Bub1 autophosphorylation feeds back to regulate kinetochore docking and promote localized substrate phosphorylation

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During mitosis, Bub1 kinase phosphorylates histone H2A-T120 to promote centromere sister chromatid cohesion through recruitment of shugoshin (Sgo) proteins. The regulation and dynamics of H2A-T120 phosphorylation are poorly understood. Using quantitative phosphoproteomics we show that Bub1 is autophosphorylated at numerous sites. We confirm mitosis-specific autophosphorylation of a several residues and show that Bub1 activation is primed in interphase but fully achieved only in mitosis. Mutation of a single autophosphorylation site T589 alters kinetochore turnover of Bub1 and results in uniform H2A-T120 phosphorylation and Sgo recruitment along chromosome arms. Consequently, improper sister chromatid resolution and chromosome segregation errors are observed. Kinetochore tethering of Bub1-T589A refocuses H2A-T120 phosphorylation and Sgo1 to centromeres. Recruitment of the Bub1-Bub3-BubR1 axis to kinetochores has recently been extensively studied. Our data provide novel insight into the regulation and kinetochore residency of Bub1 and indicate that its localization is dynamic and tightly controlled through feedback autophosphorylation.
The accurate traverse through mitosis results in equal allocation of duplicated sister chromosomes and is critical for cellular and organism health. To ensure this, eukaryotes have evolved a safeguard mechanism known as the spindle assembly checkpoint (SAC), which functions during both meiosis and mitosis, and monitors the correct attachment of kinetochores to microtubules. The activities of both the SAC and the microtubule attachment machinery are orchestrated by a network of kinases and phosphatases. SAC kinases including budding uninhibited by benzimidazole 1 (Bub1), monopolar spindle 1 (Mps1) and Aurora B play a dual and interconnected role in microtubule attachment regulation and SAC signalling. Recently, a remarkable body of work has begun to outline how these kinases (and their counteracting phosphatases) monitor the status of attachments and relay this as a diffusible biochemical signal. A clear picture of the recruitment of the checkpoint kinase Bub1 to the kinetochore is beginning to emerge. Mps1 phosphorylation of so-called MELT motifs on the KNL1 subunit of the macromolecular KMN complex together with the KI (Lys-Ile) motif of KNL1 promote the recruitment of Bub1-Bub3 in a manner that involves multiple cooperative interactions. Less well understood is how this recruitment is dynamically regulated, although recent evidence supports a role for the protein phosphatases PP2A and PP1 in determining the extent of Bub1 recruitment. The current model posits that once at the kinetochore, Bub1 acts as a stable scaffold for recruitment of anaphase promoting complex/cyclosome (APC/C) inhibitors including BubR1, Mad1 and Mad2, as well as centromere proteins E and F, and the mitotic centromere-associated kinesin; this scaffolding function of Bub1 is thought to be kinase independent.

Bub1 also has kinase-dependent functions during mitosis. Cdc20 is an in vitro target of Bub1 and this phosphorylation may directly contribute to APC/Ccdc20 inhibition. Bub1 phosphorylation of the conserved histone H2A at T120 (H2A-T120, human numbering) results in a histone mark that mediates the recruitment of MEI-S332/shugoshin (Sgo) proteins to the centromere during both meiosis and mitosis. In mammalian mitosis, Bub1 recruitment of Sgo1 in complex with protein phosphatase 2A protects cohesion at centromeres until mammalian mitosis, Bub1 recruitment of Sgo1 in complex with the KI (Lys-Ile) motif of KNL1 promotes the recruitment of Bub1–Bub3 in a manner that involves multiple cooperative interactions. Less well understood is how this recruitment is dynamically regulated, although recent evidence supports a role for the protein phosphatases PP2A and PP1 in determining the extent of Bub1 recruitment. The current model posits that once at the kinetochore, Bub1 acts as a stable scaffold for recruitment of anaphase promoting complex/cyclosome (APC/C) inhibitors including BubR1, Mad1 and Mad2, as well as centromere proteins E and F, and the mitotic centromere-associated kinesin; this scaffolding function of Bub1 is thought to be kinase independent.

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Here we use a quantitative proteomics approach to identify Bub1-specific autophosphorylation sites. We show that Bub1 is significantly autophosphorylated outside the activation segment and kinase domain, including at the conserved threonine 589 (T589). We show the Bub1 activity is primed in interphase but does not fully mature until mitosis. Immunofluorescence with a phosphospecific antibody indicates that autophosphorylation at T589 is prevalent during early mitosis. Alanine substitution of this residue (T589A) results in chromosome missegregation and incomplete sister chromatid arm resolution as a result of non-localized H2A-T120 phosphorylation and ectopic Sgo1 recruitment. Fluorescence recovery after photobleaching (FRAP) experiments reveal that Bub1-T589A and Bub1-kinase dead (D946A, hereafter referred to as KD) exhibit more rapid kinetochore turnover than wild-type (WT) protein. Forced localization of Bub1-T589A to the kinetochore (KT) refocuses H2A-T120 phosphorylation and Sgo1 localization to the kinetochore. We propose that spatially constrained H2A-T120 phosphorylation, and thus sister chromatid cohesion, are promoted by a positive feedback mechanism formed by autophosphorylation of Bub1 at T589 that regulates the dynamics of Bub1 kinetochore docking.

Identification of Bub1 autophosphorylation sites. To identify Bub1 autophosphorylation sites, we devised an approach based on stable isotope labelling in cell culture (SILAC, Fig. 1a) of Bub1 WT and KD. To enable quantitation of the changes in phosphopeptide abundance by mass spectrometry (MS), cells were labelled by growing them in medium containing either light arginine and lysine (Argo/Lys0) or the heavy isotopic variants [15C,15N2]arginine and [13C6,15N2]lysine (Arg10/Lys0). Immunoprecipitated, mitotic, Bub1-WT and Bub1-KD expressed in differentially labelled cells were separately subjected to a non-radioactive in vitro kinase assay. This autophosphorylation amplification step was introduced to increase the occupancy of phosphorylation sites within Bub1 and thus increase phosphopeptide detection and, importantly, to allow distinction between genuine autophosphorylation sites and phosphorylation incurred by co-precipitating kinases. We also considered this approach superior to an in vitro assay of recombinant proteins as Bub1 mitotic modifications, localization and binding partners may all contribute to genuine and physiologically relevant Bub1 autophosphorylation. The experiment was performed in triplicate with minor changes: the amino acid labelling was reversed in one replicate (exp2, Fig. 1b) to control for a potential effect of amino acid labelling, and in the final replicate (exp3, Fig. 1b) a combination of Lys-C, Glu-C and elastase were used instead of trypsin to diversify peptide coverage.

Data from the three independent experiments resulted in a combined coverage of 68% of Bub1 and a total of 38 unique phosphorylation sites (MASCOT score cutoff of ≥13; Class I sites Supplementary Table S1), of which 30 sites could be definitively assigned SILAC and protein ratios in at least 1 replicate. Threonine 960 phosphorylation in the activation segment of the kinase domain was identified in all three experiments, was found to have a high phosphopeptide-peak ratio and was used as reference for normalization, results of which are shown in Fig. 1b. Several additional phosphosites were identified after Lys-C, Glu-C and elastase digestion, but contained neither lysine nor arginine and no SILAC ratio could be assigned. These were thus excluded from further analysis (see Supplementary Data 1).

Of the sites we identified, 19 were novel, whereas 19 have been previously curated in PhosphoSitePlus. The majority of the phosphorylation sites identified were situated in low complexity stretches in between domains (Fig. 1c), with the exception of T960 in the kinase activation segment.

Twenty phosphosites were significantly upregulated in Bub1-WT compared with Bub1-KD and thus considered potential Bub1 autophosphorylation sites (Fig. 1c, red). These sites exhibited a fold increase in phosphopeptide-peak ratio of ≥3, considered a conservative cutoff requirement for fold change. Alignment of these sites, together with H2A-T120 (Fig. 1d), suggested a tendency for basic (mainly K at positions −1 and +5) and small non-polar (at positions +2 and +3) residues relative to the phosphoacceptor, as well as an exclusion of acidic residues surrounding the phosphosites. Of the phosphosites that were not considered to be Bub1 dependent (phosphopeptide-peak ratio <3), 50% (residues 452, 459, 596 and 655) were followed by a proline, suggesting that they may be targets of a proline-directed kinase such as CDK1 or
mitogen-activated protein kinase, in agreement with previous observations\textsuperscript{22–24}. S314 and S331 adhered to the consensus for ATM/ATR kinases; S314 was previously identified as an ATM site and may be required for Bub1 activation\textsuperscript{25,26}. Two additional sites, S247 and S250, adhered to a Plk1/Mps1 consensus, which have also been shown to phosphorylate Bub1 (refs 24,27). Thus, Bub1 is highly phosphorylated by a number of mitotic kinases, including itself.

Regulation of Bub1 activation and autophosphorylation. To investigate Bub1 autophosphorylation at sites outside the activation segment, we generated phosphospecific antibodies towards two potential autophosphorylation sites, T589 and S679. These sites (see Fig. 2a for evolutionary alignment) were consistently autophosphorylated in our MS experiments. They were also preceded by at least one basic residue at the –1 (T589) or –2 (S679) position and have been independently observed in large-scale mitotic MS screens\textsuperscript{21,28}. We thus reasoned that they were genuine \textit{in vivo} autophosphorylation sites. Anti-pT589 staining of fixed cells clearly decorated kinetochores and overlapped the Bub1 signal in prophase and prometaphase (Supplementary Fig. 1a). Anti-pT589 signal was lost on depletion of Bub1 or phosphatase treatment (Supplementary Fig. 1b), demonstrating that the pT589 signal is both Bub1 dependent and phosphospecific. Importantly, depletion and rescue experiments revealed that the pT589 signal was lost in Bub1-KD and Bub1-T589A-expressing cells (Supplementary Fig. 1c), indicating that phosphorylation at T589 is strictly dependent on Bub1 kinase activity, in agreement with its identification as an autophosphorylation site. No signal was detected by immunofluorescence with anti-pS679 antibody, although there was a clear signal on western blottings. Anti-pS679 detects Bub1 from mitotic extracts, before but not after phosphatase treatment, demonstrating phosphospecificity of this antibody (Supplementary Fig. 1d).

A number of groups have recently reported on the role of the Bub1 tetratricopeptide repeat (TPR) domain in regulating kinase

Figure 1 | Identification of Bub1 autophosphorylation sites. (a) Schematic of the SILAC protocol for identification of Bub1 autophosphorylation sites.
(b) Heat map representation of normalized phosphopeptide:peptide ratio of phosphosites identified on Bub1 from three independent MS experiments.
(c) Cartoon illustration of the position of the identified phosphorylation sites relative to the functional domains of Bub1. Autophosphorylation sites are red; other phosphorylation sites are in black. (d) Weblogo representation and amino acid enrichment of Bub1 surrounding phosphorylation sites.
activity with conflicting results. We thus sought to determine the domains of Bub1 required for kinase function as measured by autophosphorylation. We depleted endogenous Bub1 with small interfering RNAs (siRNAs) targeting the 3′-untranslated region (3′-UTR) and expressed MYC-tagged Bub1, WT, KD, the Bub3-binding mutant (Δ229–256), the checkpoint mutant in conserved motif I (Δ458–476), the kinase extension domain mutant (Δ740–766) and the ΔTPR in HeLa cells. We then determined phosphorylation at T589 (Fig. 2c). As the Bub3-binding mutant Δ229–256 does not bind to the kinetochore, we forced kinetochore localization using a Mis12-tag to determine the role of Bub3 binding in Bub1 activation independent of its role in kinetochore recruitment. As expected, Bub1-WT-expressing cells demonstrated robust pT589 and pS679 signal, whereas little or no signal was observed in cells expressing Bub1-KD or the Bub1 kinase extension domain mutant (Δ740–766, Fig. 2b,c), confirming the status of these sites as bona fide Bub1 autophosphorylation sites. Bub3 binding,

Figure 2 | Full Bub1 activation is mitotic specific and requires the kinase extension domain. (a) Evolutionary conservation of Bub1 T589 and S679. (b) Bub1 deletion mutants were expressed in HeLa cells depleted of endogenous Bub1. Mitotic cells were stained with Hoechst (Blue in merge), anti-MYC (green) and anti-pT589 (red). Scale bar, 10 μM. Quantitation of pT589 signal relative to CREST at kinetochores (mean ± s.e.) from a minimum of ten cells per condition is indicated in the right-most panel. (c) Cells were transfected with Bub1 mutants as in b and enriched in mitosis by nocodazole treatment. Anti-pT679 (upper half) and anti-pT589 (bottom half) western blottings were performed with MYC-Bub1 immunoprecipitated from equalized lysates. Anti-MYC blotting (second and fourth panels) reveals equal loading. (d) MYC-Bub1 was immunoprecipitated from HeLa cells stably expressing MYC-Bub1-WT arrested in G1/S or mitosis by thymidine (THY) or nocodazole (NOC) treatment, respectively, and blotted with anti-pT679 antibodies (upper panel) or stripped and reprobed with anti-MYC. (e) Western blottings of histones purified from thymidine- and nocodazole-arrested cells with anti-H2A-pT120 (upper panel) or anti-H2A (lower panel) antibodies. (f) U2OS cells expressing a 256-copy array of the lac operator were transfected with a LacI-GFP, 3XMYC- Δlac-Bub1-WT or KD. Fixed cells were stained with Hoechst (blue), anti-MYC or GFP in the control (green) and either anti-H2A-pT120 (red, f) or anti-Sgo1 (red, g). The overlap between the MYC and H2A-pT120 or Sgo1 is shown in the panel on the right of each figure. Error bars represent s.e. Scale bar, 5 μM.
conserved motif I and the TPR domain of Bub1 did not significantly contribute to Bub1 kinase activity, as measured by TS89 and S679 phosphorylation (Fig. 2b,c). Kinetochores recruitment is therefore not required for Bub1 activation but serves to focus Bub1 kinase activity to kinetochores.

We were also intrigued by the recent suggestion that Bub1 is a constitutively active kinase based on the persistent phosphorylation of the P + 1 autophosphorylation site S969 in G1 (ref. 19). To definitively test this, we verified Bub1 autophosphorylation at S679 (Fig. 2d) as well as H2A-T120 (Fig. 2e) in extracts from thymidine- and nocodazole-arrested cells. We find that neither Bub1-S679 nor H2A-T120 (in agreement with previous results12) was apparently phosphorylated in interphase extracts, although a clear signal was detected in extracts from mitotic cells, suggesting that Bub1 was not generally active during interphase. Nevertheless, we considered the possibility that the constitutive phosphorylation of S969 may reflect partial Bub1 activity, as has been previously suggested19. To test whether Bub1 may be further activated during interphase, we expressed 3×MYC and Lac repressor (LacI)-fused Bub1 WT and Bub1 KD in cells stably expressing a 256 copy array of the lac operator sequence (LacO) in an arm of chromosome 1 (ref. 32) in an effort to artificially increase the localized concentration of Bub1. In interphase cells, LacI-tagged Bub1 WT and KD efficiently localized to the LacO array as indicated by anti-MYC immunofluorescence. In lacI-Bub1-WT- but not lacI-Bub1-KD-expressing cells or control cells, a clear overlapping signal was detected for H2A-pT120 and Sgo1 (Fig. 2f,g). Thus, increasing the local concentration of Bub1 is sufficient to induce its activation, even in the absence of kinetochores in interphase. This is in agreement with our data above showing that Bub1 activity is not dependent on Bub3 binding (Fig. 2b,c). Collectively, our results demonstrate that Bub1 phosphorylation at TS89 and S679 occurs in vivo and establish that these are indeed autophosphorylation sites. Moreover, our data confirm and extend earlier observations demonstrating that Bub1 activation is primed already in interphase. We show that under normal circumstances, Bub1 is not sufficiently active in interphase but can be efficiently activated by increasing the local concentration.

**Bub1 TS89 autophosphorylation regulates mitotic progression.**

We next focused on the role of TS89 autophosphorylation, as this site is highly evolutionarily conserved (Fig. 2a). We generated stable isogenic HeLa lines expressing a single copy of triple MYC and green fluorescence protein (GFP)-tagged Bub1 WT, KD and TS89A12 (Supplementary Fig. S2a,b). In in vitro kinase assays, Bub1-TS89A supported efficient H2A-T120 phosphorylation and mitotic arrest in the presence of nocodazole or taxol, suggesting that TS89 phosphorylation is not implicated in regulation of the kinase activity or the SAC function of Bub1 (Supplementary Fig. 2c,d).

We sought to test whether Bub1 autophosphorylation at TS89 contributes to chromosome congression, which requires Bub1 kinase activity12,29,33. Stable Bub1 lines were depleted of endogenous Bub1 and were treated with MG132 to monitor background staining-observed Bub1-WT- and Bub1-KD-expressing cells (Fig. 4b). Similar to Sgo1, expression of Bub1-TS89A led to localization of Sgo2 to chromosome arms (Fig. 4a). Similar to Bub1-KD, expression of Bub1-TS89A led to relocalization of Sgo1 to chromosome arms and the Sgo1 signal was more intense than that detected in Bub1-KD cells, an observation that was confirmed by corrected total cell fluorescence measurements directly on the chromosome arms (Fig. 4a and quantification within). Similarly, Sgo2 signal was detected at chromosome arms in cells expressing Bub1-KD, whereas it localized as expected to the centromere in Bub1-WT cells (Fig. 4b). Similar to Sgo1, expression of Bub1-TS89A led to relocation of Sgo2 to the chromosome arms (Fig. 4b), at levels considerably higher than seen in Bub1-KD-expressing cells. Nevertheless, a significant signal for Sgo2 could be clearly detected at the kinetochore, indicating that unlike Sgo1 a pool of Sgo2 remained insensitive to Bub1-KD and Bub1-TS89A. We next examined the H2A-T120 phosphorylation under the same conditions. In cells expressing Bub1-WT, H2A-pT120 was clearly localized to the centromere but lost in Bub1-KD-expressing cells, as expected. Expression of Bub1-TS89A, surprisingly, resulted in H2A-T120 phosphorylation along the entire length of the chromosome (Fig. 4c). Quantification of the H2A-pT120 signal specifically at chromosome arms revealed a significant increase in cells expressing this mutant compared with the essentially unphosphorylated Bub1-WT and Bub1-KD-expressing cells (Fig. 4c). To test whether the scaffolding function of Bub1 is altered by the loss of TS89 phosphorylation, we verified the localization of BubR1. We found that at least steady-state levels of BubR1 are unchanged between cells expressing Bub1-WT, KD or TS89A (Fig. 4d). Similarly, recent reports have concluded that Bub1 overexpression, which leads to H2A-pT120 spread to chromosome arms, did not alter the strength of the SAC or the recruitment of mitotic regulators29. Collectively, our data indicate that TS89 autophosphorylation limits H2A-pT120 and hence Sgo to centromeres. The extended mitosis observed in Bub1-TS89A cells may thus be a result of the

Bub1 autophosphorylation restricts H2A-pT120 to centromeres. The delay in mitotic progression in Bub1-TS89A-expressing cells was somewhat surprising, considering that the more severe KD mutation exhibited normal timing. We reasoned that the effect of the TS89A mutation on mitotic timing may be masked in the Bub1-KD, in which all Bub1 phosphorylation and activity are lost. To address this possibility, we sought to determine the effect of the TS89A mutant on kinase-dependent Bub1 signalling. The H2A-pT120 centromeric mark generated by Bub1 recruits Sgo1 and Sgo2 to promote chromosome biorientation and proper chromosome segregation14; lack of Bub1 protein or Bub1 kinase activity has been reported to cause the spread of Sgo1 along the entire length of the chromosome15,34,35. In agreement with these observations, we find that Sgo1 is mislocalized to chromosome arms in cells expressing Bub1-KD, whereas Sgo1 is primarily localized to the centromere in cells expressing Bub1-WT (ref. 34 and Fig. 4a). Similar to Bub1-KD, expression of Bub1-TS89A led to relocation of Sgo1 to chromosome arms and the Sgo1 signal was more intense than that detected in Bub1-KD cells, an observation that was confirmed by corrected total cell fluorescence measurements directly on the chromosome arms (Fig. 4a and quantification within). Similarly, Sgo2 signal was detected at chromosome arms in cells expressing Bub1-KD, whereas it localized as expected to the centromere in Bub1-WT cells (Fig. 4b). Similar to Sgo1, expression of Bub1-TS89A led to relocation of Sgo2 to the chromosome arms (Fig. 4b), at levels considerably higher than seen in Bub1-KD-expressing cells. Nevertheless, a significant signal for Sgo2 could be clearly detected at the kinetochore, indicating that unlike Sgo1 a pool of Sgo2 remained insensitive to Bub1-KD and Bub1-TS89A. We next examined the H2A-T120 phosphorylation under the same conditions. In cells expressing Bub1-WT, H2A-pT120 was clearly localized to the centromere but lost in Bub1-KD-expressing cells, as expected. Expression of Bub1-TS89A, surprisingly, resulted in H2A-T120 phosphorylation along the entire length of the chromosome (Fig. 4c). Quantification of the H2A-pT120 signal specifically at chromosome arms revealed a significant increase in cells expressing this mutant compared with the essentially background staining-observed Bub1-WT- and Bub1-KD-expressing cells (Fig. 4c). To test whether the scaffolding function of Bub1 is altered by the loss of TS89 phosphorylation, we verified the localization of BubR1. We found that at least steady-state levels of BubR1 are unchanged between cells expressing Bub1-WT, KD or TS89A (Fig. 4d). Similarly, recent reports have concluded that Bub1 overexpression, which leads to H2A-pT120 spread to chromosome arms, did not alter the strength of the SAC or the recruitment of mitotic regulators29. Collectively, our data indicate that TS89 autophosphorylation limits H2A-pT120 and hence Sgo to centromeres. The extended mitosis observed in Bub1-TS89A cells may thus be a result of the
longer time required to remove the ectopic cohesion resulting from unchecked H2A phosphorylation.

Sgo1 translocation to the chromosome arms after Bub1 inactivation induces persistent cohesion along mitotic chromosomes\(^1\). We therefore tested whether Bub1-T589A expression also resulted in ectopic cohesion using chromosome spreads. In control GL2-treated cells (85%) and rescued cells expressing Bub1-WT (74%), sister chromatids were predominantly X-shaped with only the centromere connection apparently maintained (Fig. 4e). As expected, cells depleted of Bub1 or depleted of Bub1 and rescued with Bub1-KD showed a significant increase in the proportion of cells with poor resolution of sister chromatids along the entire chromosome length (57% and 62%, respectively). Similarly, and in agreement with the mislocalization of Sgo proteins, cells expressing Bub1-T589A (61%) mostly displayed incomplete resolution along the length of chromosomes, presumably owing to unscheduled protection of cohesion caused by the spread of Sgo along the entire chromosome length. Together, these results suggest that in addition to H2A-T120 phosphorylation itself, Bub1 autophosphorylation at T589 is required to restrict H2A-T120 phosphorylation to...
the centromere, thereby confining Sgo and cohesion protection to this region.

Bub1-KD and -T589A display increased cytoplasmic residency. Loss of localized H2A-T120 phosphorylation in Bub1-T589A cells was also seen in KNL1-depleted cells19 and suggested that kinetochore targeting of Bub1 enriches H2A-T120 phosphorylation at centromeres14. To independently verify these observations, we depleted Bub3, the constitutive binding partner of Bub1 that is strictly required for Bub1 kinetochore binding through interaction with KNL1 (refs 36–38) reviewed in ref. 8). Bub3 depletion results in efficient relocalization of Bub1 to the cytoplasm, as expected (ref. 39 and data not shown). Concomitant to this loss, we observed a massive spread of H2A-T120 phosphorylation along chromosome arms and a corresponding recruitment of Sgo1 (Fig. 5a,b). These results are in strong agreement with the observation that Bub3 binding is not required for Bub1 activity per se, but rather to focus Bub1 activity to kinetochores (Fig. 2b,c), and argue that loss of Bub3–Bub1 concentration at the kinetochore results in ectopic H2A-T120 phosphorylation and Sgo1 recruitment19, probably through the activity of cytoplasmic Bub1.

Figure 4 | Uniform H2A-T120 phosphorylation results in ectopic Sgo recruitment and impaired sister chromatid resolution in cells expressing Bub1-T589A. (a–d) Mitotic Bub1-WT, KD and T589A depleted of endogenous Bub1 were fixed and stained with anti-CREST (blue) and anti-MYC (green), and (a) Sgo1, (b) Sgo2, (c) H2A-pT120 and (d) BubR1 (all in red). Quantification of immunofluorescence intensity specifically at the chromosome arms (corrected total cell fluorescence) ± s.e. of Sgo1, Sgo2 and H2A-pT120 is indicated in the respective merge panel. For BubR1, fluorescence intensity relative to the CREST signal ± s.e. is shown. (e) Stable Bub1 cell lines were depleted of endogenous Bub1, arrested in mitosis using nocodazole and harvested for chromosome spreads before staining with Hoechst (blue) and anti-GFP (green). The different chromosomal conformations were quantified and indicated in the graph. Data represent the mean ± s.e. of 4 independent experiments, with 58–105 cells scored per condition per experiment. Scale bar, 10 μM.
Figure 5 | Bub1-KD and Bub1-T589A display increased residency in the cytosol. (a) Mitotic control (siGL2) and Bub3-depleted (siBub3) cells were fixed and stained with anti-H2A-pT120 (red), anti-Sgo1 (green) and anti-CREST (blue). (b) Quantification of the localization of H2A-pT120 and Sgo1 signals. Data represent the mean ± s.e. of three independent experiments. Eighty to 300 cells were scored per condition per experiment. (c) Images and (d) quantification (normalized average pixel intensity); low (1–1.2), medium (>1.2 to ≤1.3) and high (>1.3) of 3 × MYC-GFP-Bub1 signal and localization in live cells synchronized in mitosis by a thymidine release. Data represent the mean ± s.e. of 3 independent experiments, with 58–61 cells measured per condition. Significance was measured for the high group by one-way analysis of variance (ANOVA) and pairwise t-test (Holm–Sidak). (e) Scatter plot of the cytoplasm versus kinetochore GFP levels of individual cells from each of the stable cell lines. The number of cells, R² (measure of the goodness-of-fit) and significance (one-way ANOVA) are indicated. (f) Western blotting showing levels of the 3 × MYC-GFP-Bub1 proteins in the stable cell lines in whole cell extracts (left) and in cytoplasmic extracts (right). Scale bar, 10 μM.

The parallels in the phenotype observed in Bub3-depleted cells and Bub1-T589A cells were surprising, considering that Bub1-T589A localized efficiently to the kinetochore, as measured by indirect immunofluorescence, and exhibited normal activity in vitro (Supplementary Fig. 2B,C). To determine directly whether Bub1-T589A resided in the cytoplasm and to avoid potential artefacts from fixation, we monitored the localization of enhanced GFP-tagged Bub1 in our isogenic cell lines in living mitotic cells. We measured the cytoplasmic expression using three independent approaches. First, we monitored Bub1 expression in undisrupted prometaphase cells. Approximately 38% of the cells expressing Bub1-WT showed low or undetectable levels of GFP signal in the cytoplasm, in agreement with Bub1 residency being primarily at the kinetochore. Surprisingly, we found that in Bub1-KD- and Bub1-T589A-expressing cells, this percentage was much lower with ~8% and 5% of cells exhibiting low cytoplasmic GFP levels, respectively. Conversely, proportionally more Bub1-KD and T589A cells displayed high GFP signal in the cytoplasm when compared with Bub1-WT-expressing cells (Fig. 5c,d). As an alternative approach, we plotted the cytoplasmic versus kinetochore GFP signal of individual cells in a random population of mitotic cells from each of the cell lines. Linear regression analysis indicated that Bub1-KD- and Bub1-T589A-expressing cells tended to display higher cytoplasmic versus kinetochore ratios than Bub1-WT (Fig. 5e). Although no significant difference was observed between Bub1-KD and Bub1-T589A cells (P = 0.36), the cytoplasmic:kinetochore GFP ratios in these cells were found to be significantly higher than the cells expressing Bub1-WT (P < 0.001, one-way analysis of variance (ANOVA); Fig. 5e). Finally, we tested the overall expression in these Bub1 cell lines, as well as the proportion of the protein that was found in the cytoplasmic compartment after fractionation. Western blotting indicated that Bub1-WT, KD and T589A are expressed at similar overall levels (Fig. 5f, left panel). However, when taking just the cytoplasmic fraction in consideration, both Bub1-KD and...
Bub1-T589A displayed higher cytoplasmic levels (Fig. 5f), in agreement with our aforementioned results. Taken together, our observations suggest that although Bub1-WT, -KD and -T589A cell lines express similar overall levels of Bub1, Bub1-KD and Bub1-T589A exhibit higher cytoplasmic occupancy than Bub1-WT.

**Kinetochore-tethered Bub1-T589A refocuses H2A-pT120 and Sgo.** As Bub1-T589A appeared to localize normally to kinetochores (Supplementary Fig. 2a,b), we examined whether an increase in exchange at kinetochores caused aberrant cytoplasmic presence by measuring FRAP. After photobleaching at kinetochores, Bub1-WT recovered to ~52% (Fig. 6a) in agreement with previous observations in Ptk2 cells40 and fission yeast11, with a half-life of ~15 s. Recovery of Bub1-KD and Bub1-T589A increased marginally to 55% and 61%, respectively. Recovery occurred with significantly faster kinetics with half-life measurements of 7.44 s for Bub1-KD and 5.85 s for Bub1-T589A (P < 0.001, one-way ANOVA). In contrast, we found no major difference in cytoplasmic diffusion (Fig. 6a). This data suggests that Bub1 kinase activity and, in particular autophosphorylation at T589, restricts the kinetics as well as the fraction of Bub1 exchanged between kinetochores and the cytoplasm.

We next reasoned that if increased Bub1-T589A kinetochore turnover was indeed causing uniform H2A-pT120 and Sgo1 recruitment to chromatin, then stable tethering of Bub1-T589A to the kinetochore would refocus H2A-T120 phosphorylation. To test this idea, we expressed MYC-tagged Bub1 WT, the kinetochore would refocus H2A-T120 phosphorylation. Recruitment to chromatin, then stable tethering of Bub1-T589A to cytoplasm shuttling, which is in turn required for accurate mitotic progression by ensuring localized H2A-T120 phosphorylation and Sgo1 recruitment. Kinetochore tethering of either Bub1-T589A or the Bub3-binding mutant Bub1-Δ229–256 via Mis12 refocuses H2A-T120 phosphorylation and Sgo1 to the centromere. Our study reveals an additional regulatory layer controlling Bub1 localization.

Considerable evidence from the literature supports this model of Bub1 function. First, all conditions in which proper Bub1 kinetochore targeting is impaired result in the spread of the H2A-pT120 signal and/or Sgo1 displacement along chromosome arms. Our data here show that depletion of Bub3 or loss of the Bub1–Bub3 interaction result in unchecked H2A-T120 phosphorylation and Sgo recruitment. Similarly, depletion of KNL-1 or ectopic localization of the Bub1 kinase domain to chromosome arms led to uniform H2A-T120 phosphorylation on chromatin14,19. In fission yeast, expression of Bub1 lacking the amino-terminal kinase-targeting region elevated H2A-S121 phosphorylation along the entire chromosome length.14 Interestingly, depletion or inhibition of Aurora B kinase also affects Sgo1 localization by causing its redistribution to chromosome arms during mitosis and meiosis47–51. This may be at least in part due to the loss of Bub1 kinetochore targeting in the absence of Aurora B activity35,52,53. In addition to our observations with Bub1, both Aurora B and Mps1 have been reported to contribute to their own localization through autophosphorylation. In Saccharomyces cerevisiae, Ipl1 (the budding yeast Aurora B orthologue) and Cdc1 phosphorylation of a number of consensus sites in Slr115/INCENP restricted premature chromosomal passenger complex localization to the spindle54,55 but left Ipl1 activity unchanged. Similarly in vertebrates, Aurora B activity is strictly required for proper loading of the complex at centromeres56 and the central spindle57,58. Mps1 exchange is also dependent on its own kinase activity59. More inactive than active Mps1 can be detected at kinetochores by immunofluorescence and FRAP analysis indicated faster recovery (1.5-fold) of inactive Mps1 at the kinetochore59. More recently, it has been suggested that Mps1 autophosphorylation at a number of N-terminal sites outside the kinase domain reduces the affinity of Mps1 to kinetochores and may thus underlie this exchange60. The ability to regulate their own localization may therefore be a hallmark of the structurally and functionally diverse kinases that orchestrate mitosis.

Crystal structures suggest that Bub1 may be a constitutively active kinase19,61 and Bub1 is autophosphorylated at S969 in the activation segment throughout the cell cycle. However, it is clear that not all Bub1 phosphorylation events occur during interphase (Fig. 2d,e and ref. 14). To reconcile these results we propose that a critical concentration of Bub1 must be reached before activation (see model in Fig. 7). In interphase, the concentration of Bub1 is low and thus the kinase remains effectively inactive. In agreement with this, Bub1 is degraded at the end of mitosis in an APC/Cdh1-dependent manner and its levels drop rapidly on entry into G1 (refs 24,62). Indeed, increased local concentration of Bub1 is sufficient for activation and H2A-T120 phosphorylation, and Sgo1 recruitment during interphase (Fig. 2f,g). At G2/M, Bub1 protein expression increases and the critical threshold would be achieved resulting in Bub1 activation. In support of this idea, H2A-S121 is phosphorylated and Sgo2 is recruited along the entire chromosome length in G2 in a Bub1-dependent manner in vertebrates, Aurora B activity is strictly required for proper loading of the complex at centromeres56 and the central spindle57,58. Mps1 exchange is also dependent on its own kinase activity59. More inactive than active Mps1 can be detected at kinetochores by immunofluorescence and FRAP analysis indicated faster recovery (1.5-fold) of inactive Mps1 at the kinetochore59. More recently, it has been suggested that Mps1 autophosphorylation at a number of N-terminal sites outside the kinase domain reduces the affinity of Mps1 to kinetochores and may thus underlie this exchange60. The ability to regulate their own localization may therefore be a hallmark of the structurally and functionally diverse kinases that orchestrate mitosis.
fission yeast\textsuperscript{14}. On mitotic entry and the establishment of kinetochores, this activity becomes concentrated in the vicinity of its targets through Bub3–Bub1 binding to KNL1. As cytoplasmic Bub1 remains capable of phosphorylating H2A-T120 during mitosis albeit ectopically (Figs 5a and 6b, and ref. 14), kinetochore targeting spatially restricts, rather than activates, Bub1 (ref. 19). T589 autophosphorylation further restricts Bub1 shuttling between the cytoplasm and kinetochore. This is...
necessary because the increased cellular concentration of Bub1 during mitosis is sufficient to activate it and induce otherwise indiscriminate H2A-T120 phosphorylation and Sgo recruitment in these mutants. The effect of this dynamic exchange in Bub1-KD cells is masked due to the lack of H2A-T120 phosphorylation altogether. Loss of this autoregulatory phosphorylation results in ectopic cohesion protection, owing to mislocalized Sgo and a significant prolongation of mitosis, perhaps reflecting the additional time required to remove Sgo and cohesion from along chromosome arms. In support of this notion, a similar transient delay in exit was reported in cells depleted of WAPL, a protein required for the timely removal of cohesion in prophase. Thus, the role of Bub1 in Sgo localization and cohesion protection is twofold: first, Bub1 directly phosphorylates H2A-T120 to mediate Sgo recruitment and, second, through feedback autophosphorylation at T589, Bub1 ensures that H2A-T120 and Sgo are restricted to kinetochores. Constitutive autophosphorylation of S969 in the P+1 loop of Bub1 (which occurs by intramolecular phosphorylation and is independent of Bub1 concentration) may function as a priming event to ensure rapid and efficient H2A-T120 (and T589) phosphorylation on mitotic entry. Activation of Bub1 may thus not be switch-like and may involve intermediate states that exhibit varying degrees of activity.

The H2A-pT120-Sgo1 pathway serves as an adaptor to facilitate Aurora B inner centromeric accumulation. We therefore checked both localization and activation of Aurora B. We found that neither Aurora B protein levels nor Aurora B activity, as measured by autophosphorylation on T232 or phosphorylation of the canonical substrate CENPA-S7, was appreciably different between Bub1-WT and Bub1-T589A cells, although all three signals were diminished in Bub1-KD cells, as expected (Supplementary Fig. 3). Considering that depletion of both Sgo1 and Sgo2 is necessary to mislocalize Aurora B, Bub1-T589A phosphorylation may induce conformational changes that alter affinity of Bub1 to kinetochores. Binding of Bub3 as measured by immunoprecipitation revealed no difference between this mutant and Bub1-WT however, and any change caused by this mutation might be small and restricted to the shuttling pool of Bub1 (~50%) and thus not easily detected by such steady-state assays. Alternatively, it is possible the pT589 motif forms a docking site for a protein interaction motif, which would allow for the dynamic exchange of Bub1 between the cytoplasm and kinetochores. For example, 14–3–3 binding to pThr9eine motifs has been shown to control the nucleocytoplasmic exchange of a number of proteins. Whether a similar mechanism regulates Bub1 exchange remains to be explored.

How T589 phosphorylation changes the dynamics of Bub1 during mitosis remains unclear. One possibility is that phosphorylation may induce conformational changes that alter affinity of Bub1 to kinetochores. Binding of Bub3 as measured by immunoprecipitation revealed no difference between this mutant and Bub1-WT however, and any change caused by this mutation might be small and restricted to the shuttling pool of Bub1 (~50%) and thus not easily detected by such steady-state assays. Alternatively, it is possible the pT589 motif forms a docking site for a protein interaction motif, which would allow for the dynamic exchange of Bub1 between the cytoplasm and kinetochores. For example, 14–3–3 binding to pThr9eine motifs has been shown to control the nucleocytoplasmic exchange of a number of proteins. Whether a similar mechanism regulates Bub1 exchange remains to be explored.

Methods

Cell culture and transfection. All cell lines were grown at 37°C with 5% CO2 in DMEM (HyClone) supplemented with 10% fetal bovine serum or supplemented bovine growth serum (PAA). HeLa cells for the generation of stable isogenic cell lines were a generous gift of Patrick Meraldi. Stable lines were generated by electroporation essentially (Flip-in system, Life Technologies) and were selected for and grown in the presence of hygromycin (300 μg ml⁻¹). U2OS cells expressing a constitutively active form of the Tet operator sequence were a kind gift of David Spector (CSHL) and were maintained in the presence of 100 μg ml⁻¹ of hygromycin. Drug treatments were performed at the following concentrations and durations, unless otherwise indicated: thymidine (Acros Organics, 2 mM for 16 h), MG132 (Calbiochem, 20 μM for 2 h) and nocodazole (Sigma, as indicated, 16 h). Transient plasmid transfections (Figs 2 and 6) were performed with jetPRIME or TransIT (Polyplus) according to the manufacturer’s instructions. Protein depletion was performed with DsiRNAs (IDT), unless otherwise indicated, using either oligofectamine (Invitrogen) or INTERFERin (Polyplus) and analysed at ~48–72 h after transfection. The Bub1 siRNA pool targeting the 3'-UTR region has been previously reported. The DsiRNA equivalent was generated and correspond to the following sequences: Bub1-3’-UTR-1: 5’-UCGGACUUGAUAAUCUGAGU AAAA-3’; Bub1-3’-UTR-2: 5’-UCGACAGUUGAUAUGAAGUCGCT-3’; Bub1-3’-UTR-3: 5’-AAAAAAGGUUUAAGUAGGAGCAGAU-3’; and Bub1-3’-UTR-4: 5’-UUAGACUUGCAGUAUUCAAAUUU-3’. The Bub3-siRNAs used target the following sequences: sBuB3-1: 5’-UCAGAAGUAGCACAAAAA AAdTF-3’ and sBuB3-2: 5’-AGGGUUAGAUAUGAAGCGUUAUGA-3’.

Chromosome spreads. The Bub1 stable cell lines were split into six-well plates and transfected with Bub1 3'-UTR DsRed. At 36 h post transfection, cells are synchronized with 330 nM nocodazole overnight. Mitotic cells were shaken off, washed three times in PBS and incubated for 15 min at 37°C with rotation in 55 mM KCl to swell the cells. Twenty thousand cells were subsequently resuspended in 1% (vol/vol) Triton X-100, 10 mM MgCl₂, 0.1% Tween 20 and 10 μM calcein were spread onto coverslips by centrifugation at 500g for 5 min in a cytospin with slow acceleration and deceleration. Cells attached on slide were fixed in PTEFM for 10 min as before. Chromosome spreads were then stained as indicated in the figure legends.

Cloning and mutagenesis. Bub1 WT was cloned into the pcDNA3/FRT/TO expression vector modified to include an N-terminal triple MYC tag (HindIII–KpnI) followed directly in-frame by EFYP (KpnI–BamHI), generated by PCR amplification from pEGFP-N1 (Clontech). For artificial kinetochore targeting of Bub1-T589A and Bub1-Δ229–256, the enhanced GFP sequence was substituted for haspin-generated pHST3 marker that recruits survivin to centromere. It is likely to be that sufficient Aurora B is recruited and activated at centromeres in the Bub1-T589A-expressing cells. Although we cannot rule out a minor effect on Aurora B activity that is beyond the resolution offered by the phosphospecific antibodies used in this study, the congression defects observed may be due to a reduction in centromeric Sgo2, which is required for mitotic centromere-associated kinesin recruitment. We also found that steady-state Bub1R levels as measured by immunofluorescence (IF) are unchanged in the Bub1-T589A mutant. However, considering that Bub1 kinetochore binding occurs directly through Bub1 (ref. 67), it may well be that Bub1 kinetochore turnover (rather than bulk levels) is also altered in the Bub1-T589A mutant. Answering this question will require further investigation.

Immunofluorescence and antibodies. Cells were grown on cover slips and were arrested in mitosis either by nocodazole (330 nM) or by 10–12 h release from thymidine block. Fixation was performed by incubation with PTEFM (0.2% Triton X-100, TEGTA and 4% formaldehyde) before blocking in ~3% BSA in PBS-T. Coverslips were incubated with primary and secondary antibodies for 1 h at room temperature, except for CENP-A pS7, which was incubated at 4°C overnight. Rabbit polyclonal phosphospecific antibodies against T589 and S679 were generated against phosphopeptides CIRCNgTPALS and CLLIRpQPAAG, respectively. Antibodies were affinity purified with the phosphopeptide for all experiments shown here and used at 1 μg ml⁻¹. Other antibodies were used at 1 μg ml⁻¹, unless otherwise indicated, as follows:...
anti-MYC (9E10, Thermo Scientific), anti-Bub1 (ref. 70), anti-GFP (1181446001, Roche), anti-Sgo1 (H00151641-M01, Abnova), anti-H2AP-T120 (61195, Active Motif) and a generous gift of Y. Watanabe), anti–a-tubulin (DM1A, Santa Cruz), anti-Sgo2 (kind gift of Tim Yen), anti-GAPDH (used at 1:2,000, NB300–221, Novusbio), CENP-A pS7( used at 1:100, 2187, Cell Signaling technology), anti–Aurora B (611082, BD Transduction Laboratories), anti-Aurora pT232 (600–401– 677, Rockland), anti-histone H2A (67–416, Millipore) and CREST anti-centromere serum (HCT-0100, Immunovision). Dylight series secondary antibodies (Thermo) were used for immunofluorescence (1:1,000) and horseradish peroxidase–coupled secondary antibodies (Jackson Immunoresearch) were used for western blotting (1:10,000).

Protein detection and fractionation. For immunoblotting and immunoprecipi-
tations, cells were lysed with RIPA buffer containing 150 mM Tris–HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 1% NP-40, 0.1% Na deoxycholate and a protease and phosphatase inhibitor cocktail that included 1 mM aprotinin, 1 mM leupeptin, 1 mM sodium vanadate, 10 mM sodium pyrophosphate, 0.1 mM leupeptin, 1 mg/ml aprotinin and 1 mM AEBSS. Cells were lysed on orbital shaker at 4 °C for at least 30 min; lysates were centrifuged at 14,000 r.p.m. for 15 min at 4 °C. The supernatant was collected and protein concentrations were measured using the BCA kit (Pierce). The cytoskeletal and cytoskeletal fraction of Bub1, mitotic cells stably expressing Bub1–WT, KD or T589A were harvested by shake-off after thymidine release, washed twice in PBS and lysed for 10 min on ice in cytoskeletal buffer (0.5% Triton X-100, 100 mM PIPES pH 6.8, 100 mM NaCl, 1.5 mM MgCl2, 300 mM sucrose, protease inhibitor cocktail (1 mg/ml aprotinin, 1 mM leupeptin, 1 mM sodium vanadate, 10 mM AEBSS, 10 mM Tris-HCL pH 8.0, 1 mM ATP). The lysate was spun down for 4 min at 3,200 r.p.m. and the resulting supernatant (S1) constituted the cytoskeletal fraction. The original, non-cropped blots for all western blotting in this study are shown in Supplementary Fig. 4.

Microscopy and FRAP. Cells were imaged by confocal microscopy on an inverted Olympus IX80 microscope equipped with a WaterFLEX–Boreal-SC Yokagawa spinning disc (Quorum Technologies) and an Orca Flash4 camera (Hamamatsu). Imaging of live cells was performed with an inverted microscope and by laser scanning confocal microscopy. Optical sections were acquired with identical exposure times for each channel within an experiment and then projected into a single picture using ImageJ (rsb.info.nih.gov). Image processing was performed in Image J or Photoshop and images shown in the same figure have been identically scaled.

For FRAP experiments, the cells were grown on glass-bottom lab-tek chambered slides (Thermo Scientific). FRAP analysis was performed on Leica DMi6000B equipped with a heated chamber (37 °C) and a Mosaic active illumination system (Spectral Applied Research), which allowed for simultaneous bleaching and acquisition, and an ImageEM (512 × 512 camera (Hamamatsu). The microscope, and FRAP equipment used in these studies were purchased from Molecular Devices. Image acquisition (every 150 ms) began 15 frames before bleaching and continued for an additional 15 frames after bleaching. The bleached region in each channel was a circular region of 15 pixel diameter and only kinetochores that remained visible within this region for the length of the experiment were included in the analysis. Quantification of fluorescence recovery was obtained using the FRAP profiler plugin of ImageJ, which accounts for correction of overall bleaching. Recovery rates for phospho McKi67, McKi67–WT, McKi67–KD and T589A were determined after fitting a single exponential curve (which showed the best fit) using the formula $F(t) = A (1 - e^{-kt})$, where $A$ = fraction recovery. Half-time recovery was determined using the formula $t_{1/2} = \ln(0.5)/k$.

Live-cell imaging was performed on the above indicated microscopy system that is also equipped with a motorized stage (ASI) and an incubator with atmospheric CO$_2$ heated to 37 °C. Bub1 stable cell lines were subject to depletion of endogenous Bub1 for 48 h, then synchronized in mitosis after a further 16-h block with thymidine. Image acquisition was started 12 h after release. Only cells visibly expressing the GFP–tagged Bub1 were included in subsequent analysis.

SILAC labelling and MS analysis. 293T cells were cultured in heavy or light amino acid-containing medium for five generations before transfection with 3 × MYC–tagged Bub1–WT or Bub1–KD. Cells were harvested for experiment 1 after which nucodazole was added for an additional 16 h. Cells were harvested, lysed in RIPA lysis buffer and 3 × MYC–tagged Bub1–WT or Bub1–KD were immunoprecipitated with anti–MYC antibodies for 2 h. The immunoprecipitated Bub1 was washed 3 × with RIPA lysis buffer, 1 × with RIPA buffer including 300 mM NaCl and a final buffer exchange with kinase reaction buffer lacking ATP and MgCl$_2$. The immunoprecipi-
tates were then subjected to a cold in-vitro kinase assay (20 mM Tris pH 7.4, 10 mM EGTA, 100 mM sodium orthovanadate, 10 mM MgCl$_2$, 4 mM MnCl$_2$, 1 mM dithiofretirole, 5 mM NaF and 100 mM ATP) at 30 °C for 30 min. The reaction was stopped by the addition of SDS–PAGE sample buffer. The Bub1–WT and Bub1–KD immunoprecipitates were then loaded onto SDS–PAGE gels and visualized by Coomassie brilliant blue staining. The band corresponding to the size of 3 × MYC–Bub1 was excised and processed for MS analysis$^{28}$. In gel digestion was performed using either 15 ng ml$^{-1}$ of trypsin or was added in an enzyme/ substrate ratio of 1:50 of each Lys–C, GluC and elastase.

Nanol liquid chromatography–MS/MS analysis. All peptide samples were separated by online reverse-phase nano liquid chromatography and analysed by electrospray tandem MS (MS/MS). Using a nanoACQUITY ultra-performance liquid chromatography system (Waters), samples were injected onto a 14-cm fused silica capillary column with an inner diameter of 75 μm and a tip of 8 μm (New Objective) packed in-house with 3-μm ReproSil-Pur C18-AQ (Dr Maisch GmbH). The LTQ–Orbitrap mass spectrometer (Thermo Scientific) was equipped with a nanoelectrospray ion source (Proxeon Biosystems). Peptides were separated and eluted by a stepwise 180 min gradient of 0%–100% between buffer A (0.2% formic acid in water) and buffer B (0.2% formic acid in acetonitrile). Data-dependent acquisition was performed on the LTQ–Orbitrap using Xcalibur 2.0 software. As the positive ion mode, Survey full-scan MS spectra were acquired to 3000 m/z were acquired in the FT–Orbitrap with a resolution of 60,000 at m/z 400. A maximum of five peptides were sequentially isolated for fragmentation in the linear ion trap using collision-induced dissociation. The Orbitrap lock mass feature was applied to improve mass accuracy. To improve phosphopeptide analysis, the multistide activation options in PepTrad software (Molecular Devices) were used for MS/MS fragmentation spectra. Carbamidomethylation was set as fixed modification and methionine oxidation, protein N-terminal acetylation and phosphorylation (STY) were considered as variable modifications. Putative SILAC pairs were searched with their respective labelled amino acids as fixed modification, whereas peaks that were not assigned to any of the SILAC silvers were searched using R10 and K8 as variable modifications. Enzyme specificity was set to Trypsin/P, that is, allowing cleavage N-terminal to proline in the context of [KR]P. Up to two missed cleavages were allowed. The minimum required peptide length was set to six amino acids. Searches were performed against PI human (version 3.48; 71,400 protein entries) that was concatenated with reverse database sequences (142,800 protein entries in total). Further, MaxQuant filtered Mascot results using additional parameters such as the number of labelled amino acids (maximum of three) in the identified peptide sequence and the measured mass accuracy as a function of intensity. As an additional quality measure to increase identification stringencies, we only accepted phosphorylation site identifications with Mascot scores above 12 or PTM scores of at least 30. Quantitation of SILAC pairs was performed with the following parameters: re-quantify; for protein quantification, discard unmodified counterpart peptides, except for oxidation and acetyl protein N-terminal; use raor and unique peptides; minimum ratio count of 1; minimum score 0; and minimum peptides 1. The initial maximum false discovery rates were set to 0.02 and 0.05 for peptides and proteins, respectively, and further reduced by Mascot score filtering as described above. False discovery rates were calculated as (number of hits in the reversed database/number of hits in the forward database) × 100%. Whenever the set of identified peptides in one protein was equal to or completely contained in the set of identified peptides of another protein, these two proteins were joined in a single protein group. In cases where the peptides have more than one phosphorylation site, some of these phosphorylation sites are identified as multiply phosphorylated peptides, whereas others are identified on multiple singly phosphorylated peptides.

Phosphopeptide analysis. A summary of all quantifiable SILAC pairs identified on Bub1 from each of the three independent experiments is shown in Supplementary Data 1. It is worth noting that in experiment 2, in a certain number of cases, peptides corresponding to Bub1–KD were identified but lacked phosphorylation all together. In these cases, we marked the phosphopeptide:peptide ratio as 100%. Weblogo analysis was performed on the 15-mer peptides cor-

Quantification and statistical analysis. Unless otherwise stated, all experiments were performed in triplicate. Image quantification was performed using Image J. For quantification of signal intensities at kinetochores, the CREST/MYC signal was used to generate a binary mask to include kinetochore and centromere signals. Integrated signal intensity was measured in all relevant channels and intensities were normalised to the Myc channel. Unless otherwise stated, a minimum of ten cells were quantified per condition for all experiments involving kinetochore quantification. For H2A–pT210 and Sgo1 signal intensity at
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Author contributions
A.A. and A.L. performed the majority of the experiments with assistance from P.T. and G.C. Initial identification of autophosphorylation sites was done by S.E. and K.D. S.E. designed the experiments and wrote the manuscript with assistance from A.A. and K.D.

Additional information
Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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