PP2A-B56 opposes Mps1 phosphorylation of Knl1 and thereby promotes spindle assembly checkpoint silencing

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Introduction

Mps1-dependent spindle assembly checkpoint (SAC) signaling delays anaphase entry until all chromosomes have been correctly attached to the mitotic spindle (Foley and Kapoor, 2013). Knl1 is the kinetochore localized binding partner for the SAC proteins Bub1, BubR1, and Bub3 (Kiyomitsu et al., 2007). Phosphorylation of Knl1 by Mps1 is a prerequisite for the interaction of Bub1 and Bub3 with Knl1 (Schittenhelm et al., 2009; Krenn et al., 2012, 2014; London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012; Primorac et al., 2013; Vleugel et al., 2013). Once amphitelic kinetochore attachment has been achieved, the kinetochore levels of Bub1, Bub3, and BubR1 drop (Funabiki and Wynne, 2013). This SAC silencing process requires the reversal of Mps1-mediated phosphorylations on Knl1 to stop further recruitment of SAC proteins and production of the wait-anaphase signal. In yeast, PP1 is an important SAC phosphatase promoting SAC silencing by opposing Mps1, as well as Aurora B (Pinsky et al., 2009; Vanoosthuyse and Hardwick, 2009; Meadows et al., 2011; Rosenberg et al., 2011; London et al., 2012). While in mammalian cells a BubR1-associated pool of PP2A-B56 has recently been shown to oppose Aurora B (Foley et al., 2011; Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013), the phosphatase opposing Mps1 has not been identified. Here we demonstrate in vivo and in vitro that BubR1-associated PP2A-B56 is a key phosphatase for the removal of the Mps1-mediated Knl1 phosphorylations necessary for Bub1/BubR1 recruitment in mammalian cells. SAC silencing is thus promoted by a negative feedback loop involving the Mps1-dependent recruitment of a phosphatase opposing Mps1. Our findings extend the previously reported role for BubR1-associated PP2A-B56 in opposing Aurora B and suggest that BubR1-bound PP2A-B56 integrates kinetochore surveillance and silencing of the SAC.
**Results and discussion**

**PP2A opposes Mps1-dependent kinetochore localization of BubR1 and Bub1**

To investigate the role of dephosphorylation in the early events of SAC silencing (Fig. 1 A), we developed an assay in which SAC arrested cells were treated with a brief pulse of the specific Mps1 inhibitor AZ3146 to synchronously inactivate the checkpoint (Hewitt et al., 2010). Downstream events and mitotic exit are prevented by simultaneous addition of the proteasome inhibitor MG132. In control cells, Mps1 inhibition resulted in the loss of BubR1 and Bub1 from the kinetochore within 5 min (Fig. S1 A), which confirms the dependence of these SAC proteins on Mps1 activity for localization (Maciejowski et al., 2010; Sliedrecht et al., 2010). Mps1 inhibition in the presence of the general phosphoprotein phosphatase (PPP) inhibitor calyculin A resulted in retention of BubR1 and Bub1 at kinetochores (Fig. 1, B and C). Since Bub1 behaved identically to BubR1 in all experiments, BubR1 staining is representative of both Bub1 and BubR1 in all figures. Our observations indicate the presence of a highly active PPP-family phosphatase in SAC-arrested cells, promoting rapid dissociation of Bub1 and BubR1 from kinetochores in the absence of opposing Mps1 activity (see the model in Fig. 1 A).

To identify the specific PPP phosphatase, SAC inactivation assays were performed in cells depleted of the PPP family catalytic subunits, PP1–6 (Fig. 1 D). Under these conditions, BubR1 levels at the kinetochore remained similar to the control (Fig. 1 D, Mps1 active/Control), which indicates that the SAC was still active. Although all PPP-family catalytic subunits were efficiently depleted (Fig. 1 F), only depletion of the PP2A catalytic subunit α, or α and β together, resulted in quantitative retention of the BubR1 signal (Fig. 1 D, Mps1 inactive/Mps1 inhibition; and Fig. 1 E). Because Knl1-associated PP1 had previously been identified as the phosphatase opposing Knl1 phosphorylation by Mps1 in yeast (London et al., 2012), and PP1α and PP1γ had also been shown to interact with human Knl1 (Liu et al., 2010), we further investigated the potential involvement of PP1α and PP1γ. To test for potential redundancy between these two PP1 catalytic subunits, PP1α and -γ were depleted together and for longer (84 h). Under these conditions, haspin-dependent histone H3 phospho Thr3 staining, a known PP1 target (Qian et al., 2011), was efficiently retained in the presence of the haspin inhibitor 5-iodotubercidine (De Antoni et al., 2012), confirming functional knockdown of PP1α and -γ (Fig. S1 B). Nevertheless, protection of BubR1 kinetochore localization upon Mps1 inhibition was not observed (Fig. S1 C). Therefore, under these conditions a PP2A phosphatase opposes Mps1 activity in SAC-arrested human cells, whereas PP1 does not. We thus focused on PP2A as a candidate phosphatase initiating Knl1 dephosphorylation during SAC silencing.

**A PP2A-B56 holoenzyme complex regulates Bub1 and BubR1 kinetochore localization**

PP2A holoenzymes consist of a catalytic subunit, a scaffolding subunit (PP2A-A), and a member of one of four families of regulatory subunits B, B’ B”, or B”’. The B (PP2A-B55) and B’ (PP2A-B56) families are most relevant for mitotic progression in mammalian cells (Bollen et al., 2009; Barr et al., 2011). To define the PP2A holoenzyme complex promoting release of BubR1/Bub1 from the kinetochore, cells were depleted of the PP2A scaffolding subunit, PP2A-A, or all subunits of the PP2A-B55 and -B56 regulatory subunit families (Foley et al., 2011; Cundell et al., 2013; Fig. 2. A and B). Western blotting of selected PP2A-B55 or -B56 subunits demonstrated efficient depletion (Fig. 2 C). Removal of the main PP2A scaffolding subunit PP2A-A as well as depletion of all B56, but not B55 subunits, resulted in the retention of BubR1 kinetochore signal (Fig. 2, A and B). Furthermore, live cell imaging of PP2A-B56–depleted HeLa cells expressing GFP-BubR1 showed a strongly delayed loss of kinetochore GFP signal upon Mps1 inhibition (Fig. 2, D and E). Further investigation revealed that although full retention of BubR1 kinetochore localization was only achieved when all B56 subunits were depleted simultaneously, PP2A-B56 was the largest single contributor to this activity (Fig. S2, A–C). Together, these results show that the PP2A holoenzyme regulating Bub1 and Bub1 kinetochore association consists of PP2ACαβ, PP2A-A, and PP2A-B56 (Fig. 2, A and B), and point to a potential redundancy between PP2A-B56 isoforms, also observed in other studies (Foley et al., 2011; Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013).

**Delayed mitotic exit in the absence of PP2A-B56**

PP2A-B56 has recently been found to regulate microtubule–kinetochore attachments by opposing Aurora B and Plk1 activity, resulting in severe chromosome alignment problems in the absence of PP2A-B56 (Foley et al., 2011). Live-cell imaging of GFP-tubulin histone H2B-mCherry HeLa cells revealed a persistent prometaphase arrest when cells were depleted of all PP2A-B56 subunits (Fig. S2 D). Our data suggest that this arrest is caused by a combination of the inability to initiate SAC silencing by dephosphorylating Knl1, and persistent SAC activation caused by incorrect microtubule–kinetochore attachments (Foley et al., 2011; Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013). To separate the role of PP2A-B56 in SAC silencing from its role in promoting microtubule–kinetochore attachment, mitotic PP2A-B56–depleted cells were treated with the Mps1 inhibitor AZ3146 to overcome the persistent SAC activation and induce mitotic exit. In this situation, PP2A-B56–depleted mitotic cells required significantly more time to initiate sister chromatid separation than control cells (on average, 27.75 ± 4.74 min in siPP2A-B56 cells [n = 12] versus 7.27 ± 1.74 min in control cells [n = 10]; Fig. 2. F and G). This strongly suggests a requirement for PP2A-B56 in the silencing of the SAC in addition to its known role in stabilizing microtubule–kinetochore interactions.

**PP2A-B56 dephosphorylates Mps1-phosphorylated Knl1**

Bub1 and BubR1 binding to the kinetochore is dependent on Mps1-phosphorylated Knl1 (London et al., 2012; Shepperd et al.,
Figure 1. BubR1 and Bub1 localization to kinetochores is negatively regulated by PP2A. (A) Mps1 phosphorylation of Knl1 recruits Bub1, Bub3, and BubR1. An unidentified SAC phosphatase opposes Mps1 and controls kinetochore release of Bub proteins. (B) Nocodazole-arrested Hela cells were treated with MG132 [control] or an MG132/Mps1 inhibitor AZ3146 mix [Mps1 inhibition], or preincubated for 3 min with 25 nM calyculin A before AZ3146/MG132 addition [Mps1 + PPP inhibition]. Cells were fixed and stained for Bub1, BubR1, and kinetochores [CREST]. Bar, 10 µm. (C) CREST-normalized BubR1 kinetochore intensity was plotted [mean ± SD [error bars]; n ≥ 120 kinetochores per bar]. (D) HeLa cells depleted of individual PPP family catalytic subunits were nocodazole arrested, treated for 5 min with either MG132 or MG132/AZ3146, fixed, and stained for BubR1 and kinetochores [CREST]. Bar, 10 µm. (E) Quantitation of the ratio of CREST-normalized BubR1 signal in control and AZ3146-treated cells [mean ± SD [error bars]; n ≥ 120 kinetochores per bar]. Images shown are representative of three independent experiments. (F) Cell lysates of the cells used in D were blotted as indicated.
all PP2A-B56 but not PP2A-B55 regulatory subunits (Fig. 3, A and B). This suggested that PP2A-B56 dephosphorylates pKnl1-pT875. Consistent with this idea, we observed an almost twofold increase in pKnl1-pT875 signal in asynchronously cycling cells depleted of PP2A-B56 (Fig. 3, C and D).

To verify that Knl1 is a direct PP2A-B56 substrate, GST-tagged Knl1 728-1200 or Knl1 728-1200-T875A, in which the 2012; Yamagishi et al., 2012). To demonstrate that PP2A-B56 dephosphorylates this form of Knl1, an antibody was raised against one of the conserved Mps1 phosphorylation sites on Knl1, pT875 (Yamagishi et al., 2012). Staining with anti–pKnl1-pT875 was dependent on the presence of Knl1 and Mps1 activity (Fig. S3, A and B). Importantly, the loss of pKnl1-pT875 signal upon Mps1 inhibitor treatment could be prevented by depleting all PP2A-B56 but not PP2A-B55 regulatory subunits (Fig. 3, A and B). This suggested that PP2A-B56 dephosphorylates pKnl1-pT875. Consistent with this idea, we observed an almost twofold increase in pKnl1-pT875 signal in asynchronously cycling cells depleted of PP2A-B56 (Fig. 3, C and D).

To verify that Knl1 is a direct PP2A-B56 substrate, GST-tagged Knl1728-1200 or Knl1728-1200-T875A, in which the
phospho-acceptor threonine 875 had been mutated to alanine, was in vitro phosphorylated by Mps1WT or catalytically inactive Mps1D664A. The phosphorylated protein was then detected by Western blotting using the pKnl1-pT875 antibody. Equal loading of Knl1 substrate and the addition of equivalent amounts of Mps1WT and Mps1D664A was confirmed by Western blotting. The asterisk indicates the position of the phosphorylated threonine recognized by the pKnl1-pT875 antibody. (F) GST-Knl1728-1200 was phosphorylated with Mps1WT, Mps1 was inhibited by the addition of AZ3146, and phospho-Knl1 was dephosphorylated by the addition of PP2A-B56 (Cundell et al., 2013). The final amounts of PP2A complexes in the different lanes of the assay were 0, 3.6, 8.75, 17.5, and 27 nM. pKnl1-pT875 and PP2A-B56 were visualized by Western blotting. Equal loading of GST-Knl1728-1200 and Mps1 was confirmed by Western blotting with anti-GST (Knl1) and anti-His (Mps1) antibodies. (G) GFP-Mis12 complexes with associated Knl1 and SAC proteins were purified from GFP-Mis12 HeLa cells. The immunoprecipitates were incubated with PP2A-B55 or PP2A-B56 and analyzed by Western blotting.
with purified PP2A-B56γ complexes (Cundell et al., 2013; Fig. 3 F). Titration of this PP2A enzyme complex demonstrated effective dephosphorylation of phosphorylated GST-Knl1128-1200 by 27 nM PP2A-B56γ, comparable to the amount of PP2A-B55 required to achieve tau dephosphorylation (20 nM; Xu et al., 2008; Fig. 3 F). To test dephosphorylation of phospho-Knl1 by PP2A complexes in a more physiological setting, GFP-Mis12 immunoprecipitates with associated Knl1 and SAC proteins (Kiyomitsu et al., 2007; Welburn et al., 2010) were incubated with equivalent amounts of PP2A-B56 and PP2A-B55, and supernatant and beads were separated and analyzed (Fig. 3 G). Addition of PP2A-B56 but not PP2A-B55 released Mis12 complex associated Bub1, Bub3, and BubR1 into the supernatant and reduced the pKnl1-pThr75 signal on the beads, which is consistent with dephosphorylation of phospho-Knl1 by PP2A-B56 but not PP2A-B55 (Fig. 3 G and Fig. S3 C).

**BubR1-bound PP2A-B56 regulates kinetochore release of BubR1 and Bub1**

Our data so far indicate that PP2A-B56 is a key phosphatase for the dephosphorylation of Mps1-phosphorylated Knl1. Loss of localized PP2A-B56 activity should thus lead to retention of Bub1 and BubR1 at the kinetochore in the absence of Mps1 activity. Recent studies indicate that two pathways exist to localize PP2A-B56 to the centromere and kinetochore region of mammalian chromosomes. First, PP2A-B56 is targeted to the centromere by interaction with Sgo2, one of the two shugoshin proteins in mammalian cells (Kitajima et al., 2006; Xu et al., 2009; Tanno et al., 2010), and second, a distinct pool of PP2A-B56 is localized to the outer kinetochore via the SAC protein BubR1 (Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013). Although Sgo2 depletion resulted in a strong reduction of centromeric PP2A-B56α (Fig. S3, D and E; Tanno et al., 2010), loss of kinetochore BubR1 upon Mps1 inhibition was not affected (Fig. S3F), which indicates that Sgo2-bound PP2A-B56 does not contribute to phospho-Knl1 dephosphorylation. In contrast, replacement of endogenous BubR1 with equivalent, physiological levels of GFP-BubR1WT or GFP-BubR1L669A/V672A mutant for PP2A-B56 binding (Kruse et al., 2013), demonstrated that cells expressing GFP-BubR1L669A/V672A, but not GFP-BubR1WT, retained both GFP-BubR1 and endogenous Bub1 on the kinetochore after Mps1 inhibition (Fig. 4, A–C). Furthermore, when progressing through an undisturbed cell cycle, cells expressing only GFP-BubR1L669A/V672A retained high GFP-BubR1 levels on kinetochores and remained on average three times longer in mitosis than GFP-BubR1WT cells (Fig. 4, D–F). Confirming this, in fixed GFP-BubR1WT cells approximately half of all mitotic figures were in anaphase after 11 h of release from a thymidine block, as indicated by separated DNA masses and loss of securin staining (Fig. 4 G). In contrast, cells expressing GFP-BubR1L669A/V672A displayed a markedly higher proportion of prometaphase figures and almost no anaphase figures (Fig. 4 G), which indicates that the absence of BubR1-associated PP2A-B56 delayed mitotic exit due to a combined effect of impaired microtubule–kinetochore attachment formation (Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013) and an inability to release Bub1 and BubR1 from the kinetochores and silence the SAC (Fig. 4, D and G). Together, these findings suggest that BubR1-associated PP2A-B56 establishes a negative feedback loop affecting its own recruitment (Fig. 4 H) and regulating SAC silencing and anaphase entry.

**Regulation of the SAC by PP2A-B56**

We have shown that in mammalian cells, a key phosphatase for the dephosphorylation of the Bub1/Bub3/BubR1 phospho-binding sites on Knl1 is BubR1-associated PP2A-B56 (see Fig. 5 for model). This result is intriguing because in yeast, Knl1-associated PP1 has been shown to be the phosphatase responsible for reversing the Mps1-mediated phosphorylation of Knl1/spc105/spc7, and Knl1-bound PP1 had also been implicated in SAC silencing in metazoan cells (Espeut et al., 2012; London et al., 2012; Zhang et al., 2014). Although these studies indisputably indicate a requirement for PP1 in SAC silencing, it has also been demonstrated that in mammalian cells PP1γ can only associate with Knl1 once the Aurora B–phosphorylated PP1-binding SILK and RVSF motifs in the N terminus of Knl1 have been dephosphorylated upon microtubule–kinetochore attachment and physical separation of centromeric Aurora B and kinetochores localized to the centromere by interaction with Sgo2, one of the two shugoshin proteins in mammalian cells (Kitajima et al., 2006; Xu et al., 2009; Tanno et al., 2010), and second, a distinct pool of PP2A-B56 is localized to the outer kinetochore via the SAC protein BubR1 (Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013). Although Sgo2 depletion resulted in a strong reduction of centromeric PP2A-B56α (Fig. S3, D and E; Tanno et al., 2010), loss of kinetochore BubR1 upon Mps1 inhibition was not affected (Fig. S3F), which indicates that Sgo2-bound PP2A-B56 does not contribute to phospho-Knl1 dephosphorylation. In contrast, replacement of endogenous BubR1 with equivalent, physiological levels of GFP-BubR1WT or GFP-BubR1L669A/V672A mutant for PP2A-B56 binding (Kruse et al., 2013), demonstrated that cells expressing GFP-BubR1L669A/V672A, but not GFP-BubR1WT, retained both GFP-BubR1 and endogenous Bub1 on the kinetochore after Mps1 inhibition (Fig. 4, A–C). Furthermore, when progressing through an undisturbed cell cycle, cells expressing only GFP-BubR1L669A/V672A retained high GFP-BubR1 levels on kinetochores and remained on average three times longer in mitosis than GFP-BubR1WT cells (Fig. 4, D–F). Confirming this, in fixed GFP-BubR1WT cells approximately half of all mitotic figures were in anaphase after 11 h of release from a thymidine block, as indicated by separated DNA masses and loss of securin staining (Fig. 4 G). In contrast, cells expressing GFP-BubR1L669A/V672A displayed a markedly higher proportion of prometaphase figures and almost no anaphase figures (Fig. 4 G), which indicates that the absence of BubR1-associated PP2A-B56 delayed mitotic exit due to a combined effect of impaired microtubule–kinetochore attachment formation (Suijkerbuijk et al., 2012; Kruse et al., 2013; Xu et al., 2013) and an inability to release Bub1 and BubR1 from the kinetochores and silence the SAC (Fig. 4, D and G). Together, these findings suggest that BubR1-associated PP2A-B56 establishes a negative feedback loop affecting its own recruitment (Fig. 4 H) and regulating SAC silencing and anaphase entry.
period of Mps1 hypersensitivity is created at the beginning of mitosis when Mps1 can phosphorylate Knl1 in the absence of the opposing phosphatase, generating a maximal SAC protein-binding platform that recruits Bub3/Bub1/BubR1 and associated PP2A-B56. Second, by tying PP2A-B56 to BubR1, the system is poised to extinguish the Bub1/BubR1 kinetochore signal as soon as Mps1 activity drops, yet remain potentially reactivatable because the Mps1 opposing phosphatase declines together with Mps1 levels. In the future it will be interesting to determine what proportion of kinetochore-associated BubR1 is used for mitotic checkpoint complex formation and PP2A-B56 recruitment, respectively.
Technologies). DNA primers were obtained from Invitrogen. siRNA single
(DBD). Mutagenesis was performed using the QuikChange method (Agilent
rying hexahistidine-tagged full-length wt or kinase-dead Mps1 cloned into
cloned into pGEX-5X-1 (GE Healthcare). Recombinant baculoviruses car-
were made using pcDNA5/FRT/TO vectors (Invitrogen) modified to en-
Mammalian expression constructs for BubR1, PP2A-B56
amplified from ORFeome clone 100069116 (Thermo Fisher Scientific).
polymerase (Promega). A fragment (aa 728–1,200) of human Knl1 was
, Mis12, BubR1, and Mps1 were ampli-
Molecular biology
Human PP2A-B56α, PP2A-B56α, Mis12, BubR1, and Mps1 were ampli-
from human tests cDNA (Marathon cDNA; Takara Bio Inc.) using Phu
polymerase (Promega). A fragment (aa 728–1,200) of human Knl1 was
amplified from ORFeome clone 100069116 (Thermo Fisher Scientific).
Mammalian expression constructs for Bub1, PP1, PP2A-B56α, and PP2A-B55α
were made using pcDNA5/FRT/TO vectors (Invitrogen) modified to en-
code the FLAG or GFP reading frames. For the generation of HeLa cells
stably expressing GFP-Mis12 or mCherry-histone H2B and EGFP-α-tubulin,
Histone H2A and α-tubulin were cloned into Bacterial-ori-based vectors encoding the
appropriate tags, the chicken β-actin promoter in the case of α-tubulin to reduce expression, and the bacterial and puromycin selection markers. For the generation of GST-tagged Knl12861200, this fragment of Knl1 was cloned into pGEX-5X-1 (GE Healthcare). Recombinant baculoviruses car-
ying hexahistidinetagged full-length wt or kinase-dead Mps1 cloned into the
pAC5G2 vector (BD) were produced using the BaculoGold system (BD). Mutagenesis was performed using the QuikChange method (Agilent
Technologies). DNA primers were obtained from Invitrogen. siRNA single
duplexes or On-target SMARTPools were obtained from GE Healthcare (see
Table S1 for sequences of phosphatase catalytic subunit SMART-
Pools). PP2A-B55 regulatory subunits were depleted simultaneously by mixing the individual SMARTPools for PP2R2A, β-, C-, and D. PP2A-
B56 regulatory subunits were depleted simultaneously by mixing single
siRNA duplexes targeting each individual regulatory subunit (Foley et al.,
2011). The siRNA oligo target sequence for the 3′ UTR of BubR1 was
5′-GCAATCAAGTCTCAGAT-3′.

Cell culture
HeLa cells were cultured in DMEM containing 10% [vol/vol] bovine calf serum at 37°C and 5% CO2. For plasmid transfection and siRNA transfection,
Mirus LT1 (Mirus Bio LLC) and Oligofectamine (Invitrogen), respectively,
were used. Stable HeLa cell lines with single copies of the desired transgene were created using the T-Rex doxycycline-inducible Flp-In system (Invitrogen; Tighe et al.,
2004). HeLa cells stably expressing GFP-Mis12 or HeLa cells stably expressing mCherry-histone H2B and EGFP-α-tubulin (Zeng et al.,
2010) were generated using a standard transfection protocol and selection with 0.7 mg/ml genetin or 1.0 µg/ml puromycin and 2.0 µg/ml blasticidin. For synchronization, cells were treated for 20 h with 2.5 mM thymidine, washed three times in PBS and twice with growth me-
dium, and then incubated in fresh growth medium for the indicated time. SP in infected cells for the expression of Mps1 wt and kinase-dead (KD) kinase
were grown at 27°C and atmospheric CO2 in TC100 containing 10% [vol/vol] bovine calf serum and 1% [vol/vol] Glutamax (Invitrogen).

Protein expression and purification
For the purification of recombinant hexahistidine-tagged full-length wild-
type and kinase-inactive D664A Mps1 from SP insect cells, 20 × 107
insect cells per construct were infected with the respective viruses at a mul-
tiplicity of infection of 10. After 60 h of infection, the cells were collected,
washed in cold PBS, and lysed for 10 min in IMAC20 buffer (20 mM Tris-
Cl, pH 8.0, 200 mM NaCl, and 20 mM imidazole) containing 0.1% Triton
X-100 and a protease inhibitor cocktail (Sigma-Aldrich). After a 2-h
incubation in the cold, the beads were washed extensively with IMAC20,
and bound proteins were eluted with IMAC20 buffer (20 mM Tris-
Cl, pH 8.0, 200 mM NaCl, and 200 mM imidazole). The peak fractions as judged by SDS-PAGE were pooled and dialyzed against PBS.

GST-tagged Knl12861200 was expressed in Escherichia coli BL21
cells. The pellet of a 2 liter bacterial culture was resuspended in 150 mM
Hepes, pH 8.0, 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and protease
inhibitor cocktail (Sigma-Aldrich), and lysed using a homogenizer (Emulsi-
flex). The lysate was cleared by centrifugation and incubated with glutathi-
one-Sepharose (GE Healthcare) for 2 h in the cold. After extensive washing
with lysis buffer, bound proteins were eluted with 100 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 20 mM reduced glutathione. Peak fractions as judged
by SDS-PAGE were pooled and dialyzed against 150 mM Hepes, pH 8.0, and
150 mM NaCl.

Figure 5. Model of Knl1 dephosphorylation by BubR1-associated PP2A-B56. Dephosphorylation of the Mps1 phosphorylation sites on Knl1 by BubR1-
associated PP2A-B56 results in removal of the binding sites for Bub1, Bub3, and Bub1 when Mps1 activity drops upon successful microtubule–kinetochore
attachment. This leads to loss of Bub1 and BubR1 from the kinetochore and initiation of SAC silencing.
PP2A-B55 and PP2A-B56 complexes were purified from FLAG-PP2A-B55- and FLAG-PP2A-B56-transfected HEK-293T cells as described previously (Cundell et al., 2013). In brief, three 15-cm dishes of HEK-293T cells per construct (pCDNA5-FLAG-PP2A-B55, pCDNA5-FLAG-PP2A-B56C, and empty FLAG vector) were transfected with 8 µg DNA and LTI transfections reagent (Minus Bio LLC) according to the manufacturer’s instructions. After 40 h of transfection, the cells were pelleted, washed in cold PBS, and lysed for 15 min on ice in 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% [vol/vol] Triton X-100, and 1 µm protease inhibitor cocktail. The lysates were cleared by centrifugation in an Eppendorf centrifuge (14,000 rpm, 4°C, 10 min), and the cleared lysates were incubated with FLAG-agarose beads (Sigma-Aldrich) for 3 h in the cold. The beads were washed twice with lysis buffer, four times with 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 0.1% [vol/vol] Triton X-100; once with 20 mM Tris-Cl, pH 8.0, and 150 mM NaCl; and once with 100 mM Tris-Cl, pH 8.0, and 1 mM MgCl2. PP2A complexes were eluted twice with 50 µl of elution buffer (0.2 mg/ml FLAG peptide in 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, and 1 mM MgCl2), each, in a VibeR rocker at room temperature. Beads and eluates were separated by centrifugation, DTT and glycerol were added to 1 mM and 20% final concentration, respectively, and the eluted proteins were snap frozen in liquid nitrogen.

Immunofluorescence analysis
Immunofluorescence analysis was performed as described previously (Dunsch et al., 2011) using PTEMF buffer (20 mM Pipes-KOH, pH 6.8, 0.2% Triton X-100, 1 mM MgCl2, 10 mM EGTA, and 4% formaldehyde) for fixation. Antibody dilutions were performed in PBS, 2% [vol/vol] BSA, and 0.1% [vol/vol] Triton X-100, except for phospho-Knl1 antibodies that were diluted in PBS, 2% [vol/vol] BSA, and 0.3% [vol/vol] Triton X-100. Samples on 1.5-thickness coverslips were imaged using a 60× 1.35 NA oil immersion objective lens on a microscope system (BX61; Olympus) equipped with filter sets for DAPI, EGF/Alexa Fluor 488, Alexa Fluor 555, and Alexa Fluor 647 (Chroma Technology Corp.); a CoolSNAP HQ2 camera (Roper Scientific); and MetaMorph 7.5 imaging software (GE Healthcare). Image z stacks comprising 12 images 0.2 µm apart were collected and maximum projected to give a single image for each color channel. The different color channels were then combined in Meta- Morph to give a 24-bit RGB image. These were cropped in Photoshop CS3 and imported into Illustrator CS3 (both from Adobe) for figure production. Quantitations of kinetochore intensities were performed using Velocity (PerkinElmer) or ImageJ software. Background-corrected kinetochore intensities (3–4 cells; ≥40 kinetochores per cell) determined by placing a 7 × 7-pixel circular region of interest over individual kinetochores were normalized to CREST or CenP-A signal as indicated. Analysis of kinetochore intensities was performed in Excel (Microsoft). All immunofluorescence experiments shown are representative of at least three independent experiments.

Spindle checkpoint silencing assay
For SAC silencing assays, HeLa cells were seeded into 6-well dishes with 200,000 cells per well 24 h before transfection. Three tubes were prepared containing the empty vector and the indicated constructs. One tube was mock transfected, one was transfected with the empty vector and the other with the indicated construct. For transfection, the culture media were replaced with 6-well dishes with 1 ml of Opti-MEM (Invitrogen). Three micrograms of DNA and 6 µl of Lipofectamine 2000 (Invitrogen) were used per well. After 3 h, the media were replaced with 3 ml of growth media and cells were left to grow for 16 h. 1 µg/ml MG132 and 2 µM AZ3146 (MPS1 inhibitor; Hewitt et al., 2008) was then added. Dephosphorylation was assessed by loss of phospho-specific signal. Table S1 gives siRNA target sequences. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201406109/DC1.

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