A minimal number of MELT repeats supports all the functions of KNL1 in chromosome segregation

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ABSTRACT

The Bub1–Bub3 and BubR1–Bub3 checkpoint complexes, or the Bubs, contribute to the accurate segregation of chromosomes during mitosis by promoting chromosome bi-orientation and halting exit from mitosis if this fails. The complexes associate with kinetochores during mitosis, which is required for proper chromosome segregation. The outer kinetochore protein KNL1 (also known as CASC5, Blinkin and AF15014) is the receptor for Bub proteins, but the exact nature of the functional binding sites on KNL1 are yet to be determined. Here, we show that KNL1 contains multiple binding sites for the Bub proteins, with the Mps1-phosphorylated MELT repeats constituting individual functional docking sites for direct binding of Bub3. Surprisingly, chromosome segregation and the spindle assembly checkpoint (SAC) are still functional when KNL1 is deleted of all but four of its twelve MELT repeats. Systematically reducing the number of MELT repeats to less than four reduced KNL1 functionality. Furthermore, we show that protein phosphatase 1 (PP1) binding to KNL1 during prometaphase reduces the levels of Bub proteins at kinetochores to approximately the level recruited by four active MELT repeats.

KEY WORDS: Bub1, BubR1, Kinetochore, KNL1, Spindle assembly checkpoint, SAC

INTRODUCTION

Mitosis ends the cell cycle with the task of partitioning the duplicated sister chromatids equally to the new daughter cells. Failure in chromosome segregation causes aneuploidy, which can promote the formation of cancers and affect lifespan; thus, it is of great importance to understand the molecular details of the segregation process (Ricke and van Deursen, 2013). Each sister chromatid contains a centromere region that marks the building site for a large protein structure referred to as the kinetochore (Santaguida and Musacchio, 2009; Takeuchi and Fukagawa, 2012). The kinetochore plays a pivotal role in ensuring accurate chromosome segregation both by making stable end-on attachments to dynamic microtubules and by activating the spindle assembly checkpoint (SAC) if this fails (Lara-Gonzalez et al., 2012). Stable kinetochore-microtubule attachments crucially depend on a conserved outer kinetochore complex referred to as the KMN network (Cheeseman et al., 2006; Cheeseman et al., 2004; DeLuca et al., 2005; DeLuca et al., 2006). The KMN network comprises the four-subunit Ndc80 complex, the large KNL1 protein and the four-subunit Mis12 complex that connects the KMN network to the inner kinetochore (Cheeseman et al., 2006; Przewloka et al., 2011; Screpanti et al., 2011). In metazoans, KNL1 binds to the Zwint kinetochore protein that is required for binding the Rod–Zwilch–Zw10 (RZZ) complex in prometaphase (Kiyomitsu et al., 2011; Kops et al., 2005; Wang et al., 2004). The Ndc80 complex and KNL1 are able to directly bind to microtubules and, in the context of the reconstituted KMN network from C. elegans, these two microtubule-binding activities synergize (Cheeseman et al., 2006; Wei et al., 2007). It is clear that the microtubule-binding activity of Ndc80 is required in vivo for stable end-on attachment and chromosome segregation (Guimaraes et al., 2008; Miller et al., 2008). However, work in C. elegans has shown that the microtubule-binding activity of KNL1, residing in the extreme N-terminus of the protein, is not essential for chromosome segregation in vivo, but whether this is true in other organisms has not yet been tested (Espeut et al., 2012).

In addition, the KMN network is important for the SAC as illustrated by the fact that the complete removal of the Ndc80 complex or KNL1 abrogates the checkpoint (Kiyomitsu et al., 2007; Meraldi et al., 2004; Pagliuca et al., 2009); this correlates with the absence of specific checkpoint proteins at the kinetochore. The conserved SAC proteins are Mps1, Bub1, BubR1 (Mad3 in yeast), Bub3, Mad1 and Mad2. All the SAC proteins dynamically associate with improperly attached kinetochores and this is thought to be required for generating the ‘wait anaphase’ signal that halts mitotic progression (Howell et al., 2004; Shah et al., 2004).

KNL1 is the direct receptor for the Bub proteins and this interaction also contributes to chromosome congression because both Bub1 and BubR1 are required for this independently of their role in the SAC (Ditchfield et al., 2003; Kruse et al., 2013; Lampson and Kapoor, 2005; Meraldi and Sorger, 2005; Suikerbusjuk et al., 2012). The mechanism of how the Bub proteins are recruited by KNL1 in human cells is unclear, as conflicting observations exist in the literature. In the original characterization of KNL1, the binding to Bub proteins was proposed to rely on interactions between the TPR domains of the Bub proteins and the KI motifs in the N-terminus of KNL1 (see Fig. 1A) (Kiyomitsu et al., 2007). Human KNL1 contains two KI motifs, each proposed to be specific for either the Bub1 or BubR1 TPR domain. However, mutagenesis of the TPR domains based on the structures of the TPR–Bub protein complexes was unable to confirm that these interactions are required for kinetochore recruitment of the Bub proteins (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011; Krenn et al., 2012). A direct interaction between the KI motifs and Bub proteins also does not readily...
Fig. 1. See next page for legend.
**RESULTS**

**Dissecting the regions of KNL1 required for chromosome congression**

As outlined in the introduction, different motifs in KNL1 have been implicated in the kinetochore recruitment of Bub1 and BubR1 but their functions in vivo have not been carefully investigated. Here, we set out to identify the functionally relevant domains of KNL1 in chromosome segregation using RNA interference (RNAi) complementation assays.

To do this, we identified two independent small interfering RNA (siRNA) oligonucleotides that efficiently depleted the endogenous KNL1 protein without affecting the levels of several known SAC components (supplementary material Fig. S1A). Both siRNA oligonucleotides produced similar phenotypes, as described in the literature (Kiyomitsu et al., 2007; Pagliuca et al., 2009). In more than 90% of KNL1-depleted cells we observed a prolonged mitotic arrest with several chromosomes failing to congress to the metaphase plate followed by the entry into anaphase with unaligned chromosomes (supplementary material Fig. S1, type 2 cells; Table 1). Immunofluorescence analysis of cells subjected to cold (hereafter cold-treated cells), revealed that the unaligned chromosomes had not attached to microtubules (supplementary material Fig. S2). A minor pool of KNL1-depleted cells progressed rapidly through mitosis and never formed a metaphase plate, probably reflecting a population with very efficient KNL1 depletion, which completely abrogated the SAC (supplementary material Fig. S1, type 1 cells). Given the strong effect of KNL1 RNAi-mediated depletion, this allowed for RNAi complementation assays to probe for regions of KNL1 required for chromosome congression.

We generated a panel of stable inducible isogenic HeLa cell lines expressing C-terminally Venus-tagged full-length KNL1 or KNL1 with truncations from the N-terminus lacking 150, 300, 600, 1000 or 1200 amino acids, respectively (referred to as KNL1 Δ1–150, Δ1–300 etc.). Given that both siRNA oligonucleotides produced a similar phenotype, only one was chosen to proceed with, and KNL1 expression constructs that were resistant to this siRNA oligonucleotide were made. The Mis12 complex and Zwint-binding sites were maintained in all the constructs and the expressed proteins all targeted correctly to the kinetochore (Figs 1 and 2). The stable cell lines were co-transfected with a histone marker to provide a more careful analysis of chromosome congression in live-cell imaging experiments. First, we analyzed the ability of full-length KNL1 to rescue the chromosome congression defect by time-lapse microscopy using the outlined RNAi rescue protocol (Fig. 1B). In the stable cell lines, we observed heterogeneous expression of exogenous KNL1 and therefore only analyzed cells with comparable expression levels and with a clear KNL1 kinetochore signal. The exogenous KNL1 fully supported chromosome congression with timings similar to control treated cells (Fig. 1C; Table 1). In parallel, siRNA- and cold-treated cells that expressed the complementary full-length KNL1 were analyzed by immunofluorescence, and in this assay KNL1 complementation by exogenous KNL1 in relation to synchronization of cells by a double thymidine block protocol. (C) Still images from time-lapse experiments of HeLa cells complemented with KN1L1, KNL1 Δ1–1000 and KNL1 Δ1–1200 with differential interference contrast (DIC), YFP (KNL1) and CFP (histone 3) channels shown. The time at nuclear envelope breakdown (NEBD) is set as zero and time is given in minutes on the CFP channel images. Asterisks highlight unaligned chromosomes.

**Table 1. Chromosome segregation phenotype in the different KNL1 complemented cell lines**

| siLuc | siKNL1 | KN1l | Δ1–150 | Δ1–300 | Δ1–600 | Δ1–1000 | Δ1–1200 |
|-------|--------|------|--------|--------|--------|---------|---------|
| NEBD to anaphase (minutes) | 40 (n = 128) | 118 (n = 77) | 40 (n = 93) | 50 (n = 129) | 55 (n = 108) | 75 (n = 117) | 65 (n = 135) | 115 (n = 139) |
| NEBD to metaphase (minutes) | 20 | NA | 20 | 20 | 25 | 30 | 25 | NA |
| Cells with unaligned chromosomes (%) | 3% (n = 54) | 98% (n = 90) | 6% (n = 35) | ND | ND | ND | 15% (n = 52) | 84% (n = 91) |

The median NEBD to anaphase and NEBD to metaphase time are indicated for control-treated as well as KNL1-depleted cells and KNL1-depleted cells complemented with the indicated KNL1 constructs. The number of cells analyzed (n) for each condition is indicated next to the NEBD to anaphase time. The last row indicates the percentage of cells displaying unaligned chromatids as determined from the analysis of cells expressing a fluorescent histone marker. The number of cells analyzed (n) for each condition is indicated next to the percentage number. ND indicates that the number was not determined for this KNL1 construct. NA indicates that metaphase time could not be determined because under these conditions a full metaphase plate did not form.

As outlined in the introduction, different motifs in KNL1 have been shown to bind to four MELT repeats is sufficient for KNL1 function. Supports KNL1 functions, suggesting that the level of Bub proteins binding sites for the Bub proteins that are negatively regulated by MELT repeats of KNL1. Surprisingly, a binding of protein phosphatase 1 (PP1) to KNL1. This explains the need for Bub3 to bind to Bub1 and BubR1 in order for them to localize to kinetochores (Taylor et al., 1998). However, recruitment of Bub proteins through KI motifs could still be functionally important, although it might only reflect a small fraction of the kinetochore pool. Recent work in yeast and human cell lines has shown that Mps1-mediated phosphorylation of MELT repeats in the KNL1 proteins has a role in kinetochore recruitment of the Bub1–Bub3 complex (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). In several KNL1 proteins, numerous MELT repeats are distributed in the N-terminal half of the protein, the exact number varying greatly between different species (Vleugel et al., 2012). When phosphorylation of KNL1 by Mps1 is prevented in yeast through mutation of multiple MELT repeats, cells experience problems in chromosome congression and fail to mount a proper checkpoint, which correlates with the inability to recruit Bub1–Bub3 (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). The exact role of the MELT repeats and their phosphorylation is at the moment unclear, and whether distinct MELT repeats have specific functions in the SAC and chromosome congression has not been explored.

Here, we set out to understand which regions of human KNL1 are required for Bub1 and BubR1 kinetochore recruitment and their role in the SAC and chromosome congression. We find that the phosphorylated MELT repeats of KNL1 are direct binding sites for Bub3, explaining the need for Bub3 in the kinetochore recruitment of Bub1 and BubR1. In agreement with this, we show that the MELT repeats of KNL1 in vivo represent multiple independent binding sites for the Bub proteins that are negatively regulated by binding of protein phosphatase 1 (PP1) to KNL1. Surprisingly, a truncated KNL1 containing only four MELT repeats (but not less) supports KNL1 functions, suggesting that the level of Bub proteins that bind to four MELT repeats is sufficient for KNL1 function.
Fig. 2. See next page for legend.
Fig. 2. Localization of BubR1 and Bub1 in the KNL1 complemented cell lines. (A) Immunofluorescence images of cells depleted of endogenous KNL1 (siKNL1) and complemented with the indicated KNL1 constructs. Cells are stained for BubR1, GFP, CREST and DAPI. The images were deconvolved and all z-stacks through the cell were projected. Cells were synchronized as outlined in Fig. 1B and treated with 200 ng/ml nocodazole overnight before pre-extraction and fixation. sILuciferase, control cells with siRNA against Luciferase. (B) Quantification of KNL1, BubR1 and Bub1 kinetochore signals normalized to the CREST signal in control-treated (sILuciferase) or KNL1-depleted cells. More than 70 pairs of kinetochores from eight cells were analyzed. (C) Quantification of BubR1 kinetochore signals and normalization to the GFP signal in the different KNL1 expression cells. The BubR1 and GFP signals in the three z-stacks encompassing the bulk kinetochore signal were quantified using softWorx analysis software. For each condition at least 70 pairs of individual kinetochores from eight different cells were analyzed. Results are means±s.e.m. (D) A similar analysis of Bub1 to that described for BubR1 in C. (E,F) Analysis of BubR1 levels in cells expressing KNL1 Δ1–1000 and when the four remaining MELT repeats are mutated to MELA or removed. Representative images are shown in E and quantified in F. More than 70 pairs of kinetochores from eight cells were analyzed. **P<0.01; ns, not significant (Student’s t-test).

We then analyzed the ability of the different KNL1 truncations to support proper chromosome segregation using the RNAi rescue protocol. Surprisingly, we found that removal of the first 1000 amino acids of KNL1 still resulted in a functional protein that could support chromosome congression, although mitosis was delayed by 25 minutes (Fig. 1C; Table 1). This truncated KNL1 lacked the KI motifs as well as eight of the twelve MELT repeats present in human KNL1, arguing that at least the KI motifs are dispensable for chromosome congression. However, when we deleted an additional 200 amino acids, removing the remaining four MELT repeats (KNL1 Δ1–1200), the cells were unable to support chromosome congression (Fig. 1C; Table 1). Similar results were obtained in immunofluorescence analyses of cold-treated cells (supplementary material Fig. S2).

Analysis of cells expressing the shorter KNL1 truncations revealed that plate formation was delayed by 5 minutes in cells expressing KNL1 Δ1–300 and 10 minutes in KNL1 Δ1–600, indicating that domains in these regions of KNL1 contribute slightly to the efficiency of chromosome congression but are not essential (Table 1; supplementary material Fig. S3). The prolonged nuclear envelope breakdown (NEBD) to anaphase times observed for certain KNL1 truncations that cannot be ascribed to changes in metaphase plate formation timing might reflect subtle differences in kinetochore–microtubule interactions not detectable by live-cell microscopy.

In conclusion, a minimal KNL1 protein lacking the first 1000 amino acids is able to almost fully support KNL1 function.

Phosphorylated MELT repeats function as direct binding sites for Bub1–Bub3 and BubR1–Bub3 on KNL1

To dissect the underlying molecular details of how a large part of KNL1 can be removed without profoundly affecting its function, we analyzed the recruitment of Bub1 and BubR1 to the kinetochore in the cell lines that were depleted of endogenous KNL1 and expressed different truncated KNL1 proteins (Fig. 2). The cells were arrested with nocodazole to compare similar kinetochore–microtubule binding states and then the cells were stained for either Bub1 or BubR1 and for KNL1 using an anti-GFP antibody. The levels of the Bub1 and BubR1 proteins and KNL1 were quantified on individual kinetochores using three consecutive z-stacks 200 nm apart, encompassing the highest kinetochore signal. The Bub values were normalized to KNL1 values using the GFP signal to directly compare the effect of the different KNL1 truncations.

The deletion of KNL1 strongly reduced the levels of Bub1 and BubR1 on kinetochores and their localization was restored by expression of exogenous full-length KNL1 (Fig. 2A–D). When we deleted the first 150 or 300 amino acids there was an increase of close to 3-fold in the kinetochore levels of BubR1 and Bub1 compared to when full-length KNL1 was expressed, suggesting that this region negatively influences their recruitment (Fig. 2A–D; supplementary material Fig. S4A). Further deletions from the N-terminus gradually reduced Bub1 and BubR1 protein levels such that KNL1 Δ1–1000 recruited similar levels as full-length KNL1 and it was only KNL1 Δ1–1200 that was unable to recruit Bub proteins (Fig. 2A–D). These observations are in line with the live-cell analysis and could explain why KNL1 Δ1–1000 can support chromosome congression whereas KNL1 Δ1–1200 cannot. We also analyzed the kinetochore levels of Mad2 in a number of these cell lines and there was a statistical difference between cells expressing full-length KNL1 and KNL1 Δ1–1200, although the change was not as large as for BubR1 and Bub1 (supplementary material Fig. S4B,C). Although Mad2 recruitment to kinetochores depends on Bub1, our data suggest that Bub1 might not be the direct receptor.

We were struck by the fact that a gradual decline in Bub1 and BubR1 levels at kinetochores was observed when we made KNL1 deletions from the N-terminus, indicating that there were multiple binding sites present in KNL1 rather than a single specific one. Given the recent observations that phosphorylation of KNL1 by Mps1 promotes Bub1 protein recruitment (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012), we speculated that each MELT repeat might constitute a functional binding site given that the decline largely followed the number of MELT repeats removed. Indeed, when we specifically mutated the four remaining MELT repeats in KNL1 Δ1–1000 to MELA, BubR1 and Bub1 were no longer recruited to kinetochores, supporting this idea (Fig. 2E,F; see Fig. 4E for the Bub1 quantification). A KNL1 construct where we had deleted amino acids 1000–1215, removing the four MELT repeats remaining in KNL1 Δ1–1000, still recruited BubR1, indicating that there was not a specific requirement for these four MELT repeats in BubR1 recruitment (Fig. 2C).

To gain biochemical evidence for the MELT repeats functioning as receptors for the Bub proteins, we performed a peptide pulldown experiment using a biotinylated KNL1 peptide spanning residues 866–883 and encompassing the 875 phosphorylation site residing in a MELT-like motif (MDIT), which has been shown to be phosphorylated in vivo (Yamagishi et al., 2012). We used the KNL1 peptide in either an unphosphorylated form or a phosphorylated form and pre-bound the peptides to Streptavidin beads. These beads were then incubated with a nocodazole-treated cell extract to which we added the Mps1 inhibitor reversine to prevent phosphorylation of the unphosphorylated peptide. Following incubation and washing, we monitored the binding of checkpoint and kinetochore proteins by western blotting. Strikingly, the phosphorylated peptide was strongly enriched for BubR1 and Bub3, supporting the idea that the BubR1–Bub3 complex binds directly to the phosphorylated MELT repeats (Fig. 3A).

As both BubR1 and Bub1 depend on Bub3 for their kinetochore localization, and as both proteins displayed a similar dependency.
on the number of MELT repeats remaining in KNL1 for their kinetochore localization, we reasoned that Bub3 might bind directly to phosphorylated MELT repeats. To test this, we expressed and purified human Bub3 from HEK293 cells and used this in MELT-peptide-binding experiments (Fig. 3B). Purified Bub3 bound specifically to the phosphorylated MELT peptide providing a direct biochemical explanation for the role of Mps1 phosphorylation of MELT repeats in the recruitment of Bub1 and BubR1 (Fig. 3C). While this work was under revision similar observations were reported by the Musacchio laboratory and they also provided important structural insight into the recognition of phosphorylated MELT repeats by Bub3 (Primorac et al., 2013).

These results suggest that the crucial role of KNL1 in supporting chromosome congression is to recruit either Bub1 or BubR1 (or both) and that this is achieved through direct Bub3 binding to the phosphorylated MELT repeats.

**A minimal number of MELT repeats is required for KNL1 function**

As four MELT repeats supported KNL1 function in chromosome segregation, we wanted to investigate whether this was the minimal number of MELT repeats required. We therefore systematically mutated one, two or three MELT repeats in KNL1 Delta-1000 to MELA, starting from the N-terminus (Fig. 4A) and generated stable cell lines expressing these constructs. As expected, this resulted in a decrease in Bub1 and BubR1 kinetochore levels as the number of MELT repeats were reduced, and with only two MELT repeats close to background levels of Bub proteins remained (Fig. 4D,E). We then analyzed the ability of the different KNL1 proteins to support chromosome congression by live-cell imaging. As soon as we reduced the number of MELT repeats in KNL1 Delta-1000 to three or two, there was a strong increase in mitotic timing and ~50% of those cells had problems in aligning all the chromosomes to the metaphase plate (Fig. 4B,C; supplementary material Table S1). The presence of only one MELT repeat had a similar phenotype to constructs with all MELT repeats mutated or KNL1 Delta-1200 (supplementary material Table S1).

This shows that a minimal number of four MELT repeats is required in KNL1 Delta-1000 to support chromosome congression and in line with this, the KNL1 Delta-1000 truncation recruited similar levels of Bub1 and BubR1 as did full-length KNL1.

**A functional SAC is supported by a minimal number of MELT repeats**

As the complete depletion of KNL1 results in a non-functional checkpoint, this supports the hypotheses that binding of Bub1 and BubR1 to KNL1 is required for the SAC. We wanted to investigate which motifs in KNL1 were required for a functional SAC, as they could be distinct from the motifs required for chromosome segregation. In the unperturbed live-cell assays, only a small fraction of cells progress rapidly through mitosis even though the KNL1 depletion is efficient and therefore we could not make conclusions on the SAC from these assays. Therefore, to directly monitor SAC strength, we challenged cells with a low dose of the microtubule poison nocodazole and monitored the ability of cells to maintain a prolonged mitotic arrest. In control-depleted cells, we observed a prolonged mitotic arrest, and depleting KNL1 clearly reduced the duration of the arrest (Fig. 5A,B). Reintroducing exogenous full-length KNL1 to KNL1-depleted cells increased the time spent in mitosis, showing that our RNAi complementation assay worked. We then analyzed a number of KNL1 truncations in this assay and found that expressing KNL1 Delta-1000 by mutation or deletion reduced the time spent in mitosis in the nocodazole-treated cells, arguing that, as for the chromosome congression phenotype, the role of KNL1 in the SAC can be supported by the four remaining MELT repeats in KNL1 Delta-1000.
Fig. 4. See next page for legend.
Next, we analyzed the effect of gradually reducing the number of MELT repeats remaining in KNL1 Δ1–1000 to determine whether there was a minimum number required for the SAC (Fig. 5C). Cells expressing a construct containing three MELT repeats behaved similarly to KNL1 Δ1–1000, whereas reducing the number to two MELT repeats resulted in a reduction in the time spent in mitosis. However, reducing the number of MELT repeats to less than two further reduced the time, indicating that with two MELT repeats remaining, the SAC remained active although with reduced activity.

The balance between PP1 activity and Mps1 controls the levels of Bub proteins on KNL1 in prometaphase

The fact that KNL1 Δ1–150 and KNL1 Δ1–300 had increased kinetochore levels of the Bub proteins suggested that these regions contained elements that negatively regulated their recruitment. Given that, in yeast, PP1 negatively regulates the interaction between KNL1 and Bub1 and the fact that KNL1 contains a conserved binding site for PP1 required for SAC silencing in yeast, we anticipated that the role of PP1 binding to KNL1 was to remove the Bub proteins, although this had not been experimentally investigated in any system (London et al., 2012; Meadows et al., 2011; Rosenberg et al., 2011).

To analyze the role of PP1 binding to KNL1 in human cells, we mutated the conserved RVSF motif to AAAA in either full-length KNL1 or KNL1 Δ1000–1215. This mutation of the RVSF motif has previously been shown to prevent PP1 binding to human KNL1 (Liu et al., 2010). In addition, we reintroduced a similar PP1-binding site at the N-terminus of KNL1 Δ1–300. First, we analyzed the kinetochore levels of BubR1 or Bub1 in the different stable cell lines after depletion of endogenous KNL1 and found that the mutation of the PP1-binding site resulted in an approximate 1–300 reduced the levels of BubR1, although the number to two MELT repeats remaining, the SAC remained active although with reduced activity.

To determine the minimum number of MELT repeats required for KNL1 function, (A) Schematic of KNL1 Δ1–1000 constructs generated with four, three, two, one and zero MELT repeats remaining. (B) Representative still images from time-lapse movies of cells depleted of endogenous KNL1 and complemented with KNL1 Δ1–1000 2 MELT (YFP signal) and expressing a marker for histone (CFP). The time at NEBD is set to zero. Asterisks indicate unaligned chromosomes. (C) Similar to B but cells were complemented with KNL1 Δ1–1000 0 MELT. (D,E) Quantification of BubR1 and Bub1 levels in nocodazole-arrested cells complemented with the indicated KNL1 constructs normalized to the GFP signal in the different KNL1-expressing cells. Results are means±s.e.m. ****P<0.0001; ***P<0.001; *P<0.05; ns, not significant (Student’s t-test).

DISCUSSION

Here, we have addressed how the Bub proteins are recruited to and removed from the kinetochore in human cells and find that the phosphorylated MELT repeats in human KNL1 represent individual functional binding sites recognized directly by Bub3. As long as a limited number of MELT repeats in KNL1 are maintained, then the SAC and chromosome congression are functional. This potentially explains why the exact number of MELT repeats is not strongly conserved.

Interaction of the Bub proteins with KNL1

The large outer kinetochore protein KNL1 is a receptor for the Bub proteins, but in human cells, the exact mechanism in which both the N-terminal KI motifs as well as the MELT repeats in KNL1 have been suggested to play a role is debated (Kiyomitsu et al., 2007; Yamagishi et al., 2012). Here, we show that KNL1 truncations lacking the KI motifs can recruit the Bub proteins efficiently, in line with previous observations (Bolanos-Garcia et al., 2011; Krenn et al., 2012; Lara-Gonzalez et al., 2011; Yamagishi et al., 2012). Although the KI motifs are dispensable for bulk recruitment of the Bub proteins, they might still contribute to the efficiency of chromosome congression, as removing amino acids 150–300 of KNL1, encompassing the KI motifs, leads to a 5–10 minute delay in metaphase plate formation compared to KNL1 and KNL1 Δ1–150. As the total levels of Bub proteins on kinetochores is very similar in cells expressing KNL1 Δ1–150 and KNL1 Δ1–300, a small pool of proteins recruited to the KI motifs might contribute specifically to chromosome congression, for instance by precisely positioning the B56-PP2A phosphatase complexes (Foley et al., 2011; Kruse et al., 2013; Suijkerbuijck et al., 2012). However, our data do not support that the KI motifs are crucial for a functional SAC.

Phosphorylation of the MELT repeats in KNL1 by Mps1 has been shown to be important for kinetochore recruitment of Bub1–Bub3 but the exact function of these repeats had not been clarified (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). Our observation that further truncations of KNL1 Δ1–300 resulted in a gradual decrease in kinetochore levels of the Bub proteins and that the decrease matched the number of MELT repeats removed argues that the MELT repeats are capable of binding directly to the Bub1–Bub3 and BubR1–Bub3 complexes. Indeed, we have been able to show that Bub3 can bind a...
Fig. 5. See next page for legend.
phosphorylated MELT repeat peptide directly, explaining the role of Bub3 in kinetochore localization of Bub1 and BubR1. Given the requirement for phosphorylation of the MELT repeats by Mps1 to enhance Bub3 binding, and the role of PP1 binding to KNL1 in removing the Bub proteins shown in this study, the phosphate group must play a crucial role in binding to the complexes. Indeed, the recent determination of the structure of Bub1–Bub3 in complex with a phosphorylated MELT peptide shows a specific binding pocket on Bub3 for the phosphate group (Primorac et al., 2013). Given that BubR1–Bub3 and Bub1–Bub3 complexes are recruited to KNL1 by very similar mechanisms, it is puzzling why Bub1 is needed for BubR1–Bub3 localization to kinetochores and this will be important to understand.

The four MELT repeats remaining in KNL1 Δ1–1000 can, in our hands, fully support KNL1 function, which is in line with the observation that this KNL1 truncation recruits Bub proteins to a similar level as does full-length KNL1. However, lowering the number of MELT repeats in KNL1 Δ1–1000 to three severely affected chromosome congression and the SAC was affected when only two MELT repeats remained. This indicates that, in human cells, four phosphorylated MELT repeats represent the minimum required docking sites for Bub1 and BubR1, and interestingly this is close to the level we observe binding to full-length KNL1. Why human KNL1 contains twelve MELT repeats when four appears sufficient is unclear, but it could potentially allow docking of Bub proteins at specific positions on KNL1 allowing efficient chromosome congression under certain conditions. Currently we do not know whether the failure in chromosome segregation is due to the loss of Bub1 and/or BubR1, as the proteins have opposing roles in establishment of kinetochore–microtubule interactions. However, the presence of unattached kinetochores observed in our cold stability assays might suggest that it is lack of PP2A recruitment that gives rise to unaligned chromosomes.
Fig. 7. See next page for legend.
Binding of protein phosphatase 1 to KNL1 removes Bub proteins and contributes to SAC silencing. (A) Still images from cells depleted of endogenous KNL1 and complemented with KNL1ΔPP1 from live-cell recordings. The DIC, YFP (KNL1) and GFP (histone 3) signals from a representative cell is shown and time at NEBD is set to zero and time is given in minutes. (B) Quantification of NEBD to anaphase and metaphase to anaphase time in the complemented cells. The number of cells analyzed for each condition is indicated and the red line indicates the median. For the metaphase to anaphase timing only cells where it was clear when the two events occurred were included in the analysis. ***P<0.001; *P<0.05 (a Mann–Whitney test). (C,D) Cells complemented with KNL1 or KNL1ΔPP1 were arrested overnight with 200 ng/ml nocodazole and either left untreated or treated with 0.5 μM reversine for 10 minutes before fixation. The level of BubR1 was determined and then normalized to GFP under the different conditions and at least 70 pairs of kinetochores from eight individual cells were analyzed. ****P<0.0001; ns, not significant (Student’s t-test).

**PP1 antagonizes Mps1 during an active SAC**

Previous work in yeast and worms has shown that PP1 is required for SAC silencing and also that PP1-binding sites in KNL1 and kinetochore 8 family members are crucial for this, suggesting that precise targeting of PP1 is important (Espeut et al., 2012; Meadows et al., 2011; Rosenberg et al., 2011; Vanoosthuyse and Hardwick, 2009). Here, we reveal that PP1 targeting to human KNL1 is crucial for kinetochore removal of the Bub proteins but that this not only occurs upon satisfaction of the SAC but constantly during an active SAC. In line with this, PP1γ can be observed on kinetochores in prometaphase (Liu et al., 2010; Trinkle-Mulcahy et al., 2003). We speculate that the MELT repeats are constantly phosphorylated and dephosphorylated during an active SAC and that this might be the molecular mechanism giving rise to turnover of Bub1 and BubR1 at kinetochores. Interestingly, preventing PP1 binding to KNL1 not only slightly affects SAC silencing in human cells, in agreement with what has been observed in worms (Espeut et al., 2012). The reason for this is probably that the Mad1–Mdc2 complex is removed by a dynein-dependent mechanism, which does not require PP1. Without the Mad1–Mdc2 complex on kinetochores, the SAC is still silenced despite Bub1 and BubR1 persisting on kinetochores.

PP1 binding to KNL1 has been reported to affect chromosome segregation in human cells, whereas in yeast the major function relates to SAC silencing (Liu et al., 2010; Meadows et al., 2011; Rosenberg et al., 2011). We do not observe any major defects in chromosome segregation in KNL1ΔPP1 based on time-lapse imaging, and recent data suggests that instead the B56-PP2A phosphatase complexes antagonize Aurora B at kinetochores to stabilize kinetochore–microtubule interactions (Foley et al., 2011; Kruse et al., 2013; Suijkerbuijk et al., 2012). Even though PP1 and B56-PP2A are both recruited to kinetochores by KNL1, they appear to have distinct substrates, and determining how this specificity is achieved will be important to understand the dynamic regulation of kinetochores.

**MATERIALS AND METHODS**

**Cloning and cell lines**

Full-length KNL1 and all the truncated constructs were amplified by PCR (Expand Long Range, Roche) with KpnI and NotI sites in the forward and reverse primer, respectively. The PCR products were cloned into the KpnI- and NotI-digested pcDNA5/FRT/TO c-Venus vector. siRNA resistance was achieved by mutating 5′-AGATCTGATTAAAGGA-TCCACGA-3′ (4167–4188) into 5′-GGACTTATCAAGAACCCCT- CGT-3′. All the constructs have been confirmed by full sequencing.

Mutation of the four MELT motifs within the region of 1000–1200 amino acids was performed by DNA synthesis (GeneArt, Invitrogen). Details of the cloning will be provided upon request. Stable cell lines of HeLa/FRT/ TRex with inducible KNL1 constructs were generated as described previously (Zhang et al., 2012).

**Immunofluorescence**

Cells growing on coverslips were pre-extracted with 0.5% Triton X-100 in PHEM buffer (60 mM PIPES, 25 mM HEPES pH 6.9, 10 mM EGTA, 4 mM MgSO4) for 5 minutes before fixation with 4% paraformaldehyde in PHEM buffer for 20 minutes. For microtubule cold stability assay, the cells were incubated on ice for 15 minutes before the fixation as described above. The antibodies used for cell staining were against KNL1 (Bethyl, 1:200), CREST (Antibodies Incorporated, 1:400), GFP (Abcam, 1:400), BubR1 (made in-house, 1:400), Mad2 (made in-house, 1:400). All the fluorescent secondary antibodies are Alexa Fluor conjugated (Invitrogen, 1:1000). Images were collected using a 100× objective on a DeltaVision Elite microscope (GE Healthcare/Applied Precision). Protein intensity on kinetochores was quantified by drawing a circle closely along the rod-like CREST staining covering the outer kinetochore protein staining of interest. The intensity number from the peak of three continuous stacks was subtracted from the background from neighboring areas and averaged. All the intensity was normalized against the GFP fluorescence intensity.

**Knockdown and rescue of KNL1 in HeLa cells**

Stable HeLa cell lines were cultivated in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum, antibiotics and hygromycin (Invitrogen). Cells were synchronized with 2 mM thymidine for 24 hours before transfection with siRNA oligonucleotides (100 nM as final concentration). A stealth RNA oligonucleotide from Invitrogen (5′-AAGAUCGUAUAAAGGAUCCGAAA-3′) was used for all the stainings and filmings. For the SAC experiment (Fig. 5), an RNAi oligonucleotide (5′-CCACCAUUGGAACACCAAA-3′, Sigma) against the 3′ UTR of KNL1 was combined with the stealth RNA oligonucleotide to achieve a more robust KNL1 depletion. The expression of wild-type or mutant KNL1 was induced after RNAi by adding doxycycline to the medium. The cells were arrested again 12 hours later by thymidine for another 24 hours. The cells were released from thymidine and recorded by filming. For immunofluorescence, after thymidine release, nocodazole (200 ng/ml final) was added for 14 hours to arrest cells before fixation as described above.

**Live-cell imaging**

Cells grown in 8-well chamber (Ibidi) with L-15 medium were mounted on a DeltaVision Elite microscope. A 40× oil-immersion objective was used. DIC and YFP images were collected continuously for 8–12 hours at 5- or 10-minute intervals. Image J (NIH) was used to extract still images of the region of interest in cells and analysis of movies was performed using softWorx software. 100 nM nocodazole was added 5 hours after the last thymidine release for the experiment in Fig. 5.

**Bub3 expression and purification**

Bub3 was cloned into the HIS-OneStrep N-term vector pCR00053 (LIC-c-PEP4) using ligation independent cloning (LIC). The plasmid was transformed into Mach1-T1 cells (Invitrogen) and 2.7 l overnight culture was GIGAprepped with the Nucleobond PC 10000 EF kit (Macherey-Nagel). The transfection was performed into human embryonic kidney EBNA 6E cell lines (HEK293 6E) grown in Freestyle 293 F17 expression medium and Freestyle F17 (Invitrogen). At 1 day prior to transfection, HEK293 6E cells were re-suspended in fresh Freestyle 293 F17 expression medium to a cell density of 1.2×10^6 cells/ml and incubated at 37°C overnight. At ~15 minutes before transfection, cells were re-suspended in fresh unsupplemented Freestyle 293 F17 expression medium at a cell density of 20×10^6 cells/ml and incubated in the orbital shaker incubator at 37°C, under 70% humidity, 5% CO2, and at 120 rpm (Ø50 mm), until being transfected. GigaPrep DNA (50 μg/ml final) and Polyethyleneimine “MAX” (PEI) (Polysciences; 100 μg/ml final), under 70% humidity, 5% CO2, for 5 minutes before fixation with 4% paraformaldehyde in PHEM buffer for 20 minutes. For microtubule cold stability assay, the cells were incubated on ice for 15 minutes before the fixation as described above. The antibodies used for cell staining were against KNL1 (Bethyl, 1:200), CREST (Antibodies Incorporated, 1:400), GFP (Abcam, 1:400), BubR1 (made in-house, 1:400), Mad2 (made in-house, 1:400). All the fluorescent secondary antibodies are Alexa Fluor conjugated (Invitrogen, 1:1000). Images were collected using a 100× objective on a DeltaVision Elite microscope (GE Healthcare/Applied Precision). Protein intensity on kinetochores was quantified by drawing a circle closely along the rod-like CREST staining covering the outer kinetochore protein staining of interest. The intensity number from the peak of three continuous stacks was subtracted from the background from neighboring areas and averaged. All the intensity was normalized against the GFP fluorescence intensity.
solution final) were directly added to the cell suspension. Complete Freestyle 293 F17 expression medium (1% FBS) was added to a final volume of 4 of cell suspension, 4 hours after transfection. At 3 days post transfection, the pellets were collected by centrifugation at 750 rpm for 10 minutes at 4°C. Bub3 was purified using the Strep-tag/Strep-Tactin purification system provided by IBA.

Peptide pulldown and recombinant protein binding experiments

Two peptides were used for this assay: Biotin-PFG2-EDDKND-Nle-DITKSYTIEIN-amide (BIOSYNTAN) mimics the MELT motif and Biotin-PFG2-EDDKND-Nle-Dl-pT-KSYTIEIN-amide mimics the phosphorylated MELT motif. Nocodazole-arrested cells were lysed in lysis buffer containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.3% Triton X-100 with protease inhibitors, phosphatase inhibitors and the Mps1 kinase inhibitor reversin. Cell lysate was incubated with preassembled peptide–Streptavidin–agarose complex (Sigma) for 4 hours at 4°C. After washing four times with the lysis buffer, the bound proteins were eluted by 1× Laemmli buffer at 95°C. The elate was analyzed by western blotting against BubR1 (antibody made in-house), Bub3 (BD Biosciences), Mps1 (Abcam) and Ndc80 (Abcam).

Purified recombinant Bub3 was digested with TEV protease to cut away the Strep tag. 3 μg of Bub3 was incubated with the preassembled peptide–Streptavidin–agarose complex or the beads alone in 500 μl lysis buffer for 1 hour at room temperature. After washing four times, the protein was eluted by 1× Laemmli buffer at 95°C. Western blotting was performed to check for the presence of Bub3.

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Competing interests

The authors declare no competing interests.

Author contributions

G.Z. generated reagents and performed all the experiments except the experiments in Fig. 5, which were performed by T.L. T.L. also did filming to generate data for Fig. 1 and Table 1. All authors contributed to designing experiments and J.N. wrote the paper.

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Supplementary material

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