Dissecting the roles of human BUB1 in the spindle assembly checkpoint

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# Equal author contributions
Abstract

Mitotic chromosome segregation is initiated by the anaphase promoting complex/cyclosome (APC/C) and its co-activator CDC20. APC/C^{CDC20} is inhibited by the spindle assembly checkpoint (SAC) when chromosomes have not attached to spindle microtubules. Unattached kinetochores catalyze the formation of a diffusible APC/C^{CDC20} inhibitor that is composed of BUBR1, BUB3, MAD2 and a second molecule of CDC20. Kinetochore recruitment of these proteins as well as SAC activation rely on the mitotic kinase BUB1, but the molecular mechanism by which BUB1 accomplishes this in human cells is unknown. We show that BUBR1 and BUB3 kinetochore recruitment by BUB1 is dispensable for SAC activation. Unlike its yeast and nematode orthologs, human BUB1 does not associate stably with the MAD2 activator MAD1 and, although required for accelerating loading of MAD1 onto kinetochores, is dispensable for its steady-state levels there. Instead, we identify a 50 amino acid segment harboring the recently reported ABBA motif close to a KEN box as critical for BUB1’s role in SAC signaling. The presence of this segment correlates with SAC activity and efficient binding of CDC20 but not MAD1 to kinetochores.
Introduction

During mitosis, all chromosomes have to attach to microtubules of the mitotic spindle and become biorientated before cells are allowed to proceed into anaphase. Chromosome attachment is mediated by kinetochores, large multi-protein structures that form the bridge between chromosomes and spindle microtubules (Foley and Kapoor, 2013). Microtubule binding activity at kinetochores is mainly provided by the KMN network, a 10-subunit protein assembly consisting of three subcomplexes KNL1-C/MIS12-C/NDC80-C (Cheeseman et al., 2006).

In addition to kinetochore-microtubule attachment formation, the KMN network plays a key role in coupling kinetochore attachment status to SAC activity. The SAC is activated at unattached kinetochores and results in the formation of a soluble inhibitor of APC/C\(^{CDC20}\), a multisubunit E3 ligase whose activity initiates chromosome segregation and mitotic exit by targeting key mitotic regulators for proteasomal destruction (Pines, 2011). The soluble inhibitor of the APC/C is known as the mitotic checkpoint complex (MCC) and is composed of MAD2, BUBR1, BUB3 and CDC20 (Chao et al., 2012; Izawa and Pines, 2015; Kulukian et al., 2009; Sudakin et al., 2001).

The SAC response is initiated at the KMN network by MPS1-mediated phosphorylation of multiple motifs in repeat sequences of KNL1 (London et al., 2012; Shepperd et al., 2012; Vleugel et al., 2015; Yamagishi et al., 2012). When phosphorylated, these motifs recruit BUB1/BUB3 dimers that are essential for SAC signaling and chromosome biorientation (Krenn et al., 2014; Primorac et al., 2013; Vleugel et al., 2013; Vleugel et al., 2015; Zhang et al., 2014). Since the discovery of BUB1, the molecular mechanism by which it participates in SAC activation has been a matter of controversy. Studies in mice, budding yeast and fission yeast have shown that BUB1 kinase activity is required for SAC activation (Kawashima et al., 2009; Ricke et al., 2012; Yamaguchi et al., 2003) but this was contradicted by other studies using the same model organisms (Baker et al., 2009; Fernius and Hardwick, 2007; London and Biggins, 2014; Perera et al., 2007; Rischitor et al., 2007; Warren et al., 2002). Human cell studies have proven equally inconsistent: While one study proposed that BUB1 directly phosphorylates and inhibits CDC20 (Kang et al., 2008), another showed that a truncated BUB1 protein lacking the kinase domain significantly restored SAC activity to BUB1-depleted cells (Klebig et al., 2009). Recent studies showed that in budding yeast and *C. elegans* BUB1 directly binds MAD1 via a region preceding the kinase domain (*S. cerevisiae*), or via the kinase domain itself (*C. elegans*) (London and Biggins, 2014; Moyle et al., 2014). Whether this function of BUB1 is
conserved in human cells is unknown. In addition to MAD1/MAD2 and BUB3, BUB1 is essential for recruiting BUBR1 and CDC20 to kinetochores (Johnson et al., 2004; Kang et al., 2008; Klebig et al., 2009; Sharp-Baker and Chen, 2001). It is at present unknown how BUB1 accomplishes this, and it is unclear if this is functionally relevant for SAC signaling.

Here we set out to identify the molecular role of BUB1 in SAC signaling in human cells. We provide evidence that promoting BUBR1 kinetochore-localization is not a crucial aspect of BUB1’s function in the SAC and that BUB1-mediated kinetochore recruitment of MAD1 is insufficient for SAC functioning. We instead find a strong correlation between SAC activity, CDC20 kinetochore binding and the presence of 50 residues in BUB1 harboring 2 motifs known to bind CDC20 in other proteins.
Results and discussion

BUBR1 kinetochore localization is dispensable for the SAC

To uncover the contribution of BUB1 to the SAC, we examined which aspect of BUB1 is required for BUBR1 kinetochore localization. We therefore removed various domains/motifs previously implicated in SAC function from LAP-tagged BUB1 (LAP-BUB1WT), creating LAP-BUB11-318, -BUB11-500 and -BUB11-778 (Fig. 1A). All RNAi-resistant constructs were stably integrated in HeLa-FRT cells at a single doxycycline-inducible locus (Klebig et al., 2009). BUBR1 kinetochore recruitment in nocodazole-treated cells was abolished by RNAi of BUB1 and this was rescued by expression of LAP-BUB1WT (Fig. 1B,C). All BUB1 truncations localized to kinetochores to similar levels (Fig. 1B,C), as expected (Krenn et al., 2012), and were able to restore BUBR1 levels at kinetochores upon BUB1 RNAi (Fig. 1B,C). We thus conclude that the N-terminal 318 amino acids of BUB1 encompassing a TPR domain and GLEBS motif are sufficient for BUBR1 kinetochore recruitment. This redefines the region in BUB1 required for BUBR1 recruitment to a conserved alpha-helix following the GLEBS motif (amino acids 271-318) (Overlack et al., 2015).

We next asked whether the BUB1-BUB3 interaction is required to recruit BUBR1 to kinetochores. To circumvent the complicating issue that disrupting this interaction (by mutating the GLEBS motif) also prevents kinetochore binding of BUB1 (Taylor et al., 1998), BUB1 was artificially tethered to kinetochores by fusing it to the KMN network protein MIS12 (LAP-MIS12-BUB1WT) (Fig. 1A). BUBR1 localization was recovered when endogenous BUB1 was substituted with MIS12-BUB1, but not when the GLEBS motif was mutated (MIS12-BUB1E252K) (Fig. 1D,E) (Overlack et al., 2015).

To determine if BUBR1 kinetochore localization is required to SAC signaling, cells expressing MIS12-BUB1E252K were analyzed for SAC activity by filming mitotic progression in the presence of nocodazole and low dose of the MPS1 inhibitor reversine (250 nM) (Santaguida et al., 2010). Cells depleted of BUB1 exited mitosis within one hour, whereas control cells and BUB1-depleted cells expressing MIS12-BUB1WT were able to maintain a functional SAC for over five hours (Fig. 1F). Surprisingly, although MIS12-BUB1E252K did not recruit detectable levels of BUBR1 to kinetochores (Fig. 1D,E), it fully restored SAC function (Fig. 1F). Conversely, a BUB1 fragment that was sufficient for BUBR1 kinetochore localization (LAP-MIS12-BUB11-318, Supplemental Fig. 1A,B) could not support SAC activation (Fig. 1F). Taken together, these observations suggest that a primary role of kinetochore BUBR1 lies not in SAC activation but most likely in other processes such as
chromosome biorientation through recruitment of the phosphatase PP2-B56 (Kruse et al., 2013; Suijkerbuijk et al., 2012; Xu et al., 2013).

**Human BUB1 is not required for stable association of MAD1 to unattached kinetochores, but accelerates its loading**

We next asked whether human BUB1 promotes SAC activation by forming a stable complex with MAD1, as it does in budding yeast and *C. elegans* (London and Biggins, 2014; Moyle et al., 2014). LAP-BUB1<sup>WT</sup> immunoprecipitated from nocodazole-treated cells associated with known interactors such as BUB3, BUBR1 and KNL1, as observed by mass spectrometry (Fig. 2A). However, no MAD1 peptides were identified in these BUB1 purifications (Fig. 2A). Furthermore, ectopic tethering of BUB1 to a LacO array (LacI-BUB1) was insufficient to recruit MAD1, whereas endogenous BUBR1 was readily detected at those LacO foci (Fig. 2B). These observations argue against the existence of a stable BUB1-MAD1 complex in human cells. We next wished to determine the extent to which BUB1 contributes to MAD1 kinetochore localization. Cells depleted of BUB1 were treated with nocodazole for 3 hours, fixed, and stained for MAD1 by immunofluorescence. Although BUB1 kinetochore levels were reduced to < 5% in both HeLa and RPE-1 cells, we observed no difference in MAD1 kinetochore localization compared to control cells (Fig. 2C,D,E,F). SAC sensitization by partial MPS1 inhibition (250 nM reversine) reduced MAD1 kinetochore levels but, even in this case, BUB1 RNAi could not further reduce MAD1 levels (Fig. 2C,D,E,F). Together, these data argue against a role of human BUB1 as the predominant receptor for MAD1 in human cells.

Although our observed lack of correlation between BUB1 and MAD1 at kinetochores agrees with some reports (Hewitt et al., 2010; Vleugel et al., 2013), it contrasts with others (Kim et al., 2012; Klebig et al., 2009). We noted that while we assessed MAD1 kinetochore levels in cells treated with nocodazole for several hours, others did so in unperturbed cells which have likely spent less time in mitosis (Klebig et al., 2009). To test this, we measured MAD1 kinetochore levels at different times after metaphase kinetochores devoid of MAD1 were forced to detach and recruit MAD1, by addition of nocodazole (Fig. 2G,H). In control cells, BUB1 and MAD1 rapidly accumulated at kinetochores, with kinetochore levels peaking at 10 (BUB1) and 20 (MAD1) minutes (Fig. 2G,H). As expected, BUB1-depleted cells had accumulated similar amounts of MAD1 as control cells after 40 minutes. Strikingly, however, in the first 20 minutes, MAD1 levels were significantly lower in BUB1-depleted cells (Fig. 2G,H). BUB1 thus accelerates efficient MAD1 kinetochore loading, potentially through the
previously identified CD1 motif (Klebig et al., 2009), but is not essential for it. These findings show that the mechanism of MAD1 kinetochore binding in human cells is at least in part different from that in budding yeast and C. elegans, in which BUB1 is the primary kinetochore receptor for MAD1 (London and Biggins, 2014; Moyle et al., 2014). Human BUB1 appears to catalyze MAD1 kinetochore-recruitment, perhaps by forming transient interactions to bring MAD1 to kinetochores or by licensing kinetochores for MAD1 binding.

**Amino acids 501-550 in human BUB1 are essential for SAC activation**

To assess if accelerating MAD1 kinetochore loading by BUB1 affects SAC signaling, we analyzed various BUB1 truncations (BUB1\(^{1-318}\), BUB1\(^{1-500}\) and BUB1\(^{1-778}\)) for their ability to rescue SAC function and MAD1 loading after BUB1 depletion. The SAC defect induced by BUB1 depletion was rescued by expression of BUB1\(^{WT}\), BUB1\(^{1-778}\), BUB1\(^{1-696}\) and BUB1\(^{1-555}\) but not by BUB1\(^{1-318}\) or BUB1\(^{1-500}\), suggesting that that the region between amino acids 501-555 is essential for SAC functioning (Fig. 3A). In our analyses, therefore, the BUB1 kinase domain was not required for the SAC. Importantly, whereas BUB1-depleted cells had reduced MAD1 kinetochore levels 10 minutes after nocodazole addition to metaphase cells, cells expressing BUB1\(^{1-500}\) recruited normal amounts of MAD1 in that time span (Fig. 3B,C). Poor SAC signaling in BUB1\(^{1-500}\)-expressing cells cannot therefore be explained by impaired MAD1 loading, or, as shown in Figure 1, by impaired BUBR1 kinetochore binding.

**The SAC-function of BUB1 correlates with its ability to recruit CDC20**

Amino acids 501-555 of BUB1 harbor KEN-box motif 1 (Kang et al., 2008). In addition, our alignment of metazoan BUB1 orthologs revealed the presence of a conserved [FY]xx[FY]x[DE] motif (amino-acids 527-532) (Fig. 3D). The protein ACM1 utilizes a similar motif known as the A motif in addition to its KEN box to interact with and thereby inhibit the CDC20 ortholog CDH1 (Enquist-Newman et al., 2008; He et al., 2013). Since recent reports have shown the existence of a similar motif in BUBR1 (dubbed the Phe box or ICDC20BD) that is important for a stable BUBR1-CDC20 interaction and for SAC activity (Diaz-Martinez et al., 2014; Lischetti et al., 2015), we tested whether this region in BUB1 is involved in CDC20 kinetochore localization. CDC20 localizes strongly to kinetochores in nocodazole-treated cells and this depends on BUB1 (Fig. 3E,F). Whereas BUB1\(^{WT}\) and BUB1\(^{1-555}\) restored CDC20 kinetochore levels, BUB1\(^{1-500}\) did not (Fig. 3E,F), showing that indeed the region between 501-555 is responsible for CDC20 kinetochore targeting. Furthermore, LacI-BUB1 efficiently recruited CDC20 to ectopic sites in U2OS-LacO cells.
This depended on the GLEBS-motif (Fig. 3G) (Overlack et al., 2015) and therefore most likely required the presence of BUB1/BUB3, although we cannot exclude a potential separate function of the GLEBS-motif (Fig. 3G). Thus, the A box-containing segment as well as BUBR1 were required for BUB1’s ability to recruit CDC20. This agrees with a recent study by the Pines lab, published while our manuscript was under revision, which showed that CDC20 kinetochore recruitment requires the A box-like motifs (referred to as the ABBA motif in that study) of both BUB1 and BUBR1 (Di Fiore et al., 2015). To retain consistency in nomenclature, we will hereafter refer to the A box-like motif in BUB1 as the ABBA motif. Although direct (Di Fiore et al., 2015), the BUB1-CDC20 interaction may be transient since CDC20 peptides were not detected in BUB1 immunoprecipitations by mass spectrometry (Fig. 2A). We conclude that although BUB1 promotes loading of MAD1 to unattached kinetochores, its ability to activate the SAC more strongly correlates with its ability to recruit CDC20 to kinetochores. We thus propose that this constitutes the essential role of BUB1 in SAC signaling.

In order to understand SAC activation and MCC assembly, it will be important to study ABBA motif/KEN box motifs in BUB1 and the role of CDC20 kinetochore binding in the SAC. CDC20 kinetochore recruitment by BUB1 might bring CDC20 in close proximity to newly formed C-MAD2 and thus facilitate the formation of C-MAD2/CDC20 dimers, as has recently been proposed for nuclear pore-mediated C-MAD2/CDC20 dimers, as proposed for nuclear envelope breakdown (Rodriguez-Bravo et al., 2014). Alternatively, by ensuring CDC20 kinetochore localization, BUB1 might promote SAC signaling by allowing kinetochore-driven modification of CDC20 or by mediating a conformational change in CDC20 that allows it to bind to C-MAD2. Since BUBR1 is not required at unattached kinetochores for SAC activity, we propose a model in which C-MAD2/CDC20 dimers formed at kinetochores interact with cytoplasmic BUBR1/BUB3 dimers.
Materials and Methods

Plasmids
pCDNA5-LAP-BUB1\textsuperscript{WT} was made by ligation of LAP-BUB1\textsuperscript{WT} into the Xho1 and Hpa1 site of pCDNA5-LAP-KNL1 (Vleugel et al., 2013) and encodes full-length, siRNA-resistant BUB1. LAP-BUB1-truncations were made by introduction of stop-codons in LAP-BUB1\textsuperscript{WT}. LAP-MIS12-BUB1\textsuperscript{WT} was made by PCR of MIS12 with 2 Xho1 sites and ligation into the Xho1 site of LAP-BUB1\textsuperscript{WT}. LAP-MIS12-BUB1\textsuperscript{E252K} was made by introduction of a lysine substitution in LAP-MIS12-BUB1\textsuperscript{WT}.

Cells culture and transfection
Tissue culture and transfection were done as described in (Vleugel et al., 2013). Expression of constructs was induced by 2 µg/ml doxycycline for 24h. siBUB1 (5’-GAAUGUAAGCUUCAGAA-3’) was transfected twice using HiPerfect (Qiagen) for HeLa’s or Lipofectamine RNAiMAX (Life Technologies) for RPEs for 24h at 20 nM.

Immunofluorescence and antibodies
Cells were grown on 12 mm coverslips and treated as indicated in figure legends with nocodazole (830 nM), MG132 (5 uM) and reversine (250 nM). Cells were fixed and stained as described in (Vleugel et al., 2013). All images were acquired on a DeltaVision RT (Applied Precision) with a 100×/1.40 NA U Plan S Apochromat objective (Olympus) using softWoRx software (Applied Precision). Images are maximum intensity projections of deconvolved stacks and were quantified as described in (Vleugel et al., 2013). Primary antibodies used were GFP-Booster (ChromoTek), rabbit-anti-BUB1 and rabbit-anti-BUBR1 (Bethyl), mouse-anti-MAD1 (a gift from A. Musacchio, MPI, Dortmund, Germany) rabbit-anti-CDC20 (Santa Cruz), guinea pig-anti-CENP-C (MBL) and CREST/anti-centromere antibodies (Cortex Biochem.). Secondary antibodies were Alexa Fluor 488/568/647 (Molecular Probes).

Live cell imaging
Cells were grown on 24-wells plates and synchronized using 2 mM thymidine for 24h. Cells were released into nocodazole (830 nM) and reversine (250 nM). Filming started 4-6 hours after thymidine release and was done at 37°C at 5% CO\textsubscript{2} with a 20x/0.5 NA UPFLN objective on a microscope (model IX-81; Olympus) controlled by Cell-M software (Olympus). Images
were acquired using a CCD camera (ORCA-ER; Hamamatsu Photonics) and processed using Image J software.

**Mass spectrometry**

Cells were synchronized in mitosis by a 24h thymidine block, followed by o/n treatment with nocodazole. LAP-BUB1 expression was induced for 24h and cells were harvested, followed by immunoprecipitation and mass spectrometry as described in (Vleugel et al., 2013).

**Conserved motif analysis**

Conservation of BUB1 residues was determined by a Jackhmmer run with human BUB1 as query on the UniProt database. Only metazoan species were included in the analysis.

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Fig. 1. BUB1-mediated kinetochore recruitment of BUBR1 is not required for the SAC.

(A) Schematic representation of BUB1 truncation mutants used in B-E, asterisk indicates E252K mutation. (B-C) Representative images (B) and quantification (C) of BUBR1 kinetochore localization in HeLa Flp-in cells expressing LAP-BUB1 constructs.
Quantification in C shows kinetochore intensity (+SD) as a ratio over CENP-C kinetochore intensity. n=10 cells. Kinetochore levels are normalized to 1 in control cells for BUBR1 and in BUB1WT cells for LAP-BUB1. (D-E) As in A-B, with the indicated constructs. (F) Time-lapse analysis of HeLa Flp-in cells expressing LAP-MIS12-BUB1WT, -BUB1E252K and –BUB11-318. Cells were transfected with siLUC or siBUB1, synchronized with thymidine and treated with nocodazole and 250 nM reversine. Time from nuclear envelope breakdown to mitotic exit was scored by cell morphology using DIC. Data show cumulative fraction of cells that have exit mitosis. n=50 cells, representative from 3 experiments.
Fig. 2, BUB1 promotes efficient kinetochore loading of MAD1. (A) Mass spectrometry analysis of LAP-BUB1 following immunoprecipitation from nocodazole-treated cells. (B) Immunolocalization of MAD1 (top) and BUBR1 (bottom) in U2OS-LacO cells transfected with LacI-LAP-BUB1 WT. (C-F) Representative images (C and E) and quantification (D and F) of BUB1 and MAD1 kinetochore localization of HeLa Flp-in (C and D) or RPE (E and F) cells. Quantification as in Figure 1c. n=10 cells representative of 2 experiments. Kinetochore levels are normalized to 1 in siLUC cells without reversine. (G and H) Representative images (G) and quantification (H) of BUB1 and MAD1 kinetochore localization of HeLa Flp-in cells after 1 hour of MG132 treatment followed by the addition of nocodazole for the indicated times. Quantification as in Figure 1c. n=20 cells representative of 2 experiments. Kinetochore levels are normalized to 1 at the time point showing the highest localization.
Fig. 3, CDC20 kinetochore recruitment by BUB1 correlates with SAC activity. (A) Time-lapse analysis of HeLa Flp-in cells expressing LAP-BUB1 variants, as in Figure 1f. (B-C) Representative images (B) and quantification (C) of HeLa Flp-in cells expressing LAP-BUB1
variants and treated with MG132 for 1 hour followed by 10 minutes of nocodazole. Quantification as in Figure 1c. n=10 cells representative of 3 experiments. (D) Sequence logo of metazoan BUB1\textsuperscript{501-555}, depicting extent of sequence conservation of residues in 501-550. (E-F) Representative images (E) and quantification (F) of nocodazole-treated HeLa Flp-in cells expressing LAP-BUB1 variants stained for LAP-BUB1 (GFP), CDC20 and CENP-C. Quantification as in Figure 1c. n=10 cells representative of 3 experiments. Kinetochore levels are normalized to 1 in control cells for CDC20 and in BUB1\textsuperscript{WT} cells for LAP-BUB1. (G) Immunolocalization of CDC20 in U2OS-LacO cells transfected with LacI-LAP-BUB1\textsuperscript{WT} (top) or LacI-LAP-BUB1\textsuperscript{E252K} (bottom).
Fig. 4, Model of BUB1 functions. Model of BUB1 functional regions. Check marks indicate the region being essential for the process.
References


