BubR1 recruitment to the kinetochore via Bub1 enhances spindle assembly checkpoint signaling

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The spindle assembly checkpoint (SAC) is an important cell cycle control mechanism that minimizes chromosome missegregation during cell division. It is activated when unattached kinetochores are not stably attached to the plus ends of spindle microtubules. This leads to the generation of a diffusible anaphase-inhibitory signal, known as the mitotic checkpoint complex (MCC). The MCC delays anaphase onset to avert cell division in the presence of unattached kinetochores.

The rate at which an unattached kinetochore generates the MCC depends on its ability to recruit SAC signaling proteins, which include constituent proteins of the MCC: Bub1-Bub3, BubR1-Bub3, Mad1-Mad2, and Cdc20 (Figure 1A, dashed square). This signaling cascade generates either the MCC itself, its subcomplex C-Mad2-Cdc20, or both. Given this knowledge, it is reasonable to expect that the rate of MCC generation at a kinetochore will increase with higher recruitment of the MCC components to the kinetochore (Collin et al., 2013; Lara-Gonzalez et al., 2021a; Piano et al., 2021). Interestingly, however, this expectation appears to not hold true for BubR1. Disruption of BubR1 recruitment to unattached kinetochores does not reduce the duration of SAC-induced mitotic arrest in nocodazole-treated cells (Overlack et al., 2015; Zhang et al., 2015). This is because BubR1 recruits to the kinetochore protein phosphatase 2A (PP2A), which promotes SAC silencing (Foley et al., 2011; Nijenhuis et al., 2014; Qian et al., 2017). Despite this knowledge, it is crucial to determine whether BubR1 recruitment to unattached kinetochores also promotes SAC signaling by enhancing MCC assembly. This BubR1 activity can be crucial for minimizing chromosome missegregation during normal cell division wherein a small number of unattached kinetochores must activate the SAC and delay anaphase onset (Roy et al., 2020).
the other hand, the recruitment of Bub1 and BubR1 via the “KI” motifs in the kinetochore protein Knl1 does not contribute to MCC generation mediated by the “MELT” motifs within the Knl1 phosphodomain (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011; Primorac et al., 2013; Roy et al., 2019). Impor-
tantly, the extended mitosis was due to increased Mad2-Cdc20 and
Bub1-mediated SAC signaling. We also establish a mathematical model to elucidate the mechanistic details of the SAC signaling cascade that generates the MCC. Finally, we also demonstrate that BubR1 recruitment to the kinetochore via Bub1 promotes SAC signaling.

RESULTS AND DISCUSSION

The BubR1-binding domain of Bub1 promotes Bub1-mediated MCC assembly

Bub1 coordinates the rate-limiting step in MCC assembly: the formation of the closed-Mad2-Cdc20 (Mad2:Cdc20) subcomplex (Faesen et al., 2017; Lara-Gonzalez et al., 2021; Piano et al., 2021). Mad2:Cdc20 must bind BubR1 to complete MCC formation. Bub1 is recruited to the kinetochore by Bub1, and the KI motifs and MELT motifs in KNL1 (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011; Overlack et al., 2015; Zhang et al., 2016). Whether this BubR1 recruitment promotes MCC formation remains unclear. In fission yeast, Bub1 binding to Bub1 is essential for SAC activity (Leontiou et al., 2019). However, BubR1 is unlikely to be recruited to budding yeast kinetochores (Tromer et al., 2016; Roy et al., 2022), and in nocodazole-treated HeLa cells, the disruption of Bub1-mediated Bub1R1 recruitment slightly strengthens the SAC (Overlack et al., 2015; Zhang et al., 2016). The latter phenotype arises because the disruption of Bub1R1 recruitment also disrupts Bub1R1-mediated PP2A recruitment to the kinetochore (Elowe et al., 2007; Suijkerbuijk et al., 2012). Therefore, to isolate and quantitatively define the effect of Bub1-BubR1 heterodimerization on Bub1-mediated MCC assembly, we used the eSAC system.

Forced dimerization of a fragment of the central domain of Bub1 with Mps1 delays anaphase onset in HeLa cells, budding yeast, and fission yeast (Aravamudhan et al., 2015; Yuan et al., 2017; Chen et al., 2019; Leontiou et al., 2019). In HeLa cells, induced dimerization of the central domain of Bub1 (Bub1<sup>1231-620</sup>-mNG-2xFkbp12; diagram in Figure 1B) with the Mps1 kinase domain (Frβ-mCherry-Mps1<sup>500-817</sup>) produces a dosage-dependent increase in the duration of mitosis with a maximal duration of ~145 min (Figure 1C, replotted for comparison from Chen et al., 2019). Importantly, the extended mitosis was due to increased Mad2-Cdc20 and

In this study, we examine the contribution of Bub1 recruitment to kinetochore-mediated SAC signaling. Using the ectopic SAC activation (eSAC) system, we find that the binding of Bub1 to Bub1 elevates Bub1-mediated MCC generation (Chen et al., 2019). On
MCC formation in HeLa cells and fission yeast (Leontiou et al., 2019; Roy et al., 2022). To assess the contribution of Bub1-BubR1 heterodimerization to Bub1-mediated MCC assembly, we created a truncated Bub1 phosphodomain lacking the BubR1 heterodimerization domain (Bub1144-620-mNG-2xFkbp12) (Overlack et al., 2015; Zhang et al., 2016). Rapamycin-induced dimerization of this phosphodomain with Frb-mCherry-Mps11500-1617 elicited a significantly weaker eSAC activity, with a maximal mitotic duration of 105 ± 6 min (Figure 1C, estimated from a fit with the four-parameter Hill equation; the range includes 95% confidence intervals; see Materials and Methods for details). We previously found that deletion of the Bub3-binding GLEBS domain from the Bub1 phosphodomain does not decrease its eSAC activity (Roy et al., 2022). Therefore, Bub1-BubR1 heterodimerization promotes MCC formation mediated by the Bub1 phosphodomain. It should be noted that we cannot rule out the possibility that the large truncation used here affects Mad1 interaction with the eSAC to some extent.

Simulation of the signaling activity of the Bub1 phosphodoms

To quantitatively understand the dose-response dependence of the eSAC system, we constructed a mathematical model by considering the events at the Bub1 phosphodomain before MCC assembly. This model consists of two stages. In the first stage, we calculate the steady-state concentrations of signaling complexes assembled by the Bub1 eSAC phosphodoms assuming mass action kinetics (Figure 1D; Eqs. 1–4 and S6–S5j). Bub1231-620 recruits BubR1 and Cdc20 independently of its phosphorylation state or the presence of other bound proteins; Bub1144-620 recruits only Cdc20 (Di Fiore et al., 2015; Overlack et al., 2015). Both phosphodoms are activated by Mps1-mediated phosphorylation, after which they recruit Mad1-Mad2 (abbreviated as Mad1/2) (London and Biggins, 2014; Faesen et al., 2017; Ji et al., 2017; Zhang et al., 2017). Therefore, the signaling activity of each phosphodomain will be proportional to the amount of Frb-mCherry-Mps11500-1617, that is, the eSAC dosage in the cell, and it will be limited by the cellular Mad1/2 abundance when the eSAC dosage exceeds Mad1/2 abundance (shown later; see Supplemental Figure S1A). Through these interactions, Bub1231-620 can assemble two types of signaling complexes: one that contains Cdc20, BubR1, and Mad1/2 and one containing only Cdc20 and Mad1/2; Bub1144-620 forms only the signaling complex containing Mad1/2 and Cdc20 (Figure 1D, left).

We simulated MCC formation by the Bub1:BubR1:Mad1/2:Cdc20 signaling complexes as follows. Because Mad2:Cdc20 formation is the rate-limiting step in MCC assembly (Faesen et al., 2017), the Bub1231-620:Bub1:Mad1/2:Cdc20 signaling complex first assembles Mad2:Cdc20 with the rate constant kasmcc1. The newly formed Mad2:Cdc20 can bind BubR1 either within the signaling complex with the rate constant kasmcc2 or in the cytosol with the rate constant kasmcc1 (Figure 1D, bottom). The model assumes that all the Mad2:Cdc20 formed by Bub1231-620:Bub1:Mad1/2:Cdc20 binds BubR1 within the signaling complex. However, allowing a reasonable fraction of Mad2:Cdc20 to escape from the signaling complex does not affect the overall behavior of the model (unpublished data). Because Mad2:Cdc20 formation is the rate-limiting step, we reduce the number of free parameters by assuming that kasmcc1 and kasmcc2 are equal. The cumulative MCC formed by Bub1231-620 is the sum of MCC formed from these two processes. All Mad2:Cdc20 generated by Bub1144-620:Mad1/2:Cdc20 form MCC in the cytosol. In the second stage of the model (Figure 1D; Eqs. S5–S6; Materials and Methods), the MCC formed by both processes modulates cyclin B degradation and thereby controls the timing of metaphase-to-anaphase transition (Supplemental Figure S1B) (He et al., 2011; Chen et al., 2019).

We first simulated the dose-response curve for the Bub1144-620 eSAC involving only cytoplasmic MCC assembly. We retained protein concentrations used in the original model of He et al. (2011) and used reasonable rate constants for Mad2:Cdc20 formation (kasmcc1) and cytosolic MCC formation (kasmcc2). Figure 1E, red curve; see Supplemental Table S1 for protein concentrations and rate constants used). Next, we considered MCC formation by the Bub11231-620 eSAC, which can assemble the MCC within the signaling complex or the cytosol. If BubR1 recruited by the signaling complex does not participate in MCC formation (i.e., kasmcc2 = 0), the simulation produced a dose-response curve with a lower maximal response (dotted blue line in Figure 1E). This is because BubR1 bound to Bub1 cannot participate in any form of MCC assembly. Therefore, the only effect of Bub1-BubR1 heterodimerization is a reduced cytosolic BubR1 concentration and, consequently, a correspondingly reduced rate of MCC generation. This model prediction is inconsistent with the data. Therefore, BubR1 recruited by Bub1 must promote MCC formation within the eSAC-signaling complex. Following this insight, we assumed that kasmcc2 is 10-fold higher than kasmcc1 for cytosolic MCC assembly. With this change, the simulation produced a longer delay in anaphase onset, matching our observations.

This simulation provides two insights. First, it supports the observation that Bub1-BubR1 heterodimerization promotes MCC formation by the eSAC based on the Bub1 phosphodomain. It also indicates that if Bub1-BubR1 heterodimerization does not promote MCC formation, it will lead to BubR1 sequestration and a reduced rate of cytosolic MCC formation. This effect plays a critical role in the experiments that follow.

KI motifs suppress the signaling strength of the eSAC phosphodomain

The KI motifs, so named because they contain lysine and isoleucine residues critical for their activities, recruit Bub1 and BubR1 to the human kinetochore (Figure 1A). The first KI motif (K1) is thought to exclusively bind Bub1, whereas the second motif (K2) binds BubR1 (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011; Krenn et al., 2014, 2012). A prior study concluded that the KI motifs cooperate with the first MELT motif in KNL1 to strengthen SAC signaling (Niegel et al., 2013; Krenn et al., 2014). However, it is unclear whether the activity of regulatory enzymes (e.g., PLK1, PP2A) bound to Bub1 and BubR1 plays a role in these observations (Nijenhuis et al., 2014; von Schubert et al., 2015; Jia et al., 2016). Therefore, to test whether the KI motifs directly promote MCC assembly and delineate their roles, we developed an eSAC phosphodomain comprising the two KI motifs and a MELT motif (Knl1160-256) (Figure 2A). We also created a variant phosphodomain with inactive KI motifs (Krenn et al., 2012). If Bub1 and BubR1 recruitment via the KI motifs enhances MELT motif activity, this will be apparent as increased signaling strength of this new eSAC phosphodomain compared with that of the variant phosphodomain with inactive KI motifs.

We first determined the dependence of mitotic duration on the dosage of the variant phosphodomain with inactive KI motifs (M1-KI1*-K12*-mNG-2xFkbp12; Figure 2A, left). The maximal response for this phosphodomain was higher than the previously defined maximal response for the eSAC phosphodomain containing the 12th MELT motif alone (211 vs. 157 min with ±95% confidence intervals of 169–357 and 152–163. Respectively, predicted by a four-parameter sigmoidal fit to the binned data). This difference likely results from different Bub1-Bub3 binding affinities of the first and 12th MELT motifs (Chen et al., 2019). Interestingly, the phosphodomain with
FIGURE 2: Characterization of the binding of the KI motifs in the eSAC phosphodomain with Bub1 and BubR1 and its contribution to eSAC signaling. (A) Left: Schematic of the two phosphodomains used to test whether the KI motifs contribute to MCC assembly mediated by the MELT motif in the eSAC phosphodomain. K11-KI2 indicates phosphodomain with intact KI motifs; K11*-KI2* indicates phosphodomains wherein the KI motifs are inactivated using suitable point mutations (see Materials and Methods for details). The scatter plot in the middle displays the dose-response data for the two phosphodomains (n = 1888 for K11-KI2 [green] and n = 836 for K11*-KI2* [magenta] from ≥2 technical replicates; symbol usage follows the scheme established in Figure 1C). The bar graph on the right displays the maximal mitotic duration predicted by four-parameter sigmoidal fits to the binned mean values as in Figure 1C. Vertical lines display 95% confidence intervals on the fit parameter. (B) Left: Schematic of the phosphodomains consisting of four MELT motifs and either active or inactive (indicated by *) KI motifs. Right: Immunoprecipitation of the eSAC phosphodomain using mNeonGreen-Trap beads followed by immunoblot analysis to probe for the coimmunoprecipitate of the indicated proteins. This experiment was performed once. (C) Dose-response data for the indicated phosphodomains. Only the mean values of binned data are shown for clarity. Data analysis performed as in Figure 1D (n = 1019, 1024, 666, and 3219 from ≥2 technical replicates for K11-KI2, K11-KI2*, K11*-K12, and K11*-K12*, respectively). The bar graph on the right displays the maximal time in mitosis of the fitted maximal mitotic duration (error bars represent 95% confidence intervals).

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active KI motifs (M1-K11-KI2) has a significantly lower signaling strength: the eSAC concentration required for half-maximal response nearly doubled (half maximal effective concentration or EC50 = 9.7 and 19.4 a.u. respectively; Figure 2A, middle). The decreased signaling strength was eventually compensated by high eSAC dosage as evidenced by the maximal mitotic duration at high eSAC dosage (Figure 2A, middle and right). These data suggest that the Bub1 and BubR1 molecules recruited by the KI motifs do not enhance eSAC signaling mediated by the upstream MELT motif.

Bub1 and BubR1 interactions with the KI motifs do not require Mps1-mediated phosphorylation of KNL1

To better understand the observed suppression of eSAC activity by the KI motifs, we constructed a new phosphodomain by fusing an unstructured region of the Knl1 phosphodomain spanning three previously characterized MELT motifs (Knl1[1881-1014]) to the C-terminus of the phosphodomain used above (Knl1[160-256], also referred to as “M3”; see Figure 2B) (Vleugel et al., 2015; Chen et al., 2019). By incorporating multiple MELT motifs in the phosphodomain, we wanted to test whether the KI motifs influence the ability of the four MELT motifs in the phosphodomain to engage in synergistic signaling (Chen et al., 2019). On the basis of a previous study, we created mutant phosphodomains wherein the KI motifs were inactive individually or together (K11*-K12, K11-KI2*, and K11*-K12*; the asterisk denotes a loss of function; Figure 2B) (Krenn et al., 2012).

We first determined whether the two KI motifs interact with Bub1 and BubR1 exclusively and whether the phosphorylation of MELT motifs is necessary for these interactions. We immunoprecipitated the four mNeonGreen-tagged eSAC phosphodomain from whole-cell extracts of mitotic HeLa cells in the absence of the Mps1 kinase domain using mNeonTrap beads and probed the precipitates for Bub1 and BubR1 (Materials and Methods). When both KI motifs were active (K11-KI2), Bub1 and BubR1 coprecipitated with the eSAC phosphodomain. As expected, Bub1 and BubR1 did not coprecipitate with the eSAC phosphodomain containing inactive KI motifs (K11*-K12*; Figure 2B). Surprisingly, with the first KI motif inactive (K11*-K12), which interacts with Bub1 alone, Bub1 still coprecipitated with the phosphodomain, albeit at a lower level. Moreover, BubR1 coprecipitation was reduced. Inactivation of the second KI motif (K11-KI2*) made BubR1 undetectable in the precipitate and reduced the amount of Bub1. These data can be explained by the heterodimerization between Bub1 and BubR1, although it remains possible that the second KI motif can interact with Bub1. We obtained similar results from immunoprecipitation experiments involving eSAC phosphodomains with only one MELT motif and the two KI motifs (Supplemental Figure S2A). These experiments show that the KI motifs interact with Bub1 and BubR1 constitutively; this interaction does not require the phosphorylation of MELT motifs. The experiments also confirm that the mutations significantly reduce Bub1 and BubR1 binding.

An N-terminal fragment of KNL1 spanning residues 1–334 localized transiently to prometaphase kinetochores, likely by interacting with kinetochore-bound Bub1 or BubR1 or with KNL1 itself...
(Kern et al., 2015; Chen et al., 2019). Therefore, we examined the localization of the KI1-KI2 and KI1*-KI2* phosphodomain in mitotic cells treated with rapamycin using immunofluorescence (Supplemental Figure S2B). Both phosphodomains colocalized with kinetochores, although the amount of kinetochore-localized KI1*-KI2* was ∼20% lower than that of the KI1-KI2 phosphodomain (Supplemental Figure S2B).

We next tested whether the kinetochore localization of the eSAC phosphodomains affects the dose-response data for the two phosphodomains indirectly, by affecting kinetochore–microtubule attachment. For this, we obtained dose-response data for the two phosphodomains while also observing chromosome congression and segregation in these two cell lines. For both cell lines, chromosome alignment appeared normal in most cells (Supplemental Videos 1 and 2). In the case of the KI1*-KI2* eSAC system, we observed lagging chromosomes in the spindle midzone and anaphase bridges in cells that underwent anaphase after a prolonged metaphase arrest (54 out of 403 cells). These defects can be ascribed to cohesion fatigue setting in during the metaphase arrest (Daum et al., 2011). We also noted an increased incidence of unaligned or lagging chromosomes in cells with KI1-KI2 eSAC system (53 out of 373 cells examined, compared with 15 out of 403 cells for KI1*-KI2*) likely because the phosphodomain sequesters Bub1 and BubR1. To test whether the cells with chromosome missegregation affect the dose-response data, we revealed the dose-response trend by smoothing the data using LOWESS filtering by including and excluding the cells with chromosome segregation defects. The overall trend remained largely unaffected (Supplemental Figure S2C). Therefore, we conclude that the dose-response data of the eSAC systems are shaped mainly by the properties of the eSAC phosphodomain; they are minimally influenced by kinetochore-based SAC signaling.

Bub1 and BubR1 recruited by the KI motifs do not contribute to MCC assembly mediated by the MELT motifs

We first determined the baseline activity of the four MELT motifs by establishing the dose-response correlation for the eSAC phosphodomain with inactive KI motifs (KI1*-KI2* in Figure 2B, left, purple circles). The response elicited by this eSAC phosphodomain was nonmonotonic: the mitotic duration increased steeply before gradually decaying to a lower value (see Supplemental Figure S3A). A nonmonotonic response was not apparent for a previously characterized eSAC phosphodomain containing four MELT motifs (numbers 11–14) (Chen et al., 2019). The different behaviors of the two phosphodomains may be ascribed to different Bub1-Bub3 binding affinities of the MELT motifs that they contain. Notably, when both KI motifs were active (KI1-KI2), the maximal duration of mitosis was significantly reduced (∼87 min estimated by a four-parameter sigmoidal fit to the binned averages of the data; see Figure 2C). The KI motifs similarly suppressed the signaling strength of an extended phosphodomain containing seven MELT motifs (Supplemental Figure S3B).

We next determined the response elicited by the two eSAC phosphodomains containing four MELT motifs and only one active KI motif. When only the first KI motif was active (KI1-KI2*, only Bub1 depleted; see Figure 2B), the dose-response data were monotonic with a slightly lower maximal response than the maximal response elicited by KI1*-KI2* (Figure 2C, green circles and curve). When only the second KI motif was active (KI1*-KI2, Bub1, and BubR1 depleted; Figure 2C), the maximal response was significantly attenuated (blue circles and line in Figure 2C). Interestingly, the response to this eSAC was also nonmonotonic, an initial overshoot followed by decay to a lower response level (residuals from the Hill equation fit shown in Supplemental Figure S3A).

These results reinforce the conclusion that Bub1 and BubR1 recruited by the two KI motifs do not directly contribute to MCC generation by the MELT motifs within the same phosphodomain (Figure 2C). The distinctly different effects of Bub1 and BubR1 sequestration on eSAC activity also suggest that the cellular abundance of these proteins may be an important aspect of SAC signaling (Heinrich et al., 2013).

Numerical simulation of the dose-response data

The strong suppression of eSAC signaling by the second KI motif that binds BubR1 can be ascribed to two effects of BubR1 sequestration: the reduced rate of MCC assembly and a lowered limit on the maximal amount of MCC that can be generated. The latter effect is unlikely to play a major role in shaping the dose-response data. This is because the KI1*-KI2* eSAC delays mitosis by at most 300 min, significantly shorter than the ∼1500-min-long arrest seen in nocodazole-treated HeLa cells (Collin et al., 2013; Dick and Gerlich, 2013). Therefore, the amount of MCC produced by the eSAC systems is likely to be lower than the amount produced in nocodazole-treated HeLa cells. Therefore, a lower rate of MCC generation rather than a lower maximal amount of MCC that can be generated following BubR1 sequestration likely shapes the dose-response data.

An intuitive explanation for the dose-response dependence can be developed using the following four observations: 1) Bub1 and BubR1 interactions with the KI motifs do not require MELT motif phosphorylation (Figure 2B), 2) Bub1 and BubR1 recruited by the KI motifs do not contribute to the activity of the phosphorylated MELT motifs (Figure 2, A–C), 3) the eSAC phosphodomains are significantly more abundant than Bub1 and BubR1 (shown later), and 4) phosphorylated MELT motifs in an eSAC phosphodomain recruit Bub1, BubR1, and Mad1 (Chen et al., 2019). The first three observations indicate that the two KI motifs in the eSAC phosphodomains will differentially sequester Bub1 or BubR1, and the last observation suggests that the MELT motifs in the eSAC phosphodomains will form two distinct signaling complexes: MELpT:Bub1:Mad1/2 and MELpT:Bub1:BubR1:Mad1/2 (Figure 3A; Cdc20 and Mad1/2 are present in both and hence are not indicated). When taken together, these observations suggest that the differential sequestration of Bub1 and BubR1 by the KI motifs will affect the composition of the signaling complexes assembled on the phosphodomains. If the two signaling complexes assemble the MCC at different rates, the result will be different mitotic delays, explaining why the four phosphodomains produce distinctly different maximal mitotic delays. The quantitative dose-response data and numerical simulations provide an excellent opportunity to test this model and the notion that MELpT:Bub1:BubR1:Mad1/2 generates MCC at a higher rate than the MELpT:Bub1:Mad1/2.

We simulated this model in two stages (Figure 3A). In the first stage, we calculate the equilibrium concentrations of the two signaling complexes using rates governed by mass action (Eqs. 6–15; Materials and Methods); in the second stage, we calculate the rate of MCC formation and its effect on the metaphase-to-anaphase transition (Eqs. 16 and 17; Materials and Methods). For both signaling complexes, the events before Mad2:Cdc20 formation are identical. Therefore, we did not explicitly simulate them. After this step, MELpT:Bub1:BubR1 can assemble the MCC either within the signaling complex itself or in the cytosol, whereas MELpT:Bub1 must rely on cytoplasmic MCC assembly. Using these insights, we expanded our previously described eSAC model (Chen et al., 2019). This model simulates the activity of the four MELT motifs in a manner...
FIGURE 3: Numerical simulation of the dose-response data for the eSAC phosphodomains containing KI motifs. (A) Schematic of the two-stage model used to simulate the dose-response curves. (B) The influence of Bub1-BubR1 stoichiometry and the differential sequestration of Bub1 and BubR1 by the KI motifs on the equilibrium concentrations of the two signaling complexes formed on phosphorylated MELT motifs. (C) Comparison of the equilibrium concentration of the MELpT-Bub1-BubR1 complex assembled by KI1*-KI2 and KI1-KI2* (left) and the ratio (right) of their maximal responses as a function of the Bub1-BubR1 stoichiometry. For the two phosphodomains to generate different responses, the Bub1-BubR1 stoichiometry must be around 1:1 (indicated by the blue shaded area). (D) The ratio of the maximal responses produced by KI1*-KI2 and KI1-KI2* as a function of the Bub1-BubR1 stoichiometry and the ratio of the rates at which the MELpT-Bub1-BubR1 and the MELpT-Bub3 signaling complexes produce MCC (i.e., $K_{MCC}/K_{MCC}$). (E) Simulation of the dose-response curves for the four phosphodomains using the same set of parameter values.

**Dependence of the equilibrium concentrations of MELpT:Bub1 and MELpT:Bub1:BubR1 assembled by the eSAC systems on Bub1-BubR1 stoichiometry.**

The equilibrium concentrations of MELpT:Bub1 and MELpT:Bub1:BubR1 depend on two sets of parameters: 1) the concentrations of Bub1, BubR1, and the eSAC phosphodomain (quantification shown after the simulations) and 2) the affinities of Bub1 and BubR1 for KI1 and KI2, respectively, Bub1 for phosphorylated MELT motifs and BubR1 for the eSAC phosphodomain, most of the BubR1 recruited to the phosphodomain will bind to MELpT motifs rather than the KI2 motif. If this is the case, an activity of the KI2 motif should not strongly affect the eSAC signaling activity. However, this prediction is not supported by the data. For these reasons, we assume that BubR1 does not bind to MELpT. As will be seen later, our model with these simplifying assumptions adequately captures the main features of the dose-response data.
Consequently, the concentration of MELpT:Bub1 will become negligible (dashed green and blue lines, respectively, near the X-axis in Figure 3B, left). At high eSAC dosage, the two eSAC systems will form similar amounts of MELpT:Bub1:BubR1 despite the differential sequestration of Bub1 and BubR1 (converging dashed lines at high eSAC dosage in Figure 3B, right). Therefore, their eSAC signaling activities will also be similar, contrary to our observations.

When BubR1 and Bub1 concentrations are similar, a sizable fraction of MELpT:Bub1 will not recruit BubR1 (Figure 3B, right, solid lines), especially at a high eSAC dosage. Therefore, at high eSAC dosage, the concentration of MELpT:Bub1:BubR1 is lower for KI1*-KI2 than for KI1-KI2* (solid lines in Figure 3B, left). Figure 3C shows how Bub1-BubR1 stoichiometry affects MELpT:Bub1:BubR1 concentration for the high and constant eSAC dosage (Figure 3C, left). BubR1:Bub1 <2.5 ensures that the two phosphodomains assemble different concentrations of MELpT:Bub1 and MELpT:Bub1:BubR1 (blue shaded region in Figure 3C, left). This difference in the equilibrium concentrations of the signaling complexes can explain the differential eSAC activities.

A higher rate of MCC generation by MELpT:Bub1:BubR1 compared with MELpT:Bub1 can explain the differential behavior of the two phosphodomains

Differences in the concentrations of MELpT:Bub1 and MELpT:Bub1:BubR1 will translate into different activities only if they generate MCC at different rates. As before, we calculate the rate of MCC generation by assuming it to be proportional to the concentrations of MELpT:Bub1 and MELpT:Bub1:BubR1 (Figure 3B). Following our experimental results, the K1-bound Bub1 and BubR1 do not promote eSAC signaling. To simulate the effect of the MCC generated on mitotic progression, we modified the model of metaphase-to-anaphase transition described by He et al. (Supplemental Figure S4B; Materials and Methods) (He et al., 2011; Chen et al., 2019).

As discussed in the preceding sections, the maximal mitotic duration produced by the two eSAC systems depends on 1) the equilibrium concentrations of MELpT:Bub1 and MELpT:Bub1:BubR1, which are in turn dependent on the Bub1-BubR1 stoichiometry, and 2) the values of the MCC generation rate constants $k_{\text{MCC}}$ and $k'_{\text{MCC}}$. To determine the working combination of these factors, we fixed the eSAC dosage at a high value and calculated the duration of mitosis for a range of ratios of $k_{\text{MCC}}$ to $k'_{\text{MCC}}$ and Bub1:BubR1 stoichiometry. Figure 3D displays how the two ratios affect the maximal duration of mitosis achieved by KI1-KI2* and KI1*-KI2, respectively. For Bub1:BubR1 = 1 and $k_{\text{MCC}}/k'_{\text{MCC}} > 6$, the ratio of the maximal mitotic durations achieved by the KI1-KI2* and KI1*-KI2 eSAC systems exceeds 1.4.

Following this result, we used [Bub1]:[BubR1] = 1 and $k_{\text{MCC}}/k'_{\text{MCC}} = 0.1$ (rate constant for MCC assembly within the signaling complex is 10-fold higher than the rate constant for cytoplasmic MCC assembly) to simulate the dose-response curves for all four phosphodomains. This simulation captures key characteristics of the dose-response data for all four phosphodomains (Figure 3E). As before, the assumption of synergistic signaling was necessary to reproduce the nonmonotonicity of the dose-response data for the phosphomain containing four MELT motifs (Chen et al., 2019). Without it, the responses elicited by all phosphodomains become monotonic (Supplemental Figure S4D).

**Stoichiometry of Bub1, BubR1, Mad1, and the eSAC phosphodomain in HeLa cells**

The relative amounts of Bub1, BubR1, and the eSAC phosphodomain emerge as critical determinants of eSAC signaling. Therefore, for comparative protein abundance measurements, we constructed genome-edited HeLa cell lines wherein mNeonGreen (abbreviated as mNG) was fused to the N-terminus of Bub1 and BubR1, and Mad1-mNeonGreen in genome-edited HeLa cell lines. (A) Immunoblots showing that roughly half of BubR1 and Mad1 (left) and Bub1 (middle) proteins in the three partially genome-edited cell lines is tagged with mNeonGreen (asterisks on the right of each displayed blot mark the mNG fusion protein). Right: Immunoblot of whole-cell extracts of the three cell lines probed with anti-mNeonGreen antibodies. This experiment was performed once. (B) Average mNG-Bub1, mNG-BubR1, Mad1-mNG, and M4-KI1-KI2-mNG-2xFkbp12 signals from mitotic HeLa cells (left) and estimation of the relative protein abundance, assuming that the total protein abundance is twice as high as the abundance of the mNG-labeled species (mean ± SD; n = 50 each for mNG-Bub1, mNG-BubR1, and Mad1-mNG, n = 27 for M4-KI1-mNG-2xFkbp12).

**FIGURE 4:** Quantification of the relative abundances of the eSAC phosphodomain, mNeonGreen-Bub1, mNeonGreen-BubR1, and Mad1-mNeonGreen in genome-edited HeLa cell lines. (A) Immunoblots showing that roughly half of BubR1 and Mad1 (left) and Bub1 (middle) proteins in the three partially genome-edited cell lines is tagged with mNeonGreen (asterisks on the right of each displayed blot mark the mNG fusion protein). Right: Immunoblot of whole-cell extracts of the three cell lines probed with anti-mNeonGreen antibodies. This experiment was performed once. (B) Average mNG-Bub1, mNG-BubR1, Mad1-mNG, and M4-KI1-KI2-mNG-2xFkbp12 signals from mitotic HeLa cells (left) and estimation of the relative protein abundance, assuming that the total protein abundance is twice as high as the abundance of the mNG-labeled species (mean ± SD; n = 50 each for mNG-Bub1, mNG-BubR1, and Mad1-mNG, n = 27 for M4-KI1-mNG-2xFkbp12).
Recruitment of BubR1 by Bub1 per se strengthens kinetochore-based SAC signaling

Following these results and insights, we reexamined the role of Bub1-BubR1 heterodimerization in kinetochore-based SAC signaling. PP2A recruitment to the kinetochore in this manner contributes to SAC silencing directly (Kruse et al., 2013; Espert et al., 2014; Qian et al., 2017) and indirectly by either promoting Protein Phosphatase 1 recruitment (Nijenhuis et al., 2014) or stabilizing kinetochore-microtubule attachment (Suijkerbuijk et al., 2012). We confirmed these findings using the knock-in/knockdown strategy wherein endogenous BubR1 was knocked down using RNA interference (RNAi) and replaced with fluorescently tagged versions of either wild-type BubR1 or BubR1 lacking the heterodimerization domain (BubR1ΔHD, Supplemental Figure S5A). In media containing 100 nM GSK923295 (a small molecule inhibitor of the mitotic kinesin CENP-E), mCherry-BubR1ΔHD expression prolonged mitosis compared with mCherry-BubR1 expression (Supplemental Figure S5B), because the reduced BubR1 recruitment to the kinetochore also reduced PP2A activity as shown previously (Kruse et al., 2013; Overlack et al., 2015; Hertz et al., 2016; Zhang et al., 2016). Knockdown of the five isoforms of the PP2A-targeting subunit B56 in addition to BubR1 in these experiments did not affect the results, indicating that the phenotype was not caused by the activity of any residual BubR1 (Supplemental Figure S5B) (Foley et al., 2011).

To separate the effect of BubR1 recruitment to the kinetochore from the BubR1-mediated recruitment of PP2A, we created two additional BubR1 mutants: mNG-BubR1ΔKARD that lacks the PP2A-binding KARD domain and mNG-BubR1ΔHD,ΔKARD that lacks the KARD domain and the BubR1 heterodimerization domain (Figure S5A). The complete removal of the KARD domain will abolish the binding of B56αx and other isoforms of B56 onto BUBR1 (Wang et al., 2016a,b), enabling us to analyze whether the recruitment of BUBR1 to the signaling kinetochore per se contributes to the SAC activity. Importantly, we ensured that the expression level of these mutants was similar to that of wild-type BubR1 because the transient overexpression of BubR1 can deplete the cytosolic pool of Bub3 (Taylor et al., 1998) and adversely affect the SAC or induce cell death (unpublished data). Higher cytosolic BubR1 concentration may also proportionally increase the rate of cytosolic MCC formation and thus mask impaired MCC assembly within the kinetochore.

We knocked down endogenous BubR1 in HeLa-A12 using RNAi and rescued these cells with mNG-BubR1ΔKARD or mNG-BubR1ΔHD,ΔKARD. As expected, mNG-BubR1ΔKARD localized to unattached kinetochores, whereas mNG-BubR1ΔHD,ΔKARD localization to unaligned kinetochores was undetectable (Figure 5B). We also quantified the duration of mitotic arrest using nocodazole treatment following the previous studies. To ensure that the BubR1 mutants were not overexpressed, we established the physiological BubR1 expression level by quantifying cytosolic BubR1 fluorescence in the genome-edited mNG-BUBR1 HeLa-A12 cells treated with control small interfering RNA (siRNA) and imaged under identical conditions. In our knock-in/knockdown experiments, we considered only those cells exhibiting mNG intensity that is 0.5–2 times the average mNG intensity of mitotic mNG-BubR1 HeLa-A12 cells (Figure 5C, left). Quantification of mitotic duration revealed that cells expressing mNG-BubR1ΔKARD arrested significantly longer than control cells; the longer duration is attributable to the loss of PP2A activity from the kinetochores (Saurin et al., 2011). Notably, cells rescued with mNG-BubR1ΔHD,ΔKARD arrested for a significantly shorter amount of time compared with the cells rescued with mNG-BubR1ΔKARD (Figure 5C, right). Thus, the recruitment of BubR1 per se strengthens the SAC.

In conclusion, Bub1-BubR1 heterodimerization significantly enhances SAC signaling activity in human cells. This enhancement can be simply due to enrichment of BubR1 at the site of formation of Mad2-Cdc20 (Lara-Gonzalez et al., 2021a; Piano et al., 2021), although more complicated mechanisms can also be envisioned. Bub1 and BubR1 recruitment via the KI motifs does not contribute to eSAC signaling mediated by MELT motifs, indicating that their contribution to SAC signaling is likely to be minor. Although prior studies found that the KI motifs promote SAC signaling, this contribution was detectable only in the context of recombinant Knl1 variants containing either just one MELT motif (Krenn et al., 2014) or three inactive MELT motifs (Vleugel et al., 2013); the contribution...
was undetectable in a Knl1 variant containing multiple MELT motifs but lacking the N-terminus including the KI motifs (Zhang et al., 2014). These observations can be explained by the KI motifs’ relatively weak affinity for the TPR domains of Bub1 and BubR1 (Bolanos-Garcia et al., 2011; Krenn et al., 2012) compared with the affinity of phosphorylated MELT motifs for the Bub1-Bub3 complex (Primorac et al., 2013) and the fact that MELT motifs outnumber the KI motifs. Owing to these factors, the phosphorylated MELT motifs likely recruit the majority of Bub1 and BubR1 to the kinetochore (Zhang et al., 2014; Overlack et al., 2015; Vleugel et al., 2015).

Our findings highlight the dual effect of Bub1-mediated BubR1 recruitment on the SAC. BubR1 stabilizes the kinetochore–microtubule attachment by recruiting PP2A, thereby promoting the silencing of SAC. But it also promotes the SAC activity per se, which is critical for minimizing chromosome missegregation in normally dividing cells, wherein the last few unattached kinetochores need to be able to signal the cell to delay anaphase onset (Roy et al., 2020).

**MATERIALS AND METHODS**

**Request a protocol through Bio-protocol.**

### Plasmid construction

The plasmids used for the stable cell lines were based on plasmids that have been described previously (Chen et al., 2019). Briefly, the phosphodomain was integrated into either NotI or Ascl and XhoI restriction sites to create constitutively expressed phosphodomain-mNeonGreen-2xFkb12. The Mps1160-857 fragment corresponding to the Mps1 kinase domain was integrated into the Fsel and BglII restriction sites to create conditionally expressed Frb-mCherry-Mps1160-857.

eSAC phosphodomsains spanning the 1st MELT motif (M1) and the two KI motifs were created by fusing Knl1160-256 to mNeonGreen-2xFkb12. eSAC phosphodomaains containing four MELT motifs and the two KI motifs were created by fusing Knl1160-256 to Knl1 fragment Knl1181-1014 spanning MELT motifs 12–14, which has been characterized previously (Vleugel et al., 2015). The activity of the first KI motif was disrupted by changing its amino acid sequence from “KIDTTSKFLANLK” to “KADAASALANLK” (KI1*). Similarly, the activity of the second KI motif was disrupted by mutating its amino acid sequence from “KIDFNDFIKLRLK” to “KIDFNDIAIKALK” (KI2*) following Krenn et al. (2012). The relevant open reading frames in all plasmids were confirmed using Sanger sequencing.

DNA repair templates used for CRISPR/Cas9-mediated genome editing were constructed via DNA assembly using the NEB HiFi DNA assembly kit per the manufacturer’s instructions. Successfully edited alleles encode mNeonGreen-tagged SAC proteins that separate the corresponding wild-type protein and the fluorescent protein mNeonGreen by a short flexible linker (mNG-BUBR1 and mNG-BUB1: GSGGSG; MAD1-mNG: GGAGGSGG). The sequences of all homology-directed repair template plasmids and Cre-lox recombination-mediated cassette exchange plasmids are available upon request.

### Tissue culture and cell line construction for eSAC analyses

HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 1% Pen/Strep, 1×-GluMax, and 25 mM HEPE5 under standard tissue culture conditions (37°C and 5% CO₂). Stable cell lines expressing the two eSAC components were generated by integrating a bicistronic eSAC plasmid at engineered lox sites in the HeLa genome according to the protocol described in Khandelia et al. (2011). Upon transfection, DMEM supplemented with 1 μg/ml puromycin was used to select transformed cells, and all the colonies were pooled to culture the transformed cells used in the experiments. The expression of the eSAC components in each cell line was confirmed using immunoblotting with FKBP12 and mCherry antibodies.

For dose-response analysis, each eSAC cell line was plated –40–48 h before the start of the experiment in DMEM without puromycin. Doxycline was added at the time of plating to induce the expression of Frb-mCherry-Mps1. Before imaging, the cells were washed with phosphate-buffered saline (PBS). Fluorobrite media with 10% FBS, 1% Pen/Strep with or without rapamycin were added to each well.

### Genome editing HeLa cells using CRISPR/Cas9

The guide RNAs (gRNAs) for in situ BUBR1 and BUB1 N-terminal mNeonGreen-tagging were 5′-CAGAGGUGGCGGGUGUGAAGA-3′ and 5′-GGUUCAGGGUUUGCGGCUGC-3′, respectively. The gRNA for in situ MAD1 C-terminal mNeonGreen-tagging was 5′-CAGACGGUGGCGAGCCGUCG-3′. Single-guide RNAs (sgRNAs) were synthesized using the EnGen sgRNA Synthesis Kit (for the Streptococcus pyogenes–originated Cas9; New England Biolabs). The SpCas9-sgRNA ribonucleoprotein (RNP) complex was assembled at room temperature in a buffer consisting of 20 mM HEPES-KOH (pH 7.5), 150 mM KCl, 1 mM MgCl₂, 10% (by volume) of glycerol, and 1 mM dithiothreitol using 100 pmol of SpCas9-2 × NLS (the QB MacroLab) and 120 pmol of sgRNA. The RNP complex and 1.5 μg of a linearized homology-directed repair template plasmid were transfected into 2 × 10⁵–5 × 10⁵ nucodazole-arrested mitotic HeLa A12 cells using a Nucleofector and the associated Cell Line Kit R (Lonza) following the manufacturer’s instructions. After 5 wk, green fluorescent–positive mitotic cells (arrested by 330 nM nocodazole for 16 h) were sorted directly into 96-well plates at 1 cell/well. Healthy colonies were subject to further validation by genotyping and sequencing, as well as immunoblotting.

For genotyping, HeLa-A12 genomic DNAs were purified using the Wizard SV Genomic DNA Purification System (Promega). Genotyping primers (BUBR1 forward primer 5′-CCTGGTGCACATCTGAGCTAT-3′, BUB1 reverse primer 5′-CTCAATGAGACTCCAGTGTTT-3′, BUB1 forward primer 5′-CCCTCTACATGAGGCGGTACA-3′, BUB1 reverse primer 5′-GCTGCCAAGTAAACATT-3′, MAD1 forward primer 5′-GGACTTTTCAGGGACGTGGT-3′, and MAD1 reverse primer 5′-GAGTTGGGAGGAGGGACTC-3′) were designed to bind outside of homology arms to avoid false-positive colonies from the homology-directed repair template plasmid to an off-target genomic locus.

### Drug and RNAi treatments

To induce the expression of the mCherry-Frb-Mps1 kinase domain doxycycline was added to a final concentration of 2 μg/ml (stock concentration 2 mg/ml in dimethyl sulfoxide [DMSO]) –48 h before the start of the experiment. Before the start of each experiment, rapamycin was added –1 h prior to imaging to a final concentration of 500 nM (stock concentration 500 μM in DMSO) to induce theimerization of the eSAC kinase domain with the eSAC phosphodomain. Nocodazole was added to a final concentration of 330 nM (stock concentration 330 μM in DMSO). The cocktail of siRNA against five different 856 isomers was added to a final concentration of 40 nM (stock concentration 10 μM).

The siRNA sequences were obtained from Zhang et al. (2016). siRNA sequences used to knock down 856 isomers were obtained from Foley et al. (2011). Cell cycle synchronization in G1/S was achieved by treating cells with 2.5 mM thymidine (from a 100 mM stock in PBS) for 16–18 h. Cells were washed with DMEM for release from the G1/S arrest. To arrest cells in a prometaphase-like condition,
cells were released from a G1/S block and then treated with 236 nM GSK923295 (stock concentration 236 μM in DMSO)–7 h postrelease and imaged after 1 h.

**Immunoprecipitation**

HeLa A12 cells constitutively expressing either MELT1-KI or MELT1-KI-M3 were synchronized at G1/S by 2.5 mM thymidine. Cells were synchronized in metaphase using 10 μM MG132 nine hours after being released from a (double) thymidine block. After another 1.5 h, cells were scraped off the plate, washed once with PBS, pelleted, snap-frozen, and stored at −80°C. Cells were thawed, resuspended in the complete lysis buffer (75 mM HEPES-HCl [pH 7.5], 150 mM KCl, 1.5 mM EGTA, 1.5 mM MgCl2, 10% [by volume] glycerol, and 0.075% [by volume] Nonidet P-40 [AmericanBio]; immediately before use one complete protease inhibitor cocktail tablet [EDTA-free; Roche Diagnostics] and a phosphatase inhibitor cocktail [1 mM Na2P2O7, 0.1 mM Na3VO4, 5 mM NaF, and 2 mM sodium β-glycerophosphate] and lysed for 1 h at 4°C while rotating. Cell lysates were then centrifuged at 18,000 × g for 25 min at 4°C. The supernatant was subsequently cleared by control agarose beads for 1.5 h at 4°C to reduce nonspecific binding. Cleared supernatant was then mixed with mNeonGreen-Trap agarose beads (ChromoTek) and rotated for 1.5 h at 4°C. Beads were washed four times using the lysis buffer. Finally, 2x Laemmli buffer (Bio-Rad Laboratories) supplemented with β-mercaptoethanol was added to the beads. Samples were boiled in a water bath for 10 min before being subjected to SDS–PAGE.

The following antibodies and working dilutions were used in immunoblotting: SB1.3 antibody against: BUB1 (Taylor et al., 2001), 1:500 or 1:1000, sheep polyclonal; BUB3 (Sigma B7811), 1:500, rabbit polyclonal; FKBP12 (Abcam ab2918), 1:2000, rabbit polyclonal; BUBR1 (Bethyl A300-995A), 1:1000, rabbit polyclonal; and mNeonGreen (ChromoTek 32F6) 1: 500.

**Immunofluorescence**

HeLa cell lines were grown on sterile coverslips in six-well plates in media supplemented with 1 μg/ml doxycycline to induce the expression of the mCherry-Frb-Mps1 kinase domain. After ~48 h, the cells were treated with 500 nM rapamycin. After 4 h of incubation, cells were fixed with 4% paraformaldehyde and stained with ACA antibodies (1:1000; Antibodies Inc., Davis, CA) and Alexa-633-conjugated secondaries (1:5000). After staining, the cells were embedded in Diamond mounting media and stored at room temperature.

The mounted cells were imaged on an Eclipse Ti-E/B inverted microscope (Nikon) with a CFI Plan Apochromat VC 100×, 1.40 NA oil objective (Nikon). The microscope was equipped with a H117E1 motorized stage (Prior Scientific), a NanoScanZ 100 piezo stage (Prior Scientific), and an X-Light V2 L-FOV confocal unit with 60 μm pinholes (CrestOptics). A CELESTA Light Engine (LumenCor) served as the excitation laser source, featuring a 477-nm line for imaging the mNeonGreen protein, a 546-nm line for imaging mCherry, and a 647-nm line for imaging the Alexa-633–conjugated antibodies. Fluorescence emission light was filtered by ET605/52m (Chroma Technology) for the red channel and by ET525/36m (Chroma Technology) for the green channel. Images were acquired by a Prime 95B 25 mm sCMOS camera (Teledyne Photometrics). A custom MATLAB program was used to quantify kinetochore-localized fluorescence signals.

**Long-term, live-cell imaging of HeLa cells**

Imaging was conducted as described in detail previously (Chen et al., 2019). We used either the Incucyte Zoom live cell imaging system (Molecular Devices), both equipped with a 20x phase objective. To image cells on the Incucyte system, cells were plated in 12-well plastic tissue culture plates, whereas they were plated in 24-well plate glass-bottom dishes (Corning) for the ImageXpress Nano system. At each position, one phase, GFP, and mCherry image was acquired every 10 min. The exposure times for mCherry and GFP images were adjusted to minimize photobleaching while enabling accurate determination of intensity values. It should be noted that the excitation sources, optics, and detector on the ImageXpress Nano and the Incucyte microscope are entirely different. Therefore, the mCherry intensity values across different experiments are not directly comparable.

**Image analysis**

Before intensity quantification, acquired images were preprocessed using functions from the “Image Processing Toolbox” provided with MATLAB as follows. First, the phase image sequence was registered to remove any movement of the field of view between adjacent time points, and at each time point, the same transform was applied to the GFP and mCherry images to register them. Additionally, image intensity from a blank, unseeded well was used for background correction of the fluorescence channels. Next, GFP and mCherry fluorescence signals were quantified using a custom graphical user interface (GUI) written in MATLAB as described previously. Briefly, this interface uses cross-correlation of each phase image with a circular kernel to identify cells with circular shapes close to the diameter of the circular kernel. The centroids of these shapes were then linked along the time axis. These images were presented to the user via the GUI to 1) discard false-positive, nonmitotic cells or debris and 2) visually correct the time of entry into or exit from mitosis. The GUI then calculated the GFP and mCherry signals per cell as the average fluorescence intensity.

In all the dose-response assays discussed in this study, the phosphodomain is highly and constitutively expressed in all cells, whereas the kinase domain is expressed conditionally by an inducible promoter. Consequently, the amount of the kinase domain expressed varies from cell to cell, and it is lower than the amount of the phosphodomain in most cells. Because of this design of the eSAC system, the dosage of the dimerized signaling complex in a cell can be inferred from the amount of Frb-mCherry-Mps1500-817 in that cell. Therefore, we defined the dosage of the eSAC signaling complex by quantifying the average mCherry fluorescence within a cell and the response as the duration of mitosis (the amount of time that the cell spends with a spherical morphology that is characteristic of mitotic HeLa cells).

The Hill equation is used to fit the sigmoidal trend:

\[
\text{Time in mitosis} = \frac{M}{1 + \left(\frac{EC50}{\text{eSAC activator}}\right)^n}
\]

wherein \(n\) is the Hill coefficient and \(EC50\) is the level of the eSAC activator at which the time in mitosis reaches the middle between the baseline level (\(m\)) and the plateau level (\(m + M\)).

**Statistical analysis**

To determine the overall trend in the dose-response data, the data were first binned (in MATLAB), and then the mean values of each bin were overlaid on the data. The number of observations and technical replicates are noted in the figure legends. These mean values
were fitted with a four-parameter sigmoidal curve using GraphPad Prism 9 software. The statistical significance of the difference between the mean values in Figure 5C and Supplemental Figure S5 was assessed using the unpaired t test with Welch’s correction. LOWESS filtering of the data in Supplemental Figure S2C was performed using GraphPad Prism with 20 points in the smoothing window.

MATHEMATICAL MODELING

Modeling the activity of Bub1 phosphodomains

Stage 1: Calculation of the steady-state concentrations of signaling complexes. (MATLAB codes available on GitHub: https://github.com/anandbaran/eSAC-KI).

This model simulates the eSAC activity of the Bub1\textsuperscript{321-620} and Bub1\textsuperscript{1441-620} phosphodomains. In the equations below, we refer to these phosphodomains simply as “Bub1.” The eSAC activator complex is formed by the dimerization of Bub1 with the Mps1 kinase domain (Bub1:Mps1). Once Mps1 phosphorylates Bub1, Bub1 can bind Mad1/2 (Bub1:Mps1:Mad1/2). Therefore, the concentrations of different species of Bub1 are related by the equation

\[
[Bub1]_i = [Bub1]_i[Mad1/2] + [Bub1]_i[Mad1/2] + [Bub1]_i
\]  
(1)

Assuming reversible binding between phosphorylated Bub1 and Mad1/2 complex, the concentration of eSAC activator complex will saturate to a value dependent on the finite concentration of Mad1/2 (set at 100 nM; Supplemental Figure S1A). Note that Bub1 can produce MCC only if it recruits Mad1/2. Therefore, even though Bub1:Mps1 and Bub1 can both bind BubR1 and Cdc20, they do not participate in SAC signaling. The recruitment of SAC proteins, formation of signaling complexes, and MCC are calculated by assuming mass action kinetics.

Model of Bub1-mediated MCC formation. Available data suggest that a signaling complex comprising Bub1\textsuperscript{321-620}, BubR1, Mad1, and Cdc20 facilitates the formation of either Mad2:Cdc20 or the MCC. We assume that when Bub1 is present the phosphodomain assembles MCC and when BubR1 is absent the phosphodomain produces Mad2:Cdc20. Therefore, the rate of Mad2:Cdc20 and MCC formation is calculated as

\[
\text{Mad2:Cdc20 formation rate at the Bub1 phosphodomain} = k_{\text{asc2}} \cdot [\text{Bub1}]_i [\text{Mad1/2}] + [\text{Bub1}]_i [\text{Mad1/2}]
\]  
(2)

MCC formation rate at the Bub1\textsuperscript{321-620} phosphodomain

\[
= k_{\text{asc2}} \cdot [\text{Mad2}]_i [\text{Cdc20:Mad1/2:Bub1}]
\]  
(3)

where \([\text{Mad2}]_i\) = the concentration of inactive (open) form of Mad2 in the cytoplasm; \([\text{Cdc20:Mad1/2:Bub1}]_i\) = the concentration of the complex between Bub1, Mad1, and Cdc20; and \([\text{Cdc20:Bub1:Mad1/2:Bub1}]_i\) = the concentration of the complex between Bub1, BubR1, Mad1, and Cdc20.

For the sake of simplicity, we assume that the rate constant for MCC formation \(k_{\text{asc2}}\) is numerically equal to the rate constant for Mad2:Cdc20 formation. This assumption is consistent with the observation that Mad2:Cdc20 formation is the rate-limiting step in MCC formation (Faesen et al., 2017).

The cytosolic Mad2:Cdc20 molecules produced by either phosphodomain interact with cytosolic, free BubR1 to complete MCC formation. We denote the rate constant for this reaction by \(k_{\text{asc1}}\). Therefore, the rate of cytosolic MCC formation is calculated as

\[
\text{MCC formation rate in the cytoplasm} = k_{\text{asc1}} \cdot [\text{BubR1}]_i [\text{Mad2:Cdc20}]
\]  
(4)

where \([\text{BubR1}]_i\) = the concentration of free BubR1 in the cytoplasm.

Stage 2: Effect of MCC formation on the timing of metaphase-to-anaphase transition. We used a previously described model of metaphase-to-anaphase transition to simulate the effect of the MCC generated on the duration of mitosis (He et al., 2011; Chen et al., 2019). In this model (schematic at the top of Supplemental Figure S1B), cyclin B (“CycB”) is synthesized at a constant rate and degraded upon APC:Cdc20-dependent ubiquitination (denoted simply as Cdc20). The abundance of CycB determines the activity of CDK1:CycB complexes, which in turn determines the activity of the eSAC complexes via phosphorylation. CDK1:CycB activity is antagonized by the counteracting protein phosphatase PP2A:B56 (“CAPP”) (Sullivan et al., 2004; Bouchoux and Uhllmann, 2011). This scheme is consistent with recent data revealing that CDK1:CycB phosphorylates Bub1 to promote its interaction with Mad1 (Ji et al., 2017). Furthermore, Mps1 kinase activity is also down-regulated by PP2A (Espert et al., 2014; Hayward et al., 2019). This scheme regulates the amount of active eSAC.

The active eSAC ultimately produces MCC according to the scheme discussed in detail above with Bub1R1 as an MCC component. Therefore, we modified the original model to include BubR1 as well as the dissociation of MCC into its constituent proteins (shown by the red dashed arrow in Supplemental Figure S1B). Furthermore, active APC:Cdc20 promotes the inactivation of closed/active Mad2 in MCC; this positive feedback of active Cdc20 on its own release from the MCC accelerates the activation of APC:Cdc20 during the transition into anaphase (He et al., 2011; Chen et al., 2019).

The equations for this model are given below using the following notation:

- \(eS1\) = Bub1:Mps1:Mad1/2
- \(eS2\) = Bub1:Mps1:Mad1/2:Bub1

\[
\frac{d[eS1]}{dt} = k_{\text{fosc}} \cdot [eS1]_i \cdot [Cdc20] - k_{\text{rcdc}} \cdot [eS1]_i \cdot [Cdc20] + k_{\text{dis2}} \cdot [eS2]_i \cdot [Cdc20] + k_{\text{dis2}} \cdot [eS2]_i \cdot [Cdc20] \]  
(5a)

\[
\frac{d[eS1]_i}{dt} = k_{\text{ancyc}} \cdot [eS1]_i \cdot [CycB] - k_{\text{ncapp}} \cdot [eS1]_i \cdot [CAPP] - k_{\text{diss2}} \cdot [eS1]_i \cdot [BubR1]_i + k_{\text{dis2}} \cdot [eS2]_i \cdot [Cdc20] + k_{\text{asc2}} \cdot [eS2]_i \cdot [Cdc20] \cdot [Mad2] \]  
(5b)

\[
\frac{d[eS1]_i}{dt} = k_{\text{fosc}} \cdot [eS1]_i \cdot [Cdc20] - k_{\text{rcdc}} \cdot [eS1]_i \cdot [Cdc20] - k_{\text{act}} \cdot [eS1]_i \cdot [Cdc20] \]  
(5c)

\[
\frac{d[eS2]}{dt} = -k_{\text{ancyc}} \cdot [eS2]_i \cdot [CycB] + k_{\text{ncapp}} \cdot [eS2]_i \cdot [CAPP] - k_{\text{fosc}} \cdot [eS2]_i \cdot [Cdc20] + k_{\text{rcdc}} \cdot [eS2]_i \cdot [Cdc20] \]  
(5d)
Thus, the main goal is to determine the steady-state concentrations of phosphorylated MELT motifs that recruit Bub1 or both Bub1 and BubR1. The KI1 motif can bind to Bub1, whereas the KI2 motif can bind to each MELT motif (MELT 13) following previous studies (Vleugel et al., 2015; Chen et al., 2019). We also chose the dissociation constant for the strong MELT motifs to be 45 nM, [Mad2:Cdc20] = 0 nM, [MCC] = 25 nM, [CAPP] = 5 nM. In the simulation, the system evolves toward a steady state corresponding to anaphase (low [CycB] and high [Cdc20]). We assume that a cell exits mitosis when [CycB] drops below 1 nM (Supplemental Figure S1E). If the initial conditions are metaphase-like (high [CycB] and low [Cdc20]), small variations in the initial conditions do not qualitatively affect the outcome of the model. Furthermore, the main result of this analysis—Bub1:BubR1 produces MCC at a higher rate than Bub1—is robust, even though many different combinations of parameters produce similar-looking dose-response curves.

Models the activities of eSAC phosphodoms containing four MELT motifs and the KI motifs

This model simulates the close-response data for the eSAC systems involving MELT and KI motifs. As before, the simulation takes place in two stages. In the first stage, we calculate the steady-state concentrations of SAC signaling proteins (Bub1 and BubR1) bound to the phosphorylated MELT motifs and KI motifs of an eSAC phosphodomain. This is followed by calculation of MCC generation using the steady-state concentrations of MELpT:Bub1 and MELpT:Bub1:BubR1. In the second stage, we simulate the duration of mitosis, according to the overall reaction scheme developed by He et al. (2011).

Stage 1: Simulation of SAC protein recruitment by the eSAC phosphodoms

Rules for protein–protein interactions. The phosphodomain consists of four MELT motifs and either active or inactive KI motifs. All four MELT motifs in phosphodomains complexed with the Mps1 kinase domain are assumed to be phosphorylated. They can recruit Bub1, which represents Bub1:Bub3 in this model. Upon binding to the MELT motif, Bub1 recruits BubR1 representing BubR1:Bub3. The KI1 motif can bind to Bub1, whereas the KI2 motif can bind BubR1. We assume that MELT and KI motifs in the eSAC phosphodomain interact with SAC proteins independently. The KI-bound Bub1 and BubR1 do not participate in SAC signaling. Therefore, the KI motifs act as sinks that reduce free Bub1 and BubR1 concentrations. We assigned the same rate of binding of the “Bub1” protein to each MELT motif (k in Supplemental Table S2), but assigned a low unbinding rate (k; Supplemental Table S2) for the strong MELT motifs (MEL 1, 12, and 14) and a higher unbinding rate for the weak MELT motif (MELT 13) following previous studies (Vleugel et al., 2015; Chen et al., 2019). We also chose the dissociation constant for K11:Bub1 binding to be equal to the dissociation constant for the K12:BubR1 binding.

Calculation of the steady-state concentrations of signaling complexes. This model avoids unnecessary complexity by assuming that the rate of MCC formation is simply proportional to the number of phosphorylated MELT motifs that recruit Bub1 or both Bub1 and BubR1. This simplification is justified, because the recruitment of Mad1, Mad2, and Cdc20 is independent of BubR1 recruitment. Thus, the main goal is to determine the steady-state concentrations.
of the two distinct signaling complexes: MEMLpT:Bub1 or MEMLpT:Bub1:BubR1. This calculation is performed as follows.

Each phosphorylated MELT motif can be in one of three possible states: MEMLpT (unbound MELT), MEMLpT:Bub1 (MELT bound by Bub1), and MEMLpT:Bub1:BubR1 (MELT bound by Bub1 and BubR1).

Because there are four MELT motifs in each eSAC activator complex, the number of possible states for the phosphomimic becomes $3^4 = 81$. The time evolution of concentrations of different Bub1 and BubR1 bound states of eSAC activator complex is given by

$$X = AX$$

where $X = \{x_1, x_1, \ldots, x_N\}$ is a vector of concentrations of the $N = 81$ different Bub1- and BubR1-binding states of the phosphodomain and $A$ is the rate matrix.

Similarly, each KI motif of the phosphodomain can be in two states: bound or unbound. The binding of Bub1 and BubR1 to KI motifs is described by the set of equations

$$\dot{y}_1 = k_{fbub} \cdot [Bub1]_i \cdot (K_I - y_1) - k_{fbub} \cdot y_1$$

$$\dot{y}_2 = k_{fbub} \cdot [BubR1]_i \cdot (K_I - y_2) - k_{fbub} \cdot y_2$$

where $K_I$ is the total concentration of KI motifs, $y_1 = [KI1:Bub1]$, and $y_2 = [KI2:BubR1]$.

[Bub1] and [BubR1] are the cytoplastic concentrations of free Bub1 and BubR1, respectively. The parameters $k_{fbub}$ and $k_{fbub}$ are the binding and unbinding rate constants between Bub1 and the first KI, and $k_{fbub}$ and $k_{fbub}$ are the binding and unbinding rates between BubR1 and KI2.

The concentrations satisfy the constraints

$$[eSAC]_T = \sum_{i=1}^{N} x_i$$

$$[Bub1]_T = [Bub1]_i + y_1 + \sum_{i=1}^{N} n_i \cdot x_i$$

$$[BubR1]_T = [BubR1]_i + y_2 + \sum_{i=1}^{N} m_i \cdot x_i$$

Here, $x_i$ is the concentration of the $i$th species, $n_i$ = number of Bub1 bound to $i$th species of the phosphodomain, and $m_i$ = number of BubR1 units bound to the $i$th species. We assume [Bub1]$_T = 100\text{nM}$ and [BubR1]$_T = 100\text{nM}$. The equilibrium concentration of each state was obtained by numerically solving

$$X = 0, i = 1, 2, \ldots, 81 \text{ and } \dot{y}_j = 0, j = 1, 2$$

In experiments, the concentration of eSAC activator complex is measured in arbitrary units of mCherry fluorescence (a.u.), whereas in our model the unit of concentration is nanomoles (nM). In our simulations, we chose the maximum value of concentration of eSAC activator complex (the value corresponding to 20 a.u. in experiments) to be 200 nM. For easier comparison to experimental figures, in our simulation results the eSAC activator complex concentration is expressed in arbitrary units, with 1 a.u. of fluorescence corresponding to 10 nM.

Supplemental Figure S4A shows the abundance of different Bub1- and BubR1-bound states as functions of the total concentration of the eSAC activator complex for KI1*-KI2*. At low eSAC concentrations, the eSAC tends to be highly loaded, with Bub1 and BubR1 on every MELT motif. However, for cells with a high eSAC concentration, [eSAC] >> [Bub1]$_T$, the most abundant eSAC species is one that does not bind any Bub1 at all (unpublished data), followed by species that bind either Bub1 or Bub1-BubR1 at only one of the four MELT motifs. We define the sum of concentrations of MEMLpT:Bub1 and MEMLpT:Bub1:BubR1 as $[eSAC]$:

$$[eSAC]_T = [\text{MEMLpT:Bub1}] + [\text{MEMLpT:Bub1:BubR1}]$$

$$[\text{MEMLpT:Bub1}] = \sum_{i=1}^{N} (n_i - m_i) \cdot x_i$$

$$[\text{MEMLpT:Bub1:BubR1}] = \sum_{i=1}^{N} m_i \cdot x_i$$

Formation of MCC by the eSAC signaling complexes

We assume that the MEMLpT:Bub1 and MEMLpT:Bub1:BubR1 complexes catalyze the assembly of MCC at the apparent rates constants $k_{MCC}$ and $k'_{MCC}$. A schematic diagram of the molecular mechanism underlying this model is displayed in Figure 4A.

We assume that the recruitment of SAC proteins (Bub1 and BubR1) to MELT motifs of the eSAC activator complexes enables the incorporation of Cd20 into MCC. Because different species catalyze this reaction at different rates, we define the effective rate of conversion, $k_{as MCC}$, as the concentration-weighted sum of the conversion rates of each eSAC complex:

$$k_{as MCC} = \frac{1}{[eSAC]_T} \sum_{i=1}^{N} (n_i - m_i) \cdot x_i + k_{MCC} \cdot \sum_{i=1}^{N} m_i \cdot x_i$$

Stage 2: The effect of MCC produced on exit from mitosis.

To calculate the effect of MCC generated by the eSAC on mitotic exit, we used a simplified version of the model of the mitotic checkpoint proposed by the He model (He et al., 2011). Active eSAC signaling complexes (eSAC$_A$) generate MCC, as described in the preceding section. The temporal dynamics of our mitotic checkpoint model are determined by the ODEs

$$\frac{d[CycB]}{dt} = k_{ascyc} - (k_{dyc} + k_{dyc,C20}) \cdot [CycB] \cdot [Cdc20]$$

$$\frac{d[eSAC_A]}{dt} = k_{as}\cdot [CycB] \cdot ([eSAC]_T - [eSAC_A])$$

$$\frac{d[MCC]}{dt} = k_{as MCC} \cdot [Cdc20] \cdot [eSAC_A]$$

$$\frac{d[CAPP]}{dt} = k_{ascapp} \cdot ([CAPP]_T - [CAPP]) - k_{capp,Cyc} \cdot [CycB] \cdot [CAPP]$$
signaling complexes (Eq. 10), which is in either the active, signaling-
competent state $eSAC_A$ or the inactive state, $eSAC_A^\text{r} = [eSAC_A]^r -
[eSAC_A]$. [MCC] and [CAPP] refer to the concentrations of the mi-
totic checkpoint complex and the CDK-counteracting protein
phosphatase, respectively. In addition, the total concentration of 
$Cdc20$ is 

$$[Cdc20] = [Cdc20]^r + [MCC] \quad (17)$$

The values of the parameters in the model and of the fixed
concentrations of some components, as listed in Supplemental Table
S2, are taken from He et al. (2011).

**Simulation of time in mitosis**

To determine the timing of the metaphase-to-anaphase transition, we
assume that a cell exits mitosis when $[CycB]$ drops below 1 nM. We
numerically integrated ODEs to calculate the time evolution of $[CycB]$ and
the components of the cell cycle machinery. As before, the initial
conditions for the simulation are chosen to be $[CycB] = 45$ nM, $[eSAC_A] = 0$, [MCC] = 25 nM, and [CAPP] = 5 nM. The qualitative
aspects of our results do not depend on the initial conditions. Sup-
plemental Figure S4C displays typical time courses for $[CycB]$, for
$eSAC_A$, for $eSAC$ activator complex) = 10 a.u. The system
always comes out of mitosis (as seen by the drop in $[CycB]$), albeit
after different time delays.

**Code availability**

MATLAB codes used for the simulations and for generating figure
panels can be accessed on GitHub: https://github.com/anandban/
eSAC-Ki).

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