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Phosphodependent Recruitment of Bub1 and Bub3 to Spc7/KNL1 by Mph1 Kinase Maintains the Spindle Checkpoint

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Summary

The spindle assembly checkpoint (SAC) is the major surveillance system that ensures that sister chromatids do not separate until all chromosomes are correctly bioriented during mitosis. Components of the checkpoint include Mad1, Mad2, Mad3 (BubR1), Bub3, and the kinases Bub1, Mph1 (Mps1), and Aurora B [1]. Checkpoint proteins are recruited to kinetochores when individual kinetochores are not bound to spindle microtubules or not under tension [2–5]. Kinetochore association of Mad2 causes it to undergo a conformational change, which promotes its association to Mad3 and Cdc20 to form the mitotic checkpoint complex (MCC). The MCC inhibits the anaphase-promoting complex/cyclosome (APC/C) until the checkpoint is satisfied. SAC silencing derepresses Cdc20-APC/C activity. This triggers the polyubiquitination of securin and cyclin, promoting the dissolution of sister chromatid cohesion and mitotic progression [6–8]. We, and others, recently showed that association of PP1 to the Spc7/Spc105/KNL1 family of kinetochore proteins is necessary to stabilize microtubule-kinetochore attachments and silence the SAC [9–12]. We now report that phosphorylation of the conserved MELT motifs in Spc7 by Mph1 (Mps1) recruits Bub1 and Bub3 to the kinetochore and that this is required to maintain the SAC signal.

Results

Bub1 and BubR1 checkpoint proteins are thought to bind kinetochores through interaction with the N-terminal region of KNL1 [13, 14]. Crystal structures of complexes between the tetratricopeptide repeat (TPR) domains of Bub1 and BubR1 and related, but distinct, motifs in KNL1 (named KI1 and KI2, respectively) have been generated [15, 16]. However, mutations in the TPR domain of Bub1 that abrogate its association to the KI1 domain of KNL1 do not block its association with kinetochores [16]. For this reason, the role of KNL1 in the kinetochore association of Bub1 and BubR1 has been brought into question. Mutation of a region of Bub1 (known as the GLEBS domain) that mediates its interaction with Bub3 prevents its association with kinetochores [16]. Although this agrees with a previous report, the binding site for Bub3 at kinetochores remains unknown [4]. In fission yeast, the Bub1 and Bub3 checkpoint proteins form a complex that binds to kinetochores during prometaphase and metaphase [17, 18]. Kinetochore association of Bub1 and Bub3 is dependent on the presence of both proteins and on Mph1 (Mps1) kinase [18–21]. Indeed, ectopic targeting of Mph1 to the outer kinetochore causes Bub1 to bind kinetochores throughout the cell cycle in checkpoint-deficient cells [22]. In this study, we examine the mechanism by which Mph1 directs the association of Bub1 and Bub3 to kinetochores.

Mph1 Kinase Phosphorylates Spc7

Bub1 fails to form discrete foci during mitosis in either mph1 or mph1(D459A) mutants (which are defective for kinase activity [23]), indicating that catalytic activity of Mph1 is required to promote kinetochore association of Bub1 (Figure 1A). To examine whether Mph1 phosphorylates Spc7 to promote kinetochore association of Bub1, we tested whether Mph1 phosphorylates Spc7 in vitro. The N-terminal half of Spc7 (residues 1–666) was purified from bacteria and incubated in the presence of wild-type (WT) or catalytically inactive Mph1. We find that Mph1 phosphorylates Spc7 in vitro on two threonine residues (T453 and T507), which are part of a repetitive motif containing the expanded consensus sequence [M/I][E/D/N][I/L/M][S/T] (termed the MELT motif) that is present in all members of the Spc7/KNL1 family [24] (Figures 1B–1D; see also Figure S1A available online). Phosphoproteomic analysis has revealed that the threonine residues of two MELT motifs in mammalian KNL1 are phosphorylated in vivo [25, 26]. Although mutation of Spc7-T453 or Spc7-T507 alone did not substantially reduce phosphorylation of Spc7 by Mph1 in vitro, mutation of all nine threonine residues of the MELT motifs (Spc7-9TA) reduced Mph1-dependent phosphorylation of Spc7 by 63.5% (Figure S1B; data not shown). These results indicate that Mph1 phosphorylates one or more threonine residues in the MELT motifs of Spc7.

Phosphorylation of Spc7 Promotes Its Association to Bub1

To examine whether phosphorylation of Spc7 MELT motifs by Mph1 influences its association to Bub1, we constructed spc7-9TA and spc7-9TE mutants, in which the threonine residues of all nine Spc7 MELT motifs were mutated to either alanine or glutamic acid. Bacterially expressed Spc7, Spc7-9TA, and Spc7-9TE fusions proteins were incubated in extracts of bub1-SZZ cells. We find that Spc7-9TA, but neither WT Spc7 nor Spc7-9TA, fusion proteins efficiently precipitate Bub1 from cell extracts (Figure 1E). Additionally, we constructed spc7-9MA and spc7-9MA,9TA mutants, in which the methionine residues of all nine MELT motifs were mutated to alanine. When expressed from the endogenous promoter, all of these mutant Spc7 proteins localize to the kinetochore, are expressed at comparable levels to WT cells, and rescue viability in the absence of endogenous Spc7 (Figures S1C and S1D). We also find that the Spc7-9TE-GFP, but not WT Spc7-GFP, protein coimmunoprecipitates with Bub1 from extracts of log phase cells (Figure 1F). Together these data suggest that Mph1 phosphorylates Spc7 on MELT motifs to stimulate its association with Bub1 both in vitro and in vivo.
Figure 1. Mph1 Kinase Phosphorylates MELT Motifs in Spc7 to Promote Association of Spc7 with Bub1

(A) Log phase cultures of *bub1-GFP sid4-tomato* cells WT (*mph1*), lacking Mph1 (*∆mph1*), or defective in Mph1 kinase activity (*mph1[KD]*) were fixed and imaged. Representative images are shown. Cells with mitotic spindles less than 2.5 μm exhibiting localized Bub1-GFP (closed arrowheads) or lacking Bub1-GFP localization (open arrowheads) are highlighted. Scale bar represents 5 μm.

(B) Mph1 phosphorylates Spc7 in vitro. Mph1 kinase or catalytically inactive Mph1 (KD) was incubated with MBP or MBP-Spc7 (1–666) fusion protein. Kinase assay (left panel) and Coomassie stained gel of input proteins (right panel) are shown. Asterisks indicate Mph1 autophosphorylation.

(C) Domain architecture of fission yeast Spc7 and its homologs in budding yeast (*S.c. Spc105*), worm (*C.e. KNL1*), and human (*H.s. blinkin*). PP1-binding sites (blue), KI motifs (gray), MELT motifs (red), and the coiled-coil kinetochore-binding domain (green) are shown.

(D) Protein alignments of the nine MELT motifs in Spc7. Invariant methionine and threonine residues are highlighted in red. KI motifs (gray), MELT motifs (red), and the coiled-coil kinetochore-binding domain (green) are shown.

(E) Spc7-9TE interacts with Bub1 in vitro. MBP-Spc7 (WT), MBP-Spc7-9TA (9TA), MBP-Spc7-9TE (9TE), or MBP-Pic1-INbox fusion proteins were incubated in extracts of untagged WT (no tag) or *bub1-SZZ* cells. Interacting proteins were precipitated on amylase beads, separated by SDS-PAGE, and subjected to western blot with anti-PAP antibody. Note that the anti-PAP antibody cross-reacts with the MBP-Spc7 proteins.

(F) Spc7-9TE interacts with Bub1 in vivo. Extracts from log phase *bub1-3HA spc7*, *bub1-3HA spc7-GFP*, or *bub1-3HA spc7-9TE-GFP* cells were prepared. Proteins were immunoprecipitated with anti-GFP antibodies, separated by SDS-PAGE, and subjected to western blot with anti-HA antibodies.
Phosphorylation of Spc7 Recruits the Bub1-Bub3 Complex to Kinetochores

These results persuaded us to examine the influence of Spc7 phosphorylation on cell-cycle-dependent localization of Bub1. We find that Bub1 localizes between separated spindle poles in control and spc7-9TE cells during early mitosis, but is undetectable in either spc7-9TA or spc7-9MA mutants during mitosis or at any other stage of the cell cycle (Figure 2A).
Strikingly, Bub1 colocalizes with spindle poles throughout the cell cycle in spc7-9TE, but not in WT, cells (Figures 2A). To verify this, we performed chromatin immunoprecipitation (ChIP) in cycling and metaphase arrested cultures. Whereas Bub1 binds to centromeric DNA only during mitosis in WT cells, Bub1 binds strongly to centromeric DNA even in cycling spc7-9TE cells (Figure 2B). Notably, association of Bub1 to centromeric DNA is substantially reduced, but not completely abolished, in metaphase arrested spc7-9TA cells (Figure 2B).

To confirm this localization, we monitored Bub1-GFP localization in fta3-mRFP cells following overexpression of Mad2, which arrests cells in metaphase by hyperactivation of the SAC [27]. Fta3 is a component of the Mit6 complex that binds the kinetochore throughout the cell cycle [28]. Although Bub1 colocalizes with kinetochores in WT and spc7-9TE cells, we were unable to detect Bub1 at kinetochores in metaphase arrested spc7-9TA or spc7-9MA cells (Figure 2C). These data suggest that Mph1 phosphorylates the MELT motifs in Spc7 to recruit Bub1 to kinetochores during early mitosis.

We next examined the dependency of Bub1 localization on Mph1 and Bub3. Bub1 colocalizes with kinetochores throughout the cell cycle in mphp1-D459A spc7-9TE cells but not in mphp3 spc7-9TE cells. The phosphomimetic spc7-9TE mutant therefore bypasses the requirement for Mph1 kinase activity to load Bub1 to kinetochores, but Bub1 loading still requires the presence of Bub3 (Figure 3A). Consistent with this, interaction of bacterialy expressed Spc7-9TE with Bub1 in cell extracts requires Bub3, but not Mad3 (Figure S2A). Like Bub1, Bub3 localizes to the kinetochore throughout the cell cycle in spc7-9TE cells but fails to accumulate at kinetochores in mitotic spc7-9TA or spc7-9MA cells (Figure 3B). Moreover, association of Bub3 with kinetochores is dependent on Mph1 activity, and ectopic association of Bub3 to kinetochores in spc7-9TE cells bypasses the requirement for Mph1 (Figures S2B and S2C). However, Bub3 loading still requires Bub1 in these cells (Figure S2C). These data suggest that Mph1 phosphorylation of the MELT motifs in Spc7 recruits the Bub1-Bub3 complex to kinetochores. Notably, ectopic association of Bub1 and Bub3 to kinetochores in spc7-9TE cells does not by itself cause cell-cycle arrest. By contrast, when Mph1 is ectopically tethered to Ndc80, Mph1 promotes association of Bub1 to kinetochores and SAC arrest [22]. One possibility is that, in this situation, Mph1 phosphorylates other components of the SAC, such as Mad1, Mad2, and Mad3, to directly activate the SAC, or destabilizes microtubule-kinetochore interactions to indirectly activate the SAC [23, 29].

Phosphorylation of Spc7 Is Required for Normal Recruitment of Mad1 and Mad2 to Kinetochore

We previously found that association of Mad1 and Mad2 to kinetochores is substantially reduced in cells lacking Bub3 [30]. This persuaded us to examine whether phosphorylation of Spc7 MELT motifs influences the localization of other SAC proteins. In WT cells, Mad1 and Mad2 form bright foci that colocalize with kinetochores in approximately 3% of cells. We find that localization of Mad1 and Mad2 to kinetochores is substantially diminished, but not completely abolished, in mitotic spc7-9TA and spc7-9MA cells (Figures S3A and S3B). Importantly, neither Mad1 nor Mad2 is ectopically recruited to kinetochores during interphase in spc7-9TE cells, indicating that ectopic recruitment of Bub1 and Bub3 to kinetochores is not sufficient to recruit Mad1 and Mad2, consistent with previous observations [22] (Figures S3A and S3B).

Spc7-MELT Mutants Have Severe Chromosome Segregation Defects

We next examined the effect of spc7-MELT mutants on chromosome segregation during mitosis. We find that spc7-9TA, spc7-9MA, and spc7-9MA,9TA mutants are acutely sensitive to thiabendazole (TBZ), a microtubule depolymerizing agent, and profoundly defective in maintenance of an artificial mini-chromosome, similar to that observed in the absence of Mph1 [31] (Figures 4A and 4B). By comparison, spc7-9TE mutants are slightly sensitive to TBZ and have no appreciable defect in chromosome segregation compared to WT cells (Figures 4A and 4B). Congruously, we find that spc7-9TA, spc7-9MA, and spc7-9MA,9TA, but not spc7-9TE, mutants are lethal in cells lacking Dis2 (type 1 phosphatase), Klp5 (kinesin-8), or Dis1 (XMAP215), all of which are required for accurate chromosome segregation (Table S1; [32–34]). Notably, both spc7-9TA and spc7-9TE mutants are synthetically lethal with mad1 mutants, indicating that the spc7-9TE mutant is not completely WT (Table S1). Cells lacking Dam1, Dis2, Klp5, or Dis1 exhibit delayed anaphase onset and numerous synthetic lethal interactions with components of the SAC [34–38]. By contrast, none of the spc7-MELT mutants showed any synthetic interactions with cells lacking Bub1, Bub3, Mph1, Mad2, or Mad3 (Table S1). Moreover, the synthetic lethality between spc7-MELT mutants and cells lacking Dam1, Dis2, Klp5, or Dis1 is not reversed by simultaneously deleting Mad3, indicating that these synthetic lethalties are probably due to a defect in chromosome segregation rather than hyperactivation of the SAC (Table S1).
Nsk1-GFP does not appear at spindle poles at early time points in spc7-9TA mutants, indicating that phosphorylation of the MELT motifs is not required for the initial SAC response when microtubules are completely absent (2 hr time point, Figure 4D). However, at later time points, Nsk1 appears at spindle poles in ~10% of spc7-9TA cells (3–6 hr time points, Figure 4D) indicating that phosphorylation of the MELT motifs is required to maintain the SAC signal. Counting the area under this curve shows that >60% of these cells have failed to maintain the SAC arrest through the 6 hr time course. SAC proficiency is similarly affected in spc7-9MA cells, and to a lesser extent in spc7-9TE cells (Figure S4B). This checkpoint defect is stronger than that
Figure 4. Phosphorylation of Spc7 MELT Motifs Is Required for Accurate Chromosome Segregation and Maintenance of the Spindle Checkpoint
(A) spc7-9TA, spc7-9MA, and spc7-9MA,9TA cells were plated onto YEA (yeast extract with adenine) plates containing indicated TBZ concentrations and incubated for 3 days at 30°C.
(B) Loss of the Ch16 (ade6-M216) mini-chromosome was measured using a colony-sectoring assay. Error bars represent the SD of three independent experiments.
(C) Anaphase onset is profoundly delayed in spc7-9TA, spc7-9MA, and spc7-9MA,9TA but only slightly delayed in spc7-9TE mutants. Log phase cultures of wild-type (spc7+), Δspc7, Δspc7 spc7-9TA, Δspc7 spc7-9MA, Δspc7 spc7-9TE, and Δspc7 spc7-9MA,9TA cells, expressing cdc13-GFP in the presence (blue bars) or absence (red bars) of Mad3 were fixed and the percentage of cells with Cdc13 on spindles and separated spindle pole bodies was assessed. Error bars represent the SD from three independent experiments.
(D) Phosphorylation of Spc7 MELT motifs is required for maintenance of the spindle checkpoint. Log phase cultures of wild-type (spc7+), Δmad3, Δbub3, Δspc7 spc7, Δspc7 spc7-9TA, Δspc7 spc7-9MA, and Δspc7 spc7-9TE cells expressing nda3-KM311 cort1-as3 nsk1-GFP were synchronized in early G2 by lactose gradient centrifugation and incubated at 18°C for the times indicated. The cells were fixed, and the percentage of cells with spindle pole associated Nsk1 was assessed.
(E) Ectopic recruitment of Bub1 and Bub3 to Spc7 aids spindle checkpoint silencing. Log phase cultures of wild-type (spc7+), Δdis2, Δbub3, Δspc7 spc7, Δspc7 spc7-9TE, and Δspc7 spc7-9TE, Δbub3 cells expressing nda3-KM311 cort1-as3 nsk1-GFP were synchronized in prometaphase by incubating at 18°C for 6 hr, and 5 μM 1NMPP1 was added. At the times indicated, the cells were fixed and the percentage of cells with spindle pole associated Nsk1 was assessed. Error bars represent the SD from three independent experiments.
observed in  \( \delta \)bub3 fission yeast cells, where neither Bub1 nor Mad3 can be recruited to kinetochores, yet the SAC remains robust ([Figure 4D]; [30, 41]). This creates an apparent paradox: why should mutation of the kinetochore binding site for Bub1-Bub3 cause more of a SAC defect than completely abolishing Bub1 recruitment to kinetochores (in  \( \delta \)bub3)? Surprisingly, when spc7-9TA was combined with  \( \delta \)bub3, the SAC response was much improved, although this did not suppress the chromosome missegregation defects of spc7-9TA ([Figure 4D]; data not shown). This demonstrates that the SAC defect observed in spc7-9TA is not due to reduced Bub1-Bub3 recruitment at kinetochores, but it suggests that when the Bub1-Bub3 complex is not bound to Spc7, it acts as a dominant-negative factor that prevents maintenance of the SAC signal. The precise explanation of this effect requires further analysis.

Enhanced Recruitment of Bub1-Bub3 to Spc7 Aids Spindle Checkpoint Silencing

We previously showed that  \( \delta \)bub3 mutants, where Bub1 and Mad3 are entirely absent from kinetochores, have SAC silencing defects [30], so we were keen to analyze SAC silencing in spc7-MELT mutants. Unfortunately, we were unable to construct the necessary spc7-9TA or spc7-9MA strains, but we have analyzed the spc7-9TE silencing phenotype. To do this, we monitored Nsk1 association to spindle poles following chemical inactivation of Ark1 (Aurora B) in mitotically arrested nda3-KM311 ark1-as3 nsk1-GFP cells. In this assay, the SAC is inactivated by addition of 1NMP1, which selectively inhibits analog-sensitive Ark1 [42], thus promoting silencing (or over-ride) of the SAC signal. In this situation, PP1\(^{\text{disc}}\) is essential for dissociation of Mad2 and Mad3 from the APC/C and for activation of the APC/C complex, which triggers cyclin B destruction and appearance of Nsk1 on spindle poles ([Figure 4E]; [35, 43]). We find that, upon addition of 1NMP1, Nsk1 appears more rapidly on spindle poles in spc7-9TE cells than in WT cells ([Figure 4E]). Likewise, we also observe more rapid destruction of Cdc13 (cyclin B) in metaphase arrested nda3-KM311 ark1-as3 cdc13-GFP spc7-9TE cells upon addition of 1NMP1 than in control cells ([Figure S4C]). Importantly, the rapid appearance of Nsk1 on spindle poles in spc7-9TE cells is completely abolished in the absence of Bub3 ([Figure 4E]). These results suggest that enhanced recruitment of Bub1 and Bub3 to the kinetochore in spc7-9TE cells promotes silencing of the SAC to such an extent that it becomes more efficient than in WT cells.

Discussion

At present, it is unclear whether Bub1 or Bub3 interact directly with phosphorylated MELT motifs in Spc7/KNL1. Bub3 contains seven WD40 repeats that are arranged in a radial pattern to form a \( \beta \)-propeller, a three-dimensional structure that mediates protein-protein interactions [44]. Some proteins containing WD40 domains, such as Cdc4, only interact with phosphorylated target proteins. Indeed Sic1, an inhibitor of cyclin B/Cdk1 in budding yeast, is only recognized and ubiquitinated by SCF\(^{\text{Cdc4}}\) when phosphorylated on multiple sites by G1 and S phase cyclin/Cdk1 complexes [45]. In the same manner, Bub3 may only interact with Spc7/KNL1 that has been multiply phosphorylated to recruit Bub1. If this is the case, we predict that the methionine of the MELT motif is also essential for Bub3 binding because spc7-9TA, spc7-9MA, and spc7-9TA,9MA mutants have indistinguishable phenotypes. However, we observe weak binding of Bub1 to centromeric DNA in metaphase arrested spc7-9TA cells, indicating that Bub1 may form additional contacts with Spc7 that contribute to the overall stability of the Spc7-Bub1-Bub3 complex. This may be analogous to the interaction observed between the TPR domains of Bub1 and the KI1 domain of KNL1, although the KI motifs do not appear to be conserved in fission yeast Spc7 [13, 14, 16]. Alternatively, because Mph1 can phosphorylate other sites in Spc7-9TA in vitro, Mph1 may promote Bub1 binding through a mechanism that does not involve phosphorylation of the MELT motifs.

It is important to note that spc7-9TA, spc7-9MA, and spc7-9TA,9MA mutants missegregate chromosomes at high frequency, whereas cells lacking Bub3 are only marginally more defective than WT [41] ([Figure 4B]). Thus, phosphorylation of the MELT motifs is necessary for accurate chromosome segregation independently of, or in addition to, recruitment of Bub1 and Bub3. Potentially, MELT motifs could bind an unidentified factor required for chromosome segregation. Alternatively, phosphorylation of the MELT motifs may influence the dynamic architecture of the outer kinetochore KMN complex during mitosis, and thus reduce the ability of kinetochores to interact with microtubules. For example, MELT phosphorylation may be required to position PP1\(^{\text{disc}}\) bound to the N terminus of Spc7 near its substrates so that it can stabilize microtubule-kinetochore interactions. Two studies have recently demonstrated that intrakinetochore stretch is necessary and sufficient to satisfy the SAC, although it is unknown how this stretching is sensed [46, 47]. Because spc7-9TA and spc7-9MA mutants are profoundly defective in chromosome segregation and fail to accumulate SAC components at kinetochores, it is tempting to speculate that the repetitive MELT motifs in Spc7/KNL1 may act as a quantitative sensor of intrakinetochore stretch. Further experiments are needed to address this and other possibilities.

Notably, the accompanying paper by London et al. [48] in this issue of Current Biology indicates that Mps1 is the major kinase that associates with kinetochores purified from budding yeast cells and, second, that phosphorylation of the MELT motifs in Spc105 (the budding yeast homolog of Spc7) by Mps1 is required for Bub1-Bub3 recruitment to kinetochores, suggesting that the mechanism targeting the Bub1-Bub3 complex to kinetochores is conserved [48]. Although Bub3 is not critical for SAC arrest in S. pombe [30, 41, 49], disruption of the binding sites for the Bub1-Bub3 complex on Spc7 causes a defect in maintenance of the SAC signal. Conversely, ectopic recruitment of the Bub1-Bub3 complex to Spc7 in spc7-9TE cells enhances SAC silencing, providing strong confirmation of our previous observations [30]. One possibility is that association of the Bub1-Bub3 complex to the MELT motifs in Spc7/KNL1 provides a docking site for the MCC to be diphosphorylated and inactivated by PP1, which binds the N terminus of Spc7/KNL1 [11, 12]. Although this model is attractive, it is probably too simplistic because at least one other pool of PP1, bound to Klp5/Klp6 complex (kinesin-8), is also required for efficient SAC silencing in fission yeast [11]. Clearly, identifying the PP1 targets required for SAC silencing and analyzing the function of the conserved MELT motifs in KNL1 for SAC signaling in vertebrate cells are important goals for the future.

Supplemental Information

Supplemental Information includes four figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.03.051.
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