig. 6d, bottom).

These data build an activity map for Spc105Δ120-329 and demonstrate that catalytically active Mps1 kinase binds exclusively in the outer kinetochore even after the kinetochore establishes stable microtubule attachment. Strikingly, this map is the mirror image of the activity map for the anchored Mps1 kinase, with the Dam1 complex demarcating the boundary in both maps (Fig. 4f and Fig. 6e). These data strongly suggest that the Dam1 complex may contribute to SAC silencing by acting as a physical barrier that separates the phosphodomain of Spc105 from Mps1.

Separation between CH domains of Ndc80 and N-Spc105 changes with the attachment state of the kinetochore

Our data suggest that microtubule attachment to kinetochores physically separates the CH domains of Ndc80 and the phosphodomain of Spc105 to silence the SAC. By corollary, unattached kinetochores must bring them in close proximity to activate the SAC. To determine whether the separation between these two domains and the attachment state of the kinetochore are correlated, we measured FRET between N-Spc105 and either N-Nuf2 or N-Ndc80, which are proximal to the CH domains (Fig. 7a and Supplementary Fig. 7). In both cases, FRET was undetectable in metaphase as predicted by the >30 nm separation between N-Spc105 and both N-Ndc80 and N-Nuf2 (ref. 20). In contrast, moderate FRET was detected in unattached kinetochores created by treating the cells with nocodazole, indicating that mCherry and GFP fused to the respective N termini were, on average, ~8 nm apart.

Proximity between the CH domains and Spc105Δ120-329 controls SAC signalling in attached kinetochores independently of the endogenous Spc105

Finally, we investigated whether Spc105Δ120-329 can restore the SAC in attached and unattached kinetochores in a position-dependent manner in spc105-6A strains that are SAC-deficient. The kinetochore provides only the architectural scaffold in this experiment. Consistent with the previous results, Spc105Δ120-329 arrested the cell cycle when anchored proximal to the CH domains (at N-Ndc80), but not when anchored distal to the CH domains (at Spc24-C, Fig. 7b). Even within unattached kinetochores, Spc105Δ120-329 restored the SAC when it was anchored at N-Ndc80, as expected (Supplementary Fig. 8). However, Spc105Δ120-329 anchored at Spc24-C also activated the SAC in unattached kinetochores, suggesting that Mps1 can access Spc105Δ120-329, even though its anchoring position is expected to be distal to the CH domains. The inherent flexibility of Ndc80 and Spc105 and the presence of multiple molecules of these proteins in the kinetochore are probably responsible for this unexpected phenotype.

DISCUSSION

Our work yields critical insights into how the protein architecture of the budding yeast kinetochore enables attachment-sensitive SAC signalling (Fig. 8a). We find that catalytically active Mps1 binds to a site located in the outer kinetochore even when the kinetochore is attached. On the basis of our findings and published data, we propose that this site corresponds to the CH domain of Ndc80. We also demonstrate that a persistent interaction between Spc105 and Mps1 is both necessary and sufficient to activate the SAC. These findings lead to an elegant model for the attachment-sensitive operation of the SAC (Fig. 8b,c). In unattached kinetochores, close physical proximity between the CH domains of Ndc80 and the phosphodomain of Spc105 allows Mps1 to phosphorylate Spc105, and also enables subsequent steps in SAC signalling. End-on microtubule attachment to the kinetochore separates the CH domains and the phosphodomain of Spc105 probably by pulling the CH domains outwards and by restraining phosphodomain in the inner kinetochore. Additionally, the Dam1 complex, which is recruited after the formation of end-on attachment, may act as a physical barrier that prevents further interaction between Mps1 and Spc105. A combination of these events leads to SAC silencing.

The control of SAC signalling by the physical separation of two protein domains is conceptually equivalent to the operation of a mechanical switch. As the two terminals of this microtubule-operated switch, the Ndc80 complex and Spc105 must be capable of binding microtubules and changing their positions and/or conformations in response to microtubule binding. Accordingly, the Ndc80 complex binds to microtubules through the CH domains. Known flexibilities in its structure should also allow it to change conformation in response to microtubule binding. Spc105 also binds microtubules, and this may play a role in restraining its otherwise unstructured phosphodomain in the inner kinetochore. Finally, low cellular abundance of the Mps1 kinase is crucial for the effective operation of this mechanical switch. If Mps1 is highly abundant, it can phosphorylate Spc105 through diffusive interactions, cause aberrant SAC activation, and thus effectively override the kinetochore-based switch.

Our work defines the ‘off’ state of the mechanical switch, but further work is needed to define its ‘on’ state. The first key question is whether the CH domain of Ndc80 is the only Mps1-recruitment site that is necessary for SAC signalling. Our findings and published data strongly argue for this to be the case. We find that the phosphorylation of Spc105 by Mps1 is both necessary and sufficient for SAC signalling. Therefore, the only activity that the Ndc80 complex...
can contribute to the SAC is the recruitment of Mps1. Accordingly, the Ndc80 complex is necessary for SAC signalling47,50,51, and its CH domain binds Mps1 (refs 32,33). The second key question is how the architecture of the unattached kinetochore, despite its inherent flexibility, promotes optimal interaction between Mps1 and Spc105. Answers to these questions will further validate and complete the cell biological description of the mechanical-switch model for the SAC.

Whether the mechanical-switch model is applicable to the kinetochore in other eukaryotes is also an important question. Higher eukaryocytes employ additional mechanisms that promote SAC silencing33,52. Moreover, in contrast to our results from budding yeast, the forced localization of Mps1 in the outer kinetochore or Mad1 in the inner kinetochore activates the SAC in other organisms11,37,53,54. These differences may be because the budding yeast kinetochore stably binds exactly one microtubule in metaphase, whereas the kinetochores in most eukaryotes bind dynamically to many microtubules. A fraction of these microtubule-binding sites are unattached even in metaphase55, creating the possibility of cross-phosphorylation of SAC proteins localized in one attachment site by Mps1 localized within adjacent sites. Despite these differences, components of the SAC switch: Mps1, Ndc80 complex and Spc105, and their nanoscale organization are highly conserved from yeast to humans56. Intriguingly, even though the Dam1 complex is absent in humans, the human kinetochore recruits other microtubule-binding proteins in the same position as that of the Dam1 complex in the yeast kinetochore56–59. This striking conservation of key proteins and their architecture suggests that the kinetochore in other eukaryotes may encode a similar mechanical switch to control the SAC.

METHODS

Methods and any associated references are available in the online version of the paper.

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METHODS

Strain and plasmid construction. Budding yeast strains and plasmids used in this study are listed in Supplementary Tables 1 and 2 respectively. Strains used in the anchoring experiments were constructed as described in ref. 9. Briefly, we deleted FBI1 in wild-type strains to eliminate the rapamycin-binding protein product of this gene. These strains also express tor1-1, which encodes the dominant-negative, rapamycin-resistant form of the Tor1 kinase. We ensured that at least one copy of TOR1 in diploid strains was mutated to tor1-1.

Fbr–GFP(S65T) (or Frb alone) was fused to the C terminus of selected SAC proteins with a 24- or 7-amino-acid linker (with the amino acid sequence ‘RIGPLKSGGGGSGGGGSGGAG’ or ‘SGGGGAG’ respectively). Two tandem copies of Fkp12 (2xFkp12) were fused to the C terminus of kinetochore proteins with the linker coding ‘RIGPLKL’ 2xFkp12 was fused to the N terminus of Ndc80 through the linker sequence ‘GAAAAAG’. A 7-amino-acid linker (sequence: ‘RIGPLKIN’ was used to fuse fluorescent proteins (either GFP(S65T) or mCherry) to the amino or carboxy terminus of selected proteins.

Spc105-6A strains were constructed using plasmid shuffling. Briefly, we deleted the genomic copy of SPC105 in a parent strain containing a centromeric plasmid containing SPC105 and the URA3 gene as the auxotrophic marker (p237A4). Next, pSB1878 linearized with NsiI was integrated at the his3 locus (ref. 12). Finally, the centromeric plasmid carrying the wild-type SPC105 was kicked out by counter-selecting for URA3 on the drug 5-FOA.

Plasmids containing the minimal phosphodomain of Spc105, pAJ349 and pAJ350 were constructed by subcloning the PCR amplification product of the phosphodomain of Spc105 (amino acids: 120–329 from pSB1332 for wild-type, or from pSB1878 for the phosphonull version17) into pAFS144 carrying the frb domain using AatII and KslI sites. These plasmids, after linearization with NsiI, were integrated at the his3 locus. For integration at the LEU2 locus, the HIS3 gene in pAJ349 and pAJ350 was replaced with LEU2 to construct pAJ351 and pAJ352, respectively. The plasmids were linearized with BstEII for integration at the leu2 locus.

Cell culture. Cells were grown in yeast extract, peptone and dextrose (YPD) media at 32 °C and imaged at room temperature in synthetic media supplemented with essential amino acids and an appropriate carbon source. To express N-terminally tagged kinetochore proteins from the galactose promoter (pGAL1), strains were grown in YP Raffinose media supplemented with 0.1–0.4% galactose. The galactose concentration was adjusted empirically as described previously28.

Stock solution (1 mg ml−1) of rapamycin in DMSO was diluted 1,000 to achieve 1 µg ml−1 final concentration in all experiments involving rapamycin-induced dimerization.

To depolymerize metaphase spindles with nocodazole26, mid-log-phase cells were synchronized in G1 with α-factor (2 µg ml−1) for 2 h and then released into nocodazole-containing media (15 µg ml−1) for 1.5–2 h.

Benomyl sensitivity assay. Tenfold serial dilutions of log-phase cultures were spread on YPD or plates containing (30 µg ml−1) of 5-FOA. The plates were then treated with nocodazole to depolymerize the spindle and activate the SAC.

Metaphase arrest by CDC20 repression. Cells expressing Cdc20 from a methionine-repressible promoter (pMET3) were synchronized in G1 by treatment with 100 µg ml−1 of liquid cultures measured at 660 nm) were plated on control and rapamycin-containing plates. After allowing the colonies to grow for 3 days at 30 °C, we determined the colony number. We ensured that the strains used in this experiment were rapamycin-resistant, by verifying that the parental haploid strains expressing either the Frb-fused SAC protein or the Fkbp12-fused kinetochore protein produced the same number of colonies on both control and rapamycin-containing plates.

Microscopy and image acquisition. A Nikon Ti-E inverted microscope with a 1.4 NA, 100×, oil-immersion objective was used in imaging3. A ten-plane Z-stack was acquired (200 nm separation between adjacent planes). The total fluorescence from each kinetochore cluster with GFP- or mCherry-tagged protein was measured using ImageJ, or a semi-automated MATLAB program as described earlier31. The copy numbers of kinetochore proteins and anchored proteins were calculated from the known copy number of the Ndc80 complex per kinetochore—8 molecules per kinetochore32.

For photobleaching, an argon-ion laser (Photonics Instruments) beam filtered with the ET-GFP filter cube was focused on the sample by the objective. The target was manually aligned with the pre-determined location of the laser focus, and then exposed to 488 nm light for 50 ms. Five-plane Z-stacks were acquired starting immediately after bleaching for 14 min, at 2 min intervals. Fluorescence was quantified from the images as above. FRET, high-resolution co-localization and fluorescence distribution analyses were conducted as previously described26,29,30.

Time-lapse imaging was used to follow the Mps1–Frb–GFP that autonomously bound to the kinetochore clusters in metaphase-arrested cells. Cells were released from the metaphase arrest by activates CDC20 expression, and a 6-plane Z-stack was acquired every 1 min intervals for 20 min. Anaphase entry was inferred from spindle elongation tracked from the spindle pole body protein (Spc97–mCherry). The change in Mps1–Frb–GFP intensity during this period was quantified, after correcting for two factors: GFP photobleaching expected from imaging and; fluorescence emission from Spc97–mCherry due to cross-excitation while imaging GFP. The representative images in Supplementary Fig. 1f have not been corrected for two factors: GFP photobleaching expected from imaging and; fluorescence emission from Spc97–mCherry due to cross-excitation while imaging GFP. The representative images in Supplementary Fig. 1f have not been corrected for two factors: GFP photobleaching expected from imaging and; fluorescence emission from Spc97–mCherry due to cross-excitation while imaging GFP.

Statistical methods. Comparisons of proximity ratio measurements were conducted using the non-parametric Mann–Whitney test. The statistical tests used are described in the respective figure legends.

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Supplementary Figure 1 Effects of anchoring key SAC regulators to Mtw1-C on the cell cycle. (a) Top: Representative images display the expected localization of SAC proteins tagged with Frb-GFP in untreated cells and one hour after the addition of rapamycin. Bottom: Benomyl sensitivity of indicated strains. (b) Representative transmitted light micrographs of four strains treated with rapamycin for 135 minutes to anchor Mps1, Ipl1, Mad1, or Glc7, at Mtw1-C. The bar graph displays the percentage of large-budded in each case averaged from two independent experiments (more than 50 cells were scored in each trial). Source data are shown in Supplementary Table 3. (c) Effect of the ATP analog 1-NAPP1 on the localization of the Ipl1 substrate Sli15-GFP in cells expressing ipl1-as6, an analog-sensitive allele of the Ipl1 kinase. Representative pre-anaphase cells expressing Sli15-GFP are shown on the right. Quantification of Sli15-GFP fluorescence on the spindle (mean ± s.d. from a single experiment; number of cells scored are displayed at the top) shows that the analog inhibits ipl1-as6. (d) Cell cycle kinetics following the release of S-phase synchronized cells into media containing 1-NAPP1 and rapamycin. Blocking ipl1-as6 activity did not have any effect on SAC activation induced by Mps1 anchored at Mtw1-C. The experiment was performed once and more than 70 cells were scored for each time point (source data are shown in Supplementary Table 3). (e) Bar graph: Frequency of prometaphase and metaphase cells with kinetochore-localized Mps1 (representative micrographs displayed at the top; 18, 12 and 45 cells were analyzed, from left to right). Spindle length was used to classify cells as prometaphase or metaphase cells. Scatter plot (mean ± 95% confidence interval; n = 21, 46 and 66 kinetochore clusters from left to right) displays the abundance of kinetochore-localized Mps1-Frb-GFP in prometaphase, metaphase-arrested cells (by repressing CDC20), and when it is anchored to Mtw1-C in heterozygous diploid strains. Each experiment was performed once. (f) Quantification of Mps1 localization to kinetochores soon after release from metaphase compared to that in anaphase (n = 10 from one experiment). Micrographs on the right show localization of Mps1 relative to spindle pole bodies over a period of 6 minutes during the metaphase to anaphase transition.
Supplementary Figure 2 Kinase activity of the kinetochore-anchored Mps1 is sufficient for SAC activation. (a) Cells expressing the analog-sensitive Mps1 allele\textsuperscript{65}, mps1-as1 or wild type Mps1 were treated as indicated at the top. Bar graph displays the percentage of two-budded cells (which form when a mitotic cell fails to sustain the SAC in the presence of a damaged spindle and produce a new bud by re-entering the cell cycle). Bars indicate the average value based on data from two independent experiments. The total number of cells scored is indicated at the top, and the source data are shown in Supplementary Table 3. (b) Inhibition of the diffusible mps1-as1 does not affect SAC activation by Mps1-Frb anchored at the kinetochore. Heterozygous diploid strains expressing mps1-as1 and Mps1-Frb were synchronized in S-phase and released into 1-NMPP1 for 15 minutes. Rapamycin was then added to anchor Mps1-Frb at Mtw1-C. The anchored Mps1 arrested the cell cycle robustly, and the cells retained the large-budded morphology for a prolonged period of time. The experiment was performed once, and more than 50 cells scored for each time point (the source data are shown in Supplementary Table 3). (c) Micrographs: Mad1 localization relative to the spindle pole body in haploid cells that have Mps1 anchored to the indicated subunit. Bar graph displays the percentage of cells with visible Mad1 localization in between the spindle poles in each case (number of cells scored in one experiment indicated on top). The corresponding metaphase spindle length in each case is presented in the scatter plot (range: 1.8-1.9 ± 0.3 μm; mean ± 95% confidence interval from n = 15, 14, 25, 23 and 33 cells from left to right).
Supplementary Figure 3 Cell cycle effects of anchoring Mps1, Ipl1 or Mad1 constitutively within the kinetochore. (a) Cell cycle kinetics of asynchronous cultures where Mps1 is anchored at the C-termini of indicated kinetochore subunits in wild-type or SAC null strains (mad2Δ). The experiment was performed once. More than 50 cells were scored for each time point. The source data are shown in Supplementary Table 3. (b) Quantification of Mps1-Frb-GFP (mean ± 95% confidence interval; n = 35, 36, 47, 43, 27, 26, 33, 31, 66, 35 and 46 kinetochore clusters from left to right) anchored at indicated kinetochore subunits measured 45 minutes after rapamycin treatment and normalized relative to endogenous Mps1 in metaphase-arrested cells. Note that the recruitment of Mps1 at Dad4-C and Ctf19-C that activates the SAC is comparable to that at Ask1-C which does not activate the SAC (see Supplementary Fig. 2c). (c) Reducing the length of the flexible linker connecting Mps1 and Frb (from 24 to 7 amino acids) did not change the effect of anchoring Mps1 on colony growth. Bars represent the average values calculated from two independent experiments. Cumulative number of colonies counted is displayed at the bottom. The source data are shown in Supplementary Table 3. (d-e) The number of colonies formed on rapamycin-containing plates relative to control plates, when Ipl1 (in d) or Mad1 (in e) is constitutively anchored at the indicated positions. Bars represent the average values calculated from 3 or 4 independent experiments. The source data are shown in Supplementary Table 3. The number of colonies on rapamycin-containing plates exceeding that on the control is likely due to pipetting errors. Reduced number of colonies upon anchoring Ipl1-Frb-GFP at Ndc80-C and N-Ndc80 may be due to attachment defects caused by Ipl1-mediated phosphorylation of kinetochore proteins. The loss of colony formation upon Mad1 anchoring at N-Ndc80 is also likely due to attachment defects (see f-g). (f) Mad1 anchored at N-Ndc80 generates unattached kinetochores (arrowheads) in a large fraction of cells (60 out of 108 cells had visible defects in kinetochore cluster morphology). (g) Graph presents the fraction of cells expressing Spc105-6A or Spc105 that arrested with large-buds when Mad1 was anchored at N-Ndc80 (rapamycin treatment for 4 hours). The experiment was performed once. The number of cells scored is indicated on top of bars.
Supplementary Figure 4 Anchoring Mps1 to Dam1 subunits leads to different phenotypes. (a) Subunit organization of the Dam1 complex defined in ref. 27 is color-coded according to the scheme displayed on the right. The untested subunits are Duo1 and Hsk4. Bar graph displays the number of colonies formed on rapamycin relative to the control plates. Bars represent averages from two independent experiments. The total numbers of colonies scored are displayed at the bottom. The source data are also shown in Supplementary Table 3. (b) Cell cycle kinetics of rapamycin treated (to anchor Mps1 at indicated subunits) or untreated (control) cells. The experiment was performed once and more than 60 cells were scored for each time point; the source data are shown in Supplementary Table 3. Since Mps1 anchored at Dad2-C, Dad4-C or Spc34-C transiently activated the SAC, we tested if shortening the linker connecting Mps1 and Frb (from 24 to 7 amino acids, also See Supplementary Fig. 3c) eliminates this transient SAC activation by restricting access to N-Spc105. However, anchored Mps1 activated the SAC in spite of the shorter linker.
**Supplementary Figure 5** SAC signaling induced by rapamycin-induced dimerization of Spc105\(^{120.329}\) and Mps1 does not require functional kinetochores. (a) Representative images show Spc105\(^{120.329}\) anchored to Mps1 (rapamycin treatment for 45 minutes) localizing to the kinetochores. Mad1 also co-localizes with these kinetochore clusters. (b) Cells carrying the temperature-sensitive ndc10-1 allele and expressing Spc105\(^{120.329}\) and Mps1-Fkbp12 were treated as indicated at the top. When released at the restrictive temperature from G1 arrest, these cells go through the cell cycle without assembling functional kinetochores and fail in cytokinesis\(^{66}\), and give rise to cells with two buds (black bars; also see transmitted light micrograph top-right). However, when the same experiment was conducted in rapamycin containing media, the emergence of two budded cells was delayed by an hour (light gray bars). We attribute this delay to SAC activation, which is also observed when Spc105\(^{120.329}\) is anchored to Mps1 in NDC10 cells at 37°C (dark gray bars). Bars represent averages from 2 independent experiments. The source data are shown in Supplementary Table 3.
**Supplementary Figure 6** SAC signaling induced by Mps1 anchored at N-Ndc80 depends on the attachment-state of the kinetochore. S-phase synchronized cells were treated as indicated in the schematic at the top and the percentage of large-budded cells formed after 100 minutes was measured as an indicator of cell cycle arrest. Mps1 anchored at Mtw1-C constitutively activated the SAC in the presence of attachments and in nocodazole. However, Mps1 anchored at N-Ndc80 allowed normal cell cycle progression and caused cell cycle arrest only in the presence of unattached kinetochores in nocodazole. Bars represent the average from 2 independent experiments. More than 50 cells were scored for each condition in each experiment. The source data are shown in Supplementary Table 3.
Supplementary Figure 7 Effect of spindle disruption on SAC protein recruitment and kinetochore architecture. (a) Spindle disruption with nocodazole generates two or three kinetochore clusters within the nuclei of most budding yeast cells as reported previously. The cluster that contained majority of the kinetochores (large, asterisks) localized proximal to the collapsed spindle pole bodies (visualized by Spc97-GFP). One or two smaller kinetochore clusters (small, arrowheads) were found distal to the spindle pole bodies. Bar graph displays the percentage of large or small clusters that are proximal to the spindle pole body. The cumulative numbers of clusters scored in 2 independent experiments are indicated at the bottom. (b) Consistent with Gillett et al. 2004, the smaller kinetochore clusters (arrowheads) in nocodazole recruit significantly higher levels of Mps1 and Bub1 than the large cluster. Mad1 was undetectable at the large clusters (bars indicate average from 2 independent experiments). 76 out of 82 smaller clusters recruited Mad1, but only 3 out of 65 large clusters had detectable Mad1. Therefore, we classify the smaller clusters as SAC-active and the larger clusters as SAC-inactive. (c) Dam1 complex (visualized with Ask1-mCherry) is retained at the SAC-inactive cluster, whereas it is significantly reduced at the SAC-active clusters in nocodazole. Quantification of Ask1-mCherry fluorescence measured relative to Spc24-mCherry fluorescence is displayed on the right. The experiment was performed once and horizontal bars represent mean ± 95% confidence intervals, n = 33, 72, 45 and 81 kinetochore clusters (left to right). Since microtubule attachment is necessary for Dam1 recruitment to the kinetochore, this observation suggests that the kinetochores located proximal to the collapsed spindle pole bodies retain microtubule attachment. (d) Measurement of FRET between GFP-Spc105 and either mCherry-Nuf2 or mCherry-Ndc80 in SAC-active and SAC-inactive kinetochore clusters. The horizontal bars represent mean ± 95% confidence interval; data pooled from more than two independent experiments. P-values were computed using non-parametric Mann-Whitney test; n = 121, 39, 110, 87, 101, 49, 90 and 47 kinetochore clusters from left to right). FRET between mCherry-Nuf2 or mCherry-Ndc80 and GFP-Spc105 in the SAC-inactive kinetochore cluster is higher than metaphase FRET value, and significantly lower than the FRET observed in the SAC-active cluster (~50%, p-value ~ 0.04). Note that the significantly higher FRET in anaphase compared to metaphase clusters is consistent with the previously reported reduction in the length of Ndc80 complex in anaphase.
Supplementary Figure 8 Spc105\textsuperscript{120,329} restores the SAC when it is anchored to unattached kinetochores in SAC-null strains. Top: Experimental scheme. Bar graph: Fraction of nocodazole-treated cells with two buds in the presence and absence of rapamycin in cells expressing spc105-6A (see micrographs on the left). Note the diffuse nuclear localization of Spc105\textsuperscript{120,329} in the absence of rapamycin. When Spc105\textsuperscript{120,329} was anchored either at N-Ndc80 or at Spc24-C, it restored the SAC. The cells arrested with large buds (transmitted light micrograph on the right). In this condition, Spc105\textsuperscript{120,329} is visible as multiple puncta corresponding to kinetochore clusters that form when budding yeast cells are treated with nocodazole (see Supplementary Fig. 7). Data represent mean from 2 independent trials. More than 100 cells were scored for each treatment. The source data are shown in Supplementary Table 3.
**Supplementary Table 1** Strains used in this study

- Strains derived from YEF473 background.  
- Strains made by crossing BY4741 and S288C. All other strains used in this study were derived from BY4743 (AJY24). In haploid strains generated by crossing BY474 background parents, we did not keep track of the LYS2 and MET15 auxotrophic markers. Supplementary figures are referred as S1 through S8.
**Supplementary Table 2** Plasmids used in this study
See experimental methods for details on plasmid construction.

| Plasmid     | Description                                                                 | Source         |
|-------------|-----------------------------------------------------------------------------|----------------|
| pAJ274      | yCP50-SPC105                                                                | This study     |
| pSB1878     | pSPC105-spc105(T149A, T172A, T211A, T235A, T284A, T313A)-12MYC               | Biggens lab   |
| pAJ248      | pRS306 mps1-as1                                                             | Winey lab     |
| pAFS144     | pHIS3-GFP(S65T)-LacI2-HIS3                                                  | Murray lab    |
| pAJ349      | Spc105<sup>120-329</sup> (pRS303 GFP(S65T)-Spc105(120-329)-FRB-NLS)         | This study     |
| pAJ350      | Spc105<sup>120-329</sup>-6A (pRS303 GFP(S65T)-Spc105-6A(120-329: T149A, T172A, T211A, T235A, T284A, T313A)-FRB-NLS) | This study     |
| pAJ351      | Spc105<sup>120-329</sup> (pRS305 GFP(S65T)-Spc105(120-329)-NLS-FRB)          | This study     |
| pAJ352      | Spc105<sup>120-329</sup>-6A (pRS305 GFP(S65T)-Spc105-6A(120-329: T149A, T172A, T211A, T235A, T284A, T313A)-FRB-NLS) | This study     |
| pAJ389      | Spc105<sup>2-329</sup> (pRS303 GFP(S65T)-Spc105(2-329)-FRB-NLS)              | This study     |
| pAJ390      | Spc105<sup>2-329</sup>-6A (pRS303 GFP(S65T)-Spc105-6A(2-329: T149A, T172A, T211A, T235A, T284A, T313A)-FRB-NLS) | This study     |
Supplementary Table 3  Source data used in generating the figures
#Cells - the total number of cells scored or analyzed in each experiment; %L.B. - percentage of cells that were large-budded; %Arrested - Percentage of cells arrested in metaphase; %meta - percentage of cells in metaphase; S. fig - Supplementary figure

| #Cells | %L.B. | %Arrested | %meta | S. fig |
|--------|-------|-----------|-------|--------|
| 100    | 0.5   | 0.2       | 0.4   |        |
| 200    | 0.7   | 0.3       | 0.5   |        |
| 300    | 0.8   | 0.4       | 0.6   |        |
| 400    | 0.9   | 0.5       | 0.7   |        |
| 500    | 1.0   | 0.6       | 0.8   |        |

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