REGULATION AND MECHANISM OF BUB1-MEDIATED SPINDLE CHECKPOINT SIGNALING

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DEDICATION

To my parents, my husband and my dearest daughter

REGULATION AND MECHANISM OF BUB1-MEDIATED SPINDLE CHECKPOINT SIGNALING

by

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REGULATION AND MECHANISM OF BUB1-MEDIATED SPINDLE CHECKPOINT SIGNALING

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The spindle checkpoint is a surveillance mechanism that ensures the fidelity of chromosome segregation during mitosis and meiosis. Bub1 is a highly conserved protein serine/threonine kinase that plays multiple roles in the spindle checkpoint. The regulation and mechanism of Bub1 in spindle checkpoint were investigated. Bub1 is degraded during mitotic exit and the degradation of it is mediated by APC/C in complex with its activator Cdh1 (APC/C^{Cdh1}). Overexpression of Cdh1 reduces the protein levels of ectopically expressed Bub1 whereas depletion of Cdh1 by RNA interference (RNAi) increases the level of the endogenous Bub1 protein. Two KEN-box motifs on Bub1 are required for its

degradation *in vivo* and ubiquitination *in vitro*. A Bub1 mutant protein with both KEN-boxes mutated is stable in cells.

Kinetochore is the origin of spindle checkpoint signal and contains the catalytic machinery for generating the signal. We identify an ATP-dependent APC/C^{Cdc20} inhibitory activity on metaphase chromosomes with unattached kinetochores. The Cdc20-S153A that cannot be phosphorylated by Bub1 is not inhibited by metaphase chromosomes, suggesting Bub1 is likely responsible for the inhibitory activity. Bub1 on unattached kinetochores is hyperphosphorylated and activated. Furthermore, the kinase-dead mutant of Bub1 cannot restore spindle checkpoint in Bub1-RNAi cells, demonstrating that the kinase activity of Bub1 is required for the spindle checkpoint.

Plk1 is required for the generation of the tension-sensing 3F3/2 kinetochore epitope and facilitates kinetochore localization of Mad2 and other spindle checkpoint proteins. We investigate the mechanism by which Plk1 is recruited to kinetochores. We show that Plk1 binds to Bub1 in mitotic cells. The Plk1–Bub1 interaction requires the polo-box domain (PBD) of Plk1 and is enhanced by Cdk1-mediated phosphorylation of Bub1 at T609. The PBD-dependent binding of Plk1 to Bub1 facilitates phosphorylation of Bub1 by Plk1 *in vitro*. Depletion of Bub1 in HeLa cells by RNAi diminishes the kinetochore localization of Plk1. Ectopic expression of the wild-type Bub1, but not the Bub1-T609A mutant, in Bub1-RNAi cells restores the kinetochore localization of Plk1. Our results suggest that phosphorylation of Bub1 at T609 by Cdk1 creates a docking site for the PBD of Plk1 and facilitates the kinetochore recruitment of Plk1.

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PRIOR PUBLICATIONS

1. **Qi, W.** & Yu, H. KEN-box dependent degradation of Bub1 spindle checkpoint kinase by anaphase promoting complex/cyclosome. ***J. Biol. Chem.*** In revision.

2. **Qi, W.**, Tang, Z., & Yu, H. (2006) Phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 is required for the kinetochore localization of Plk1. ***Molecular Biology of the Cell*** 17:3705-16.

3. Tang, Z., Shu, H., **Qi, W.**, Mahmood, N., Mumby, M.C. & Yu, H. (2006) PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. ***Developmental Cell*** 10**:** 1-10.

4. Song, B.L., Wang, C.H., Yao, X.M., Yang, L., Zhang, W.J., Wang, Z.Z., Zhao, X.N., Yang, J.B., **Qi, W.**, Yang, X.Y., Inoue, K., Lin, Z.X., Zhang, H.Z., Kodama, T., Chang, C.C., Liu, Y.K., Chang, T.Y., Li, B.L. (2006) Human acyl-CoA: cholesterol acyltransferase 2 gene expression in intestinal Caco-2 cells and in hepatocellular carcinoma. ***Biochem J***, 394: 617-626.

5. **Qi, W.** & Yu, H. (2005) The spindle checkpoint and chromosome stability. In: ***Genome and disease, Genome Dynamics,*** *Vol 1* (Volff, J.-N. ed.), Karger Publ. Co., Basel, Switzerland, pp. 116-130.

6. Bharadwaj, R., **Qi, W.** & Yu, H. (2004) Identification of two novel components of the human NDC80 kinetochore complex. ***J. Biol. Chem.*** 279: 13076-85.

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LIST OF ABBREVIATIONS

APC/C	Anaphase-promoting Complex or Cyclosome
ATP	Adenosine Triphosphate
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad50 related
BSA	Bovine Serum Albumin
Bub1	Budding Uninhibited by Benzimidazole 1
BubR1	Bub1 related protein
Cdk1	Cyclin-dependent kinase 1
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
GST	Glutathione S-transferase
INCENP	Inner centromere protein
IP	Immunoprecipitation
KD	Kinase-dead
MAPK	Mitogen activated kinase
MCC	Mitotic checkpoint complex
PI	Propidium Iodide
PBD	Polo-box domain
Plk1	Polo-like kinase 1
WT	Wild-type

CHAPTER ONE

INTRODUCTION

Normal human somatic cells contain 46 chromosomes (22 pairs of autosomes and two sex chromosomes). Chromosome missegregation leads to abnormal numbers of chromosomes or aneuploidy. This form of genetic instability alters the dosages of large subsets of genes, which can result in severe disease phenotypes. Cancer has long been recognized as a disease associated with genetic instabilities. A prevalent form of genetic instability in human cancers is chromosome instability (CIN) [1, 2]. As compared to normal cells, cancer cells with CIN gain or lose their chromosomes at a higher rate and contain abnormal numbers of chromosomes (aneuploidy). The molecular basis of CIN is not yet fully understood. However, it has become increasingly clear that malfunction of a cell-cycle surveillance mechanism called the spindle checkpoint contributes to CIN and aneuploidy.

Overview of Chromosome Segregation and the Spindle Checkpoint

Chromosomes are duplicated once and only once during each cell division [3]. The duplicated chromosomes are physically tethered together by the cohesin protein complex and are packaged into sister chromatids during mitosis [3]. In mitosis of animal cells, microtubules emanating from the two spindle poles are dynamically growing and shrinking [4]. The microtubules that are captured by the kinetochores (protein complexes that are assembled at the centromeres) of sister chromatids become selectively stabilized and link the

chromosomes to that pole [4]. Once the two opposing kinetochores of a given pair of sister chromatids are captured by microtubules from the two opposite spindle poles, that pair of sister chromatids becomes bi-orientated and aligned at the equator of the mitotic spindle [3, 4]. The spindle pulling force exerted by kinetochore microtubules counteracts the cohesion of the sister chromatids so that the bi-oriented kinetochores are under tension. After all pairs of sister chromatids achieve bi-orientation and their kinetochores are under tension, an E3 ubiquitin ligase complex called the anaphase-promoting complex or cyclosome (APC/C) tags the securin protein with polyubiquitin chains and targets it for degradation by the proteasome (Fig. 1) [3, 5]. Securin is an inhibitory chaperone of a cysteine protease called separase, because securin facilitates the folding of separase and also inhibits its protease activity [5]. Degradation of securin leads to the activation of separase, which then cleaves the Scc1 subunit of cohesin [5]. This resolves the linkage between sister chromatids (Fig. 1). The separated sister chromatids are then pulled to opposite spindle poles through their attachment with spindle microtubule fibers. This elegant process ensures that the two daughter cells inherit identical sets of chromosomes [3, 5].

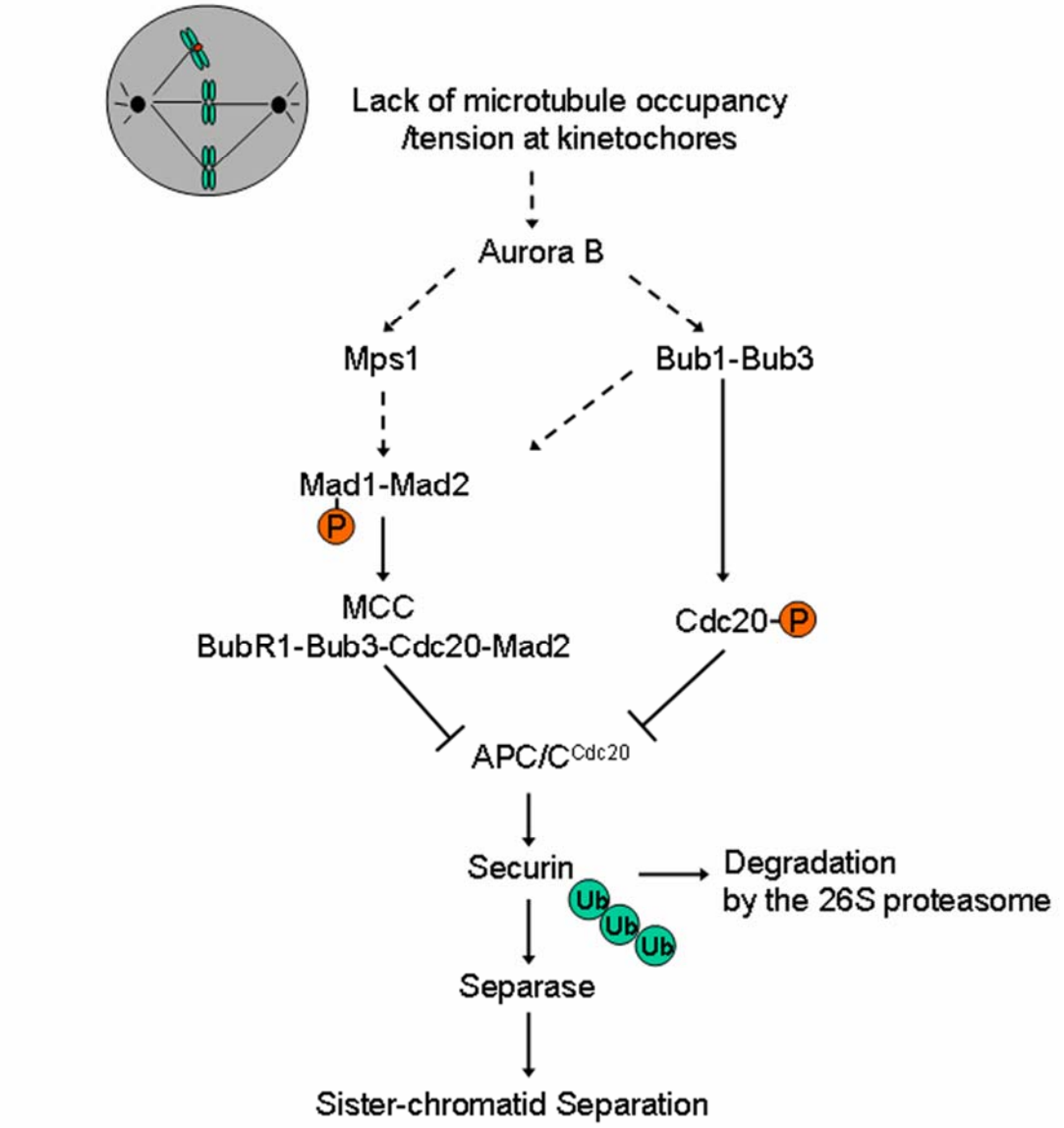


Figure 1. Molecular Pathways of the Spindle Checkpoint. At the metaphase–anaphase transition, APC/C^{Cdc20} ubiquitinates securin. Degradation of securin activates separase. Separase then cleaves the Scc1 subunit of cohesin, allowing chromosome segregation. In response to improper sister chromatid attachment to the mitotic spindle, the spindle checkpoint promotes the assembly of MCC that inhibits the activity of APC/C in a stoichiometric manner. The Bub1 spindle checkpoint kinase also becomes active, phosphorylates Cdc20, and inhibits the activity of APC/C catalytically. Inhibition of APC/C stabilizes securin, preserves sister chromatid cohesion, and delays the onset of anaphase until all sister chromatids have achieved bi-orientation on the mitotic spindle.

On the other hand, the stochastic "search-and-capture" mechanism of kinetochore-microtubule attachment implies that not all sister chromatids can achieve bi-orientation synchronously [4]. Thus, cells employ a surveillance mechanism called the spindle checkpoint to prevent premature sister chromatid separation prior to the bi-orientation of all pairs of sister chromatids. Because APC/C-mediated degradation of securin is the first irreversible step in initiating sister chromatid separation, it is not surprising that APC/C is the critical molecular target of the spindle checkpoint [6, 7]. Kinetochores that are not attached by microtubules and not under tension emit a diffusible signal that inhibits the cytoplasmic pool of APC/C, thereby preventing premature chromosome segregation. Thus, the spindle checkpoint is active in each and every cell cycle, and is important for cells to maintain the accuracy and fidelity of chromosome segregation [6-8].

The activity of APC/C is controlled by two related activators: Cdc20 and Cdh1. Both Cdc20 and Cdh1 contain a C-terminal WD40-repeat domain, and are involved in recruiting substrates to APC/C [5]. APC/C^{Cdc20} and APC/C^{Cdh1} perform distinct functions and are differentially regulated during the cell cycle [5]. APC/C^{Cdc20} is required for sister chromatid separation and the metaphase–anaphase transition by ubiquitinating securin whereas APC/C^{Cdh1} mediates the degradation of a broader spectrum of substrates in late anaphase and early G1 [5]. The spindle checkpoint selectively inhibits the activity of APC/C^{Cdc20}, stabilizes securin, delays the activation of separase, and prevents premature sister chromatid separation [6-8].

Molecular Components of the Spindle Checkpoint

The molecular players of the spindle checkpoint include Mitotic Arrest Deficiency 1 (Mad1), Mad2, Mad3/BubR1, Budding Uninhibited by Benzimidazole 1 (Bub1), Bub3, and Monopolar Spindle 1 (Mps1). The Bub and Mad genes were identified in two separate genetic screens in budding yeast [7, 9, 10]. Yeast cells that harbor mutations in these genes fail to arrest in mitosis upon transient exposure to microtubule destabilizing drugs, such as nocodazole and benzimidazole, and therefore lose viability quickly due to chromosome missegregation. Bub3 was found as a multi-copy suppressor of the *bub1-1* allele in a suppressor screen [9]. *MPS1* was originally identified as a gene required for spindle pole body duplication [11]. The *mps1* mutant cells contain monopolar spindle, but do not arrest the cell cycle at metaphase [11]. Thus, Mps1 is also required for the spindle checkpoint. Homologues of the yeast spindle checkpoint genes were later identified in higher organisms [6-8]. Disruption of these checkpoint genes in mammalian cells by antibody injection, antisense oligonucleotides, or RNA interference (RNAi) increases the frequency of chromosome missegregation during the normal cell cycle and causes a failure of these cells to undergo prolonged mitotic arrest in the presence of microtubule poisons, such as nocodazole and taxol [6-8].

In addition to these core components, several other proteins play important roles in the spindle checkpoint. For example, Aurora B is a protein kinase that is required for proper chromosome segregation and cytokinesis. The Aurora B–INCENP complex senses the lack of tension at kinetochores and destabilizes erroneous kinetochore–microtubule attachments [12, 13]. Recent evidence supports the notion that the Aurora B kinase complex senses lack

of tension and transforms the erroneous attachments into unattachment, which then potently activates canonical spindle checkpoint signaling [14]. Centromere-associated protein E (CENP-E) is a mitotic kinesin required for efficient, stable microtubule capture at kinetochores [15, 16]. CENP-E depletion by RNAi in mammalian cells causes chromosome congression defect and impairs the spindle checkpoint [17]. CENP-E directly binds to BubR1 and activates the kinase activity of BubR1 [18]. The exact function of the CENP-E–BubR1 interaction in the spindle checkpoint is unclear at present.

Kinetochore Localization of Spindle Checkpoint Proteins

Kinetochore is the origin of the "wait-anaphase signal" and its correct assembly is required for proper spindle checkpoint function [4]. All known spindle checkpoint proteins are dynamically associated with kinetochores in mitosis [4, 19]. The concentrations of many checkpoint proteins at unattached kinetochores are higher than those of attached ones. This correlates well with the on-and-off status of the spindle checkpoint. Moreover, the kinetochore localization of checkpoint proteins has been shown to be essential for the spindle checkpoint [4, 7]. However, few direct interactions between the spindle checkpoint proteins and core components of the kinetochore have been identified. In budding yeast, Skp1, an essential component for kinetochore assembly, interacts with Bub1 directly and is responsible for recruiting Bub1 to the kinetochore [20]. The Skp1-interacting domain of Bub1 was mapped to a 181-360 a.a. fragment [20]. A separation-of-function mutant allele of Skp1, *Skp1-AA*, was further identified by random mutagenesis. *Skp1-AA* retains intact

kinetochore function, but fails to interact with Bub1. The *Skp1-AA* cells show hypersensitivity to benomyl and defective kinetochore localization of Bub1. Using this allele, the kinetochore localization of Bub1 was shown to be required for checkpoint signaling caused by lack of tension, but not by lack of attachment induced by high dosage of microtubule destabilizing drugs [20]. Unfortunately, Skp1 proteins in other organisms have not been shown to be involved in kinetochore functions. It remains to be determined whether the Skp1-Bub1 interaction is conserved in organisms other than the budding yeast.

Another kinetochore protein Ndc80/Hec1 has been shown to interact with Mad1 in a yeast two-hybrid screen. However, direct interaction between Ndc80/Hec1 and Mad1 has not been demonstrated [21]. The kinetochore localization of Mad1 is diminished in Ndc80/Hec1-RNAi cells. Later studies by Bharadwaj *et al.* and DeLuca *et al.* showed that the kinetochore localization of Mad1 in Ndc80/Hec1-depleted cells is restored when microtubules are depolymerized by nocodazole treatment, suggesting that the effects of Ndc80/Hec1 in Mad1 kinetochore recruitment is indirect [22, 23].

On the other hand, studies in budding yeast and *Xenopus* egg extracts have revealed the hierarchy and temporal order of binding of checkpoint proteins to kinetochores in mitosis [24-28]. Aurora B lies most upstream in this process and is required for the kinetochore localization of all other checkpoint proteins. Bub1, Mps1, and CENP-E are recruited next and their kinetochore localizations are interdependent [25, 29]. Bub1 interacts with Bub3 throughout the cell cycle and their kinetochore localization is interdependent [26]. Mad1 and Mad2 also form a complex throughout the cell cycle [28]. Mad1 is required for the kinetochore association of Mad2 [28]. Mad2, Cdc20, and APC/C are the downstream

components[25]. They are recruited to kinetochores separately and their kinetochore localization is dependent on the upstream components [25]. Similar results were obtained in mammalian cells by using RNAi to knockdown certain spindle checkpoint protein and examining the kinetochore localization of others [30-33]. Aurora B is required for the kinetochore localization of Bub1 [32]. Bub1, BubR1, CENP-E, and Mad2 are then recruited to kinetochores sequentially [32, 33]. Together with genetic analysis in yeast, these results indicate that Aurora B, Bub1, and Mps1 act upstream in the spindle checkpoint pathway (Fig 1). These studies also suggest that kinetochore-dependent conformational changes or post-translational modifications of these upstream checkpoint proteins are required for the kinetochore recruitment and activation of downstream proteins.

Mechanism of the Spindle Checkpoint

Inhibition of APC/C by the Mitotic Checkpoint Complex (MCC)

Among all the spindle checkpoint proteins, the biochemical function of Mad2 was revealed first. Deletion of Mad2 in yeast abolishes the spindle checkpoint whereas overexpression of it results in mitotic arrest [10]. Later, it was demonstrated that Mad2 binds directly to Cdc20 in both budding and fission yeast [34, 35]. Fission yeast cells harboring a mutant Slp1 (the Cdc20 ortholog in fission yeast) that cannot bind to Mad2 escape from the mitotic arrest exerted by Mad2 overexpression [34]. These findings suggest that Cdc20 is a critical downstream target of Mad2. Subsequent biochemical studies in *Xenopus* egg extracts and mammalian cells showed that Cdc20 is an activator of the ubiquitin ligase activity of APC/C

[36]. Mad2, Cdc20, and APC/C form a ternary complex upon checkpoint activation and the ubiquitin ligase activity of APC/C is inhibited in this complex [37]. Moreover, purified recombinant Mad2 protein exists in both monomeric and dimeric forms. Mad2 dimer, but not Mad2 monomer, inhibits APC/C^{Cdc20} in *Xenopus* egg extracts, suggesting that either dimerization and/or a conformational change of Mad2 accompanied with dimerization are required for APC/C inhibition [37].

The structures of apo-Mad2 and Mad2 in complex with a peptide that mimics the Mad2-binding motifs of Mad1 and Cdc20 were then determined by nuclear magnetic resonance (NMR) spectroscopy [31, 38]. These studies revealed a dramatic conformational change between apo-Mad2 and Mad2 bound to Mad1 or Cdc20. The crystal structure of Mad2 bound to a 120-residue fragment of Mad1 was then determined and confirmed that Mad1 and Cdc20 trigger similar conformational changes of Mad2 [39]. Recently, Luo *et al*. have shown that the monomeric and dimeric forms of Mad2 interconvert slowly *in vitro* in the absence of ligands [40]. This interconvertion is accelerated by a sub-stoichiometric amount of Mad2-binding peptide of Mad1. The dimeric form of Mad2 has a conformation similar to the Cdc20-bound form of Mad2 and is thus more active in inhibiting APC/C^{Cdc20} [40]. These and other studies led to a "two-state Mad2" model. In this model, Mad2 exists in two conformations, one of which is more active in inhibiting APC/C^{Cdc20}. Upon checkpoint activation, Mad1 facilitates the formation of the "activated" conformation of Mad2, leading to inhibition of APC/C. Very recently, a related but distinct model called the "Mad1–Mad2 template" model has been proposed [41]. In this model, the Mad1–Mad2 heterodimer recruits another Mad2 molecule through a Mad2–Mad2 interaction. The loosely bound Mad2

molecule is passed on to Cdc20, resulting in APC/C inhibition. Obviously, more studies are needed to test both models.

BubR1, the vertebrate homolog of yeast Mad3, was next shown to be another direct inhibitor of APC/C [42]. Similar to Mad3, BubR1 associates with Bub3 constitutively via its conserved GLEBS (GLE2p Binding Sequence) motif (Fig. 2A) [30]. Unlike Mad3 that does not have any catalytic domain, BubR1 has a C-terminal kinase domain (Fig. 2A). Purified recombinant BubR1 protein inhibited the activity of APC/C immunoprecipitated from *Xenopus* egg extracts or mammalian cells in the absence of Mad2 [42]. Surprisingly, direct binding between BubR1 and Cdc20, but not the kinase activity of BubR1, is responsible for the APC/C-inhibitory effect of BubR1 [42]. Although both Mad2 and BubR1 can inhibit Cdc20 independently, there is synergism between them in inhibiting APC/C *in vitro* [43]. Moreover, BubR1/Mad3, Mad2, Bub3, and Cdc20 form a single complex called the mitotic checkpoint complex (MCC) in mitosis [27, 44, 45]. Thus, the MCC is a key stoichiometric checkpoint inhibitor of APC/C *in vivo* (Fig. 1). On the other hand, the fact that the BubR1–Bub3–Cdc20 and Mad2–Cdc20 sub-complexes are sufficient to inhibit APC/C *in vitro* suggests that these sub-complexes might also be involved in APC/C inhibition in response to different spindle defects *in vivo*.

The multiple functions of Bub1 in the spindle checkpoint

Early studies in *Saccharomyces cerevisiae* have firmly established the requirement of Bub1 for proper spindle checkpoint function [7, 9]. The Bub1 gene was first cloned in budding

yeast by complementation cloning. Later, the orthologs of Bub1 in other species were found by sequence homology. Bub1 contains three major domains: an N-terminal tetratrico peptide repeat (TPR) domain that mediates its kinetochore localization, a GLEBS motif that binds to Bub3, and a C-terminal serine/threonine kinase domain (Fig. 2A). Roberts *et al.* showed by i*n vitro* kinase assays that the budding yeast Bub1 possesses kinase activity toward itself (autophosphorylation) [46]. Although Bub3 interacts with Bub1, Bub3 is not required for the kinase activity of Bub1 since Bub1 protein from *Bub3Δ* cells still undergoes autophosphorylation [46]. A dominant allele of Bub1, *Bub1-5*, that harbors a point mutation in its kinase domain was isolated based on its ability to cause a mitotic delay after overexpression [47]. The *Bub1-5* allele complements *bub1Δ* for growth after benomyl exposure. More importantly, a point mutation that abolishes the kinase activity of *Bub1-5* completely eliminates the mitotic delay phenotype, indicating that *Bub1-5* may exhibit higher kinase activity [47]. This finding supports the notion that the kinase activity of Bub1 is important for the spindle checkpoint.

It has been shown that Mad1 becomes hyperphosphorylated and up-shifted in SDS-PAGE in response to spindle checkpoint activation in budding yeast [48]. Furthermore, the phosphorylation of Mad1 is lost in *bub1Δ* cells [48]. Brandy *et al.* performed biochemical studies in yeast to analyze Mad1 associated proteins and found that Bub1-Bub3 forms a complex with Mad1 in mitosis [49]. The formation of this complex is greatly enhanced upon checkpoint activation [49]. In addition, recombinant human Bub1 phosphorylates Mad1 *in vitro*, although the *in vivo* relevance and function of this activity have not been established [50]. As discussed above, Bub1 is also required for the kinetochore localization of BubR1,

Mad1, and Mad2 in vertebrates. These results strongly suggest that Bub1 acts upstream of MCC (Fig. 1).

A recent study by Tang *et al.* modified the classical view of Bub1 and revealed a downstream function of Bub1 [51]. Tang *et al.* showed that Bub1 inhibits the APC/C^{Cdc20} activity in an ATP-dependent fashion by directly phosphorylating Cdc20 *in vitro*. Furthermore, Cdc20 is phosphorylated at six serine/threonine residues *in vivo*. Recombinant Bub1 phosphorylates Cdc20 at the same six sites *in vitro*. A Cdc20 mutant that lacks these phosphorylation sites (referred to as $Cdc20^{BPM}$) is refractory to phosphorylation and inhibition by Bub1 *in vitro*. When expressed in cells, $Cdc20^{BPM}$ also abrogates the ability of these cells to undergo mitotic arrest in the presence of spindle poisons [51]. Moreover, $Cdc20^{BPM}$ is still capable of interacting with and being inhibited by BubR1 or Mad2, although it remains possible that Bub1-mediated phosphorylation of Cdc20 might facilitate the formation of MCC *in vivo* [51]. These results indicate that phosphorylation of Cdc20 by Bub1 is required for efficient checkpoint signaling (Fig. 1).

Consistent with an involvement of the Bub1 kinase activity in the spindle checkpoint, Bub1 itself is rapidly phosphorylated upon spindle damage caused by brief treatments with nocodazole or taxol [52]. The kinase activity of Bub1 toward Cdc20 is enhanced in mitosis [51]. Furthermore, in *Xenopus* egg extracts Bub1 becomes hyperphosphorylated when bound to chromatin, and the autophosphorylation of Bub1 immunoprecipitated from nocodazole-treated chromosomes is enhanced [53]. The mechanism by which Bub1 is activated upon checkpoint activation is unknown. Chen *et al.* showed that phosphorylation of Bub1 on *Xenopus* mitotic chromosomes can be partially inhibited by MAPK inhibitor treatment. *In*

vitro kinase assays showed that MAPK phosphorylates Bub1 [53]. The mutant Bub1 that contains mutations on the 5 conserved S/T-P sites (MAPK consensus sites) has less autophosphorylation and cannot restore the checkpoint in Bub1-depleted extract [53]. However, the enhancement of Bub1 activity toward Cdc20 upon MAPK phosphorylation has not been demonstrated directly. Additionally, Bub1 phosphorylation by MAPK has not been shown in other systems. It remains possible that Cdk1 or other S/T-P kinases phosphorylate Bub1 on those residues. Along this vein, Bub1 has also been shown to be phosphorylated by Cdk1 in fission yeast [54]. However, it is unclear whether Cdk1-mediated phosphorylation enhances the kinase activity of Bub1.

In summary, the spindle checkpoint uses multiple mechanisms to inhibit APC/C. MCC (BubR1–Bub3–Cdc20–Mad2) or its sub-complexes inhibit APC/C in a stoichiometric manner whereas Bub1 inhibits APC/C catalytically. The dynamics of the kinetochore localization of the spindle checkpoint proteins in live cells has been measured using fluorescence recovery after photobleaching (FRAP) [55, 56]. On the unattached kinetochores, GFP/YFP fused Bub1 and Mad1 are mostly stably associated, while Mad2, Mps1, Bub3 and BubR1 undergo dynamic exchange [55, 56]. Consistent with the idea that multiple mechanisms regulate Cdc20, GFP-Cdc20 displays biphasic dynamics at unattached kinetochores: a slow phase that is likely to reflect the formation and release of MCC and a fast phase that might be due to the release of free Cdc20 [55]. It is conceivable that the more dynamic population of Cdc20 is phosphorylated and inhibited by Bub1 and released before it can be incorporated into MCC. This catalytic mechanism for APC/C^{Cdc20} inhibition may help

to explain how only one unattached kinetochore can generate enough "wait-anaphase signal" to inhibit the entire pool of APC/C^{Cdc20} in the cell.

Physiological Functions of Spindle Checkpoint Proteins

In yeast, cells with spindle checkpoint genes deleted are still viable, indicating that the spindle checkpoint is not required for normal mitosis. Intriguingly, the Bub1- and Bub3-null cells are very sick and have higher rate of chromosome loss, while the Mad1-, Mad2- and Mad3- deleted cells grow normally [57, 58]. Therefore, Bub1 may have additional roles in regulating chromosome segregation. Very recently, Bub1 has been shown to protect centromeric cohesion in mitosis by targeting Sgo1 to kinetochores [59, 60].

Studies in *Drosophila* also showed that the spindle checkpoint is not required for normal mitosis [61]. Mad2-null homozygous flies are viable and do not show dramatic aneuploidy, although these mutants do not arrest their cell cycle upon spindle damage caused by colchicine treatment [61]. However, flies homozygous for P element–induced, near-null mutations of *BubR1* die during late larval/pupal stages due to chromosome missegregation and apoptosis [62, 63]. It has been suggested that Mad2 is uniquely required for the spindle checkpoint while the other checkpoint proteins may acquire other functions, such as chromosome segregation or developmental roles [61].

In contrast to yeast and fly, the spindle checkpoint genes are essential in mammals. *MAD2*-null mice are embryonic lethal [64]. The *MAD2*-null mouse embryonic fibroblasts (MEF) are unable to arrest in response to spindle disruption [64]. Widespread chromosome

missegregation and apoptosis occur in *MAD2*-null embryos at E6.5 when the cells of the epiblast begin rapid cell divisions [64]. *BUBR1*-deficient mice also die before E8.5 due to massive chromosome missegregation and apoptosis [65]. Thus, the spindle checkpoint is required for accurate chromosome segregation in mice and human cells in the absence of spindle damage. Why has the spindle checkpoint become essential in higher organisms? One possibility is the larger number of chromosomes in mammals. Drosophila has only 4 pairs of chromosomes while human has 23 pairs of chromosomes. The increased number of chromosomes elevates the chance of erratic attachment between microtubules and the kinetochores. It has also been proposed that the spindle assembly is very efficient in fly so that the spindle checkpoint is normally not needed [61]. Nonetheless, the spindle checkpoint functions in every cell cycle in mammals to allow the cells more time to achieve bi-orientation on all sister kinetochores.

Defective Spindle Checkpoint and Aneuploidy

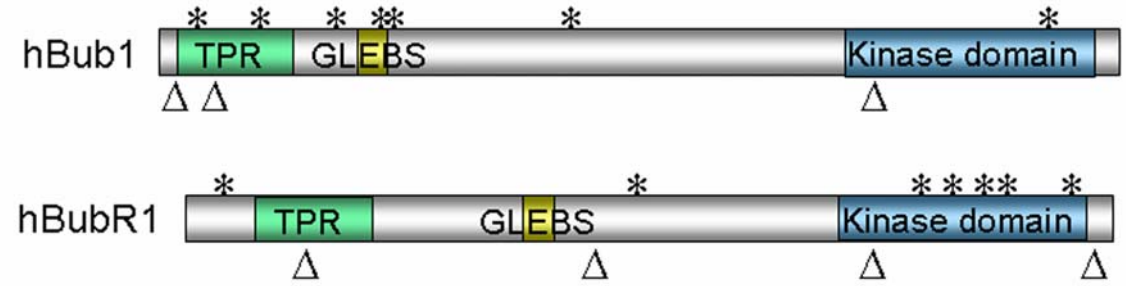
Mutations of Spindle Checkpoint Genes in Cancer

A growing body of evidence indicates that defects in spindle checkpoint signaling might contribute to tumorigenesis. It is well accepted that cancer formation requires multiple genetic alterations [2]. Cells harboring mutations that allow them to gain growth advantage will expand in the population. The number of mutations that are necessary for clonal expansion and cancer formation is estimated by mathematical extrapolation to be between 6 and 10 [2]. However, the DNA mutation rate in normal cells is too low for this number of

mutations to occur in the lifetime of a given cell. If the spindle checkpoint is compromised, cells may gain or lose chromosomes in each and every division. This may lead to the loss-of-heterozygosity (LOH) of tumor suppressors and/or the acquisition of extra copies of proto-oncogenes, thus accelerating the transformation process. Thus, the spindle checkpoint is proposed to be a barrier that cells need to overcome to become cancerous [66].

Indeed, mutations in *BUB1* might be involved in the pathogenesis of human cancers, especially colorectal cancer. Mutations in one of the two copies of *BUB1* were found in CIN-type colon cancer cells [67](Fig. 2). Cahill ***et al.*** described two *BUB1* mutations: one encodes a truncated Bub1 protein containing only the N-terminal 75 residues while the other introduces a single point mutation at residue 492 that changes a serine to tyrosine [67]. Interestingly, expression of the mutated Bub1 protein containing only residues 1-75 disrupted the spindle checkpoint in euploid colon cancer cells and caused aneuploidy, presumably through blocking the functions of the wild-type Bub1 in a dominant-negative fashion [67]. Though the exact mechanism of this dominant-negative effect of Bub1 is unknown, it is conceivable that the activation of Bub1 involves autophosphorylation through a dimerization event at the kinetochores, akin to the activation of receptor tyrosine kinases [68]. The N-terminal 75-residue fragment of Bub1 may bind to the wild-type Bub1 and prevents its activation and/or kinetochore localization.

A



B

Protein	Mutation	Source tissues or cell lines	Reference
Bub1	Δ10-32 a.a.	Lymphoid leukemia cell line Molt3	[77]
	H51D	Lung adenocarcinoma	[76]
	Δ76-1085 a.a.	Colorectal cancer cell line V400	[67]
	A130S	Colorectal cancer	[84]
	R209Q	Lung cancer cell line NCI-H345	[79]
	Y259C, H265N	Pancreatic cancer cell line Hs766T	[78]
	S492Y	Colorectal cancer cell line V429	[67]
	Δ827-1085 a.a.	Thyroid follicular lymphoma	[75]
	S950G	Rectal cancer	[80]
BubR1	T40M	Colorectal cancer cell line V531	[67]
	ΔT1023	Colorectal cancer cell line V1394	[67]
	Δ194-1050 a.a.	MVA	[89]
	Δ483-1050 a.a.	MVA	[89]
	R550Q	MVA	[89]
	Δ738-1050 a.a.	MVA	[89]
	R814H	MVA	[89]
	L844F, Q921H	MVA	[89]
	I909T	MVA	[89]
	L1012P	MVA	[89]

Figure 2. Domain Structure and Mutations of Human Bub1 and BubR1. (A) Bub1 and BubR1 contain an N-terminal tetratrico peptide repeat (TPR) motif, a GLEBS motif for association with Bub3, and a C-terminal kinase domain. Missense and nonsense mutations

found in human cancers are indicated by asterisks and triangles, respectively. (B) List of Bub1 and BubR1 mutations found in human tumors and MVA patients.

Subsequently, mutations of *BUB1* were extensively studied in different cancer samples and cell lines with aneuploidy. However, mutations of *BUB1* were found to be rare in glioblastomas [69], bladder tumors, soft-tissue sarcomas, hepatocellular carcinomas [70], head and neck squamous cell carcinomas [71], gastric carcinomas [72], and breast carcinomas [73]. On the other hand, the Bub1 genomic locus on 2q14 was shown to be relatively unstable in 14.5% of colorectal cancer samples [74]. The Bub1 mutations identified so far in tumor samples and cell lines are mainly located in its the N-terminal kinetochore localization domain [75-78](Fig. 2). However, it has not been clearly demonstrated whether these mutations affect the kinetochore localization of Bub1. Mutations in *BUBR1*/*BUB1B* and *MAD2* were also examined in cancer cell lines and tumor samples. Similar to *BUB1*, *BUBR1* and *MAD2* are not frequently mutated in multiple cancers [70, 79, 80].

Involvement of the spindle checkpoint genes in cancer formation was also confirmed in mouse models. As mentioned above, *MAD2*-null mice are not viable. However, $MAD2^{+/-}$ heterozygous mice are viable and develop lung cancers after a long latency period [81]. Similarly, *BUBR1*-null mutations also cause lethality in mice. The $BUBR1^{+/-}$ mice also develop aneuploidy and form lung and intestinal adenocarcinomas when they are challenged with carcinogens [82]. Finally, *BUB1* mutations foster growth and cellular transformation of cells derived from *BRCA2*-deficient mice. Tumors from *BRCA2*-deficient mice showed spindle checkpoint dysfunction and contained mutations in *BUB1* and *BUBR1* [83]. These

results suggest that defects in the spindle checkpoint are involved in the initiation and/or progression of cancers.

Epigenetic changes

Epigenetic changes instead of genomic mutations might also lead to a defective spindle checkpoint in cancer cells and contribute to tumor progression. Alterations of expression of the spindle checkpoint genes were examined in tumor samples and cancer cell lines. A reduction in the Bub1 protein level was observed in surgically dissected colorectal carcinomas, which positively correlated with metastasis and shorter relapse-free survival [84]. The expression levels of Bub1 and Mad2 were also changed in certain breast cancer cell lines [85, 86]. Consistently, *MAD2* is a direct transcriptional target of the BRCA1 tumor suppressor. BRCA1 binds to the promoter of *MAD2* and up-regulates its transcription. Overexpression of *MAD2* in *BRCA1*-deficient cells partially rescues the spindle checkpoint defects [87]. Intriguingly, *MAD2* is also a transcriptional target of E2F. The function of E2F is often up-regulated in cancers either through its own overexpression or through the loss of its inhibitor, pRb. Consequently, *MAD2* is constitutively expressed and deregulated in *RB*-deficient cells and these cells exhibited accelerated mitosis and chromosome instability [88]. Therefore, either up- or down-regulation of Mad2 levels can lead to chromosome instability and aneuploidy. It is thus crucial to maintain the correct steady state levels of Mad2 and other checkpoint proteins in cells.

Germline Mutation of BUBR1 and Cancer Predisposition

Although the spindle checkpoint genes are mutated in human cancers, it is unclear whether these somatic cell mutations are causal events that lead to cancer or simply secondary consequences of cancer progression. Very recently, studies on a rare human disease called mosaic variegated aneuploidy syndrome (MVA) provided the first direct evidence for a causal role of a defective spindle checkpoint in human cancers [89]. MVA is an autosomal recessive condition and >25% cells in multiple tissues of these patients are aneuploid. Some MVA patients have intrauterine growth retardation and microcephaly. Importantly, many MVA patients develop cancer at a young age. Hanks *et al.* sequenced the *BUBR1* gene in eight MVA pedigrees and found biallelic *BUBR1* mutations in five families. In each family, there is a missense mutation in one allele and a mutation that results in a truncated protein or no transcripts in the other. The five missense mutations all affect conserved residues in the kinase domain of BubR1 [89]. Because a complete loss of BubR1 function is expected to cause lethality, these missense mutations are not likely to severely disrupt the biochemical function of the remaining copy of *BUBR1*. Two of the five patients developed embryonic rhabdomyosarcomas in different tissues at the age of 7 years and 5 months, respectively [89]. These results provide the strongest evidence so far that a defective spindle checkpoint results in cancer predisposition in humans.

Conclusion

The spindle checkpoint is a cell-cycle surveillance mechanism that ensures the fidelity of chromosome segregation in mitosis and meiosis. In response to misaligned sister chromatids, the checkpoint uses multiple pathways to block the activity of APC/C, thus delaying the onset of sister chromatid separation. Malfunction of the spindle checkpoint leads to aneuploidy and contributes to cancer formation. A better understanding of the spindle checkpoint at the molecular level will be valuable for identifying new drug targets for treating human cancers.

References

1. Lengauer, C., K.W. Kinzler, and B. Vogelstein, *Genetic instabilities in human cancers.* Nature, 1998. **396**(6712): p. 643-9.

2. Rajagopalan, H. and C. Lengauer, *Aneuploidy and cancer.* Nature, 2004. **432**(7015): p. 338-41.

3. Nasmyth, K., *Segregating sister genomes: the molecular biology of chromosome separation.* Science, 2002. **297**(5581): p. 559-65.

4. Cleveland, D.W., Y. Mao, and K.F. Sullivan, *Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling.* Cell, 2003. **112**(4): p. 407-21.

5. Peters, J.M., *The anaphase-promoting complex: proteolysis in mitosis and beyond.* Mol Cell, 2002. **9**(5): p. 931-43.

6. Yu, H., *Regulation of APC-Cdc20 by the spindle checkpoint.* Curr Opin Cell Biol, 2002. **14**(6): p. 706-14.

7. Bharadwaj, R. and H. Yu, *The spindle checkpoint, aneuploidy, and cancer.* Oncogene, 2004. **23**(11): p. 2016-27.

8. Musacchio, A. and K.G. Hardwick, *The spindle checkpoint: structural insights into dynamic signalling.* Nat Rev Mol Cell Biol, 2002. **3**: p. 731-741.

9. Hoyt, M.A., L. Totis, and B.T. Roberts, *S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function.* Cell, 1991. **66**(3): p. 507-17.

10. Li, R. and A.W. Murray, *Feedback control of mitosis in budding yeast.* Cell, 1991. **66**(3): p. 519-31.

11. Winey, M., et al., *MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication.* J Cell Biol, 1991. **114**(4): p. 745-54.

12. Tanaka, T.U., *Bi-orienting chromosomes on the mitotic spindle.* Curr Opin Cell Biol, 2002. **14**(3): p. 365-71.

13. Tanaka, T.U., et al., *Evidence that the Ipl1-Sli15 (Aurora kinase-INCENP) complex promote chromosome bi-orientation by altering kinetochore-spindle pole connections.* Cell, 2002. **108**: p. 317-329.

14. Pinsky, B.A., et al., *The Ipl1-Aurora protein kinase activates the spindle checkpoint by creating unattached kinetochores.* Nat Cell Biol, 2006. **8**(1): p. 78-83.

15. Yao, X., et al., *CENP-E forms a link between attachment of spindle microtubules to kinetochores and the mitotic checkpoint.* Nat Cell Biol, 2000. **2**(8): p. 484-91.

16. Abrieu, A., et al., *CENP-E as an essential component of the mitotic checkpoint in vitro.* Cell, 2000. **102**: p. 817-826.

17. Tanudji, M., et al., *Gene silencing of CENP-E by small interfering RNA in HeLa cells leads to missegregation of chromosomes after a mitotic delay.* Mol Biol Cell, 2004. **15**(8): p. 3771-81.

18. Mao, Y., A. Abrieu, and D.W. Cleveland, *Activating and silencing the mitotic checkpoint through CENP-E-dependent activation/inactivation of BubR1.* Cell, 2003. **114**(1): p. 87-98.

19. Taylor, S.S., M.I. Scott, and A.J. Holland, *The spindle checkpoint: a quality control mechanism which ensures accurate chromosome segregation.* Chromosome Res, 2004. **12**(6): p. 599-616.

20. Kitagawa, K., et al., *Requirement of Skp1-Bub1 interaction for kinetochore-mediated activation of the spindle checkpoint.* Mol Cell, 2003. **11**(5): p. 1201-13.

21. Martin-Lluesma, S., V.M. Stucke, and E.A. Nigg, *Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2.* Science, 2002. **297**(5590): p. 2267-70.

22. Bharadwaj, R., W. Qi, and H. Yu, *Identification of two novel components of the human NDC80 kinetochore complex.* J Biol Chem, 2004. **279**(13): p. 13076-85.

23. DeLuca, J.G., et al., *Nuf2 and Hec1 are required for retention of the checkpoint proteins Mad1 and Mad2 to kinetochores.* Curr Biol, 2003. **13**(23): p. 2103-9.

24. Gillett, E.S., C.W. Espelin, and P.K. Sorger, *Spindle checkpoint proteins and chromosome-microtubule attachment in budding yeast.* J Cell Biol, 2004. **164**(4): p. 535-46.

25. Vigneron, S., et al., *Kinetochore localization of spindle checkpoint proteins: who controls whom?* Mol Biol Cell, 2004. **15**(10): p. 4584-96.

26. Sharp-Baker, H. and R.H. Chen, *Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity.* J Cell Biol, 2001. **153**(6): p. 1239-50.

27. Chen, R.H., *BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1.* J Cell Biol, 2002. **158**(3): p. 487-96.

28. Chen, R.H., et al., *Spindle checkpoint protein Xmad1 recruits Xmad2 to unattached kinetochores.* J Cell Biol, 1998. **143**: p. 283-295.

29. Abrieu, A., et al., *Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint.* Cell, 2001. **106**(1): p. 83-93.

30. Taylor, S.S., E. Ha, and F. McKeon, *The human homologue of Bub3 is required for kinetochore localization of Bub1 and a Mad3/Bub1-related protein kinase.* J Cell Biol, 1998. **142**(1): p. 1-11.

31. Luo, X., et al., *The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20.* Mol Cell, 2002. **9**(1): p. 59-71.

32. Johnson, V.L., et al., *Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression.* J Cell Sci, 2004. **117**(Pt 8): p. 1577-89.

33. Meraldi, P., V.M. Draviam, and P.K. Sorger, *Timing and checkpoints in the regulation of mitotic progression.* Dev Cell, 2004. **7**(1): p. 45-60.

34. Kim, S.H., et al., *Fission yeast Slp1: an effector of the Mad2-dependent spindle checkpoint.* Science, 1998. **279**(5353): p. 1045-7.

35. Hwang, L.H., et al., *Budding yeast Cdc20: a target of the spindle checkpoint.* Science, 1998. **279**(5353): p. 1041-4.

36. Fang, G., H. Yu, and M.W. Kirschner, *Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1.* Mol Cell, 1998. **2**(2): p. 163-71.

37. Fang, G., H. Yu, and M.W. Kirschner, *The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation.* Genes Dev, 1998. **12**(12): p. 1871-83.

38. Luo, X., et al., *Structure of the Mad2 spindle assembly checkpoint protein and its interaction with Cdc20.* Nat Struct Biol, 2000. **7**(3): p. 224-9.

39. Sironi, L., et al., *Crystal structure of the tetrameric Mad1-Mad2 core complex: implications of a 'safety belt' binding mechanism for the spindle checkpoint.* Embo J, 2002. **21**(10): p. 2496-506.

40. Luo, X., et al., *The Mad2 spindle checkpoint protein has two distinct natively folded states.* Nat Struct Mol Biol, 2004. **11**(4): p. 338-45.

41. De Antoni, A., et al., *The Mad1/Mad2 complex as a template for Mad2 activation in the spindle assembly checkpoint.* Curr Biol, 2005. **15**(3): p. 214-25.

42. Tang, Z., et al., *Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1.* Dev Cell, 2001. **1**(2): p. 227-37.

43. Fang, G., *Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex.* Mol Biol Cell, 2002. **13**(3): p. 755-66.

44. Hardwick, K.G., et al., *MAD3 encodes a novel component of the spindle checkpoint which interacts with Bub3p, Cdc20p, and Mad2p.* J Cell Biol, 2000. **148**(5): p. 871-82.

45. Sudakin, V., G.K. Chan, and T.J. Yen, *Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2.* J Cell Biol, 2001. **154**(5): p. 925-36.

46. Roberts, B.T., K.A. Farr, and M.A. Hoyt, *The Saccharomyces cerevisiae checkpoint gene BUB1 encodes a novel protein kinase.* Mol Cell Biol, 1994. **14**(12): p. 8282-91.

47. Farr, K.A. and M.A. Hoyt, *Bub1p kinase activates the Saccharomyces cerevisiae spindle assembly checkpoint.* Mol Cell Biol, 1998. **18**(5): p. 2738-47.

48. Hardwick, K.G. and A.W. Murray, *Mad1p, a phosphoprotein component of the spindle assembly checkpoint in budding yeast.* J Cell Biol, 1995. **131**(3): p. 709-20.

49. Brady, D.M. and K.G. Hardwick, *Complex formation between Mad1p, Bub1p and Bub3p is crucial for spindle checkpoint function.* Curr Biol, 2000. **10**(11): p. 675-8.

50. Seeley, T.W., L. Wang, and J.Y. Zhen, *Phosphorylation of human MAD1 by the BUB1 kinase in vitro.* Biochem Biophys Res Commun, 1999. **257**(2): p. 589-95.

51. Tang, Z., et al., *Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint.* Mol Cell, 2004. **16**(3): p. 387-97.

52. Taylor, S.S., et al., *Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells.* J Cell Sci, 2001. **114**(Pt 24): p. 4385-95.

53. Chen, R.H., *Phosphorylation and activation of Bub1 on unattached chromosomes facilitate the spindle checkpoint.* Embo J, 2004. **23**(15): p. 3113-21.

54. Yamaguchi, S., A. Decottignies, and P. Nurse, *Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint.* Embo J, 2003. **22**(5): p. 1075-87.

55. Howell, B.J., et al., *Spindle checkpoint protein dynamics at kinetochores in living cells.* Curr Biol, 2004. **14**(11): p. 953-64.

56. Shah, J.V., et al., *Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing.* Curr Biol, 2004. **14**(11): p. 942-52.

57. Vanoosthuyse, V., et al., *Kinetochore targeting of fission yeast Mad and Bub proteins is essential for spindle checkpoint function but not for all chromosome segregation roles of Bub1p.* Mol Cell Biol, 2004. **24**(22): p. 9786-801.

58. Warren, C.D., et al., *Distinct chromosome segregation roles for spindle checkpoint proteins.* Mol Biol Cell, 2002. **13**(9): p. 3029-41.

59. Tang, Z., et al., *Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis.* Proc Natl Acad Sci U S A, 2004. **101**(52): p. 18012-7.

60. Yu, H. and Z. Tang, *Bub1 multitasking in mitosis.* Cell Cycle, 2005. **4**(2): p. 262-5.

61. Richardson, H.E., *Vinegar flies turn to porto for cell division cycle meeting.* Dev Cell, 2006. **11**(2): p. 141-6.

62. Basu, J., et al., *Mutations in the essential spindle checkpoint gene bub1 cause chromosome missegregation and fail to block apoptosis in Drosophila.* J Cell Biol, 1999. **146**(1): p. 13-28.

63. Logarinho, E., et al., *Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in Drosophila cells.* J Cell Sci, 2004. **117**(Pt 9): p. 1757-71.

64. Dobles, M., et al., *Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2.* Cell, 2000. **101**(6): p. 635-45.

65. Wang, Q., et al., *BUBR1 deficiency results in abnormal megakaryopoiesis.* Blood, 2004. **103**(4): p. 1278-85.

66. Lopes, C.S. and C.E. Sunkel, *The spindle checkpoint: from normal cell division to tumorigenesis.* Arch Med Res, 2003. **34**(3): p. 155-65.

67. Cahill, D.P., et al., *Mutations of mitotic checkpoint genes in human cancers.* Nature, 1998. **392**(6673): p. 300-3.

68. Kim, D.W., B. Lu, and D.E. Hallahan, *Receptor tyrosine kinase inhibitors as anti-angiogenic agents.* Curr Opin Investig Drugs, 2004. **5**(6): p. 597-604.

69. Reis, R.M., et al., *Mutation analysis of hBUB1, hBUBR1 and hBUB3 genes in glioblastomas.* Acta Neuropathol (Berl), 2001. **101**(4): p. 297-304.

70. Hernando, E., et al., *Molecular analyses of the mitotic checkpoint components hsMAD2, hBUB1 and hBUB3 in human cancer.* Int J Cancer, 2001. **95**(4): p. 223-7.

71. Yamaguchi, K., et al., *Mutation analysis of hBUB1 in aneuploid HNSCC and lung cancer cell lines.* Cancer Lett, 1999. **139**(2): p. 183-7.

72. Shigeishi, H., et al., *No mutations of the Bub1 gene in human gastric carcinomas.* Oncol Rep, 2001. **8**(4): p. 791-4.

73. Langerod, A., et al., *BUB1 infrequently mutated in human breast carcinomas.* Hum Mutat, 2003. **22**(5): p. 420.

74. Jaffrey, R.G., et al., *Genomic instability at the BUB1 locus in colorectal cancer, but not in non-small cell lung cancer.* Cancer Res, 2000. **60**(16): p. 4349-52.

75. Ouyang, B., et al., *Mechanisms of aneuploidy in thyroid cancer cell lines and tissues: evidence for mitotic checkpoint dysfunction without mutations in BUB1 and BUBR1.* Clin Endocrinol (Oxf), 2002. **56**(3): p. 341-50.

76. Gemma, A., et al., *Somatic mutation of the hBUB1 mitotic checkpoint gene in primary lung cancer.* Genes Chromosomes Cancer, 2000. **29**(3): p. 213-8.

77. Ru, H.Y., et al., *hBUB1 defects in leukemia and lymphoma cells.* Oncogene, 2002. **21**(30): p. 4673-9.

78. Hempen, P.M., et al., *A double missense variation of the BUB1 gene and a defective mitotic spindle checkpoint in the pancreatic cancer cell line Hs766T.* Hum Mutat, 2003. **21**(4): p. 445.

79. Sato, M., et al., *Infrequent mutation of the hBUB1 and hBUBR1 genes in human lung cancer.* Jpn J Cancer Res, 2000. **91**(5): p. 504-9.

80. Imai, Y., et al., *Mutational inactivation of mitotic checkpoint genes, hsMAD2 and hBUB1, is rare in sporadic digestive tract cancers.* Jpn J Cancer Res, 1999. **90**(8): p. 837-40.

81. Michel, L.S., et al., *MAD2 haplo-insufficiency causes premature anaphase and chromosome instability in mammalian cells.* Nature, 2001. **409**(6818): p. 355-9.

82. Dai, W., et al., *Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency.* Cancer Res, 2004. **64**(2): p. 440-5.

83. Lee, H., et al., *Mitotic checkpoint inactivation fosters transformation in cells lacking the breast cancer susceptibility gene, Brca2.* Mol Cell, 1999. **4**(1): p. 1-10.

84. Shichiri, M., et al., *Genetic and epigenetic inactivation of mitotic checkpoint genes hBUB1 and hBUBR1 and their relationship to survival.* Cancer Res, 2002. **62**(1): p. 13-7.

85. Doak, S.H., et al., *Differential expression of the MAD2, BUB1 and HSP27 genes in Barrett's oesophagus-their association with aneuploidy and neoplastic progression.* Mutat Res, 2004. **547**(1-2): p. 133-44.

86. Myrie, K.A., et al., *Mutation and expression analysis of human BUB1 and BUB1B in aneuploid breast cancer cell lines.* Cancer Lett, 2000. **152**(2): p. 193-9.

87. Wang, R.H., H. Yu, and C.X. Deng, *A requirement for breast-cancer-associated gene 1 (BRCA1) in the spindle checkpoint.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17108-13.

88. Hernando, E., et al., *Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control.* Nature, 2004. **430**(7001): p. 797-802.

89. Hanks, S., et al., *Constitutional aneuploidy and cancer predisposition caused by biallelic mutations in BUB1B.* Nat Genet, 2004. **36**(11): p. 1159-61.

CHAPTER TWO

KEN-BOX-DEPENDENT DEGRADATION OF THE BUB1 SPINDLE CHECKPOINT KINASE BY ANAPHASE-PROMOTING COMPLEX/CYCLOSOME

Summary

The spindle checkpoint is a cell-cycle surveillance mechanism that ensures the fidelity of chromosome segregation during mitosis and meiosis. Bub1 is a protein serine/theronine kinase that plays multiple roles in chromosome segregation and the spindle checkpoint. In response to misaligned chromosomes, Bub1 directly inhibits the ubiquitin ligase activity of the anaphase-promoting complex or cyclosome (APC/C) through phosphorylating its activator Cdc20. The protein level and the kinase activity of Bub1 are regulated during the cell cycle; they peak in mitosis and are low in G1/S phase. Here, we show that Bub1 is degraded during mitotic exit and that degradation of Bub1 is mediated by APC/C in complex with its activator Cdh1 (APC/C^{Cdh1}). Overexpression of Cdh1 reduces the protein levels of ectopically expressed Bub1 whereas depletion of Cdh1 by RNA interference (RNAi) increases the level of the endogenous Bub1 protein. Bub1 is ubiquitinated by immunopurified APC/C^{Cdh1} *in vitro*. We further identify two KEN-box motifs on Bub1 that are required for its degradation *in vivo* and ubiquitination *in vitro*. A Bub1 mutant protein

with both KEN-box mutated is stable in cells, but fails to elicit a cell-cycle phenotype, indicating that degradation of Bub1 by APC/C^{Cdh1} is not required for mitotic exit. Nevertheless, our study clearly demonstrates that Bub1, an APC/C inhibitor, is also an APC/C substrate. The antagonistic relationship between Bub1 and APC/C may help to prevent the premature accumulation of Bub1 during G1.

Introduction

Ubiquitin- and proteasome-dependent proteolysis is one of the key mechanisms that ensure the uni-directional progression of the cell cycle (1,2). The ubiquitin molecule is activated by the ubiquitin-activating enzyme (E1), transferred to a ubiquitin-conjugating enzyme (E2), and finally attached to a lysine residue in the substrate with the help of a ubiquitin ligase (E3). These reactions can be repeated to allow the formation of ubiquitin chains on substrates. Polyubiquitinated substrates are then recognized by proteasome for destruction (3). The efficiency and specificity of substrate ubiquitination are usually determined by the E3s (3).

The anaphase-promoting complex or cyclosome (APC/C) is a multisubunit E3 ubiquitin ligase and is required for the proper segregation of sister chromatids and for the exit from mitosis (4). Prior to anaphase, the sister chromatids are bound together by the cohesin protein complex that consists of Smc1, Smc3, a kleisin subunit (Scc1/Rad21/Mcd1), and Scc3 (known as SA1 and SA2 in vertebrates). At the metaphase-anaphase transition, APC/C together with its activator Cdc20 ubiquitinates securin and targets it for degradation (5,6). Securin is an inhibitor of separase, the protease that cleaves the Scc1 subunit of cohesin to

allow sister chromatid separation. Therefore, the activation of APC/C^{Cdc20} triggers the degradation of securin, activation of separase, cleavage of cohesin, and the onset of sister chromatids separation (7). The activity of APC/C is controlled by two related activators, Cdc20 and Cdh1. Both Cdc20 and Cdh1 contain a C-terminal WD40-repeat domain, and are involved in recruiting substrates to APC/C (6). APC/C^{Cdc20} and APC/C^{Cdh1} perform distinct functions. APC/C^{Cdc20} is required for sister chromatid separation and the metaphase-anaphase transition by ubiquitinating securin and cyclin B1 whereas APC/C^{Cdh1} mediates the degradation of a broader spectrum of substrates in late anaphase and early G1 (4). Many APC/C substrates contain cis-elements or degrons called the destruction box (D-box) or KEN box that are required for their ubiquitination and degradation by APC/C.

The spindle checkpoint is a cell-cycle surveillance mechanism that ensures the accuracy of chromosome segregation and helps to maintain genetic stability (6,7). For sister chromatids to separate correctly, the two opposing kinetochores of a pair of sister chromatids must be captured by microtubules emanated from the two opposite poles of the mitotic spindle (bi-orientation). Once the pair of sister chromatids achieves biorientation, the kinetochores are under tension, because sister chromatid cohesion resists the spindle pulling force and holds the two sisters together. The spindle checkpoint senses the lack of attachment and tension at the kinetochore and transduces the signal to prevent premature sister chromatid separation until the correct kinetochore-microtubule attachment is established for all sister chromatids (7). The spindle checkpoint selectively inhibits the activity of APC/C^{Cdc20}, stabilizes securin, delays the activation of separase, and prevents premature sister chromatid separation (7).

Bub1 is a key kinase involved in the spindle checkpoint signaling (8). Genetic studies in yeast have firmly established the requirement of Bub1 for proper spindle checkpoint function (9,10). Bub1 localizes to kinetochores from early prophase to metaphase, and the kinetochore localization of Bub1 is diminished after anaphase onset (11). Bub1 is also required for the kinetochore localization of other components of the spindle checkpoint, such as BubR1 and Mad2 in vertebrates and yeast (12-14). Furthermore, Bub1 phosphorylates Cdc20 directly and therefore inhibits the activity of APC/C (15). Phosphorylation of Cdc20 by Bub1 is required for efficient checkpoint signaling (15). Consistently, the kinase activity of Bub1 is enhanced in mitosis (15). The mechanism by which Bub1 is regulated is still unknown. Bub1 is phosphorylated in mitosis in yeast (16), *Xenopus* (17,18), and mammalian cells (15,19). In *Xenopus* egg extract, the chromosome-associated Bub1 is hyperphosphorylated and exhibits higher autophosphorylation activity (17). However, it is unclear whether phosphorylation regulates the kinase activity of Bub1 and whether there are additional mechanisms that regulate Bub1.

Here, we show that the Bub1 protein level oscillates during the cell cycle. Bub1 is degraded in late anaphase and G1 by APC/C^{Cdh1}. A fragment of Bub1 is ubiquitinated *in vitro* by APC/C^{Cdh1}, but not by APC/C^{Cdc20}. We further identify two KEN-boxes in human Bub1 that are required for its degradation *in vivo* and for its APC/C-dependent ubiquitination *in vitro*. A stable, inducible cell line that expresses a Bub1 mutant containing mutations in these two KEN boxes undergo mitotic exit with regular kinetics during the normal cell cycle or during the recovery from nocodazole-mediated mitotic arrest. Therefore, APC/C^{Cdh1}-dependent degradation of Bub1 is not required for the normal progression through mitosis.

Instead, it may safeguard cells from prematurely accumulating Bub1 during G1 or quiescence.

Materials and methods

Plasmids, antibodies, and immunoblotting

The production of the Bub1, APC3 (Cdc27) and APC2 antibodies was described previously (5,15,20). The cyclin B1 antibody was purchased from Santa Cruz Biotechnology. The Cdh1 antibody was from Neomarker. HA and Myc antibodies were from Roche. For immunoblotting, the antibodies were used at 1:1000 dilution for crude sera or 1 μg/ml for purified IgG.

Tissue culture, drug treatments, and transfection

HeLa Tet-on (Clontech) and HCT116 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and 10 mM L-glutamine. To arrest cells at G1/S, cells were incubated in the growth medium containing 2 mM thymidine (Sigma) for 18 hrs. To obtain mitotic cells, the cultured cells were treated with 100 ng/ml nocodazole (Sigma) for 16-18 hrs.

To analyze the stability of the Bub1 protein in late anaphase, the cells were treated with nocodazole for 16 hrs. Nocodazole was then washed out and the cells were replated for 1 hr before cycloheximide (50 μM) was added to the medium. Cells were harvested at different time points after cycloheximide addition. To analyze the protein stability in metaphase, cycloheximide was added without washing out nocodazole.

Transfection was performed when the cell reached about 50% confluency using the Effectene reagent (Qiagen) according to the manufacturer's instructions. The plasmids were derived from pCS2-Myc or pCS2-HA. For RNAi experiments, the siRNA oligonucleotides targeting human Cdh1 (GAAGGGUCUGUUCACGUAUTT) was chemically synthesized by Dharmacon. HeLa cells were transfected as described (15) and analyzed 48 hrs after transfection.

To establish cell lines that stably express Myc-Bub1 and Myc-Bub1-KdM, HeLa Tet-on cells at 40% confluency were transfected with pTRE2-hygro-Myc-Bub1 and pTRE2-hygro-Myc-Bub1-KdM plasmids. The cells were then selected with 300 μg/ml of hygromycin (Clonetech). The surviving clones were expanded and screened for the induced expression of Myc-tagged Bub1 proteins in the absence or presence of 1 μg/ml doxycycline (Clonetech).

FACS analysis

HeLa Tet-on cells were harvested by trypsin digestion and then washed once with PBS. The cells were fixed with 70% ethanol that had been pre-cooled to –20˚C. After overnight fixation on ice, the cells were stained with propidium iodide, and analyzed by flow cytometry. The data were acquired and analyzed using the CellQuest software.

***In vitro* translation and ubiquitination assays**

The *in vitro* transcription and translation system was purchased from Promega and the reactions were performed according to manufacturer's instructions. Briefly, 80 ng of plasmid DNA, nuclease-free water, 0.2 μl ^{35}S-Methionine (10 μCi/μl), and 4 μl rabbit reticulocyte

lysate were mixed to give a final volume of 5 μl. The reaction mixture was incubated at 30˚C for 90 minutes.

The expression and purification of human Cdc20 and Cdh1 proteins from Sf9 cells were described previously (20). The interphase APC/C was purified from interphase *Xenopus* egg extracts using anti-APC3/Cdc27 antibody coupled to protein-A support. After washing with high salt XB buffer (10 mM HEPES pH 7.7, 500 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM sucrose, and 0.5% NP-40) 5 times and with XB buffer (10 mM HEPES pH 7.7, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Sucrose) twice, the APC/C beads were incubated with recombinant Cdc20 or Cdh1 proteins for 1 hr at room temperature. The APC/C beads were again washed twice with XB buffer and assayed its ability to ubiquitinate an N-terminal fragment of human cyclin B1 or various Bub1 fragments. Each ubiquitination assay contained a 5 μl mixture of an energy-regenerating system, 150 μM ubiquitin, 5 μM recombinant E1, 2 μM recombinant UbcH10, 1 μl in vitro transcribed and translated substrates, and 3 μl of the APC/C beads. The reactions were incubated under constant shaking for 1 hr at room temperature, quenched with SDS sample buffer, resolved by SDS-PAGE, and analyzed using a phosphoimager.

Results

Bub1 is degraded in late anaphase and G1

Previous studies showed that the Bub1 protein level is high in nocodazole-arrested mitotic cells but low in G1/S cells enriched by a thymidine block (21). We hypothesized that Bub1 is

regulated at the protein level and thus examined the steady state levels of Bub1 in synchronized human tissue culture cells. Cells were arrested at metaphase by nocodazole treatment, released into normal growth medium, and then harvested at the indicated time points. The level of Bub1 protein was then analyzed by SDS-PAGE and Western blotting. As shown in Figure 1A, the protein level of Bub1 decreased sharply as cells underwent mitotic exit whereas the level of an APC/C subunit, APC2, remained constant (Figure 1A, upper panel). The expression pattern of Bub1 was similar to that of cyclin B1, a well-established APC/C substrate (Figure 1A, bottom panel). The timing of Bub1 down-regulation was identical in HCT116 (Figure 1A) and HeLa cells (data not shown). To rule out the possibility that the decrease of the Bub1 protein level only occurred during mitotic exit following the recovery from nocodazole-mediated mitotic arrest, we synchronized HeLa cells at the G1/S boundary using a double-thymidine block. The cells were then released into fresh medium and harvested at different time points after release. The Bub1 protein accumulated gradually during G1 and S, peaked at G2/M, and dropped dramatically after mitosis (Figure 1B). The expression pattern of Bub1 protein again mirrored that of cyclin B1 during this experiment.

The reduction in the protein level of Bub1 during mitotic exit may be caused by increased protein degradation or decreased protein synthesis. To assess whether the reduction in the Bub1 protein level was due to protein degradation, we measured the half-life of Bub1 by cycloheximide (CHX) chase in nocodazole-arrested prometaphase cells and in late anaphase or early G1 cells released from nocodazole block for 1 hour. We found that Bub1 was stable in prometaphase cells whereas it was degraded with a half-life of about 1 hr in late

anaphase/early G1 cells (Figure 1C). This result indicated that Bub1 was rapidly degraded during mitotic exit.

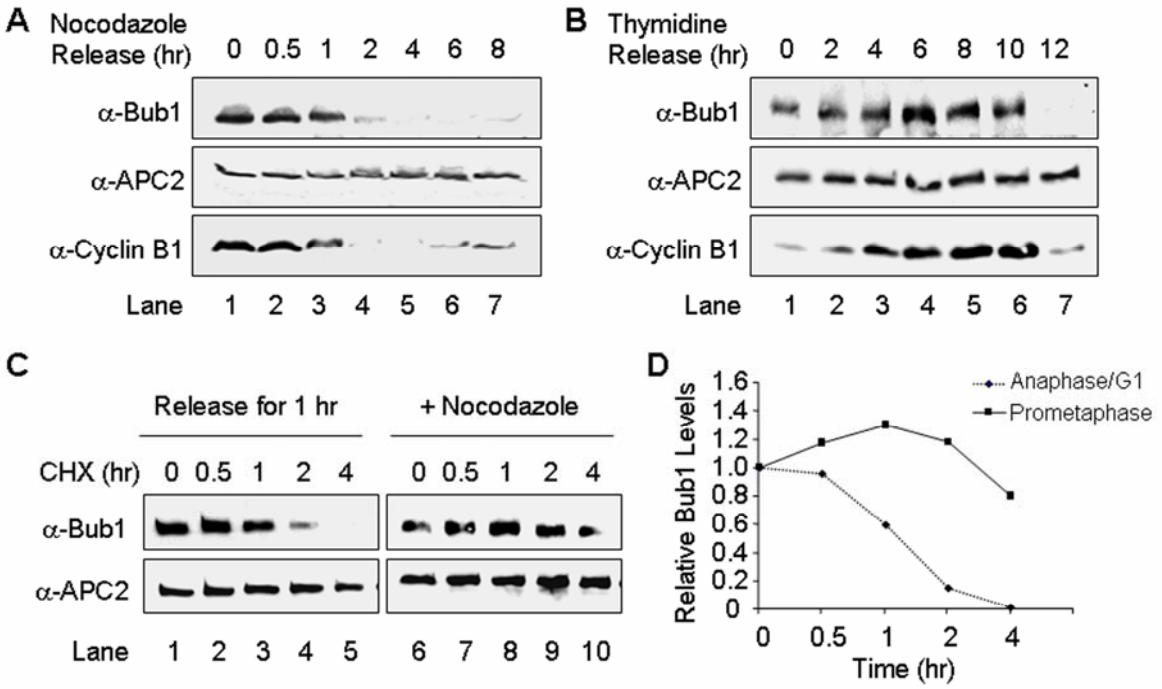


Figure 1. Bub1 is rapidly degraded during mitotic exit. (A) HCT116 cells were arrested in mitosis by 18 hrs of nocodazole treatment, released into fresh growth medium, and collected at the indicated times for Western blot analysis with anti-Bub1 antibody. APC2 blot was used as a loading control. Cyclin B1 was also blotted to indicate proper mitotic exit. (B) HeLa cells were blocked at the G1/S boundary by thymidine, released into fresh medium, and harvest for Western blot with antibodies against Bub1, APC2, and cyclin B 1 at the indicated times. (C) HeLa cells arrested in mitosis were replated for 1 hr in the presence or absence of nocodazole. Cycloheximide (CHX) was then added. Cell lysates were prepared at the indicated times and subjected to immunoblotting with the indicated antibodies. (D) Quantification of the relative Bub1 protein levels in (C).

Cdh1 overexpression reduces the level of Bub1 in cells

The oscillation pattern of the Bub1 protein during the cell cycle is very similar to those of known substrates of APC/C^{Cdh1}, such as cyclin B1 (22), Cdc20 (23), and Plk1 (24), suggesting that Bub1 may also be an APC/C^{Cdh1} substrate. We thus tested whether Cdh1 promoted the degradation of Bub1 in cells. HeLa cells were co-transfected with vectors encoding Myc-Bub1 and HA-Cdh1 and treated with cycloheximide. The protein level of Myc-Bub1 was examined by α-Myc blot. The protein level of Myc-Bub1 was decreased considerably when Cdh1 was co-expressed (Figure 2A, compare lanes 1 and 4). Furthermore, Myc-Bub1 was relatively stable in the absence of HA-Cdh1 whereas co-expression of HA-Cdh1 reduced the half-life of Myc-Bub1 to about 30 min (Figure 2A). These results suggested that Bub1 might be a substrate of APC/C^{Cdh1}.

The KEN-box motifs of Bub1 are required for its Cdh1-dependent degradation *in vivo*

Previous studies revealed that APC/C recognizes two types motifs in its substrates: the D-box (RxxLxxxxN) and the KEN-box (KEN) (25). By sequence analysis, no D-boxes were found in human Bub1. However, two putative KEN-box motifs were identified in the central region of Bub1 (Figure 2B and 2C). We thus constructed Bub1 mutants in which the first KEN-box (K1M), the second KEN-box (K2M), or both KEN-boxes (KdM) were mutated to alanine residues and tested whether the protein levels of these Bub1 mutants were reduced by Cdh1 overexpression. HeLa cells were transfected with plasmids encoding Myc-Bub1 or the three Myc-Bub1 KEN-box mutants with or without the HA-Cdh1 expression vector. The protein levels of Myc-Bub1 mutants were examined by α-Myc blot. The protein levels of Bub1-K1M and Bub1-KdM were not significantly reduced in the presence of Cdh1 (Figure 2D).

Bub1-K2M also showed partial stabilization as compared to the wild-type Bub1 protein (Figure 2D). These results indicated that both KEN-boxes are involved in the degradation of Bub1 and that the first KEN-box is the major degron of Bub1. The fact that degradation of Bub1 in the presence of excess amount of Cdh1 depended on KEN-boxes further suggested that Bub1 might be a substrate of APC/C^{Cdh1}.

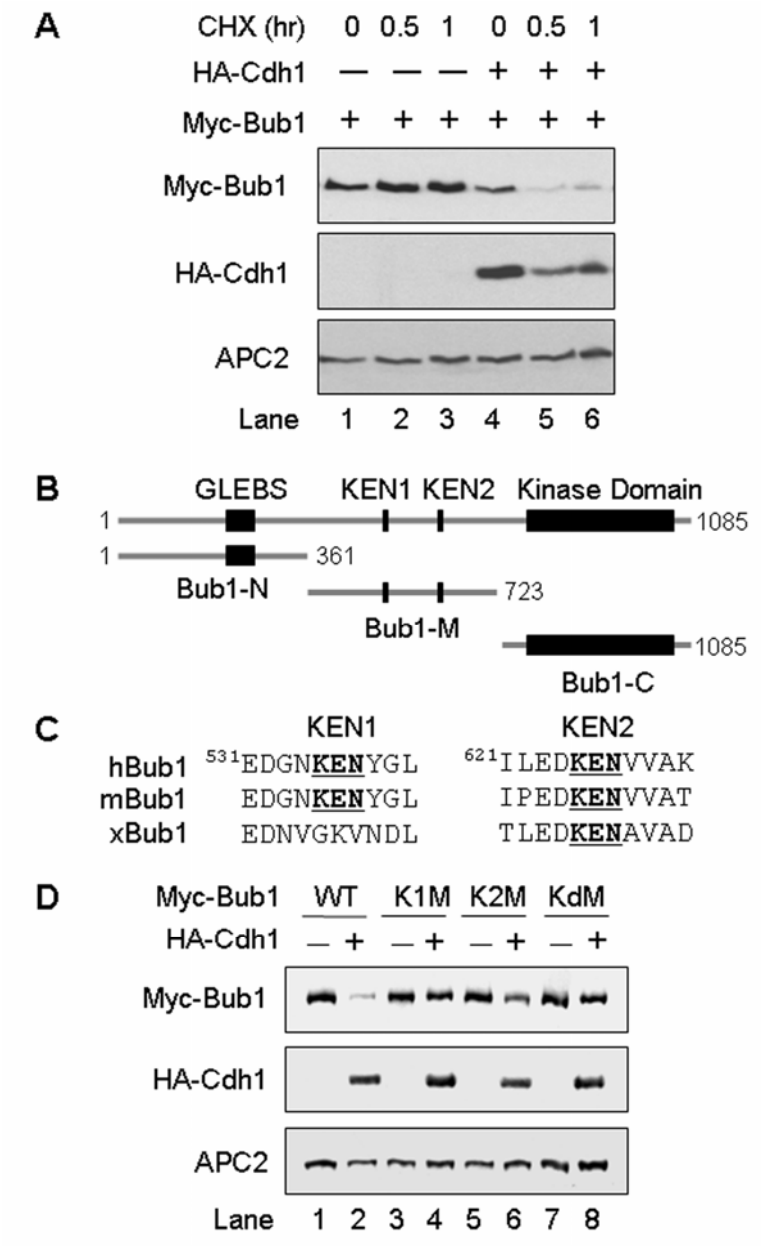


Figure 2. Ectopically expressed Cdh1 reduces the levels of Bub1 in a KEN-box-dependent manner. (A) HeLa cells were cotransfected with Myc-Bub1 with or without HA-Cdh1 constructs. Twenty-four hours after transfection, cycloheximide (CHX) was added for the indicated times. Cells were collected and subjected to Western blot with the indicated antibodies. (B) Domain structure of human Bub1. The GLEBS motif, two putative KEN-boxes, and the kinase domain are indicated. Three Bub1 fragments used in later experiments are also shown. (C) Alignment of the sequences surrounding the two putative KEN-box motifs of the Bub1 orthologs. (D) HeLa cells were transfected with Myc-Bub1 wild-type (WT) or mutant constructs (K1M, with K535, E536, and N537 mutated to alanines; K2M, with K625, E626, and N627 mutated to alanines; KdM, with both KEN-boxes mutated to alanines) together with or without HA-Cdh1, dissolved in SDS sample buffer, separated on SDS-PAGE and blotted with the indicated antibodies.

Bub1 is ubiquitinated by APC/C^{Cdh1} *in vitro*

To confirm that Bub1 was a substrate of APC/C, we tested whether immuno-purified APC/C^{Cdh1} directly catalyzed the ubiquitination of Bub1 *in vitro*. Human Bub1 is a large protein with 1085 residues. It would be difficult to resolve ubiquitin conjugates of the full-length Bub1 protein on SDS-PAGE. We thus designed three fragments of Bub1: Bub1-N, Bub1-M, and Bub1-C, each containing one third of the Bub1 protein (Figure 2B). The fragments were translated *in vitro* in the presence of ^{35}S-methionine and used as substrates in the ubiquitination assays. The N-terminal fragment of human cyclin B1 (residues 1-102) was used as the positive control (20). Cyclin B1 was efficiently ubiquitinated by both APC/C^{Cdh1} and APC/C^{Cdc20} (Figure 3A, left panel). As APC/C^{Cdh1} was often more efficient in stimulating the ubiquitination of many APC/C substrates, we had purposefully used less APC/C^{Cdh1} in this assay. Thus, under these experimental conditions, APC/C^{Cdc20} was active in promoting cyclin B1 ubiquitination (Figure 3A, compare lanes 2 and 3). Of the three Bub1 fragments, only Bub1-M was ubiquitinated by APC/C^{Cdh1}, but not by APC/C^{Cdc20} (Figure

3A). This indicates that Bub1 is a substrate of APC/C^{Cdh1} and that the APC/C recognition elements of Bub1 are located in the central region of the protein.

Both KEN-boxes are located in the central fragment of Bub1. We therefore introduced the same KEN-box mutations into Bub1-M and tested the ability of these mutants to be ubiquitinated by APC/C^{Cdh1} *in vitro*. Consistent with the results from the *in vivo* degradation assay, mutation of the first KEN-box in Bub1-M dramatically decreased the amount of the poly-ubiquitinated species (Figure 3B, compare lanes 4 and 6). Mutation of the second KEN-box had a minor effect (Figure 3B, compare lanes 4 and 8). Mutation of both KEN-boxes completely eliminated the ubiquitination of Bub1-M by APC/C^{Cdh1} (Figure 3B, lane 10). Taken together, our results clearly establish that Bub1 is ubiquitinated by APC/C^{Cdh1} *in vitro* in a KEN-box-dependent manner.

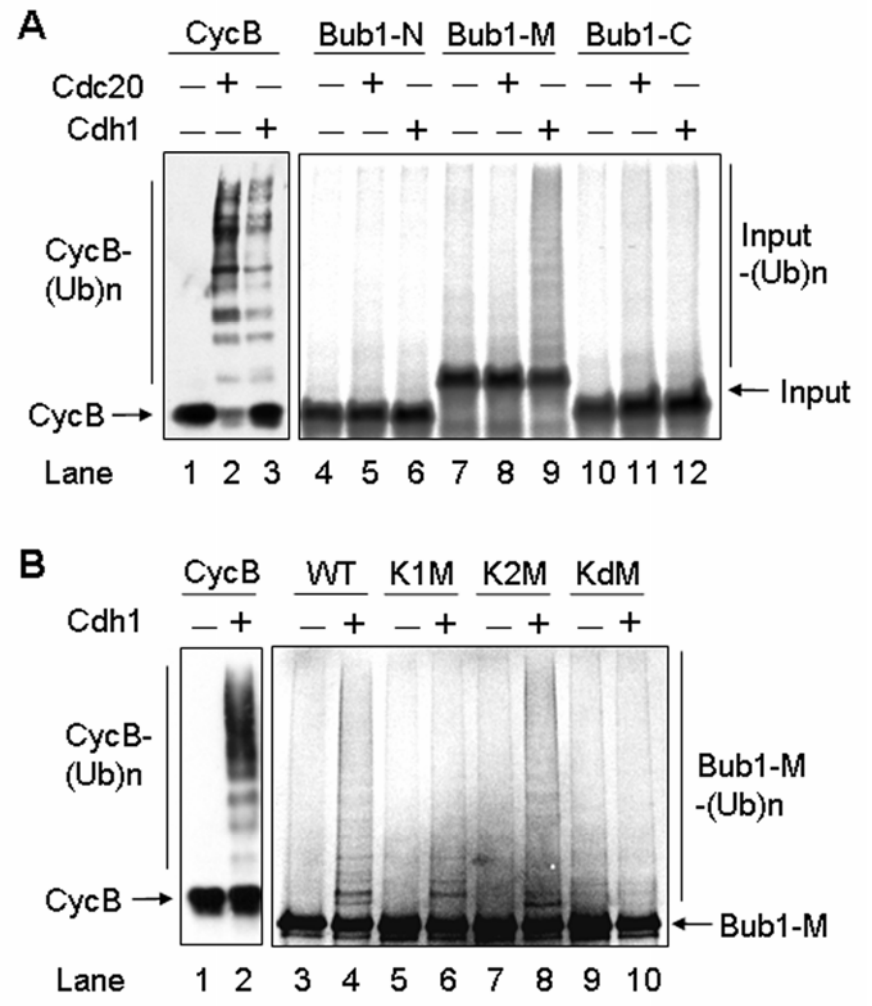


Figure 3. A fragment of Bub1 is ubiquitinated by APC/C^{Cdh1} *in vitro*. (A) APC/C was immunoprecipitated from *Xenopus* egg extracts and supplemented with recombinant Cdc20 or Cdh1. The recombinant Myc-tagged human cyclin B1 fragment (residue 1-102) or *in vitro* translated ^{35}S-labeled Myc-Bub1 fragments (Bub1-N, Bub1-M, and Bub1-C) were used as substrates. The reaction mixtures were separated on SDS-PAGE and analyzed by α-Myc blot in the case of cyclin B1 fragment or autoradiography in the case of Myc-Bub1 fragments. (B) Same as (A) except that the wild-type (WT) and the mutant Myc-Bub1-M fragments were used as substrates for ubiquitination.

Cdh1 mediates the degradation of Bub1 *in vivo*

To further test whether Cdh1 was required for the degradation of the endogenous Bub1 *in vivo*, we used small interfering RNA (siRNA) to deplete Cdh1 and measured the levels of the endogenous Bub1 and other known APC/C substrates, such as cyclin B1, by immunoblotting (Figure 4A). The Cdh1 siRNA efficiently knocked down the expression of Cdh1 (Figure 4A). The steady state levels of Bub1 and cyclin B1 were significantly elevated in Cdh1-RNAi cells (Figure 3A). This result suggested that APC/C^{Cdh1} was a major ubiquitin ligase for the degradation of Bub1 *in vivo*. Because the protein level of Bub1 was higher in mitosis, an alternative explanation of this result was that depletion of Cdh1 by RNAi caused a mitotic arrest/delay, which in turn caused the accumulation of Bub1 as a secondary effect. To rule out this possibility, we performed FACS analysis of mock transfected and Cdh1-RNAi cells (Figure 4B). No significant differences were observed between the cell cycle profiles of the mock transfected and Cdh1-RNAi cells (Figure 4B). Therefore, our results are consistent with the notion that APC/C^{Cdh1} mediates the degradation of Bub1 *in vivo*. We could not determine whether APC/C^{Cdc20} is involved in Bub1 degradation *in vivo*, because Cdc20-RNAi causes a mitotic arrest in human cells.

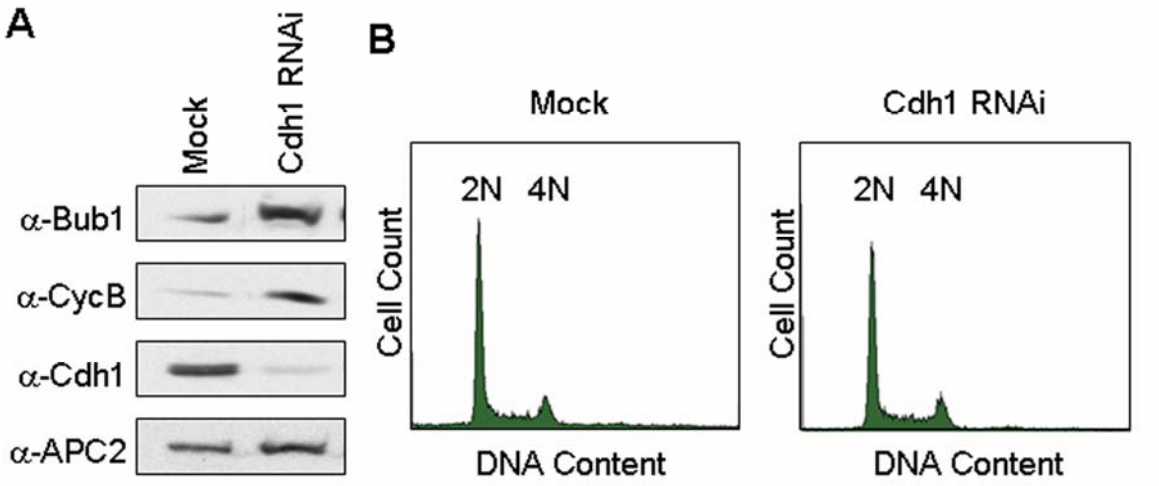


Figure 4. Depletion of Cdh1 from HeLa cells by RNAi causes accumulation of Bub1. (A) HeLa cells transfected with control or Cdh1 siRNA were harvested. The total cell lysates were immunoblotted with the indicated antibodies. (B) HeLa cells transfected with control or Cdh1 siRNA were fixed by ethanol and subjected to FACS analysis.

The KEN-box mutant of Bub1 is stabilized during mitotic exit

We next tested whether the KEN boxes of Bub1 are required for its degradation *in vivo* during mitotic exit. For this purpose, we established HeLa cell lines that stably expressed either Myc-Bub1 or Myc-Bub1-KdM driven by a tetracycline-inducible promoter. The stability of the Myc-Bub1 and Myc-Bub1-KdM proteins after nocodazole arrest-release was examined by blotting with anti-Myc antibody. As expected, Myc-Bub1 protein was degraded during mitotic exit after the recovery from the nocodazole-mediated mitotic arrest (Figure 5A, lane 1 to 6). In contrast, the Myc-Bub1-KdM protein was stable even at the 6 hr time point after nocodazole release, when cells had already progressed to the G1 phase (Figure 5A, lane 7 to 12). By using cycloheximide treatment, we also measured the half-lives of the Myc-Bub1 and Myc-Bub1-KdM proteins in the cells that were released from nocodazole

arrest for 1 hr. As shown in Figure 5B and 5C, Myc-Bub1 had a half-life of about 1.5 hrs while Myc-Bub1-KdM was stable during the course of the experiment. These results demonstrate that the KEN-box mutant of the Bub1 protein is not degraded during mitosis exit *in vivo*.

We noticed that securin and cyclin B1 were degraded with normal kinetics in Bub1-KdM expressing cells (Figure 5A and data not shown), suggesting that degradation of Bub1 is not required for the exit from mitosis following the recovery from a nocodazole-mediated mitotic arrest.

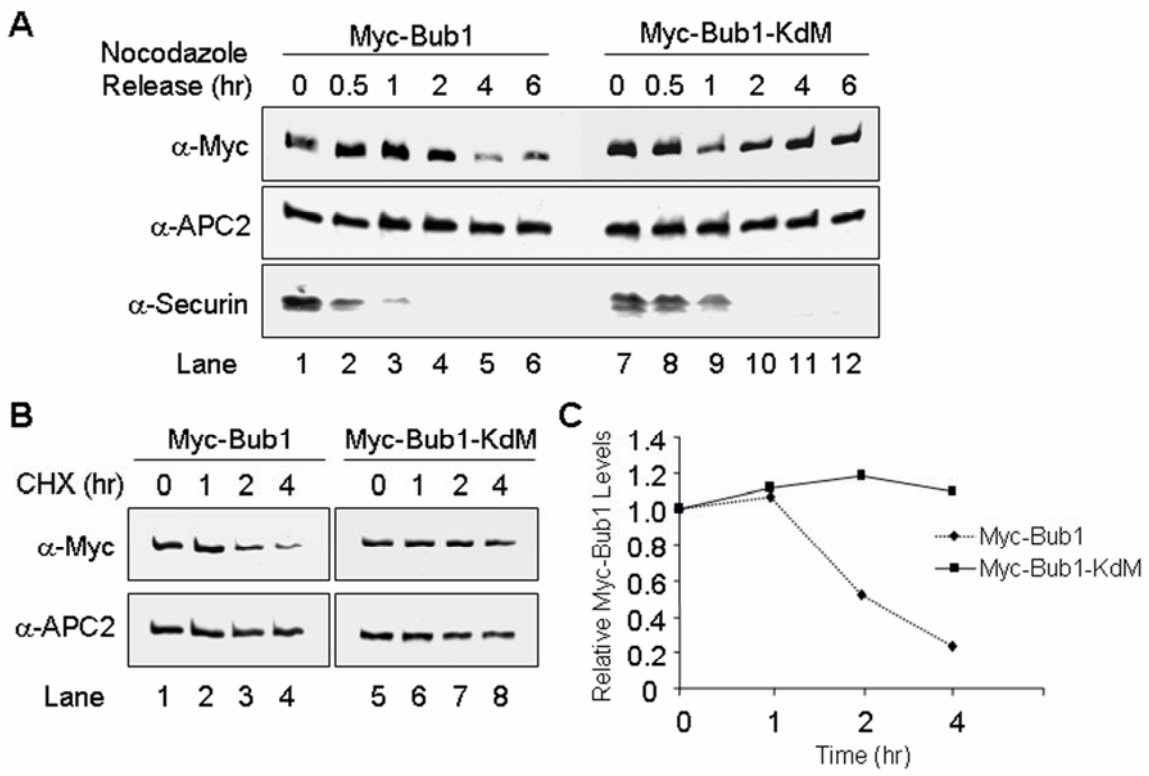


Figure 5. A KEN-box mutant of Bub1 is not degraded during mitotic exit. (A) HeLa Tet-On cells stably transfected with Myc-Bub1 or Myc-Bub1-KdM were cultured in the presence of doxycycline, treated with nocodazole for 18 hrs, harvested and analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. (B) HeLa Tet-On cells stably transfected with Myc-Bub1 or Myc-Bub1-KdM were cultured in the presence of doxycycline, treated with nocodazole for 18 hrs, and released into fresh medium for 1 hr.

Cycloheximide (CHX) was then added into medium for the indicated times. Cells were collected and analyzed by SDS-PAGE followed by immunoblotting with the indicated antibodies. (C) Quantification of the relative Myc-Bub1 protein levels in (B).

Degradation of Bub1 is not required for cellular adaptation upon prolonged exposure to nocodazole

We did not observe acute cell-cycle arrest/delay as induced by Myc-Bub1-KdM. The protein level of Myc-Bub1-KdM in the presence of Dox was much higher than that of the endogenous Bub1, as the anti-Bub1 antibody only detected Myc-Bub1-KdM, not the endogenous Bub1, in this particular Western blot (Figure 6A). Consistently, when the proliferative property of the Bub1-KdM-expressing clone was monitored, the growth curves of these cells in the absence or presence of doxycycline were very similar. Cells in both conditions divided with a doubling time of approximately 25 hrs (Figure 6B).

It has recently been reported that another spindle checkpoint protein, BubR1, is degraded in polyploid cells that had adapted and survived a prolonged nocodazole treatment and re-introduction of BubR1 causes apoptosis of these polyploid cells (26). Re-introduction of Bub1 into these polyploid cells also causes apoptosis, although the apoptosis-inducing effect of Bub1 was less dramatic than that of BubR1 (26). We therefore tested whether induction of the expression of Bub1-KdM caused apoptosis in polyploid HeLa cells that had adapted during prolonged nocodazole treatment. HeLa cells were treated with nocodazole for the indicated duration in the presence or absence of doxycycline and were then harvested for FACS analysis. As shown in Figure 6C, after 18 hours of treatment, most of the cells in both

conditions had 4N DNA content, indicating they were arrested in G2/M. By 54 hours, almost all the cells were in the sub-G1 population, which corresponded to apoptotic cells. A minor population of cells was able to adapt and possessed 8N DNA contents (polyploid) (Figure 6C). However, doxycycline-induced expression of Bub1-KdM did not significantly alter the percentage of polyploid cells (Figure 6C). Therefore, degradation of Bub1 does not appear to be required for the adaptation process when cells are subjected to a prolonged mitotic arrest.

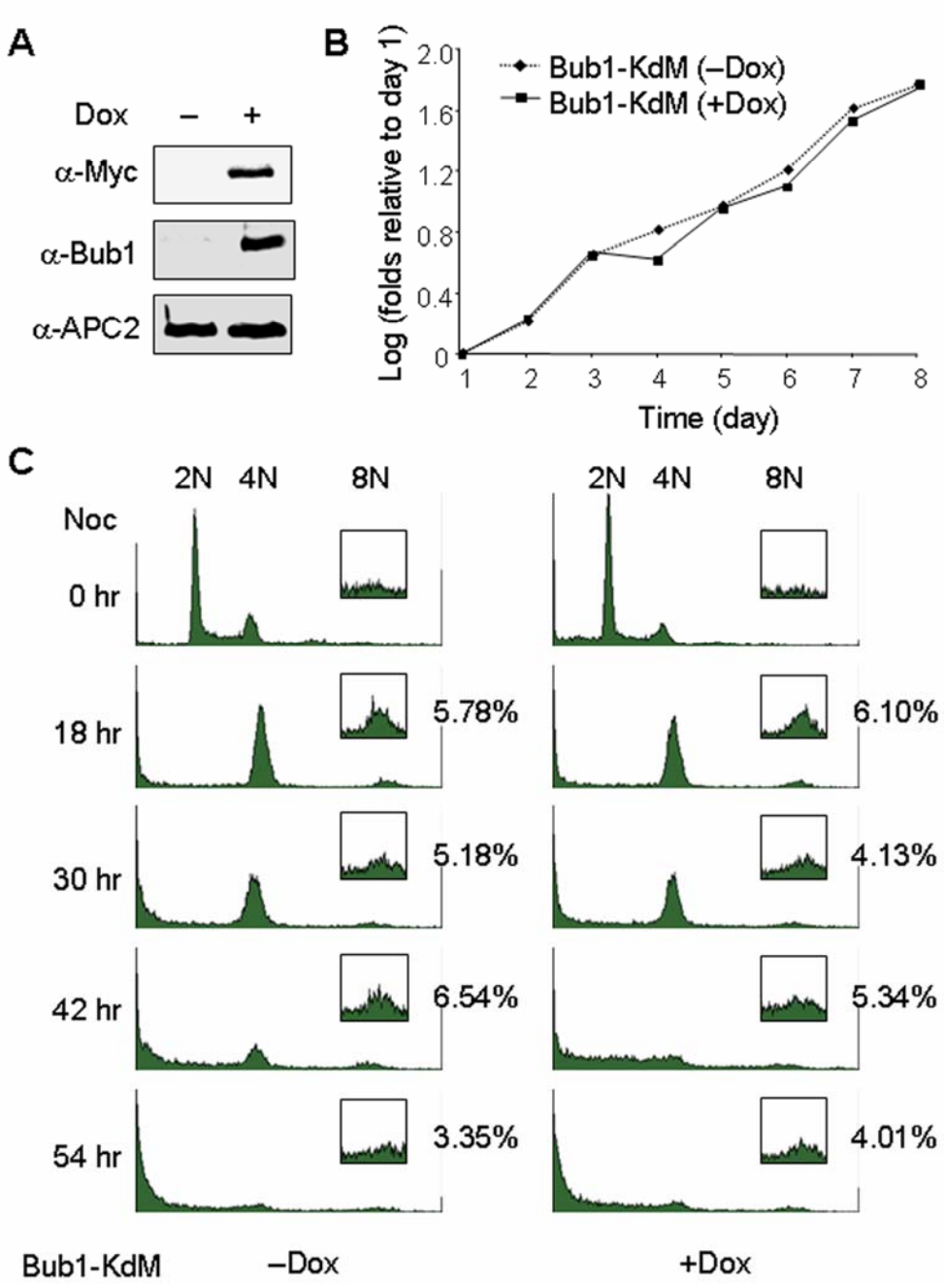


Figure 6. Overexpression of a non-degradable form of Bub1 does not cause mitotic arrest or prevent cellular adaptation after prolonged nocodazole treatment. (A) HeLa cells stably transfected with Myc-Bub1-KdM construct were cultured in the absence (–Dox) or presence (+Dox) of doxycycline. The total cell lysates were blotted with the indicated antibodies. (B) Growth curves of HeLa cells stably transfected with Myc-Bub1-KdM construct in the absence (–Dox) or presence (+Dox) of doxycycline. (C) HeLa cells stably transfected with Myc-Bub1-KdM construct were cultured in the nocodazole-containing

medium with (+Dox) or without (–Dox) doxycycline for the indicated times and analyzed by FACS. The 8N peaks were enlarged with a different scale in insets and the percentage of 8N cells were indicated.

Discussion

Bub1 is a substrate of APC/C^{Cdh1}

We have presented several lines of evidence to indicate that Bub1 is degraded during the exit from mitosis and that APC/C^{Cdh1} is the E3 ubiquitin ligase for the ubiquitination of Bub1. First, the oscillation pattern of Bub1 closely resembles that of other APC/C^{Cdh1} substrates, such as cyclin B1 (22) and Plk1 (24). Second, overexpression of Cdh1 decreases the level of ectopically expressed Bub1 in cells. Third, APC/C^{Cdh1} ubiquitinates Bub1 *in vitro*. Fourth, mutation of KEN-boxes in Bub1 stabilizes Bub1 *in vivo* and abrogates Bub1 ubiquitination *in vitro*. Finally and most importantly, depletion of Cdh1 by RNAi leads to an accumulation of the Bub1 protein in living cells.

The two related activators of APC/C, Cdc20 and Cdh1, are involved in recruiting substrates to APC/C (4,27). However, APC/C^{Cdc20} and APC/C^{Cdh1} perform distinct functions and are differentially regulated during the cell cycle (23,27,28). The functional difference between Cdc20 and Cdh1 is partly due to their different substrate specificities (6). Cdc20 recognizes the D-box in substrates (29) while Cdh1 is less selective, recognizing a variety of elements, including the D-box, KEN-box and other types of motifs (29-31). We have identified two KEN-boxes in Bub1 that are required for its ubiquitination and degradation, again consistent with APC/C^{Cdh1} being the major form of APC/C that mediates Bub1

degradation. On the other hand, the involvement of APC/C^{Cdc20} in Bub1 degradation *in vivo* cannot be formally excluded because Cdc20 RNAi is known to cause mitotic arrest.

Both KEN-boxes of human Bub1 are required for its efficient degradation, although the first KEN-box appears to be the major degron. Interestingly, the first KEN-box is not conserved in *Xenopus* Bub1. One possibility is that the second KEN-box in *Xenopus* Bub1 functions as the degron. Alternatively, *Xenopus* Bub1 is not efficiently degraded following mitotic exit.

Bub1 degradation and spindle checkpoint inactivation

Bub1 is required for the inhibition of APC/C when the spindle checkpoint is activated (15). Therefore, the degradation of Bub1 during mitotic exit is expected to contribute to the inactivation of the spindle checkpoint and may help to prevent the reactivation of the spindle checkpoint in late anaphase. However, expression of the non-degradable Bub1-KdM in cells does not result in a mitotic arrest. Cells expressing Bub1-KdM also undergo mitotic exit with normal kinetics during the recovery of a nocodazole-mediated mitotic arrest. These results suggest that degradation of Bub1 is not the sole mechanism to inactivate the spindle checkpoint in mammalian cells.

Recently, the yeast spindle checkpoint kinase, Mps1, has been shown to be degraded by APC/C (32). Forced expression of Mps1 in anaphase re-establishes the spindle checkpoint. It has been proposed that the mutual inhibition of Mps1 and APC/C forms a feedback loop to control the inactivation of the spindle checkpoint in anaphase (32). Intriguingly, overexpression of Mps1 is sufficient to cause mitotic arrest that is dependent on

the spindle checkpoint in yeast (33). However, overexpression of Mps1 does not result in mitotic arrest in mammalian cells (unpublished data) (34). This important difference suggests that mechanisms regulating the activation and inactivation of the spindle checkpoint are more complex in mammalian cells than in yeast. Nevertheless, Mps1 is degraded during mitotic exit in mammalian cells (unpublished data). Degradation of Mps1 might contribute to checkpoint inactivation in mammalian cells. In addition, several other spindle checkpoint proteins, including Aurora B and Plk1, have been shown to be degraded in an APC/C-dependent manner. It is thus possible that degradation of multiple spindle checkpoint proteins by APC/C mediates spindle checkpoint inactivation and mitotic exit. On the other hand, $Cdh1^{-/-}$ chicken DT40 cells are viable and proliferate with very similar rate as the wild-type DT40 cells (35). This argues against a major role of APC/C^{Cdh1} in mitotic exit and against a requirement for APC/C^{Cdh1}-mediated degradation of spindle checkpoint proteins in mitotic exit.

In addition to regulation of its protein levels, Bub1 is phosphorylated in mitosis by Cdk1 (19) and MAPK (17). The activity of Bub1 is regulated by phosphorylation in *Xenopus* egg extracts (17). Therefore, dephosphorylation of Bub1 may be another mechanism to inactivate the spindle checkpoint. Indeed, Bub1 is rapidly dephosphorylated when Cdk1 is inhibited by roscovitine (19). Recently, it has been shown that phosphorylation of Cdc6 protects it from APC/C^{Cdh1}-mediated degradation (36). It is conceivable that degradation of Bub1 might also be regulated by the phosphorylation state of Bub1. In supporting of this notion, the phosphorylation sites identified on Bub1 are in the vicinity of the KEN boxes (17,19). In the future, it will be interesting to examine whether dephosphorylation of Bub1 is

a key mechanism for its inactivation and whether dephosphorylation and degradation of Bub1 are two coupled events during mitotic exit.

References

1. Hershko, A. (1997) *Curr Opin Cell Biol* **9**(6), 788-799

2. Nakayama, K. I., and Nakayama, K. (2006) *Nat Rev Cancer* **6**(5), 369-381

3. Tanaka, K., Suzuki, T., and Chiba, T. (1998) *Mol Cells* **8**(5), 503-512

4. Peters, J. M. (2002) *Mol Cell* **9**(5), 931-943

5. Fang, G., Yu, H., and Kirschner, M. W. (1998) *Mol Cell* **2**(2), 163-171

6. Yu, H. (2002) *Curr Opin Cell Biol* **14**(6), 706-714

7. Bharadwaj, R., and Yu, H. (2004) *Oncogene* **23**(11), 2016-2027

8. Yu, H., and Tang, Z. (2005) *Cell Cycle* **4**(2), 262-265

9. Farr, K. A., and Hoyt, M. A. (1998) *Mol Cell Biol* **18**(5), 2738-2747

10. Bernard, P., Hardwick, K., and Javerzat, J. P. (1998) *J Cell Biol* **143**(7), 1775-1787

11. Taylor, S. S., and McKeon, F. (1997) *Cell* **89**(5), 727-735

12. Johnson, V. L., Scott, M. I., Holt, S. V., Hussein, D., and Taylor, S. S. (2004) *J Cell Sci* **117**(Pt 8), 1577-1589

13. Sharp-Baker, H., and Chen, R. H. (2001) *J Cell Biol* **153**(6), 1239-1250

14. Gillett, E. S., Espelin, C. W., and Sorger, P. K. (2004) *J Cell Biol* **164**(4), 535-546

15. Tang, Z., Shu, H., Oncel, D., Chen, S., and Yu, H. (2004) *Mol Cell* **16**(3), 387-397

16. Yamaguchi, S., Decottignies, A., and Nurse, P. (2003) *Embo J* **22**(5), 1075-1087

17. Chen, R. H. (2004) *Embo J* **23**(15), 3113-3121

18. Schwab, M. S., Roberts, B. T., Gross, S. D., Tunquist, B. J., Taieb, F. E., Lewellyn, A. L., and Maller, J. L. (2001) *Curr Biol* **11**(3), 141-150

19. Qi, W., Tang, Z., and Yu, H. (2006) *Mol Biol Cell* **17**(8), 3705-3716

20. Tang, Z., Bharadwaj, R., Li, B., and Yu, H. (2001) *Dev Cell* **1**(2), 227-237

21. Taylor, S. S., Hussein, D., Wang, Y., Elderkin, S., and Morrow, C. J. (2001) *J Cell Sci* **114**(Pt 24), 4385-4395

22. King, R. W., Peters, J. M., Tugendreich, S., Rolfe, M., Hieter, P., and Kirschner, M. W. (1995) *Cell* **81**(2), 279-288

23. Prinz, S., Hwang, E. S., Visintin, R., and Amon, A. (1998) *Curr Biol* **8**(13), 750-760

24. Lindon, C., and Pines, J. (2004) *J Cell Biol* **164**(2), 233-241

25. Pfleger, C. M., and Kirschner, M. W. (2000) *Genes Dev* **14**(6), 655-665

26. Shin, H. J., Baek, K. H., Jeon, A. H., Park, M. T., Lee, S. J., Kang, C. M., Lee, H. S., Yoo, S. H., Chung, D. H., Sung, Y. C., McKeon, F., and Lee, C. W. (2003) *Cancer Cell* **4**(6), 483-497

27. Peters, J. M. (2006) *Nat Rev Mol Cell Biol* **7**(9), 644-656

28. Visintin, R., Prinz, S., and Amon, A. (1997) *Science* **278**(5337), 460-463

29. Pfleger, C. M., Lee, E., and Kirschner, M. W. (2001) *Genes Dev* **15**(18), 2396-2407

30. Littlepage, L. E., and Ruderman, J. V. (2002) *Genes Dev* **16**(17), 2274-2285

31. Araki, M., Yu, H., and Asano, M. (2005) *Genes Dev* **19**(20), 2458-2465

32. Palframan, W. J., Meehl, J. B., Jaspersen, S. L., Winey, M., and Murray, A. W. (2006) *Science* **313**(5787), 680-684

33. Hardwick, K. G., Weiss, E., Luca, F. C., Winey, M., and Murray, A. W. (1996) *Science* **273**(5277), 953-956

34. Stucke, V. M., Sillje, H. H., Arnaud, L., and Nigg, E. A. (2002) *Embo J* **21**(7), 1723-1732

35. Sudo, T., Ota, Y., Kotani, S., Nakao, M., Takami, Y., Takeda, S., and Saya, H. (2001) *Embo J* **20**(22), 6499-6508

36. Mailand, N., and Diffley, J. F. (2005) *Cell* **122**(6), 915-926

CHAPTER THREE

BUB1 ON UNATTACHED KINETOCHORES INHIBITS APC/C BY PHOSPHORYLATING CDC20

Summary

In response to activation of the spindle checkpoint, a wait-anaphase signal is generated on unattached kinetochores and diffuses away to inhibit APC/C in the cytoplasm. A singal unattached kinetochore is sufficient to delay anaphase onset and the spindle checkpoint is rapidly inactivated when the unattached kinetochore is eliminated by laser. This high sensitivity suggests that a catalytic machinery on unattached kinetochore is involved. Here we study the potential wait-anaphase signal catalyst using metaphase chromosomes with unattached kinetochores. We identify an ATP-dependent activity that inhibits APC/C^{Cdc20} in the chromosome lysate. The Bub1 phosphorylation site mutant Cdc20, Cdc20-S153A is not inhibited by the metaphase chromosomes. Bub1 protein on the unattached kinetochores is hyperphosphorylated and highly active. Furthermore, wild-type Bub1, but not the kinase-dead Bub1, restores the spindle checkpoint response in cells depleted of Bub1 by RNA interference (RNAi). Our results suggest that Bub1 on unattached kinetochores likely facilitates the formation of wait-anaphase signal by phosphorylating Cdc20 and the kinase activity of Bub1 is required for spindle checkpoint signaling.

Introduction

Chromosome segregation in mitosis is mediated by the pulling force of spindle microtubules attached to sister chromatids via kinetochores, the proteinaceous structure assembled on centromeres (Bharadwaj and Yu, 2004; Cleveland et al., 2003). To accurately separate sister chromatids into two sets, the sister kinetochores have to be attached by the microtubules emanated from the opposite poles of the spindle, a state termed bi-orientation (Bharadwaj and Yu, 2004; Pinsky and Biggins, 2005). However, the microtubules reach and attach to the kinetochores through an error-prone "search and capture" mechanism (Pinsky and Biggins, 2005). Therefore, cells utilize a surveillance system called the spindle checkpoint to secure the fidelity of chromosome segregation (Bharadwaj and Yu, 2004). The spindle checkpoint prevents sister chromatid separation and anaphase onset until all sister kinetochores are correctly attached and achieve bi-orientation (Bharadwaj and Yu, 2004; Yu, 2002). Inactivation of the spindle checkpoint leads to aneuploidy, which is manifested in genetic disorders and cancer (Rajagopalan and Lengauer, 2004).

At the metaphase-anaphase transition, an E3 ligase Anaphase-promoting Complex/Cyclosome (APC/C) and the associated substrate-binding subunit Cdc20 targets separase inhibitor securin to degradation and allows separase to resolve the sister chromatids cohesion (Peters, 2002). The spindle checkpoint inhibits APC/C^{Cdc20} until all chromosomes are properly attached to spindle microtubules (Yu, 2002). The spindle checkpoint proteins have been identified across eukaryotes, including Mad 1 (mitosis arrest deficient), Mad2, Bub 1 (budding uninhibited by benomyl), Bub3, BubR1/Mad3, and Mps 1 (monopolar spindle) (Hoyt et al., 1991; Li and Murray, 1991; Winey et al., 1991). There are two branches

of spindle checkpoint signals (Bharadwaj and Yu, 2004). One branch is mediated by various Cdc20 containing complexes: Mad2-Cdc20 (Fang et al., 1998a), BubR1-Bub3-Cdc20 (Fang, 2002; Tang et al., 2001), and BubR1-Bub3-Cdc20-Mad2 (also termed MCC for mitotic checkpoint complex) (Sudakin et al., 2001). These complexes bind to and stoichiometrically inhibit APC/C in mitosis. The other branch involves Bub1 serine/threonine kinase, which phosphorylates Cdc20 and inhibits APC/C catalytically (Tang et al., 2004).

Kinetochores perform important functions in mitosis, including microtubule attachment and spindle checkpoint signaling (Biggins and Walczak, 2003; Cleveland et al., 2003). Nearly all spindle checkpoint proteins localize to kinetochores in mitosis (Abrieu et al., 2001; Jablonski et al., 1998; Luo et al., 2002; Taylor and McKeon, 1997). It has been hypothesized that there is an inhibitory signal generated on the unattached kinetochores by the spindle checkpoint and the signal is emitted to inhibit APC/C in the cytoplasm (McIntosh, 1991). Elegant experiments using PtK1 cells have shown that a single unattached kinetochore is sufficient to generate the inhibitory "wait-anaphase signal" to delay anaphase onset (Rieder et al., 1995). When the unattached kinetochore is ablated by laser, the spindle checkpoint is relieved and the cell rapidly initiates chromosome segregation after congression (Rieder et al., 1995). A large body of evidence suggests that Mad2, Cdc20 and Cdc20-containing complexes are candidates for this signal. These complexes directly inhibit APC/C and shows dynamic localization on unattached kinetochores (Howell et al., 2004; Shah et al., 2004). Studied by fluorescence recovery after photobleaching (FRAP), Mad2, BubR1, and Bub3 were found dynamically exchanged on unattached kinetochores with a half-life of 21-23s (Howell et al., 2004). Cdc20 shows biphasic kinetics (Howell et al., 2004). 50% of Cdc20

exhibits rapid kinetics at unattached kinetochores with a half-life of 1-3s, and the other 50% exchanges at a similar rate as Mad2, BubR1 and Bub3 (Howell et al., 2004).

How the wait-anaphase signal is generated is still largely unknown. The fact that a single unattached kinetochore is sufficient to inhibit the entire cytosolic pool of APC/C suggests that a catalytic mechanism is likely employed to generate the wait-anaphase signal(s). Contrary to wait-anaphase signals, the catalytic components may not dynamically associate with kinetochores. Based on the observation that Mad1 and Bub1 show almost no exchange on unattached kinetochores by FRAP (Howell et al., 2004; Shah et al., 2004), it has been proposed that Bub1, Mad1 and part of Mad2 form a catalytic platform for generation of wait-anaphase signals (Shah et al., 2004). Mad1/Mad2 heterodimer facilitates Mad2 to adopt the Cdc20-inhibitory conformation (Luo et al., 2002; Luo et al., 2004). Although Bub1 also inhibits APC/C by phosphorylating Cdc20 directly (Tang et al., 2004), the role of its kinase activity in the spindle checkpoint has not been addressed. Here we purify chromosomes with unattached kinetochores from nocodazole arrested HeLa cells and identify an ATP-dependent activity that inhibit APC/C^{Cdc20}. The mutant Cdc20 that is not phosphorylated by Bub1 cannot be inhibited by the metaphase chromosomes, indicating that Bub1 is likely the inhibitory kinase for APC/C^{Cdc20} from unattached kinetochores. Furthermore, wild-type Bub1 rescues the spindle checkpoint response in cells depleted of Bub1 by RNA interference (RNAi), but the kinase-dead Bub1 cannot, demonstrating that the kinase activity of Bub1 is required for proper spindle checkpoint signaling.

Materials and methods

Antibodies, and immunoblotting

The production of the Bub1, BubR1, Mad1, Mad2, APC3 (Cdc27) and APC2 antibodies were described previously (Fang et al., 1998b; Tang et al., 2001). The cyclin B1, Plk1 and Cdk1 antibody were purchased from Santa Cruz Biotechnology. Myc antibody (9E10) was from Roche. For immunoblotting, antibodies were used at 1:1000 dilution for crude sera or 1 μg/ml for purified IgG.

Tissue culture, purification of metaphase chromosomes and transfection

HeLa S3 and Tet-on (Clontech) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and 10 mM L-glutamine. To arrest cells at G1/S, cells were incubated in the growth medium containing 2 mM thymidine (Sigma) for 18 hours. To obtain mitotic cells, the cultured cells were treated with 100 ng/ml nocodazole (Sigma) for 16-18 hours.

To purify the metaphase chromosomes, metaphase arrested HeLa S3 cells were swollen for 5 minutes in ten volumes of PME buffer (5 mM Pipes/NaOH pH 7.2, 5 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA) two times and kept at 4°C for the following steps. Cells were pelleted at 1000g for 5 minutes and the pellet was resuspended in five volumes of lysis buffer (1×PME, 1% thiodiethylene glycol, 2.5 μM microcystin-LR, 1 μM okadaic acid, 1 mM ATP, 10 μg/ml cytochalasinB, 0.2% digitonin, and 10 μg/ml each of leupeptin, pepstatin and chymostatin) and lysed using a Dounce homogenizer. The lysate was then put on top of 30 ml sucrose step gradients consisting of 2 ml of HSS (1×PME, 1% thiodiethylene glycol, 1

μM microcystin-LR, 1 mM ATP, 1.8 M sucrose, and 10 μg/ml each of leupeptin, pepstatin and chymostatin) at the bottom and 28 ml of LSS (1×PME, 1% thiodiethylene glycol, 1 μM microcystin-LR, 1 mM ATP, 0.9 M sucrose, 0.02% digitonin and 10 μg/ml each of leupeptin, pepstatin and chymostatin) at the top. After centrifugation for 30 minutes at 400 rpm, the chromosome pellet was recovered from the bottom and combined with wash buffer (1×PME, 0.25% thiodiethylene glycol, 1 μM microcystin-LR, 1 mM ATP, and 10 μg/ml each of leupeptin, pepstatin and chymostatin), 1.6 ml of 0.2 M spermedine, and 0.8 ml of 0.2 M spermine to give a final volume of 49 ml. After incubation for 5 minutes, 31 ml of percoll was added. The mixture was dounced and then centrifuged for 30 minutes at 21,000 rpm. Chromosomes were recovered from a diffuse band 1 centimeter above the bottom of the tubes. They were mixed with 35 ml of wash buffer in a 50 ml conical tube. After putting 0.3 ml storage solution (1×PME, 70% glycerol) to the bottom, the tube was spun for 30 minutes at 3300 rpm. The wash was repeated once but in a 15 ml tube and centrifuge at 2200 rpm. The chromosomes were then solubilized by sonication in XB buffer (10 mM HEPES pH 7.7, 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 50 mM Sucrose) with or with out 1 mM ATP. The chromosome fraction was then cleared by centrifugation at 13,000 rpm for 30 minutes and the supernatant was used in *in vitro* ubiquitination assays or stored in aliquots at -80°C.

For the rescue experiments, HeLa Tet-on cells were transfected with Bub1 expressing plasmids and Bub1 siRNA, treated with nocodazole at 30 hours after transfection and then analyzed additional 18 hours later. Transfection was performed when the cell reached about 30% confluency using the Effectene reagent (Qiagen) according to the manufacturer's

instructions. The siRNA oligonucleotides targeting human Bub1 (GAGUGAUCACGAUUUCUAUTT) was chemically synthesized by Dharmacon. The plasmids were derived from pCS2-Myc.

In vitro ubiquitination assays

The expression and purification of human BubR1/Bub3 complex, Bub1/Bub3 complex, Cdc20 and Cdh1 proteins from Sf9 cells were described previously (Tang et al., 2001). The interphase APC/C was purified from ten volumes of interphase *Xenopus* egg extracts using anti-APC3/Cdc27 antibody coupled to protein-A support. After washing with high salt XB buffer (10 mM HEPES pH 7.7, 500 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM sucrose, and 0.5% NP-40) five times and with XB buffer two times, the APC/C beads were incubated for 1 hour at room temperature with 100 nM of recombinant Cdc20 or Cdh1 proteins that had been preincubated with 100 nM of Bub1/Bub3, BubR1/Bub3, or 2.5-10 μg of metaphase chromosome lysate. The APC/C beads were again washed twice with XB buffer and assayed by ubiquitinating a fragment of human cyclin B (residue 1-102) fused with Myc tag. Each ubiquitination assay contained a 5 μl mixture of an energy-regenerating system, 150 μM of bovine ubiquitin, 5 μM of the Myc-tagged N-terminal fragment of cyclin B, 5 μM of recombinant E1, 2 μM of recombinant UbcH10, and 3 μl of the APC/C beads. The reactions were incubated under constant shaking for 1 hour at room temperature, quenched with SDS sample buffer, resolved by SDS-PAGE, and analyzed by anti-Myc western blot.

In vitro kinase assays

The expression and purification of kinase domain of human Bub1 (Bub1C) from Sf9 cells were performed exactly as described previously (Tang et al., 2001). The 6×His tagged Cdc20 N-terminal fragment (1-170) and mutant fragments were expressed in bacterial and purified. The kinase assays were performed in 20 μl reactions. Each reaction contains 100 μM cold ATP, 0.1 μCi/μl ^{32}P-ATP, 1 μM Cdc20NT mutant protein substrates and 100 nM Bub1-Bub3 or Bub1C in kinase buffer (50 mM Tris-HCl pH7.7, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT).

For the immunoprecipitation (IP) kinase assays, affinity-purified rabbit α-Bub1 was coupled to Affi-Prep Protein A beads (Bio-Rad) at a concentration of 1 mg/ml. HeLa cells were lysed with the lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM DTT, 0.5 μM okadaic acid, and 10 μg/ml each of leupeptin, pepstatin and chymostatin). After clearing by centrifugation for 30 minutes at 4˚C at 13,000 rpm, the lysate was incubated with the antibody beads for 2 hours at 4˚C. The beads were washed with the wash buffer (50 mM Tris-HCl, pH 7.7, 500 mM NaCl, 0.5% NP-40) for three times and with kinase buffer for two times. The proteins bound to the beads were then used directly in the kinase reactions instead of the recombinant kinases. The reactions were incubated at room temperature for 30 minutes, quenched with SDS sample buffer, and analyzed by SDS-PAGE followed by autoradiography and immunoblot with Bub1 antibody.

Results

An APC/C^{Cdc20} inhibitory activity exists in purified metaphase chromosomes

To study the wait-anaphase signal generating mechanism, we purified metaphase chromosomes from a large scale of nocodazole arrested HeLa S3 cells using a modification of a method developed by Gasser and Laemmli (Figure 1A, see Experimental procedures for details) (Gasser and Laemmli, 1987). Metaphase chromosomes solubilized in the presence of 1 mM ATP were tested for the activity to inhibit APC/C^{Cdh1} and APC/C^{Cdc20} in a reconstituted *in vitro* ubiquitination system. APC/C with basal activity was immunopurified from interphase *Xenopus* egg extracts and incubated with human Cdc20 or Cdh1 in the presence of chromosome lysate, Bub1-Bub3 or BubR1-Bub3 complexes. Then purified ubiquitin-activating enzyme (E1), UbcH10, Ubiquitin, ATP regenerating system and a cyclin B fragment (residue 1-102) were mixed in the reaction to support the ubiquitination reaction. Both Cdc20 and Cdh1 stimulated the activity of interphase APC/C, evidenced by a decrease of unubiquitinated cyclin B and the appearance of ubiquitinated species (Figure 1B, lane 2 and 6). Consistent with earlier reports, BubR1 and Bub1 inhibited the activity of APC/C^{Cdc20} but not that of APC/C^{Cdh1} (Figure 1B, lane 4, 5, 8, and 9) (Tang et al., 2001; Tang et al., 2004). Interestingly, the chromosome lysate showed a similar inhibitory effect as Bub1 and BubR1 (Figure 1B, lane 3 and 7).

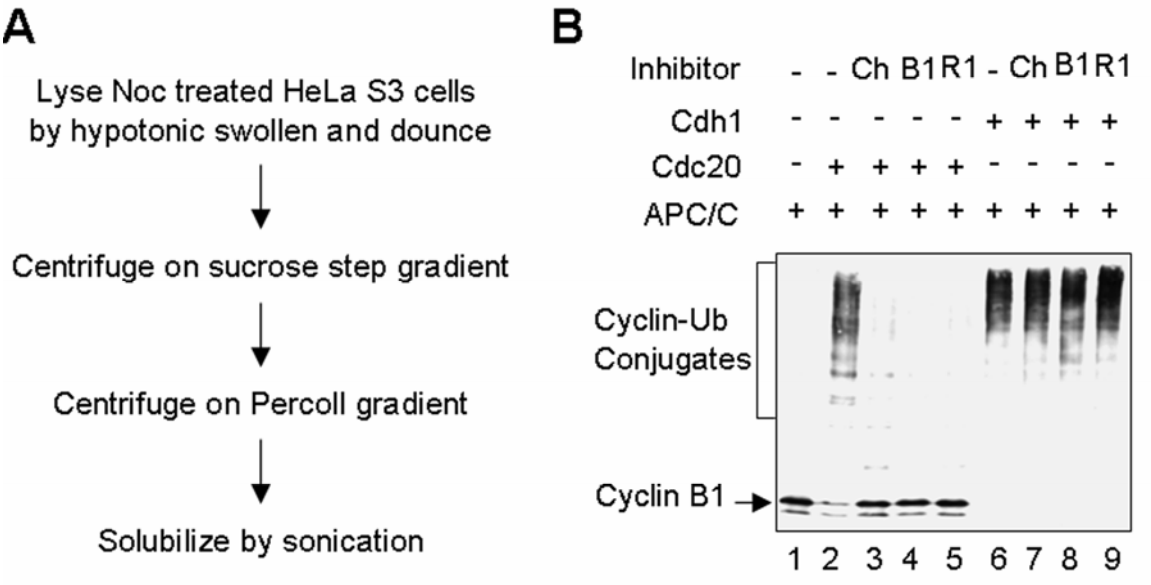


Figure 1. An APC/C^{Cdc20} inhibitory activity is copurified with the metaphase chromosomes (A) Scheme of the biochemical fractionation method to isolate metaphase chromosomes. See Experimental procedures for details. (B) APC/C was isolated from interphase *Xenopus* egg extracts and incubated with recombinant human Cdc20 or Cdh1 proteins, which were preincubated with buffer (lane 2 and 6), or 5 μg of chromosome fraction (Ch, lane 3 and 7), or recombinant Bub1-Bub3 (B1, lane 4 and 8), or BubR1-bub3 (R1, lane 5 and 9) in the presence of ATP. The ubiquitination activity was assayed with a Myc-tagged fragment (residue 1-102) of human cyclin B1. The reaction mixture was separated on SDS-PAGE and blotted with anti-Myc antibody. The positions of the cyclin B1 substrate and the cyclin B1-ubiquitin conjugates are labeled.

The APC/C^{Cdc20}-inhibitory activity is ATP-dependent and acts directly on Cdc20

Since we used ATP in the chromosome solubilization buffer, we next omitted it in solubilization buffer to test whether the inhibitory activity was ATP-dependent. We also preincubated the chromosome lysate with immunoprecipitated APC/C or Cdc20 protein first to examine whether the APC/C inhibitory activity on the chromosomes directly worked on

APC/C or Cdc20. To rule out the effect of ATP brought into the system during the ubiquitination assay, we used AMP-PNP in the following ubiquitination assays. AMP-PNP supports ubiquitination reactions, but does not support kinase reactions. When interphase APC/C was preincubated with the chromosome lysate in the presence or absence of ATP, the activity of APC/C ^{Cdc20} was not inhibited (Figure 2, lane 3-8). In contrast, preincubation of Cdc20 with the chromosome lysate in the presence of ATP caused a great reduction in the activity of APC/C ^{Cdc20} (Figure 2, lane 12-14), which was not observed when the preincubation was performed in the absence of ATP (Figure 2, lane 9-11). This result suggests that the activity from metaphase chromosomes inhibits APC/C ^{Cdc20} in an ATP-dependent manner and it directly acts on Cdc20.

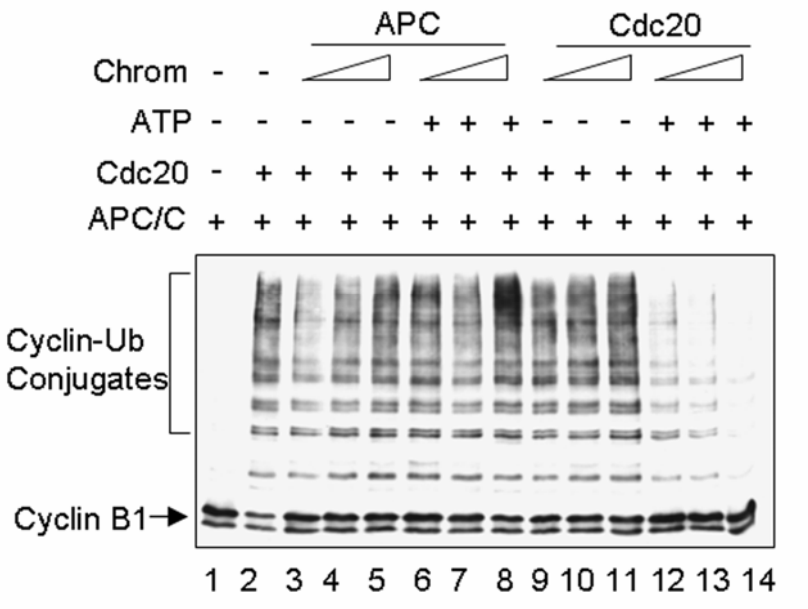


Figure 2. The APC/C^{Cdc20} inhibitory activity from metaphase chromosomes requires ATP and Cdc20. APC/C isolated from interphase *Xenopus* egg extracts and recombinant human Cdc20 were incubated respectively with solubilized metaphase chromosome fraction in the absence (lane 3-5 and 9-11) or presence (lane 6-8 and 12-14) of ATP. Then human Cdc20 protein (lane 3-8) or interphase APC/C (lane 9-14) were added and further incubated. The ubiquitination activity was assayed with a Myc-tagged fragment (residue 1-102) of

human cyclin B1. The reaction mixture was separated on SDS-PAGE and blotted with anti-Myc antibody. The positions of the cyclin B1 substrate and the cyclin B1-ubiquitin conjugates are labeled.

S153 of Cdc20 is the major Bub1 phosphorylation site and it is required for inhibition by metaphase chromosomes

Previous studies have shown that the Mad2, BubR1-Bub3, and Bub1-Bub3 complexes can inhibit the activity of APC/C ^{Cdc20} in *in vitro* ubiquitination assays (Fang et al., 1998a; Tang et al., 2001; Tang et al., 2004). Mad2 and BubR1-Bub3 inhibit APC/C ^{Cdc20} by direct interaction and do not depend on the presence of ATP. Bub1-Bub3 inhibits APC/C ^{Cdc20} by phosphorylating Cdc20, which requires ATP. To test whether the APC/C inhibitory activity from metaphase chromosomes is caused by Bub1, we need a mutant Cdc20 that cannot be phosphorylated by Bub1. Bub1 phosphorylates Cdc20 on six serine/threonine residues, all of which are located in the N-terminal domain of Cdc20 (Figure 3A) (Tang et al., 2004). It is unclear whether the six residues are phosphorylated similarly or there are one or several major phosphorylation sites. We thus mapped the major Bub1 phosphorylation sites by *in vitro* kinase assay. A human Bub1 fragment (residue 724-1085) containing the kinase domain (Bub1C) is expressed and purified from Sf9 cells. An N-terminal fragment of Cdc20 (residue 1-170, named Cdc20NT) was constructed and used as the positive control (Figure 3A). A mutant Cdc20NT with all the six residues mutated to alanine (Cdc20NT-M) was used as the negative control (Figure 3A). On the basis of Cdc20NT-M, individual residue was mutated back to serine or threonine and their phosphorylation by Bub1C was tested (Figure 3B). Significantly, the Cdc20NT-MS153 was phosphorylated by Bub1 similarly as Cdc20NT,

while the other mutants showed little phosphorylation, although similar amounts of proteins were present in the reaction (Figure 3B). Similar results were obtained using full length Bub1 protein from Sf9 cells (data not shown). Therefore, S153 is the major Bub1 phosphorylation site on Cdc20.

We next tested whether the metaphase chromosomes inhibited the S153A mutant Cdc20, which cannot be phosphorylated by Bub1. The Cdc20-S153A mutant was purified from Sf9 cells and used in the APC/C assay. The activity of Cdc20-S153A was similar as wild type Cdc20, but was not inhibited by purified Bub1 or the metaphase chromosome lysate (Figure 3C, lane 2, 5-7). This result suggests that Bub1 is very likely responsible for the inhibitory activity from metaphase chromosomes.

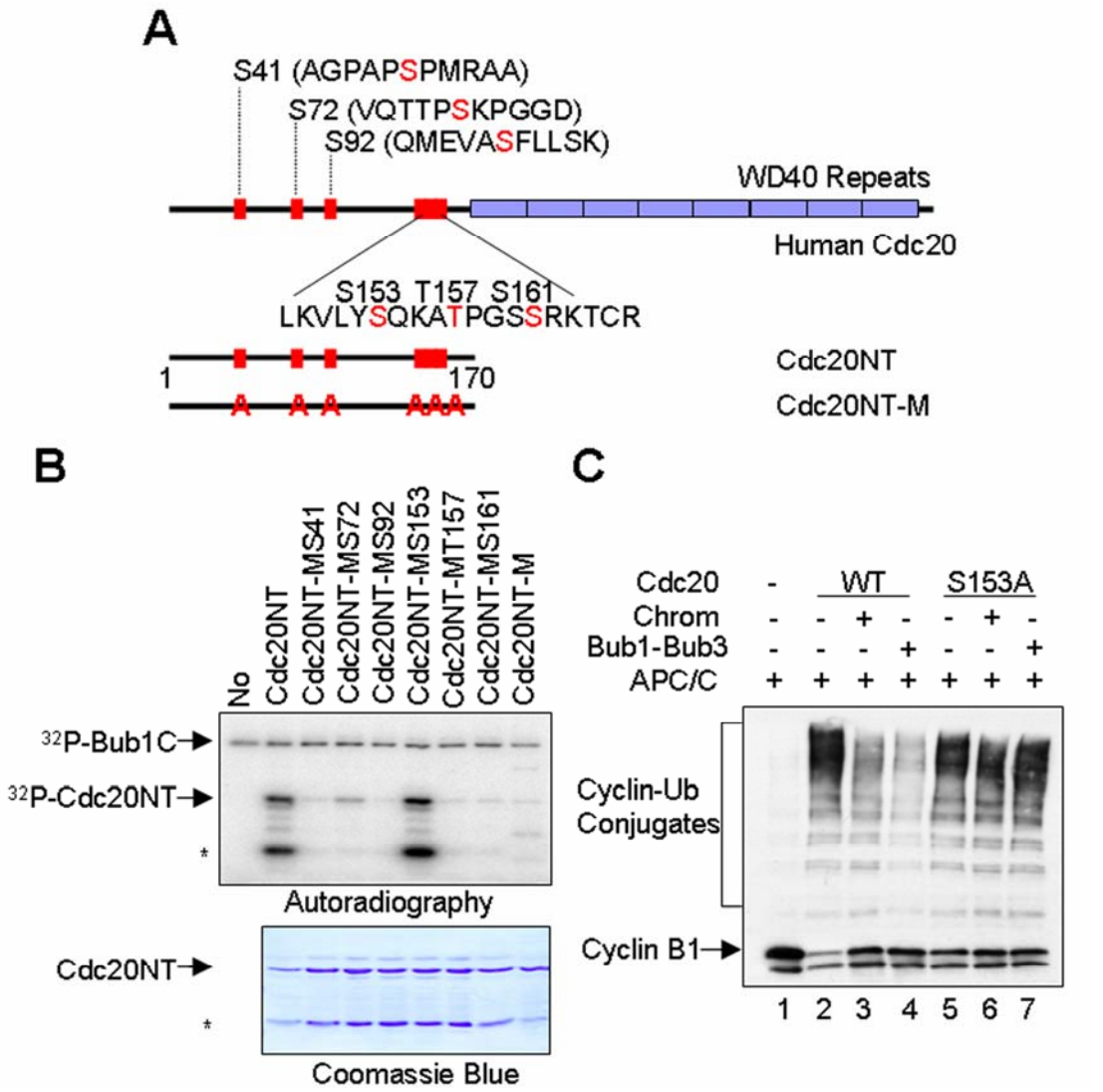


Figure 3. S153 is the major Bub1 phosphorylation site on Cdc20 and is required for inhibition by metaphase chromosomes. (A) Schematic diagram of Cdc20 showing the Bub1 phosphorylation sites. The Cdc20 truncations used in the following experiments are labeled. (B) Bub1 kinase domain (Bub1C) was incubated with Cdc20NT mutant proteins in the presence of γ-^{32}P-ATP for 30 minutes at room temperature. The reaction was quenched with SDS sample buffer, separated on SDS-PAGE, and analyzed by autoradiography. The positions of phosphorylated Bub1C and Cdc20NT are indicated. The bottom panel is a Coomassie stained gel showing similar amounts of Cdc20NT were in the reactions. (C) Interphase APC/C was isolated and incubated with recombinant human Cdc20 or Cdc20-S153A proteins, which were preincubated with buffer (lane 2 and 5), or 5 μg of chromosome fraction (lane 3 and 6), or recombinant Bub1-Bub3 (lane 4 and 7) in the presence of ATP. The ubiquitination activity was assayed with a Myc-tagged fragment (residue 1-102) of human cyclin B1. The reaction mixture was separated on SDS-PAGE and blotted with anti-

Myc antibody. The positions of the cyclin B1 substrate and the cyclin B1-ubiquitin conjugates are labeled.

Human Bub1 on unattached kinetochores is hyperphosphorylated and highly activated

It has been shown previously that the Bub1 protein from nocodazole-arrested HeLa cells is phosphorylated and upshifted on SDS-PAGE (Tang et al., 2004; Taylor et al., 2001). Furthermore, the *Xenopus* Bub1 protein on chromosome from nocodazole arrested egg extracts is hyperphosphorylated and shows higher auto-phosphorylation activity (Chen, 2004). Next, we detected the Bub1 protein in the metaphase chromosome lysate. Bub1 was upshifted in nocodazole arrested mitotic cells, compared with the Bub1 from thymidine arrested G1/S cells (Figure 4A, lane 1 and 2). Interestingly, the mobility of Bub1 on metaphase chromosomes with unattached kinetochores was further reduced (Figure 4A, lane 3) compared to that of Bub1 from mitotic cells. This upshift was completely abolished when the chromosome fraction was treated with λ–phosphatase (Figure 4A, lane 4), indicating it was a result of phosphorylation. Similarly, BubR1 and Mps1 proteins from metaphase chromosomes were also hyperphosphorylated and dramatically upshifted (Figure 4A). This is consistent with earlier reports by Chen *et al* (Chen, 2004). In contrast, the mobilities of Mad1, Mad2 (Figure 4A) and Bub3 (data not shown) were not drastically changed, suggesting that events other than phosphorylation, such as interaction with other proteins, are responsible for their activation upon spindle checkpoint activation. Furthermore, cyclin B, Cdk1 and Mad2 were enriched on metaphase chromosomes. Previous immunostaining studies have shown that the kinetochore localization of Mad2 is enhanced when microtubules

are depolymerized by nocodazole (Waters et al., 1998). However, the Cdk1-cyclin B complex has not been reported to localize to unattached kinetochores or mitotic chromosomes (Yang et al., 1998). It requires further study to demonstrate why and how Cdk1-cyclin B is recruited to metaphase chromosomes.

To further test whether the hyperphosphorylated Bub1 was more active, we immunoprecipitated Bub1 protein from G1/S cells, mitotic cells and metaphase chromosome lysate using Bub1 antibody and performed kinase assays. There was little phosphorylation of Cdc20NT-MS153 by interphase Bub1 (Figure 4B, lane 4). The activity of mitotic Bub1 was increased about two fold (Figure 4B, lane 5). Bub1 from metaphase chromosomes was about 7 fold more active than similar amount of Bub1 from mitotic cells. Our result suggests that Bub1 is hyperphosphorylated and highly activated on unattached kinetochores of metaphase chromosomes.

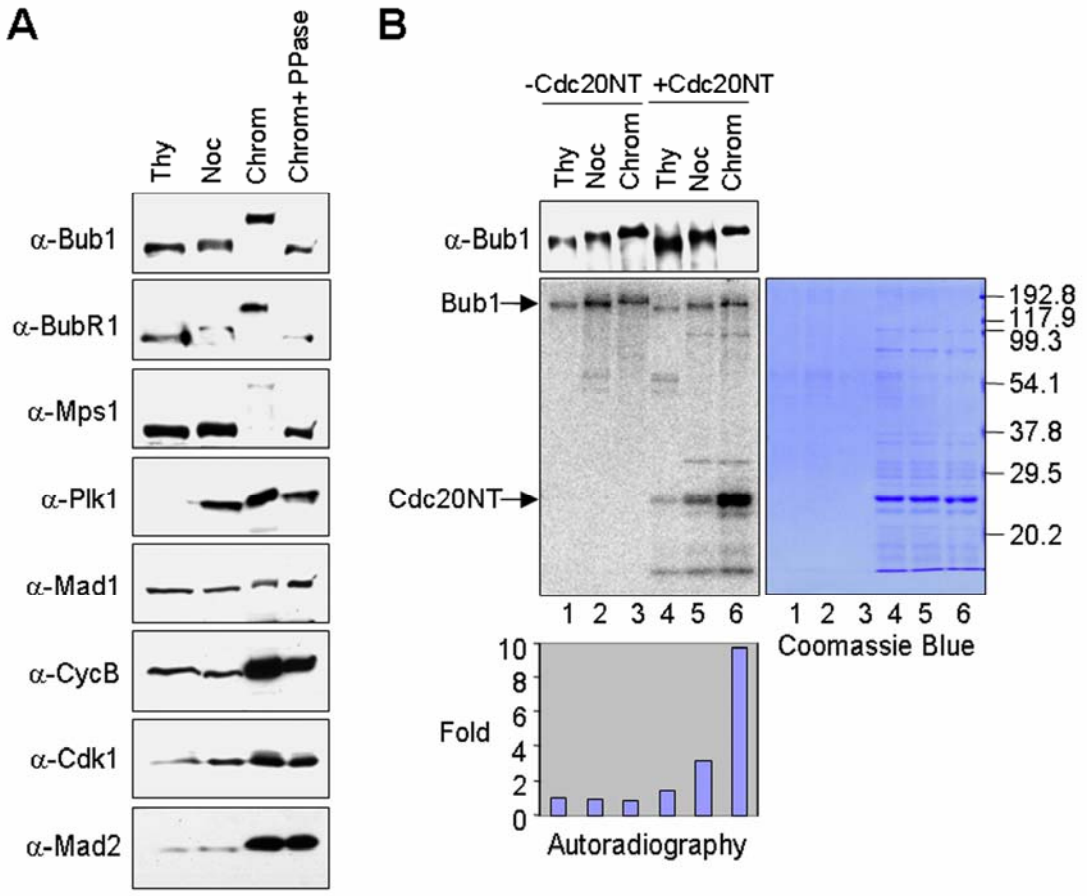


Figure 4. Bub1 on unattached kinetochores is hyperphosphorylated and activated. (A) Thymidine-arrested (Thy) HeLa cells, nocodazole-arrested (Noc) cells, metaphase chromosome lysate (chrom), or λ-phosphatase treated metaphase chromosome lysate (chrom+PPase) were dissolved in SDS sample buffer, separated on SDS-PAGE, and blotted with the indicated antibodies. (B) Endogenous Bub1 protein was immunoprecipitated from thymidine-arrested HeLa cells, nocodazole-arrested cells, or solubilized metaphase chromosome and incubated with (lane4-6) or without (lane 1-3) Cdc20NT-MS153 in the presence of γ-^{32}P-ATP for 30 minutes at room temperature. The reaction was quenched with SDS sample buffer, separated on SDS-PAGE, and analyzed by autoradiography (left middle panel), Coomassie staining (right panel), and anti-Bub1 blot (left top panel). The positions of phosphorylated Bub1 and Cdc20NT-MS153 are indicated. The right panel shows similar amounts of Cdc20NT-MS153 protein existed in each reaction and the protein size markers are indicated. The top panel shows the same amounts of Bub1 protein were present in each reaction. The bands on the radiogram were quantified and the results are shown in the left bottom panel.

The kinase activity of Bub1 is required for spindle checkpoint signaling in HeLa cells

Although it has been shown that Bub1 is required for the spindle checkpoint and Bub1 phosphorylates Cdc20 and inhibit its activity (Tang et al., 2004), the role of the kinase activity of Bub1 in spindle checkpoint signaling has not been addressed. There were conflicting results about the requirement of the kinase activity of Bub1 (Sharp-Baker and Chen, 2001; Yamaguchi et al., 2003). We sought to answer this question by rescuing Bub1 RNAi. Myc-Bub1-WT (wild type) and Myc-Bub1-KD (kinase dead) constructs with silent mutations resistant to Bub1 RNAi were transfected together with Bub1 siRNA into HeLa cells and mitotic arrests by nocodazole were analyzed. The endogenous Bub1 protein was significantly depleted by the siRNA transfection and the transfected Myc-Bub1-WT and Myc-Bub1-KD were expressed at similar levels as the endogenous Bub1 (Figure 5A, top panel). In the presence of nocodazole, Bub1-RNAi caused decrease of the securin protein and phosphorylated histone H3 levels (Figure 5A) and mitosis index (Figure 5B and C). Ectopic expression of Myc-Bub1-WT in Bub1 depleted cells partially rescued all phenotypes. However, the expression of the Myc-Bub1-KD did not restore them in Bub1-RNAi cells (Figure 5A, B, and C), although both Myc-Bub1-KD and Myc-Bub1-WT proteins were localized to the kinetochores (data not shown). Thus, the kinase activity of Bub1 is required for efficient spindle checkpoint signaling in mammalian cells.

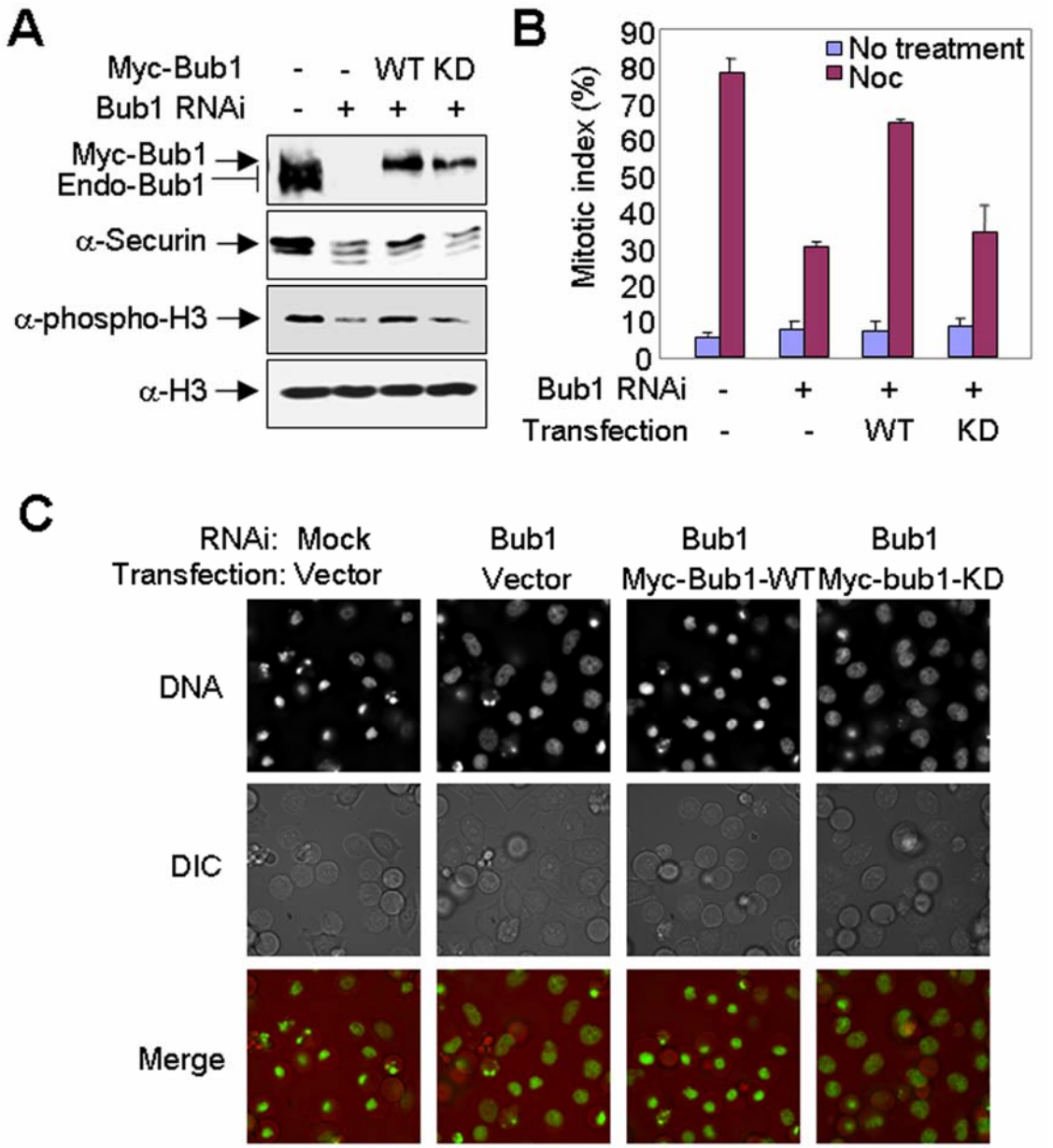


Figure 5. The kinase-dead mutant of Bub1 can not restore the spindle checkpoint in Bub1-RNAi cells. (A) HeLa Tet-on cells were transfected with the combination of Bub1 constructs and Bub1 siRNA for 30 hours, treated with nocodazole for 18 hours, followed by lysis in SDS sample buffer, separation on SDS-PAGE and analysis using anti-Bub1, anti-securin, anti-phospho-Histone H3, and anti-Histone antibodies. (B) HeLa cells similarly transfected and treated as in (A) were stained with Hoechst 33342 and directly visualized using an inverted fluorescence microscope. The mitotic index was measured by counting three fields of more than 100 cells. (C) Representative fields of the cells described in (B). Both the cell (DIC) and DNA morphologies are shown.

Discussion

Bub1 is a wait-anaphase signaling catalyst

We have isolated metaphase chromosomes with unattached kinetochores and studied the mechanism of generating spindle checkpoint signals. Our results show that Bub1 is involved in this mechanism by phosphorylating Cdc20. First, the ATP-dependent inhibitory activity from unattached kinetochores works on Cdc20 directly, rather than APC/C (Figure 1 and 2). Second, the Cdc20-S153A protein, which cannot be efficiently phosphorylated by Bub1, is not inhibited by the activity from unattached kinetochores (Figure 3). Third, the kinase activity of Bub1 is greatly enhanced on unattached kinetochores compared to Bub1 from interphase cells (Figure 4B). Fourth, the kinase-dead Bub1 fails to restore spindle checkpoint in Bub1-RNAi cells (Figure 5). Taken together, our data strongly suggest Bub1 phosphorylates Cdc20 and inhibits APC/C ^{Cdc20} on the unattached kinetochore. The fact that Bub1 from nocodazole-arrested cells is only about two folds higher than Bub1 from interphase cells indicates that the Bub1 molecules in metaphase cell are not equally active; the cytosolic pool of Bub1 is less active although it constitutes the majority of the Bub1 population.

There are some controversial results about whether the kinase activity of Bub1 is required for the spindle checkpoint. A budding yeast strain harboring the kinase-dead point mutation of Bub1 is only partially defective in mitotic spindle checkpoint arrest (Warren et al., 2002). Chen and colleagues reported that the kinase-dead Bub1 is able to recruit BubR1

and Mad2 protein to unattached kinetochores and support the spindle checkpoint in the presence of 10 ng/μl of nocodazole in *Xenopus* egg extracts (Sharp-Baker and Chen, 2001), but it cannot support the spindle checkpoint in the presence of 1 ng/μl of nocodazole (Chen, 2004). Since the *Xenopus* egg extract is an *in vitro* system, there is no easy way to evaluate which concentration of nocodazole is more physiologically relevant. However, the kinase domain of Bub1 is highly conserved among species (Farr and Hoyt, 1998). Yamaguchi and colleagues have shown that the kinase activity of fission yeast Bub1 is required for the spindle checkpoint signaling to maintain cell viability in both mitosis and meiosis I (Yamaguchi et al., 2003). We showed here that the wild-type, but not kinase-dead mutant of Bub1 restores the spindle checkpoint response in Bub1-RNAi cells by three criteria: the degradation of securin protein, the dephosphorylation of histone H3, and the decondensation of chromosome in the presence of nocodazole (Figure 5). Therefore, our results demonstrate that the kinase activity of Bub1 is required for the spindle checkpoint response in mammalian cells.

How is Bub1 activated by phosphorylation? There are two non-exclusive possibilities. Phosphorylation may directly increase its intrinsic activity (increase k_{cat}). Alternatively, phosphorylation can enhance the interaction between Bub1 and its substrates (decrease K_m). All known phosphorylation sites are located outside of the kinase domain. These phosphorylation events may alter the conformation of Bub1 to relief autoinhibition, or promote the interaction of Bub1 to its substrates, such as Cdc20.

Cdc20 containing complexes and modified Cdc20 constitute the wait-anaphase signals

There are multiple candidates for the nature of the wait-anaphase signal. Mad2, BubR1, and MCC complex have all been demonstrated to inhibit APC/C^{Cdc20} directly by interacting with Cdc20 (Bharadwaj and Yu, 2004; Yu, 2002). Our lab also showed that Bub1 can phosphorylate Cdc20 and inhibit APC/C^{Cdc20} (this study) (Tang et al., 2004). Since Cdc20 is the target of the spindle checkpoint, it is likely that the Cdc20 containing complexes, including Mad2-Cdc20, Bub3-BubR1-Cdc20, and MCC, and the phosphorylated Cdc20 constitute the wait-anaphase signals. The evidence gained by studying the dynamic kinetochore-association of spindle checkpoint proteins strongly supports this notion. The recovery of GFP-Cdc20 signal on kinetochore after photobleaching shows two phases: a slower phase has similar time as the recovery of Mad2, BubR1 and Bub3 that may reflect the formation and release of Cdc20 containing complexes; a fast phase likely corresponds to free Cdc20 that is phosphorylated by Bub1 on the kinetochores (Howell et al., 2004).

That Cdc20-S153A mutant cannot be inhibited by metaphase chromosome lysate strongly suggests that Bub1 is the inhibitory kinase from metaphase chromosomes. However, we cannot rule out the possibility that other enzymes are involved. Since the inhibition requires S153 and is ATP-dependent, it is very likely that the inhibition is mediated by phosphorylation on S153. Although Cdk1 and MAPK have been reported to phosphorylate Cdc20 (Chung and Chen, 2003; D'Angiolella et al., 2003), S153 is in an SQ motif and it does not fit in the consensus phosphorylation site for either kinases. It fits the consensus sequence of ATM/ATR kinase (Kim et al., 1999). However, our system does not contain Mn^{2+}, which

is required for ATM/ATR (Kim et al., 1999). Further study will clarify whether these kinases play any role in Cdc20 inhibition.

How can phosphorylation block the activity of Cdc20? Since Cdc20 is involved in substrate recognition of APC/C, one possibility is the phosphorylated Cdc20 can no longer interact with APC/C or substrates. The WD40 repeats of Cdh1 have been reported to constitute the substrate-binding surface. The major Bub1 phosphorylation site on Cdc20 (S153) is on the N-terminus and outside of the WD40 repeats. Therefore, it is likely that the phosphorylation impairs the Cdc20-APC/C interaction. Consistently, it has been reported that Cdk1 phosphorylates Cdc20 and inhibits its activity by preventing APC/C binding (D'Angiolella et al., 2003). The interaction between Cdc20 and APC/C can be tested by *in vitro* binding assays following *in vitro* Bub1 phosphorylation. The co-immunoprecipitation of Cdc20 and Cdc20-S153A with APC/C can also be compared using transfected mitotic cells. It is also possible that phosphorylation of Cdc20 leads to a conformational change in Cdc20. The interaction between Cdc20 and D-box containing Cyclin B fragment can be examined using *in vitro* binding assays. Meanwhile, an anti-phospho-S153-Cdc20 antibody will be useful for obtaining crucial *in vivo* evidence for the mechanisms of Cdc20 inhibition.

It has been proposed that the APC/C complex is hyperphosphorylated by unattached kinetochores in mitosis and may be sensitized to the inhibition of the spindle checkpoint (Sudakin et al., 2001). Indeed, APC/C is phosphorylated by Cdk1 and Plk1 in mitosis (Kraft et al., 2003). However, this phosphorylation activates APC/C^{Cdc20} rather than inhibiting it (Kraft et al., 2003). Consistently, metaphase chromosome lysate does not affect the activity of APC/C when it is preincubated with APC/C (Figure 2). This result rules out the possibility

that the inhibitory activity on metaphase chromosomes functions by modifying APC/C. Our system is an *in vitro* biochemical purified ubiquitination assay. It remains possible that some other enzymes modify APC/C and sensitize it to checkpoint inhibition in cells.

Although BubR1 and Mad2 were detected in the metaphase chromosome lysate (Figure 4A), we were not able to capture their inhibitory activity in our ubiquitination assays. One possibility is that the molecular ratio between BubR1 and Cdc20, and Mad2 and Cdc20 in the reaction is too low, since BubR1 and Mad2 function in a stoichiometric manner. To further test this possibility, a quantitative western blot has to be carried out to quantify the amount of BubR1 and Mad2 in the metaphase chromosome fraction. By calculation using the quantitative western blot data from Tang et al (Tang et al., 2001), the amount of BubR1 and Mad2 in metaphase chromosome fraction used in our ubiquitination system was estimated as about 12.31 fmol and 833.33 fmol. Both these concentrations are lower than the amount of Cdc20 (2000 fmol) in the system.

In summary, we have identified an activated form of Bub1 kinase as a potential APC/C^{Cdc20} inhibitor from metaphase chromosomes and demonstrated the importance of the kinase activity of Bub1 for spindle checkpoint signaling.

References

Abrieu, A., Magnaghi-Jaulin, L., Kahana, J. A., Peter, M., Castro, A., Vigneron, S., Lorca, T., Cleveland, D. W., and Labbe, J. C. (2001). Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. Cell *106*, 83-93.

Bharadwaj, R., and Yu, H. (2004). The spindle checkpoint, aneuploidy, and cancer. Oncogene *23*, 2016-2027.

Biggins, S., and Walczak, C. E. (2003). Captivating capture: how microtubules attach to kinetochores. Curr Biol *13*, R449-460.

Chen, R. H. (2004). Phosphorylation and activation of Bub1 on unattached chromosomes facilitate the spindle checkpoint. Embo J *23*, 3113-3121.

Chung, E., and Chen, R. H. (2003). Phosphorylation of Cdc20 is required for its inhibition by the spindle checkpoint. Nat Cell Biol *5*, 748-753.

Cleveland, D. W., Mao, Y., and Sullivan, K. F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell *112*, 407-421.

D'Angiolella, V., Mari, C., Nocera, D., Rametti, L., and Grieco, D. (2003). The spindle checkpoint requires cyclin-dependent kinase activity. Genes Dev *17*, 2520-2525.

Fang, G. (2002). Checkpoint protein BubR1 acts synergistically with Mad2 to inhibit anaphase-promoting complex. Mol Biol Cell *13*, 755-766.

Fang, G., Yu, H., and Kirschner, M. W. (1998a). The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. Genes Dev *12*, 1871-1883.

Fang, G., Yu, H., and Kirschner, M. W. (1998b). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol Cell *2*, 163-171.

Farr, K. A., and Hoyt, M. A. (1998). Bub1p kinase activates the Saccharomyces cerevisiae spindle assembly checkpoint. Mol Cell Biol *18*, 2738-2747.

Gasser, S. M., and Laemmli, U. K. (1987). Improved methods for the isolation of individual and clustered mitotic chromosomes. Exp Cell Res *173*, 85-98.

Howell, B. J., Moree, B., Farrar, E. M., Stewart, S., Fang, G., and Salmon, E. D. (2004). Spindle checkpoint protein dynamics at kinetochores in living cells. Curr Biol *14*, 953-964.

Hoyt, M. A., Totis, L., and Roberts, B. T. (1991). S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. Cell *66*, 507-517.

Jablonski, S. A., Chan, G. K., Cooke, C. A., Earnshaw, W. C., and Yen, T. J. (1998). The hBUB1 and hBUBR1 kinases sequentially assemble onto kinetochores during prophase with hBUBR1 concentrating at the kinetochore plates in mitosis. Chromosoma *107*, 386-396.

Kim, S. T., Lim, D. S., Canman, C. E., and Kastan, M. B. (1999). Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem *274*, 37538-37543.

Kraft, C., Herzog, F., Gieffers, C., Mechtler, K., Hagting, A., Pines, J., and Peters, J. M. (2003). Mitotic regulation of the human anaphase-promoting complex by phosphorylation. Embo J *22*, 6598-6609.

Li, R., and Murray, A. W. (1991). Feedback control of mitosis in budding yeast. Cell *66*, 519-531.

Luo, X., Tang, Z., Rizo, J., and Yu, H. (2002). The Mad2 spindle checkpoint protein undergoes similar major conformational changes upon binding to either Mad1 or Cdc20. Mol Cell *9*, 59-71.

Luo, X., Tang, Z., Xia, G., Wassmann, K., Matsumoto, T., Rizo, J., and Yu, H. (2004). The Mad2 spindle checkpoint protein has two distinct natively folded states. Nat Struct Mol Biol *11*, 338-345.

McIntosh, J. R. (1991). Structure and mechanical control of mitotic progression. Cold spring Harb Symp Quant Biol *56*, 613-619.

Peters, J. M. (2002). The anaphase-promoting complex: proteolysis in mitosis and beyond. Mol Cell *9*, 931-943.

Pinsky, B. A., and Biggins, S. (2005). The spindle checkpoint: tension versus attachment. Trends Cell Biol *15*, 486-493.

Rajagopalan, H., and Lengauer, C. (2004). Aneuploidy and cancer. Nature *432*, 338-341.

Rieder, C. L., Cole, R. W., Khodjakov, A., and Sluder, G. (1995). The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. J Cell Biol *130*, 941-948.

Shah, J. V., Botvinick, E., Bonday, Z., Furnari, F., Berns, M., and Cleveland, D. W. (2004). Dynamics of centromere and kinetochore proteins; implications for checkpoint signaling and silencing. Curr Biol *14*, 942-952.

Sharp-Baker, H., and Chen, R. H. (2001). Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. J Cell Biol *153*, 1239-1250.

Sudakin, V., Chan, G. K., and Yen, T. J. (2001). Checkpoint inhibition of the APC/C in HeLa cells is mediated by a complex of BUBR1, BUB3, CDC20, and MAD2. J Cell Biol *154*, 925-936.

Tang, Z., Bharadwaj, R., Li, B., and Yu, H. (2001). Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. Dev Cell *1*, 227-237.

Tang, Z., Shu, H., Oncel, D., Chen, S., and Yu, H. (2004). Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. Mol Cell *16*, 387-397.

Taylor, S. S., Hussein, D., Wang, Y., Elderkin, S., and Morrow, C. J. (2001). Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. J Cell Sci *114*, 4385-4395.

Taylor, S. S., and McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell *89*, 727-735.

Warren, C. D., Brady, D. M., Johnston, R. C., Hanna, J. S., Hardwick, K. G., and Spencer, F. A. (2002). Distinct chromosome segregation roles for spindle checkpoint proteins. Mol Biol Cell *13*, 3029-3041.

Waters, J. C., Chen, R. H., Murray, A. W., and Salmon, E. D. (1998). Localization of Mad2 to kinetochores depends on microtubule attachment, not tension. J Cell Biol *141*, 1181-1191.

Winey, M., Goetsch, L., Baum, P., and Byers, B. (1991). MPS1 and MPS2: novel yeast genes defining distinct steps of spindle pole body duplication. J Cell Biol *114*, 745-754.

Yamaguchi, S., Decottignies, A., and Nurse, P. (2003). Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint. Embo J *22*, 1075-1087.

Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998). Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. Genes Dev *12*, 2131-2143.

Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. Curr Opin Cell Biol *14*, 706-714.

CHAPTER FOUR

PHOSPHORYLATION- AND POLO-BOX-DEPENDENT BINDING OF PLK1 TO BUB1 IS REQUIRED FOR THE KINETOCHORE LOCALIZATION OF PLK1

Summary

Plk1 is required for the generation of the tension-sensing 3F3/2 kinetochore epitope and facilitates kinetochore localization of Mad2 and other spindle checkpoint proteins. Here we investigate the mechanism by which Plk1 itself is recruited to kinetochores. We show that Plk1 binds to Bub1 in mitotic human cells. The Plk1–Bub1 interaction requires the polo-box domain (PBD) of Plk1 and is enhanced by Cdk1-mediated phosphorylation of Bub1 at T609. The PBD-dependent binding of Plk1 to Bub1 facilitates phosphorylation of Bub1 by Plk1 *in vitro*. Depletion of Bub1 in HeLa cells by RNA interference (RNAi) diminishes the kinetochore localization of Plk1. Ectopic expression of the wild-type Bub1, but not the Bub1-T609A mutant, in Bub1-RNAi cells restores the kinetochore localization of Plk1. Our results suggest that phosphorylation of Bub1 at T609 by Cdk1 creates a docking site for the PBD of Plk1 and facilitates the kinetochore recruitment of Plk1.

Introduction

Polo-like kinases (Plks) are evolutionarily conserved protein serine/threonine kinases that play crucial roles during multiple stages of the cell cycle, especially in mitosis (Barr *et al*., 2004; Glover, 2005; Lee *et al*., 2005; Liu and Maller, 2005; Lowery *et al*., 2005; van Vugt and Medema, 2005; Xie *et al*., 2005). There are four Plk family members in mammals, Plk1-4 (Barr *et al*., 2004). Plk1 is the ortholog of *Drosophila* Polo and yeast Cdc5 and is the best studied Plk family member in mammals (Barr *et al*., 2004). Plk1 contains two signature domains: an N-terminal kinase domain and a C-terminal polo-box domain (PBD) that consists of two tandem polo-boxes (Lowery *et al*., 2005). The PBD of Plk1 functions as an independently folded domain that specifically recognizes phospho-serine/threonine containing peptides (Elia *et al*., 2003a; Elia *et al*., 2003b; Lowery *et al*., 2005). Binding of PBD to phospho-peptides has been shown to mediate the phosphorylation-dependent targeting of Plk1 to substrates, to activate the kinase activity of Plk1 allosterically, and to regulate the subcellular localization of Plk1 (Elia *et al*., 2003a; Elia *et al*., 2003b; Lowery *et al*., 2005; van Vugt and Medema, 2005).

Plk1 exerts its multiple functions in mitosis, including centrosome maturation, spindle assembly, cohesin removal, and cytokinesis, by phosphorylating a multitude of substrates (Barr *et al*., 2004; Lowery *et al*., 2005; van Vugt and Medema, 2005). For example, phosphorylation of cyclin B1, Cdc25, and Wee1 by Plk1 contributes to the activation of Cdk1, which in turn promotes the entry into mitosis (Kumagai and Dunphy, 1996; Toyoshima-Morimoto *et al*., 2001; Watanabe *et al*., 2004). Plk1 regulates centrosome maturation by phosphorylating the centrosomal protein, Nlp (ninein-like protein) and blocks

it targeting to centrosomes (Casenghi *et al*., 2003). In prophase, Plk1 is required for the removal of cohesin from chromosomal arms by phosphorylating the SA2 subunit of cohesin (Sumara *et al*., 2002; Hauf *et al*., 2005). Plk1 also phosphorylates Emi1, an inhibitor of the anaphase-promoting complex or cyclosome (APC/C), and mediates the degradation of Emi1 in early mitosis (Hansen *et al*., 2004; Moshe *et al*., 2004). During late mitosis, Plk1 interacts with and phosphorylates the central spindle proteins, MKLP1/2, Nir2, and NudC, which is required for the completion of cytokinesis (Neef *et al.*, 2003; Zhou *et al.*, 2003; Litvak *et al.*, 2004; Liu *et al.*, 2004).

More recently, several studies have revealed functions of Plk1 at the kinetochores and in the spindle checkpoint (Ahonen *et al*., 2005; Wong and Fang, 2005), a cell-cycle surveillance mechanism that ensures the fidelity of chromosome segregation (Cleveland *et al*., 2003; Bharadwaj and Yu, 2004). The spindle checkpoint prevents premature sister-chromatid separation by inhibiting the ubiquitin ligase activity of APC/C until all sister kinetochores have achieved bipolar attachment to the mitotic spindle and are therefore under mechanical tension (Yu, 2002). Lack of tension across sister kinetochores creates a yet unidentified phospho-epitope at these kinetochores that is recognized by the 3F3/2 monoclonal antibody (Cyert *et al*., 1988; Gorbsky and Ricketts, 1993; Nicklas *et al*., 1995). Plk1 has recently been shown to be responsible for generating the tension-sensing 3F3/2-phosphoepitope at the kinetochores (Ahonen *et al*., 2005; Wong and Fang, 2005). Furthermore, Plk1 facilitates the localization of Mad2 and other spindle checkpoint proteins to the kinetochores (Ahonen *et al*., 2005; Wong and Fang, 2005).

In keeping with its multiple mitotic functions, Plk1 localizes to key mitotic structures during various stages of mitosis (Barr *et al*., 2004; van Vugt and Medema, 2005). During early mitosis, Plk1 is localized at the centrosomes (Golsteyn *et al*., 1995). During late anaphase and telophase, Plk1 is recruited to the central spindle through its interactions with MKLP1/2 (Golsteyn *et al*., 1995; Neef *et al*., 2003; Liu *et al*., 2004). Finally, consistent with its kinetochore functions, Plk1 localizes to the kinetochores during mitosis (Arnaud *et al*., 1998; Ahonen *et al*., 2005; Wong and Fang, 2005). It is thus important to establish the mechanism by which Plk1 is recruited to the kinetochores.

Bub1 is a protein serine/threonine kinase and has two well-established roles in the spindle checkpoint (Hoyt *et al*., 1991; Taylor and McKeon, 1997; Yu and Tang, 2005). First, Bub1 localizes to the kinetochores in mitosis and is required for the kinetochore localization of other spindle checkpoint proteins, including BubR1 and Mad2 (Taylor and McKeon, 1997; Sharp-Baker and Chen, 2001; Johnson *et al*., 2004). Surprisingly, the kinase activity of Bub1 is dispensable for its function in targeting BubR1 and Mad2 to kinetochores (Sharp-Baker and Chen, 2001). Second, human Bub1 directly phosphorylates Cdc20 and inhibits APC/C (Tang *et al*., 2004a). Bub1 itself is hyperphosphorylated and its kinase activity is enhanced in mitosis (Chen, 2004; Tang *et al*., 2004a). In addition to these two functions in the spindle checkpoint, Bub1 is required for the kinetochore localization of the Shugoshin/MEI-S332 protein and protects centromeric cohesion (Tang *et al*., 2004b; Kitajima *et al*., 2005). Bub1 also promotes stable bipolar kinetochore-microtubule attachment in mammalian cells (Meraldi and Sorger, 2005).

In an effort to study the regulation of Bub1 during mitosis, we immunoprecipitated Bub1 from nocodazole-arrested mitotic HeLa cells and identified Plk1 as a Bub1-binding protein by mass spectrometry. Binding of Plk1 to Bub1 requires the polo-box domain of Plk1 and phosphorylation of Bub1 at T609. Bub1 is phosphorylated at T609 in mitosis *in vivo*. Phosphorylation of Bub1 by Cdk1 at T609 enhances Plk1-binding and Plk1-mediated phosphorylation of Bub1 *in vitro*. Depletion of Bub1 by RNAi diminishes the kinetochore localization of Plk1. Ectopic expression of the wild-type Bub1, but not the Bub1-T609A mutant, rescues the kinetochore localization of Plk1 in Bub1-RNAi cells. Therefore, our results suggest that Plk1 directly interacts with and phosphorylates Bub1 in mitosis and that the polo-box- and phosphorylation-dependent interaction between Bub1 and Plk1 helps to recruit Plk1 to kinetochores.

Very recently, Goto *et al.* reported that inner centromere protein (INCENP) is phosphorylated by Cdk1 in mitosis and interacts with Plk1 in a polo-box-dependent manner (Goto *et al*., 2006). The INCENP-Plk1 interaction was postulated to recruit Plk1 to kinetochores (Goto *et al*., 2006). However, as suggested by its name, INCENP localizes to inner centromeres during mitosis (Cooke *et al*., 1987). In contrast, it has been reported that Plk1 localizes to mid-to-outer kinetochores (Ahonen *et al*., 2005). In this study, we have confirmed that INCENP localizes to inner kinetochores and there is little overlap between the INCENP-staining and Plk1-staining at the kinetochores. Thus, the physical interaction between Plk1 and INCENP is unlikely to be directly responsible for the kinetochore localization of Plk1. Furthermore, INCENP is a component of the so-called “chromosome passenger complex” that also contains Aurora B, survivin, and Borealin (Pinsky and Biggins,

2005). The kinetochore localization of INCENP and Aurora B is interdependent and Aurora B is required for the kinetochore localization of Bub1 and BubR1 (Ditchfield *et al*., 2003; Honda *et al*., 2003; Vigneron *et al*., 2004). We confirm that RNAi-mediated depletion of INCENP using the same siRNA as Goto *et al.* diminishes the kinetochore localization of Bub1. Our findings along with a wealth of published data are consistent with the notion that INCENP controls the kinetochore localization of Bub1, which in turn facilitates the recruitment of Plk1 to kinetochores.

Materials and methods

Antibodies, immunoblotting, and immunoprecipitation

The production of rabbit α-Bub1, α-BubR1, α-Sgo1, and α-APC2 antibodies was described previously (Fang *et al*., 1998; Tang *et al*., 2001; Tang *et al*., 2004b). The following antibodies were purchased from the indicated sources: monoclonal α-Bub1 (ImmuQuest), α-Plk1 and α-cyclin B1 (Santa Cruz Biotechnology), the MPM-2 antibody (Cell Signaling), the CREST antibody (ImmunoVision), α-HA and α-Myc (Roche), and rabbit α-Aurora B and α-INCENP (Bethyl). For immunoblotting, the antibodies were used at 1:1000 dilution for crude sera or 1 μg/ml for purified IgG. Monoclonal α-Bub1 antibody is only used in the experiments of co-staining of Bub1 with BubR1 or INCENP.

For immunoprecipitation, affinity-purified rabbit α-Bub1 or α-Myc were coupled to Affi-Prep Protein A beads (Bio-Rad) at a concentration of 1 mg/ml. HeLa cells were lysed with the lysis buffer (50 mM Tris-HCl, pH 7.7, 150 mM NaCl, 0.5% NP-40, 1 mM DTT,

10% glycerol, 0.5 μM okadaic acid, and 10 μg/ml each of leupeptin, pepstatin and chymostatin). After clearing by centrifugation for 30 min at 4˚C at 13,000 rpm, the lysate was incubated with the antibody beads for 2 hrs at 4˚C. The beads were washed with the lysis buffer for five times. The proteins bound to the beads were dissolved in SDS sample buffer, separated by SDS-PAGE, and blotted with the desired antibodies. For the large-scale purification of Bub1-containing protein complexes, the bound proteins were eluted with 100 mM Glycine (pH 2.5), separated by SDS-PAGE, and visualized by silver staining. Protein bands were excised from the gel and subjected to analysis by mass spectrometry.

Mammalian cell culture, RNAi, and transfection

HeLa Tet-On (Clontech) cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum and 10 mM L-glutamine. To arrest cells at G1/S, cells were incubated in the growth medium containing 2 mM thymidine (Sigma) for 18 hr. To arrest cells in mitosis, cells were treated with 100 ng/ml nocodazole (Sigma) for 16-18 hrs. For the roscovitine treatment, cells were first treated with nocodazole for 18 hrs to arrest them in mitosis and roscovitine (50 μM) was added in the medium for the indicated durations.

Plasmid transfection was performed when the cell reached a confluency of about 50% using the Effectene reagent (Qiagen) according to the manufacturer's instructions. For RNAi experiments, the siRNA oligonucleotides were chemically synthesized at Dharmacon. HeLa cells were transfected as described (Tang *et al*., 2004a) and analyzed 48 hrs after transfection. The sequences of the siRNAs used in this study are: Bub1 (CCAUGGGAUUGGAACCCUGTT and GAGUGAUCACGAUUUCUAUTT), Plk1

(GGGCGGCUUUGCCAAGUGCTT), BubR1 (GGUGGGAAGGAGAGUAAUATT), INCENP (GAAGAGACGGATTTCTTAT), and Aurora B (CGCGGCACUUCACAAUUGATT). To establish cell lines stably expressing Myc-Bub1 or Myc-Bub1-T609A, HeLa Tet-on cells were transfected with pTRE2-Myc-Bub1 or pTRE2-Myc-Bub1-T609A constructs and then selected with 300 μg/ml of hygromycin (Clontech). The surviving clones were screened for induced expression of Myc-Bub1 or Myc-Bub1-T609A in the absence or presence of 1 μg/ml doxycycline (Clontech).

Identification of phosphorylation sites by tandem mass spectrometry

Cdk1 phosphorylation sites on Bub1 were identified by a combination of precursor ion scanning and nanoelectrospray tandem mass spectrometry (MS/MS). Briefly, Bub1 was phosphorylated by Cdk1 *in vitro* and separated on SDS-PAGE. The protein band was excised and digested with trypsin. The dried protein digests were dissolved in 5% formic acid and loaded onto a pulled capillary filled with POROS R2 resin. After washing, the peptides were eluted into a nanoelectrospray needle for either precursor ion scanning in negative ion mode, or MS/MS in positive ion mode. All MS analyses were performed on a QSTAR Pulsar-I quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, ON, Canada) equipped with a nanoelectrospray ion source (MDS Proteomics, Odense, Denmark). For precursor ion scanning experiments, the instrument was set in negative ion mode, with the quadrupole Q2 pulsing function turned on, to detect the PO^{3-} fragment ion at m/z –79. The optimum collision energies were determined

for each experiment by gradually increasing the voltage of Q0 in steps corresponding to one-twentieth of the m/z value of the precursor ion. After data acquisition by precursor ion scanning, the instrument was switched to positive ion mode, and the phosphopeptide sequence and sites of phosphorylation were identified by nanoelectrospray MS/MS. In the MS/MS scan mode, precursor ions were selected in quadrupole Q1 and fragmented in the collision cell (q2), using argon as the collision gas.

Immunofluorescence microscopy

HeLa Tet-On cells or various RNAi cells were cultured in chambered cover slides (Nunc) and transfected with siRNAs at about 40% confluency. After 24 hrs, thymidine (at final concentration of 2 mM) was added to the medium. After another 18 hrs, cells were washed and released into fresh medium for 7 hrs and treated with nocodazole (100 ng/ml) and MG132 (50 μM) for 4 hrs to arrest cell in mitosis. Cells were washed once with PBS and fixed with freshly made 4% paraformaldehyde for 15 min at room temperature. After washing with PBS three times, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed with the same buffer, and blocked with 5% non-fat milk in permeabilizing solution for 30 min. Cells were then incubated with the appropriate primary antibodies (diluted to 1 μg/ml in blocking solution) for 1 hr, washed three times with 0.2% Triton X-100 in PBS, and incubated with cross-absorbed fluorescent secondary antibodies (Molecular Probes) at 1:500 dilution. After washing and staining with DAPI, slides were mounted, sealed, and examined using a 63X objective on a Zeiss Axiovert 200M microscope. Images were acquired and processed with the Slidebook software (Intelligent Imaging) and pseudo-colored in Photoshop. A series of z-stack images are captured at 0.2 μm intervals and

deconvolved using the nearest neighbor algorithm. The maximum z-projection is then created for the deconvolved images. For quantification of kinetochore staining, a mask is generated to mark all kinetochores based on CREST-staining in the projected image. After background subtraction, the mean intensity for each channel and for each object in the mask is measured. These values are then exported and plotted with the Prism software. For each condition, kinetochore-staining of at least 10 cells is measured with the average and standard deviation plotted.

In vitro kinase and protein binding assays

The expression and purification of human Bub1-ΔKD, Bub1-ΔKD-S99A, Bub1-ΔKD-T609A, Plk1-T210D, and the Δ90-cyclin B1/Cdk1 complex from Sf9 cells were performed exactly as described (Tang and Yu, 2004). The kinase assay was carried out in the kinase buffer (50 mM Tris-HCl, pH7.7, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) containing 200 μM cold ATP, 0.1 μCi/μl ^{32}P-ATP, 1 μM Bub1-ΔKD and 100 nM Plk1 with or with out 100 nM Cdk1. For the two-step kinase assay, the purified Bub1-ΔKD and mutant proteins were first immobilized on α-Bub1 beads. After washing, the proteins bound to beads were used as substrates in the first step reaction in the presence of 200 μM cold ATP. The Bub1-ΔKD-containing beads were washed three times with the high-salt buffer (lysis buffer plus 300 mM KCl) and twice with the kinase buffer to completely remove Cdk1. The proteins bound to beads were subjected to the second kinase reaction with Plk1-T210D and ^{32}P-ATP. The

reactions was then incubated at room temperature for 30 min, stopped by SDS sample buffer, separated by SDS-PAGE, and analyzed using a phosphoimager (Fuji).

GST, GST-PBD, and GST-PBD-H538A/K540M proteins were expressed in bacteria and purified using glutathione-agarose beads. In protein-binding assays, the proteins were immobilized on the glutathione-agarose beads, blocked with TBS plus 5% non-fat milk, and incubated with the lysate of HeLa cells transfected with Myc-Bub1 for 2 hrs at 4˚C. The bound proteins were dissolved in SDS sample buffer, separated by SDS-PAGE, and blotted with α-Myc. For the binding assays that involved Cdk1-phosphorylated Bub1-ΔKD, Bub1-ΔKD was incubated first with 100 nM Cdk1 in the kinase buffer with or without 200 μM cold ATP for 1 hr. The reaction mixtures were then applied to beads containing various GST proteins that had been blocked with TBS plus 5% non-fat milk.

Results

Plk1 interacts with Bub1 in mitosis

To identify proteins that interacted with Bub1 in mitosis, we immunoprecipitated Bub1-containing protein complexes from nocodazole-arrested mitotic HeLa cells. As shown in Figure 1A, in addition to Bub1, Bub3, and several degradation products of Bub1, a protein band that migrated around 65 kDa was present in the α-Bub1 immunoprecipitates (IP), but not in the IP of control IgG. Mass spectrometry analysis revealed that this band belonged to human Plk1 (data not shown). To confirm the interaction between Plk1 and Bub1, we performed IP-Western type of experiments. Lysates of HeLa cells that were arrested at G1/S

or mitosis by thymidine or nocodazole, respectively, were immunoprecipitated with α-Bub1. The α-Bub1 IP was blotted with α-Plk1. Plk1 was clearly detected in the α-Bub1 IP, but not in the IPs of control IgG or α-Mps1 (a spindle checkpoint kinase) (Fisk *et al*., 2004), from mitotic HeLa cells (Figure 1B and Figure 2). The interaction between Bub1 and Plk1 was not observed in cells arrested at the G1/S boundary by thymidine (Figure 1B). Similar levels of Bub1 were present in the lysates from thymidine- or nocodazole-treated cells and in the α-Bub1 IPs from these cells. Plk1 was also present in the lysate of thymidine-arrested cells, albeit at a lower level as compared to nocodazole-treated cells. However, the difference in the amounts of Plk1 present in the α-Bub1 IPs from thymidine- and nocodazole-arrested cells was much greater than the difference between Plk1 levels in the lysates of the two types of cells. Thus, our results suggest that Bub1 specifically interacts with Plk1 in mitosis.

To further confirm the interaction between Plk1 and Bub1, HeLa cells were transfected with plasmids that encoded HA-Plk1 and Myc-Bub1 and treated with nocodazole. Lysates of these cells and α-Myc IP were blotted with α-HA. HA-Plk1 was efficiently co-immunoprecipitated with Myc-Bub1 (Figure 1C). We next performed immuno-staining experiments to determine the localization of Plk1 and Bub1 in mitosis. Earlier findings have established that both Plk1 and Bub1 localized to outer kinetochores during mitosis (Taylor and McKeon, 1997; Arnaud *et al*., 1998). By co-staining mitotic HeLa cells with a polyclonal α-Bub1 antibody and a monoclonal α-Plk1 antibody, we detected that Plk1 and Bub1 closely co-localized at the kinetochores (Figure 1D), which further supported the notion that Plk1 and Bub1 interacted in mitosis.

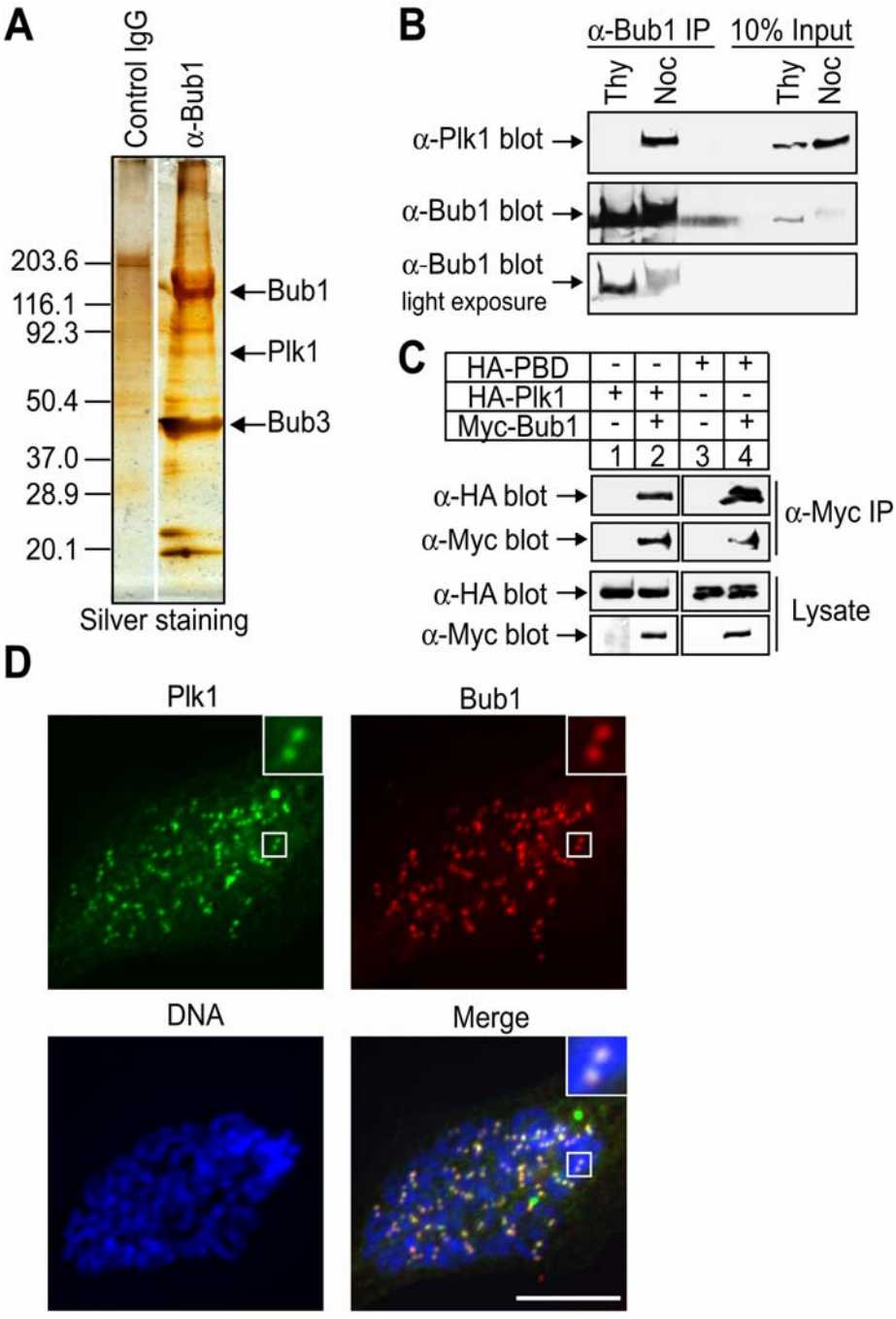


Figure 1. Bub1 and Plk1 interact and co-localize at the kinetochores in mitosis. (A) Lysate of nocodazole-arrested mitotic HeLa cells was immunoprecipitated with control IgG or α-Bub1. The IPs were separated on SDS-PAGE and stained with silver. (B) Lysates of HeLa cells treated with thymidine (Thy) or nocodazole (Noc) were immunoprecipitated with α-Bub1. The lysates and α-Bub1 IP were blotted with α-Bub1 and α-Plk1. (C) HeLa cells were transfected with plasmids encoding Myc-Bub1 together with HA-Plk1 or HA-Plk1-

PBD (polo-box domain). The cell lysates and the α-Myc IP were blotted with α-Myc and α-HA. (D) A HeLa cell at prometaphase was stained with α-Plk1 (green), α-Bub1 (red), and DAPI (blue). The boxed areas are magnified and shown in insets. The scale bar indicates 10 μm.

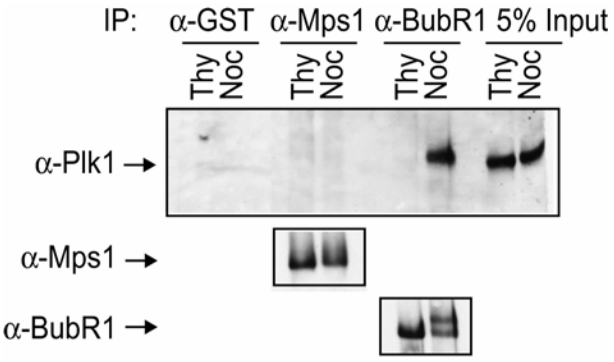


Figure 2. Plk1 binds to BubR1, but not Mps1, in mitosis. The endogenous Mps1 and BubR1 were immunoprecipitated from thymidine- or nocodazole-arrested HeLa cell lysates and blotted with α-Plk1 (top panel), α-Mps1 (middle panel), and α-BubR1 (bottom panel). The α-GST IP was included as a negative control. The input lysates were also blotted with α-Plk1.

The polo-box domain of Plk1 mediates its interaction with Bub1

Plk1 contains an N-terminal kinase domain and two polo-boxes at its C-terminal region (Figure 3A). The two polo-boxes of Plk1 have been shown to fold into one intact domain (PBD) that binds to phosphorylated Ser/Thr motifs and targets Plk1 to its substrates and proper subcellular locations (Elia *et al*., 2003a; Elia *et al*., 2003b). We tested whether the PBD of Plk1 mediated its interaction with Bub1. HA-Plk1-PBD bound to Bub1 as efficiently as did the full-length HA-Plk1, indicating that the PBD of Plk1 was sufficient for Bub1-

binding (Figure 1C). Two residues in the second polo-box of Plk1, H538 and K540, form direct contact with the phosphate group and are required for the selective binding between the PBD and phosphopeptides (Elia *et al*., 2003b). To determine whether the Bub1–Plk1 interaction required the intact PBD of Plk1, we introduced two mutations into the PBD, H538A and K540M, which were known to disrupt the phosphopeptide-binding activity of the PBD (Figure 3A) (Elia *et al*., 2003b). As shown in Figure 2B, HA-Plk1-H538A/K540M bound to Bub1 much more weakly than did the wild-type HA-Plk1. The kinase-inactive mutant of Plk1, Plk1-K82R, bound to Bub1 as efficiently as Plk1-WT (Figure 3B), suggesting that the secondary phosphorylation on Bub1 by Plk1 (see Figure 5C below) might not be required for the Bub1–Plk1 interaction.

We also performed an *in vitro* protein-binding assay. Purified recombinant GST-PBD and GST-PBD-H538A/K540M fusion proteins were bound to glutathione-agarose and incubated with lysate of nocodazole-arrested HeLa cells that had been transfected with Myc-Bub1. Myc-Bub1 selectively bound to beads containing the wild-type GST-PBD, but did not bind to beads containing GST or GST-PBD-H538A/K540M (Figure 3C). These results indicated that binding of Plk1 to Bub1 required an intact PBD. Moreover, it has been shown previously that Bub1 is hyperphosphorylated in mitosis and the hyperphosphorylated species of Bub1 migrated slower on SDS-PAGE (Chen, 2004; Tang *et al*., 2004a). Myc-Bub1 that was bound to the wild-type GST-PBD migrated slower on SDS-PAGE, as compared to Myc-Bub1 in the cell lysate (Figure 3C). This suggests that the PBD of Plk1 might preferentially bind to phosphorylated Bub1.

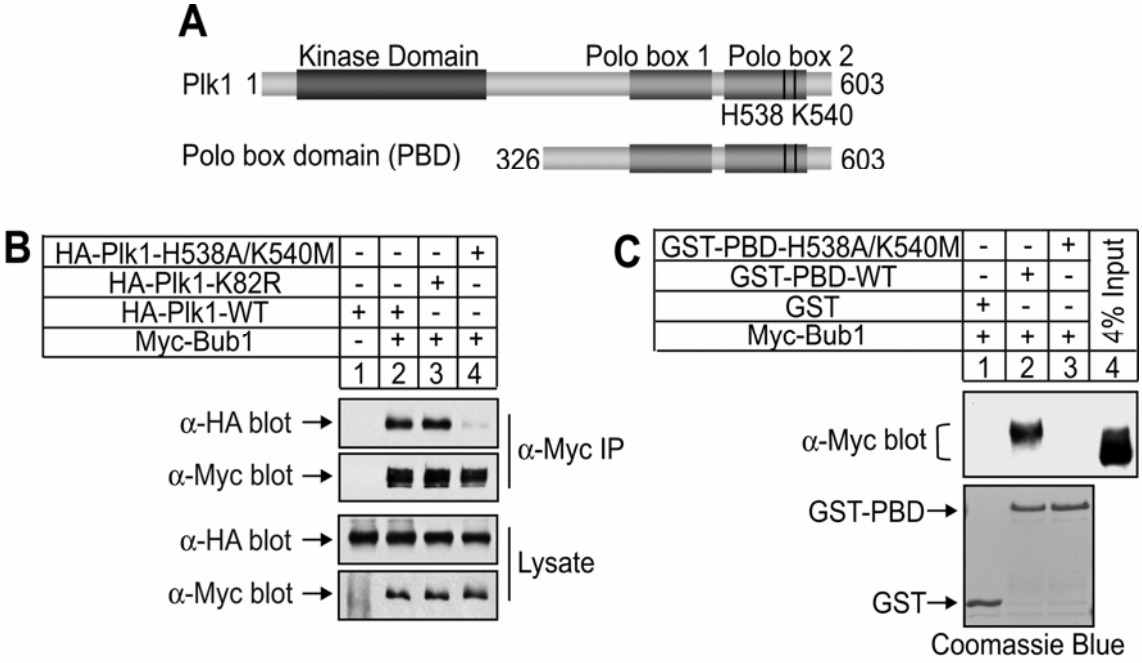


Figure 3. The PBD of Plk1 mediates the binding between Plk1 and Bub1. (A) Schematic drawing of the domain structure of Plk1 and the locations of two critical phosphate-binding residues. (B) HeLa cells were transfected with plasmids encoding Myc-Bub1 together with HA-Plk1-WT, HA-Plk1-K82R (kinase-inactive mutant), or HA-Plk1-H538A/K540M. The cell lysates and α-Myc IP were blotted with α-Myc and α-HA. (C) HeLa cells were transfected with plasmids encoding Myc-Bub1 and arrested in mitosis with nocodazole. The cell lysates were incubated with glutathione-agarose beads that contained GST, GST-PBD-WT, or GST-PBD-H538A/K540M. After washing, the proteins bound to beads were blotted with α-Myc (top panel) or stained with Coomassie blue (bottom panel).

Bub1 is phosphorylated on T609 *in vivo* and phosphorylation of T609 is required for the Plk1–Bub1 interaction

Yaffe and coworkers have shown that the PBD of Plk1 prefers to bind to phosphorylated S-pS/pT-P motifs (Elia *et al*., 2003a). Inspection of the amino acid sequence of Bub1 revealed that human Bub1 contains two such S-S/T-P motifs, one of which (T609) is conserved

among vertebrate Bub1 proteins (Figure 4A). To determine whether any of the two S-S/T-P motifs of Bub1 were phosphorylated *in vivo*, we tested whether either of the two sites was recognized by the MPM-2 phospho-specific monoclonal antibody that can detect certain pS/pT-P motifs (Yaffe *et al*., 1997). The wild-type Myc-Bub1 from mitotic HeLa cells contained a phospho-epitope that was detected by the MPM-2 antibody (Figure 4B). The S99A mutation did not alter the MPM-2 antigen within Bub1 while the T609A mutation significantly attenuated the MPM-2 reactivity of Myc-Bub1 (Figure 4B). This suggests that Bub1 is phosphorylated at T609 in mitosis, and this phospho-epitope on Bub1 can be detected by the MPM-2 antibody. However, the MPM-2 antibody still recognized Bub1-T609A to some extent, suggesting that Bub1 contained other MPM-2 epitopes in addition to T609.

Our results in Figure 4B cannot rule out the possibility that the MPM-2 antibody does not directly detect phosphorylation at T609, but detects phosphorylation at other S/T-P sites whose phosphorylation might be in turn dependent on phosphorylation at T609. Human Bub1 contains 13 S/T-P sites, seven of which are conserved in vertebrates, including T441, T452, S459, S593, T609, S655 and S661. To determine whether T609 of Bub1 was phosphorylated in mitosis and whether phospho-T609 was detected by MPM-2, we constructed a Bub1-7A mutant in which the serines/threonines in all seven conserved S/T-P motifs are mutated to alanines and a Bub1-6A-T609 mutant in which T609 was not mutated but the other six sites were mutated. Myc-Bub1-7A immunoprecipitated from nocodazole-arrested HeLa cells was not recognized by MPM-2 (Figure 4C). Bub1-6A-T609 was detected by MPM-2 (Figure 4C), suggesting that Bub1 is most likely phosphorylated at T609

and this phosphorylation event can be recognized by MPM-2. The MPM-2 reactivity of Bub1-6A-T609 was much weaker as compared to Bub1-WT (Figure 4C), consistent with the notion that Bub1 contains additional MPM-2 epitopes. Alternatively, mutations of serine/threonine residues nearby might perturb the conformation of Bub1, thereby reducing the affinity of MPM-2 toward phospho-T609. Interestingly, while the gel mobility of Bub1-WT and Bub1-6A-T609 was retarded in mitotic cell lysates, Bub1-T609A and Bub1-7A did not exhibit this gel mobility shift (Figure 4C). This suggests that phosphorylation of T609 itself or phospho-T609-dependent phosphorylation events are responsible for the gel mobility shift of Bub1. Taken together, our data suggest that phospho-T609 of Bub1 is recognized by MPM-2.

We next determined whether the endogenous Bub1 protein was recognized by MPM-2 in mitosis. The endogenous Bub1 protein was immunoprecipitated from either thymidine-treated G1/S or nocodazole-treated mitotic HeLa cells and blotted with MPM-2 and α-Bub1 (Figure 4D). The Bub1 protein from mitotic HeLa cells, but not from G1/S cells, was recognized by MPM-2, suggesting that the endogenous Bub1 protein was phosphorylated at T609 in mitosis. Moreover, while Myc-Bub1-WT and Myc-Bub1- S99A interacted strongly with HA-Plk1, Myc-Bub1-T609A failed to interact with HA-Plk1 in mitotic HeLa cells (Figure 4E). This suggests that phosphorylation of Bub1 at T609 creates a docking site for the PBD of Plk1 and is required for efficient Plk1-binding.

Interestingly, the T609-containing S-S/T-P motif is conserved in BubR1 proteins (Figure 4A). Co-IP experiments confirmed that the endogenous BubR1 interacted with Plk1 in mitosis (Figure 2). BubR1 is also hyperphosphorylated in mitosis (Taylor *et al.*, 2001).

Although we do not know whether the corresponding S-S/T-P motif of BubR1 is phosphorylated, it is very likely that Plk1 interacts with BubR1 in a manner similar to its binding to Bub1. The functional consequence of the association between Plk1 and BubR1 is not further explored in this study.

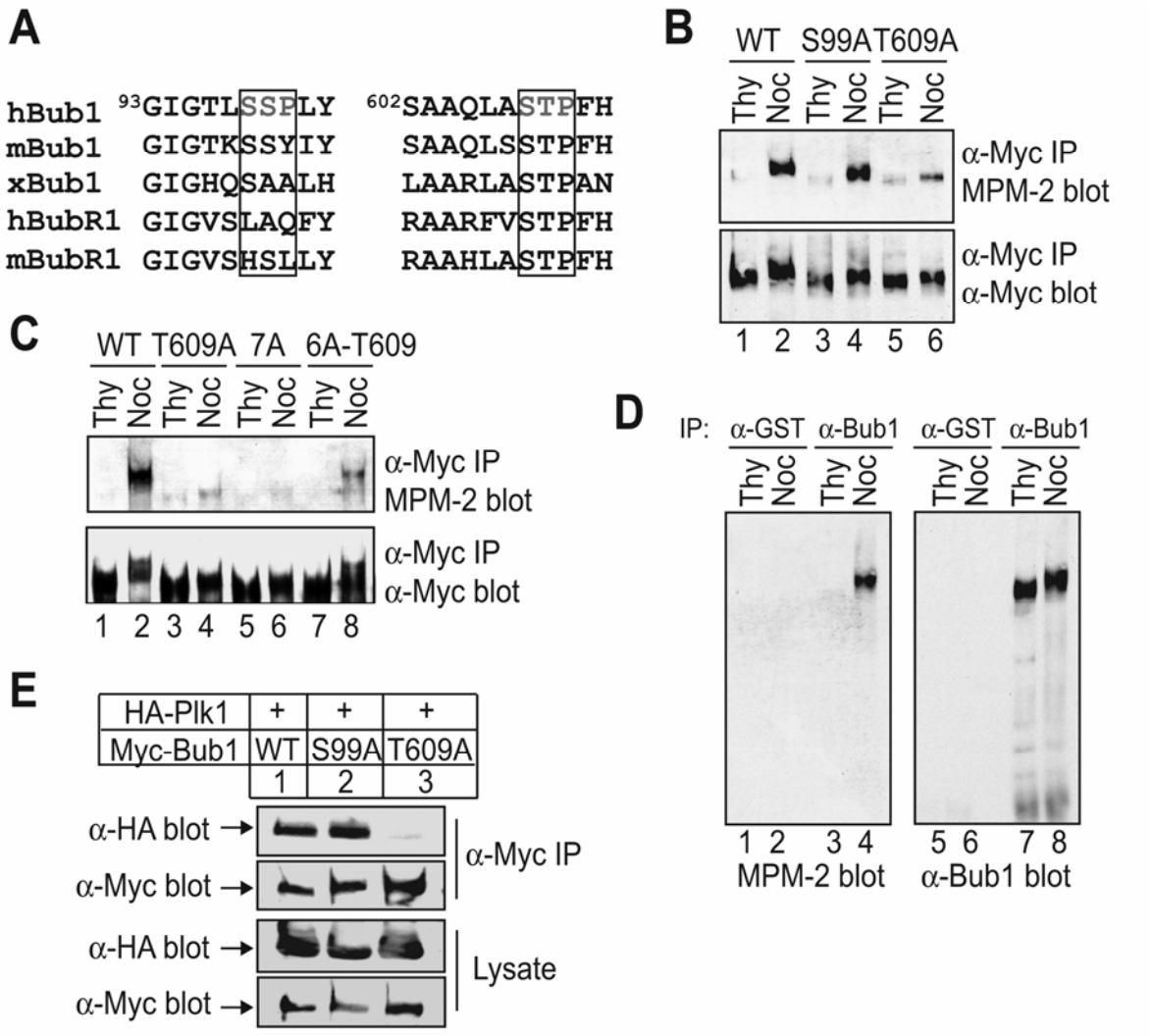


Figure 4. Phosphorylation of Bub1 at T609 occurs *in vivo* and is required for the Plk1–Bub1 interaction. (A) Sequence alignment of the two S-S/T-P motifs in human Bub1 (hBub1), mouse Bub1 (mBub1), *Xenopus* Bub1 (xBub1), human BubR1 (hBubR1), and mouse BubR1 (mBubR1). (B) HeLa cells were transfected with plasmids encoding Myc-Bub1-WT, Myc-Bub1-S99A, or Myc-Bub1-T609A and treated with thymidine (Thy) or

nocodazole (Noc). The α-Myc IP of lysates from the transfected cells was blotted with the MPM-2 antibody (top panel) and α-Myc (bottom panel). (C) HeLa cells were transfected with plasmids encoding Myc-Bub1-WT, Myc-Bub1-T609A, Myc-Bub1-7A, or Myc-Bub1-6A-T609 and treated with thymidine (Thy) or nocodazole (Noc). The α-Myc IP of lysates from the transfected cells was blotted with the MPM-2 antibody (top panel) and α-Myc (bottom panel). (D) Lysates of HeLa cells treated with thymidine or nocodazole were IPed with α-GST or α-Bub1. The IPs were blotted with the MPM-2 antibody (left panel) and α-Bub1 (right panel). (E) HeLa cells were transfected with plasmids encoding HA-Plk1 together with Myc-Bub1-WT, Myc-Bub1-S99A, or Myc-Bub1-T609A and treated with nocodazole. The lysates and α-Myc IP from the transfected cells were blotted with α-HA and α-Myc.

Phosphorylation of Bub1 by Cdk1 promotes the Plk1–Bub1 interaction and facilitates Plk1-mediated phosphorylation of Bub1 *in vitro*

Earlier studies have revealed that Cdk1 is the "priming" kinase that initially phosphorylates S-S/T-P motifs within several Plk1 substrates, such as Nir2, GRASP65, and Cdc25C, and generates the docking sites for the polo-box domain of Plk1 (Elia *et al*., 2003a; Litvak *et al*., 2004; Preisinger *et al*., 2005). We thus tested whether Cdk1 phosphorylated Bub1 *in vitro*. Because Bub1 is itself a kinase and undergoes autophosphorylation, we expressed and purified from Sf9 cells a truncation mutant of human Bub1 (residues 1-726) that lacked the kinase domain, referred to as Bub1-ΔKD, and used it as the substrate in the kinase assays. Purified recombinant cyclin B1/Cdk1 complex (referred to as Cdk1 for simplicity) phosphorylated Bub1-ΔKD (Figure 5A). This phosphorylation was blocked by roscovitine, a chemical inhibitor of Cdk1 (Figure 5A). Using mass spectrometry, we mapped the phosphorylation sites of Bub1 that had been phosphorylated by Cdk1 *in vitro*. Two major Cdk1 phosphorylation sites on Bub1 were identified, including S593 and T609 (Figure 5B).

This result demonstrates that Cdk1 can phosphorylate Bub1 on T609 *in vitro*. We next examined the effect of Cdk1-mediated phosphorylation of Bub1 on the Plk1–Bub1 interaction. Bub1-ΔKD was first incubated with Cdk1 in the presence or absence of cold ATP. The reaction mixtures were then incubated with beads containing GST, GST-PBD, or GST-PBD-H538A/K540M. After washing, the proteins bound to beads were blotted with α-Bub1 (Figure 5C). A pre-incubation of Bub1-ΔKD with Cdk1 in the presence of ATP enhanced its binding to GST-PBD (Figure 5C, compare lanes 2 and 5). The Cdk1-enhanced binding between Bub1-ΔKD and PBD required the intact phosphopeptide-binding pocket of the PBD, as less Bub1-ΔKD was bound to GST-PBD-H538A/K540M (Figure 5C, compare lanes 2 and 3). These results suggest that phosphorylation of Bub1 by Cdk1 facilitates the polo-box domain-dependent binding of Plk1 to Bub1.

We next tested whether Plk1 phosphorylated Bub1-ΔKD and whether the Cdk1-enhanced interaction between Plk1 and Bub1 also facilitated the phosphorylation of Bub1 by Plk1. To do so, we developed a two-step sequential kinase assay. In this assay, Bub1-ΔKD, Bub1-ΔKD-S99A, or Bub1-ΔKD-T609A proteins were first incubated with or without Cdk1 in the presence of cold ATP. The Bub1 proteins were immunoprecipitated by α-Bub1 beads. After extensive washing to remove Cdk1, the Bub1 proteins bound to beads were further incubated with or without Plk1-T210D (a constitutively active mutant of Plk1) in the presence of γ-^{32}P-ATP (Qian *et al.*, 1999). The removal of Cdk1 by the washing procedure was complete, as Bub1 was not phosphorylated in the absence of Plk1 (Figure 5D, lanes 3, 7, and 11). Plk1 phosphorylated Bub1 in the absence of a pretreatment by Cdk1 (Figure 5D, lane 2). A pre-incubation with Cdk1 enhanced the Plk1-mediated phosphorylation of Bub1-

ΔKD and Bub1-ΔKD-S99A, but not Bub1-ΔKD-T609A, as judged by the retarded gel mobility and the increased amount of ^{32}P-incorporation (Figure 5D). Although phosphorylation of Bub1-ΔKD by Plk1 retarded the gel mobility of Bub1-ΔKD in the autoradiograph, Bub1-ΔKD was not upshifted in the corresponding western blot (Figure 5D), indicating that only a small fraction of Bub1-ΔKD was phosphorylated in these reactions. We also tested whether Bub1 phosphorylated Plk1-K82R and failed to detect any such phosphorylation (data not shown). Our results are consistent with the notion that phosphorylation of Bub1 at T609 by Cdk1 creates a binding site for the polo-box domain of Plk1, thus enhancing the Plk1–Bub1 interaction and phosphorylation of Bub1 by Plk1.

We also noticed that Plk1 phosphorylated Bub1-ΔKD and Bub1-ΔKD-S99A more efficiently than Bub1-ΔKD-T609A even in the absence of a pre-incubation with Cdk1 (Figure 5C, compare lanes 2 and 6 with lane 10). Consistently, recombinant Bub1-ΔKD expressed and purified from Sf9 cells was detected by the MPM-2 antibody (Figure 5E). The MPM-2 reactivity of Bub1-ΔKD was abolished by λ-phosphatase treatment and greatly diminished by the T609A mutation (Figure 5E). These results suggested that a fraction of Bub1-ΔKD had already been phosphorylated at T609 by kinase(s) in Sf9 cells.

We next sought to obtain evidence to suggest that Cdk1 phosphorylated Bub1 *in vivo* and that this phosphorylation was required for the Bub1–Plk1 interaction. Nocodazole-arrested mitotic HeLa cells were treated briefly with the Cdk1 inhibitor, roscovitine (Mishima *et al*., 2004). The MPM-2 reactivity of Bub1 was lost in cells treated with roscovitine for only 15 min (Figure 5F, upper panel). Consistently, Bub1 from roscovitine-treated cells migrated faster on SDS-PAGE (Figure 5F, middle panel) and failed to interact with Plk1 (Figure 5F,

bottom panel). Thus, our data are consistent with the notion that Bub1 is phosphorylated by Cdk1 on T609 and this phosphorylation is required for its interaction with Plk1.

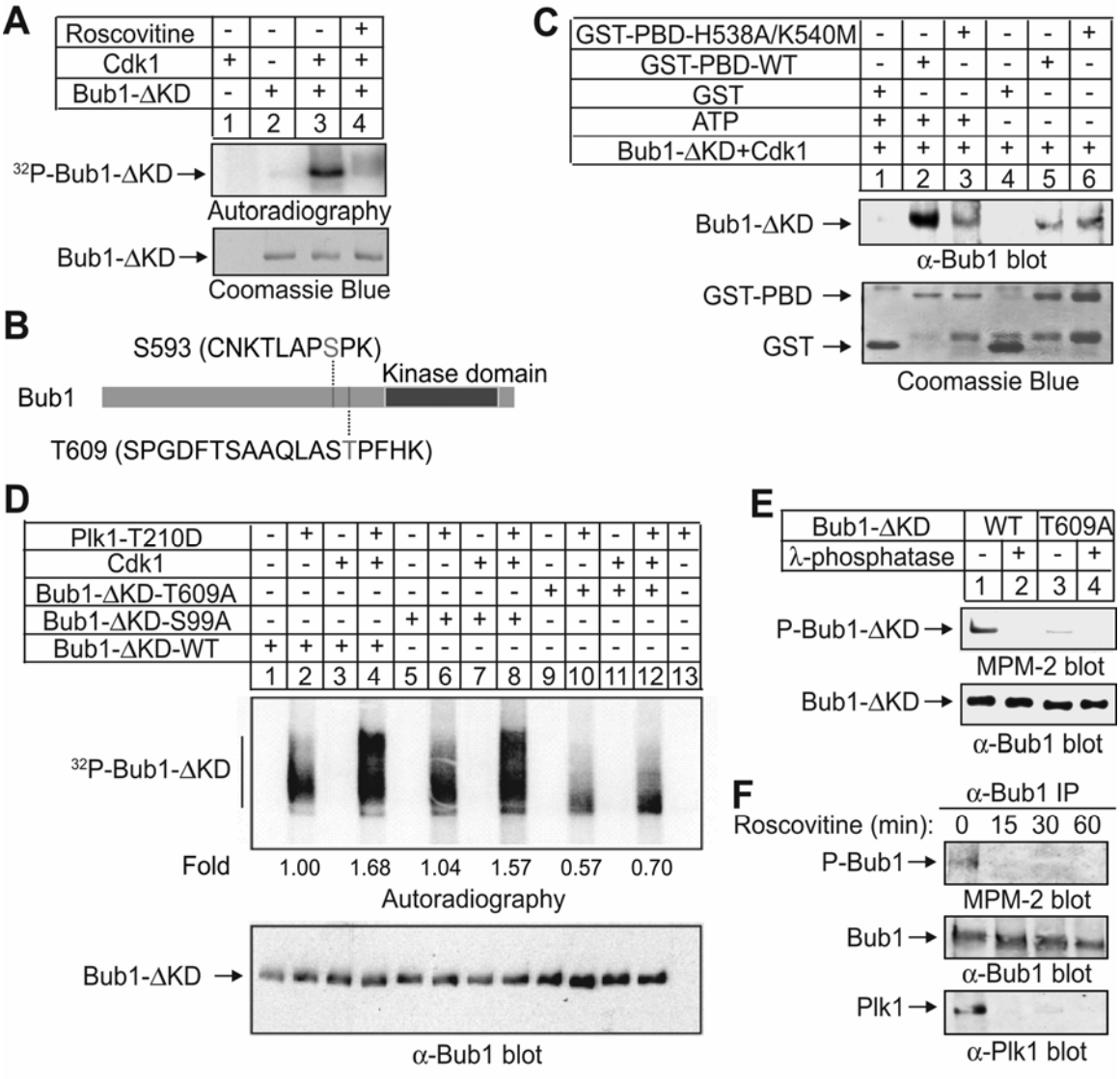


Figure 5. Cdk1 phosphorylates Bub1 and promotes Plk1-binding and Plk1-mediated phosphorylation of Bub1. (A) Recombinant purified Bub1-ΔKD (a Bub1 truncation mutant with its kinase domain deleted) was incubated with γ-^{32}P-ATP and cyclin B1/Cdk1 in the absence or presence of roscovitine (10 μM) for 30 min at room temperature. The reaction was quenched and analyzed by SDS-PAGE followed with autoradiography. The bottom panel shows a Coomassie-stained gel of the same reactions to indicate that equal amounts of

Bub1-ΔKD were included in the reactions. (B) Two major *in vitro* Cdk1 phosphorylation sites on recombinant Bub1 mapped by mass spectrometry. (C) The Bub1-ΔKD protein was incubated with Cdk1 in the presence or absence of cold ATP. The reaction mixtures were incubated with glutathione-agarose beads that contained GST, GST-PBD-WT, or GST-PBD-H538A/K540M. After washing, the proteins bound to beads were blotted with α-Bub1. The bottom panel shows a Coomassie-stained gel of the same reactions to indicate that similar amounts of GST, GST-PBD-WT and GST-PBD-H538A/K540M were present in the reactions. (C) Bub1-ΔKD and mutant proteins were incubated with Cdk1 in the kinase buffer containing cold ATP for 1 hr at room temperature. The Bub1 proteins were then immunoprecipitated and subjected to a second kinase reaction with purified Plk1-T210D and γ-^{32}P-ATP. After 1 hr, the reactions were quenched and analyzed by SDS-PAGE followed by autoradiography (top panel). The same samples were blotted with α-Bub1 to show that similar amounts of Bub1 proteins were present (bottom panel). (D) Recombinant Bub1-ΔKD or Bub1-ΔKD-T609A from Sf9 cells were either untreated or treated with λ-phosphatase and blotted with MPM-2. (E) Nocodazole-arrested HeLa cells were incubated with roscovitine (50 μM) for the indicated times and then lysed and subjected to α-Bub1 IP. The IPs were blotted with the MPM-2 antibody (top panel), α-Bub1 (middle panel), and α-Plk1 (bottom panel).

Bub1 is required for the kinetochore localization of Plk1

To explore the functions of the binding between Plk1 and Bub1, we examined whether the Plk1–Bub1 interaction is required for the kinetochore localization of Bub1 or Plk1 in mitosis. It has been shown that improper kinetochore-microtubule attachment in certain situations can cause a microtubule-dependent depletion of kinetochore proteins. For example, the kinetochore concentrations of Mad1 and Mad2 are much lower in human cells that are depleted for components of the Ndc80 complex by RNAi (Martin-Lluesma *et al.*, 2002; DeLuca *et al.*, 2003; Bharadwaj *et al.*, 2004). However, the kinetochore localization of Mad1 and Mad2 is restored when these cells are treated with nocodazole to depolymerize their microtubules (DeLuca *et al.*, 2003; Bharadwaj *et al.*, 2004). Because both Bub1 and

Plk1 have been implicated in proper kinetochore-microtubule attachment (Ahonen *et al*., 2005; Meraldi and Sorger, 2005; Wong and Fang, 2005) and because Bub1-RNAi cells do not undergo mitotic arrest efficiently in the presence of nocodazole (Tang *et al.*, 2004a; Meraldi and Sorger, 2005), we adopted the following experimental scheme (Figure 6A). Twenty-four hours after HeLa cells were transfected with siRNA against Bub1, they were arrested at the G1/S boundary by the addition of thymidine for 18 hrs. The cells were then released into fresh medium to allow cell cycle progression. Nocodazole and the proteasome inhibitor, MG132, were added 7 hrs later to arrest cells in mitosis with depolymerized microtubules and were fixed for immunostaining 4 hrs later.

Transfection of HeLa cells with small interfering RNA (siRNA) against Bub1 or Plk1 efficiently knocked down the protein levels of Bub1 or Plk1 in mitosis (Figure 6B). Depletion of Bub1 did not affect the protein levels of Plk1, and vice versa (Figure 6B). Interestingly, Bub1 from Plk1-RNAi cells migrated faster on SDS-PAGE, consistent with the notion that Plk1 was involved in the phosphorylation of Bub1 in nocodazole-arrested mitotic HeLa cells (Figure 6B). Mitotic Plk1- and Bub1-RNAi cells were stained with α-Bub1 (Figure 7A) and α-Plk1 (Figure 7A), respectively. Consistent with earlier reports (Ahonen *et al*., 2005), depletion of Plk1 by RNAi did not significantly affect the kinetochore localization of Bub1 in mitosis (Figure 7A and C). In contrast, the kinetochore localization of Plk1 was significantly reduced in mitotic Bub1-RNAi cells (Figure 7A). Similar results were obtained in prometaphase and metaphase cells from asynchronized Bub1-RNAi cells (data not shown). Co-staining of Bub1-RNAi cells with Plk1 and γ-tubulin showed that the centrosomal localization of Plk1 was unaffected by Bub1-RNAi (Figure 7B). These results indicate that

Bub1 is required for the kinetochore localization, but not the centrosomal localization, of Plk1 in mitosis.

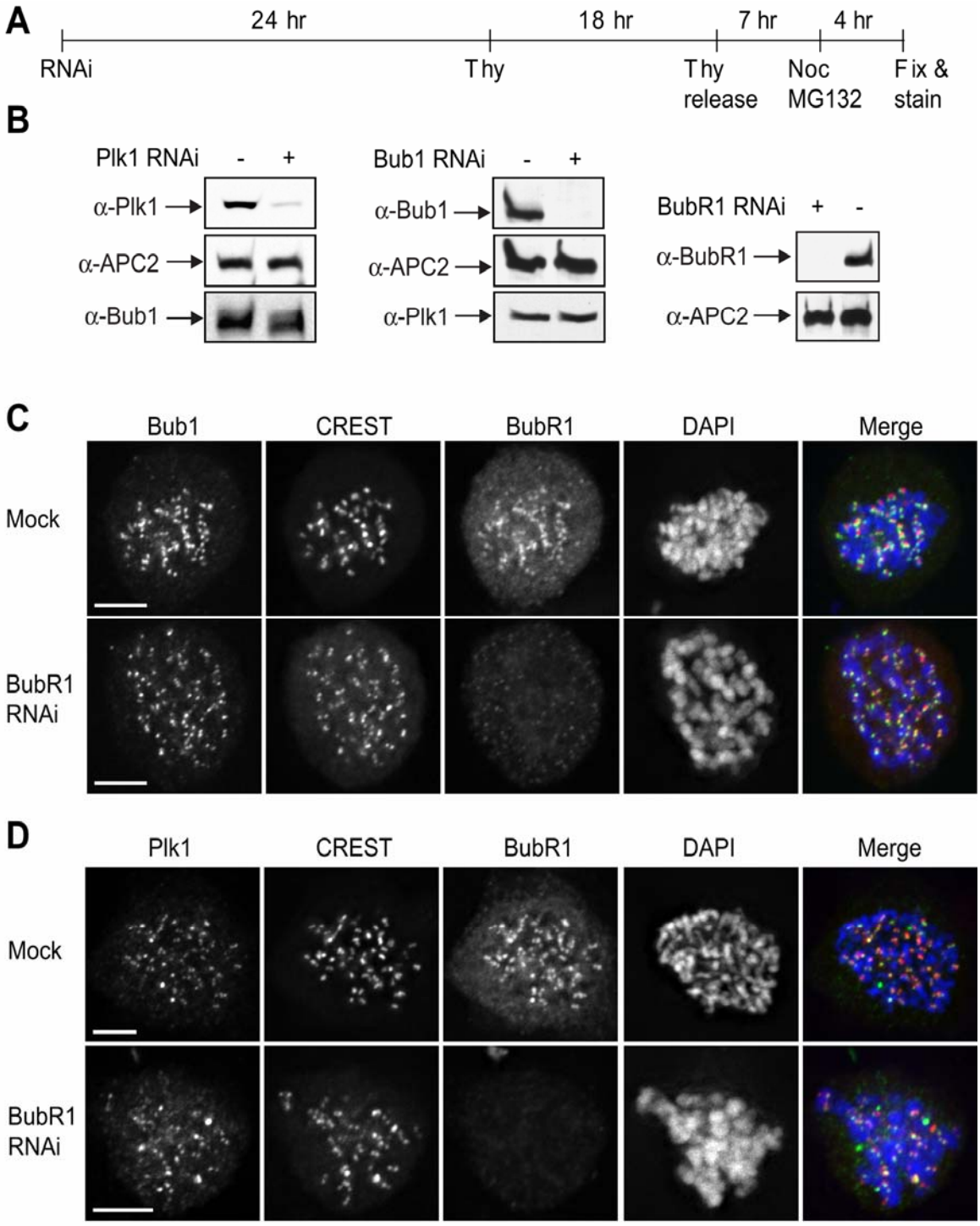


Figure 6. Depletion of BubR1 by RNAi does not significantly perturb the kinetochore localization of Bub1 and Plk1. (A) The time line of experiments described in Figures 5-7 and Supplementary data Figures S2-S4. RNAi transfection occurs at 0 hr. Thy, thymidine; Noc, nocodazole. (B) HeLa cells that were either mock transfected or transfected with siRNAs against Bub1, Plk1, or BubR1 were harvested and blotted with the indicated antibodies. (C) HeLa cells that were either mock transfected or transfected with siRNA against BubR1 were stained with α-Bub1, CREST, α-BubR1, and DAPI. In the merge, Bub1-staining is shown in green, CREST in red, and DAPI in blue. The scale bar indicates 5 μm. (D) The cells described in (C) were stained with α-Plk1, CREST, α-BubR1, and DAPI. In the merge, Plk1-staining is shown in green, CREST in red, and DAPI in blue. The scale bar indicates 5 μm.

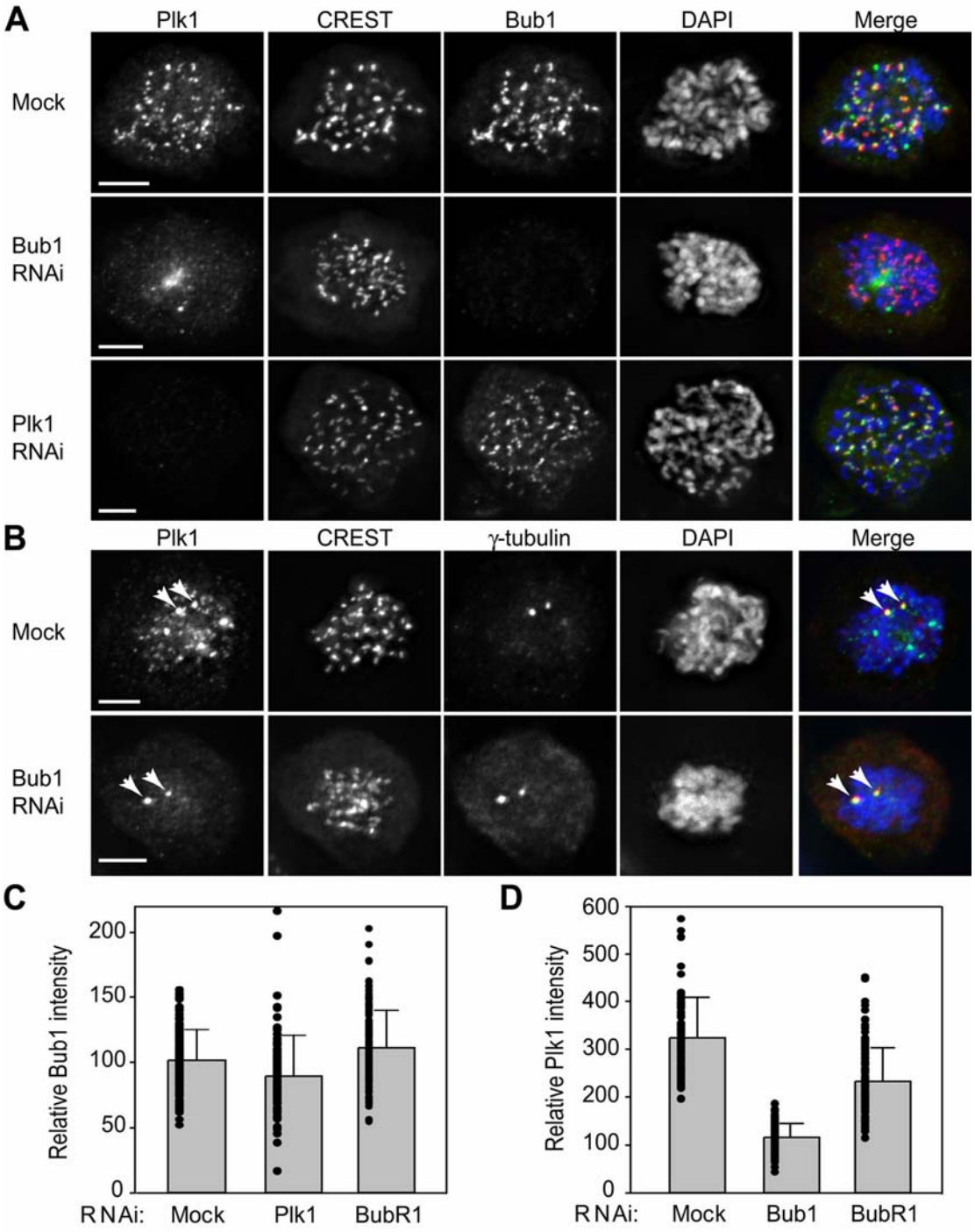


Figure 7. Bub1-RNAi diminishes the kinetochore localization of Plk1. (A) HeLa cells that were either mock transfected or transfected with siRNAs against Bub1 or Plk1 were fixed and stained with α-Plk1, α-Bub1, CREST, and DAPI. In the merge, DAPI is pseudo-colored blue, CREST in red, and Plk1 (top two panels) or Bub1 (bottom panel) in green. The scale bar indicates 5 μm. (B) HeLa cells that were either mock transfected or transfected

with siRNA against Bub1 were fixed and stained with α-Plk1, CREST, γ-tubulin, and DAPI. In the merge, DAPI is pseudo-colored blue, γ-tubulin in red, and Plk1 in green. The centrosomes are marked by arrows. The scale bar indicates 5 μm. (C) The kinetochore signal of Bub1 was quantified in mock, Plk1- and BubR1-RNAi cells. Kinetochores from more than 10 mitotic cells were analyzed and normalized using the CREST staining. The mean and standard deviation are shown. (D) The kinetochore staining of Plk1 was quantified in mock, Bub1- and BubR1-RNAi cells. Kinetochores from more than 10 mitotic cells were analyzed and normalized using the CREST staining. The mean and standard deviation are shown.

The kinetochore localization of Plk1 does not require BubR1

Bub1 is required for the kinetochore localization of other checkpoint proteins, such as BubR1 and Mad2 (Sharp-Baker and Chen, 2001; Johnson *et al*., 2004). We have also detected an interaction between BubR1 and Plk1 (Figure 2). To test whether the kinetochore localization of Plk1 was also dependent on BubR1, we examined the kinetochore localization of Plk1 in HeLa cells transfected with siRNA against BubR1. RNAi-mediated depletion of BubR1 was efficient (Figure 6B). Both Bub1 and Plk1 localized normally to mitotic kinetochores in BubR1-RNAi cells (Figure 6C, D and Figure 7C, D), indicating that BubR1 is not required for the kinetochore localization of Plk1. Consistent with earlier reports (Sharp-Baker and Chen, 2001; Johnson *et al*., 2004; Ahonen *et al*., 2005; Wong and Fang, 2005), the kinetochore localization of BubR1 was significantly reduced in Bub1-RNAi and Plk1-RNAi cells (Figure 8), suggesting that BubR1 lies downstream of Bub1 and Plk1 in the hierarchy of kinetochore association.

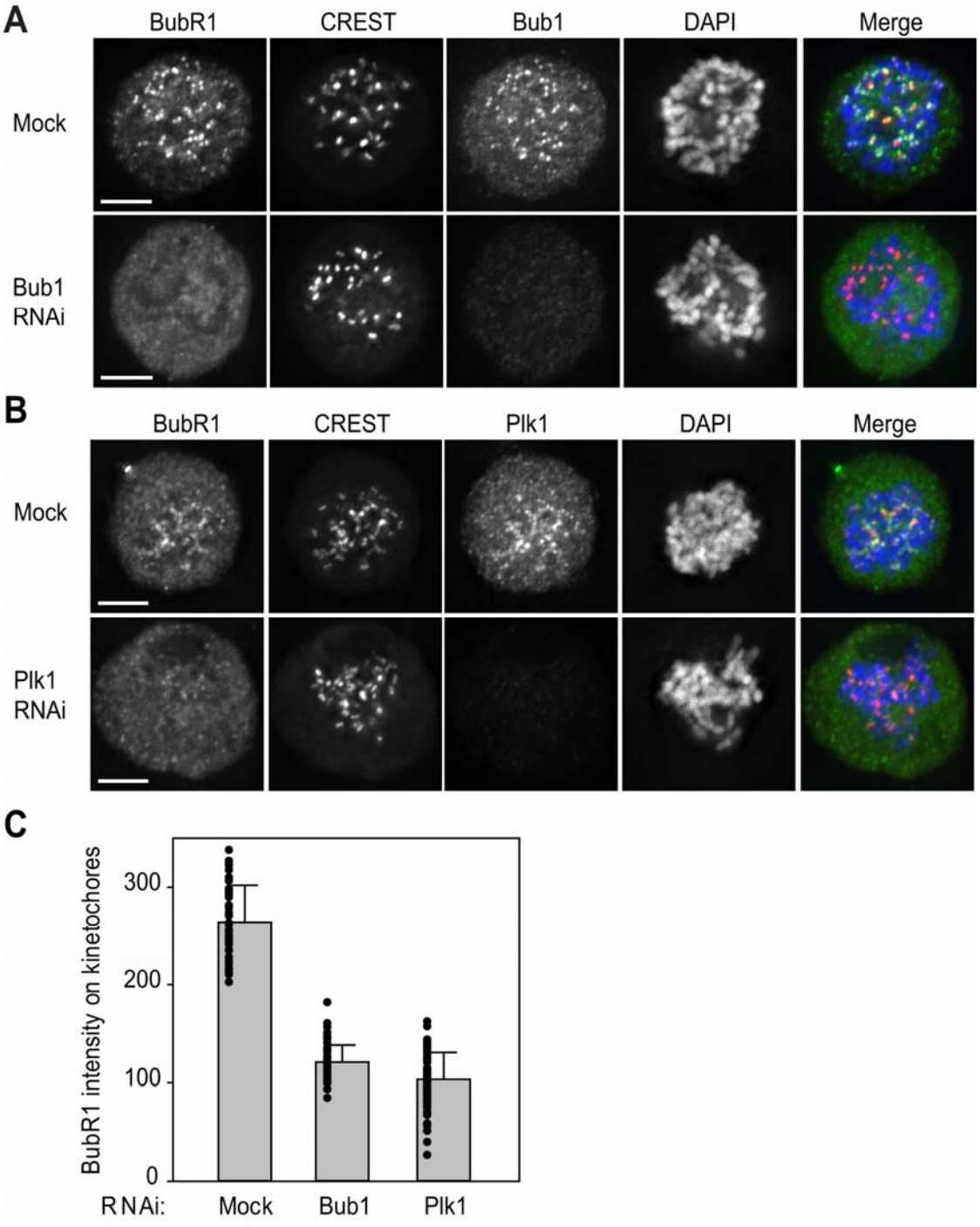


Figure 8. The kinetochore localization of BubR1 is diminished in the Bub1- and Plk1-RNAi cells. (A) HeLa cells that were either mock transfected or transfected with siRNA against Bub1 were stained with α-BubR1, CREST, monoclonal α-Bub1, and DAPI. In the merge, BubR1-staining is in green, CREST in red, and DAPI in blue. The scale bar indicates 5 μm. (B) HeLa cells that were either mock transfected or transfected with siRNA against

Plk1 were stained with α-BubR1, CREST, monoclonal α-Bub1, and DAPI. In the merge, BubR1-staining is in green, CREST in red, and DAPI in blue. The scale bar indicates 5 μm. (C) Quantification of BubR1 immunofluorescence signals at the kinetochores in cells described in (A) and (B).

The kinetochore localization of Plk1 requires the intact polo-box-binding motif on Bub1

The kinetochore localization of Plk1 is dependent on Bub1 in HeLa cells. We next sought to test whether the PBD- and phosphorylation-dependent binding between Plk1 and Bub1 was required for the kinetochore localization of Plk1. To do so, we attempted to rescue the defective kinetochore localization of Plk1 in Bub1-RNAi cells by stable transfection of plasmids that encoded Myc-Bub1-WT and Myc-Bub1-T609A (the Bub1 mutant that lacked the priming phosphorylation site required for Plk1-binding). Because these Bub1-expressing plasmids also contained silent mutations in the region that was targeted by Bub1-RNAi, the expression of the Bub1 transgenes was not knocked down by Bub1-RNAi. Both Myc-Bub1-WT and Myc-Bub1-T609A were expressed at levels comparable to that of the endogenous Bub1 (Figure 9A) and localized normally to kinetochores in mitosis (Figure 9B). The finding that Myc-Bub1-T609A exhibits normal kinetochore localization is consistent with the notion that Plk1 is not required for the kinetochore localization of Bub1 (Figure 7C) (Ahonen *et al*., 2005). Ectopic expression of Myc-Bub1-WT in Bub1-RNAi cells largely restored the kinetochore localization of Plk1 (Figure 9B and D). In contrast, expression of Myc-Bub1-T609A failed to restore the kinetochore localization of Plk1 in Bub1-RNAi cells (Figure 9B and D). Because Myc-Bub1-T609A localizes to kinetochores normally, this result strongly suggests that the Bub1–Plk1 interaction is required for the kinetochore localization of Plk1.

In addition to its function in the spindle checkpoint, Bub1 also protects centromeric cohesion by targeting the Sgo1–PP2A complex to kinetochores (Tang *et al.*, 2004b; Yu and Tang, 2005; Tang *et al.*, 2006). We thus examined the kinetochore localization of Sgo1 in our rescue experiments. Expression of either Myc-Bub1-WT or Myc-Bub1-T609A restored the localization of Sgo1 in Bub1-RNAi cells (Figure 9C and E). Consistent with an involvement of Plk1 in the removal of Sgo1 from kinetochores (Clarke *et al.*, 2005; Tang *et al.*, 2006), the intensity of Sgo1 at kinetochores was slightly higher in Myc-Bub1-T609A-expressing cells (Figure 9E). Importantly, because Myc-Bub1-T609A supports the kinetochore localization of Sgo1, this indicates that the kinetochore function of Bub1 is not grossly affected by the T609A mutation.

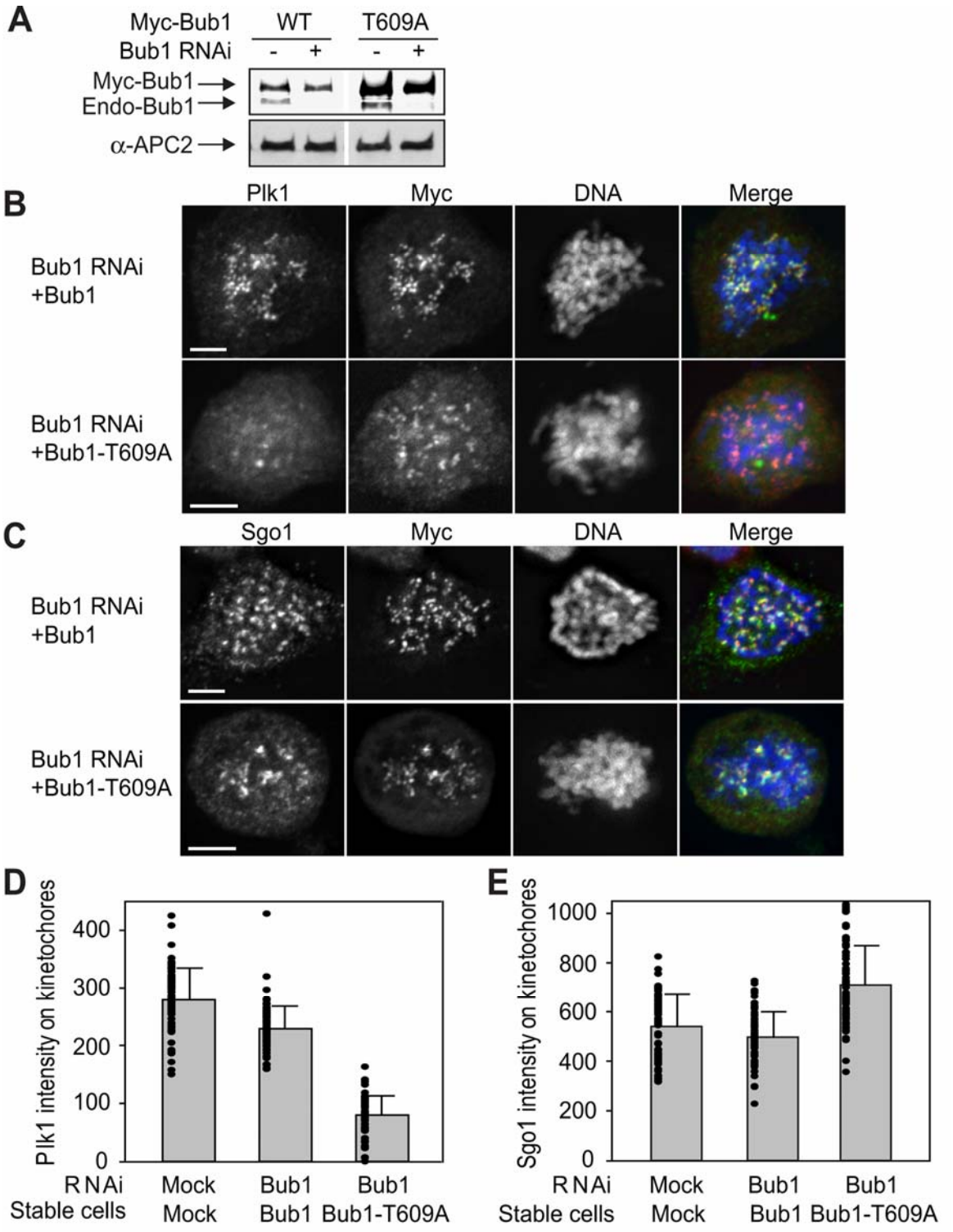


Figure 9. Expression of Bub1-T609A fails to restore the kinetochore localization of Plk1 in Bub1-RNAi cells. (A) HeLa Tet-On cells stably transfected with pTRE2-Myc-Bub1-WT or pTRE2-Myc-Bub1-T609A were cultured in the presence of doxycycline and

treated with mock or Bub1-RNAi. The cell lysates were blotted with α-Bub1 and α-APC2. The positions of the endogenous (Endo) Bub1 and Myc-Bub1 are indicated. (B) The cells described in (A) were stained with α-Plk1 (green), α-Myc (red), and DAPI (blue). The scale bar indicates 5 μm. (C) The cells described in (A) were stained with α-Sgo1 (green), α-Myc (red), and DAPI (blue). (D) Quantification of Plk1 immunofluorescence signals at the kinetochores. (E) Quantification of Sgo1 immunofluorescence signals at the kinetochores.

The kinetochore localization of Bub1 is impaired in INCENP-RNAi cells

While this manuscript was under review, Goto *et al.* reported that INCENP interacts with Plk1 in a phosphorylation- and PBD-dependent manner and is required for the kinetochore localization of Plk1 (Goto *et al*., 2006). We therefore investigated the regulation of Plk1 by INCENP. We first examined the localization of INCENP and Plk1 by immunofluorescence. INCENP localized to a single dot in the inner kinetochore between the two Plk1 dots and there was little overlap between the INCENP- and Plk1-staining (Figure 10A). Therefore, while Bub1 and Plk1 co-localize at the outer kinetochores (Figure 1D), INCENP does not co-localize with Plk1, which is inconsistent with the notion that the INCENP–Plk1 interaction directly recruits Plk1 to kinetochores. We next used the same siRNA that had been used by Goto *et al.* to deplete INCENP from HeLa cells (Figure 10E). Consistent with Goto *et al.*, INCENP-RNAi reduced the kinetochore localization of Plk1 (Figure 10A and C), albeit to a lesser extent than Bub1-RNAi. Importantly, the kinetochore localization of Bub1 was greatly reduced in INCENP-RNAi cells (Figure 10B and D). These results suggest that INCENP might regulate the localization of Plk1 through Bub1.

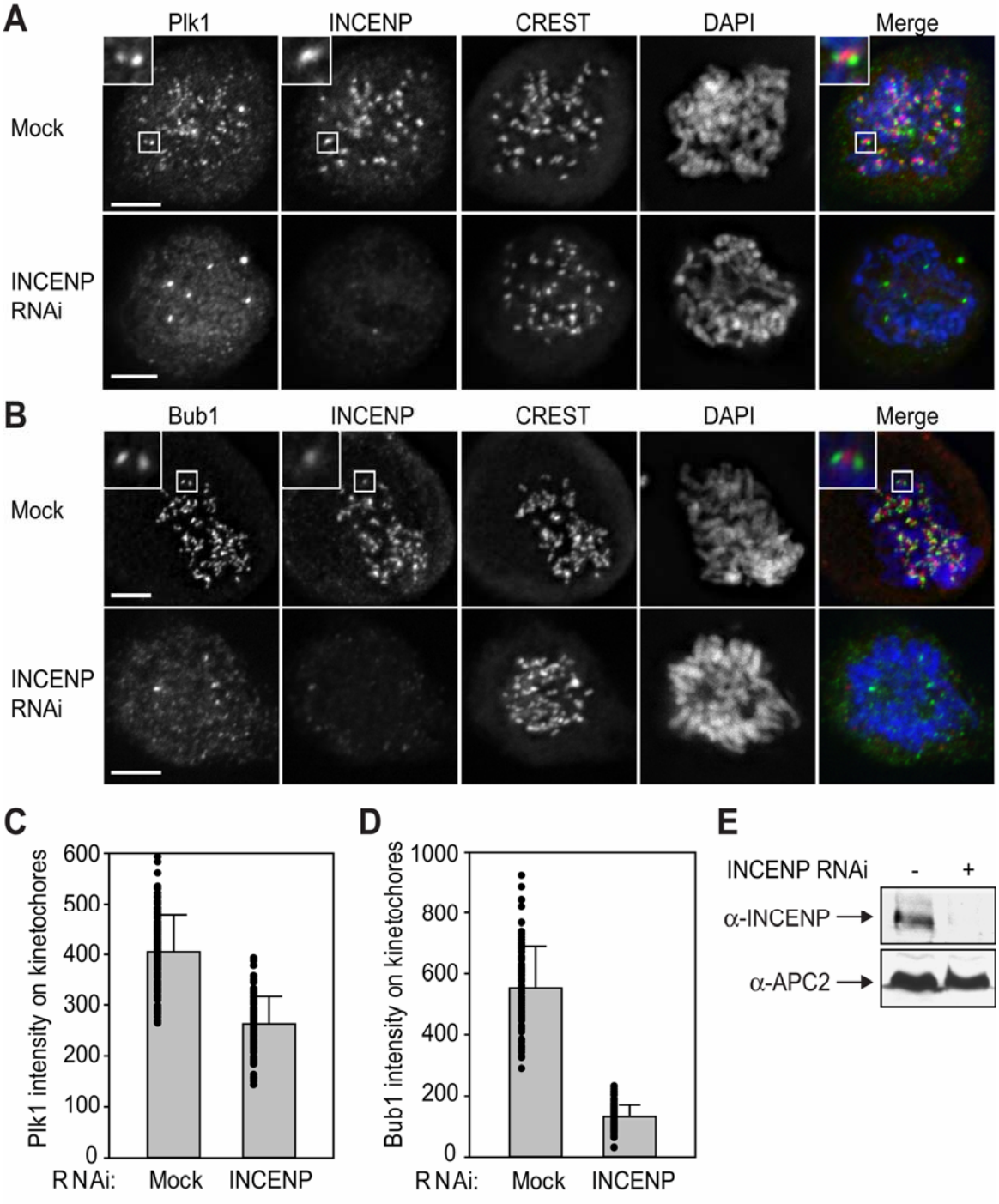


Figure 10. INCENP localizes to inner centromeres and is required for the kinetochore localization of Bub1 and Plk1. (A) HeLa cells that were either mock transfected or transfected with siRNA against INCENP were fixed and stained with α-Plk1, α-INCENP, CREST, and DAPI. In the merge, Plk1-staining is in green, INCENP-staining in red, and DAPI in blue. The boxed areas are magnified and shown in insets. The scale bar indicates 5

μm. (B) HeLa cells that were either mock transfected or transfected with siRNA against INCENP were fixed and stained with α-Bub1, α-INCENP, CREST, and DAPI. In the merge, Bub1-staining is in green, INCENP-staining in red, and DAPI in blue. The boxed areas are magnified and shown in insets. The scale bar indicates 5 μm. (C) Quantification of Plk1 immunofluorescence signals at the kinetochores in cells described in (A). (D) Quantification of Bub1 immunofluorescence signals at the kinetochores in cells described in (B). (E) Lysates of cells described in (A) were blotted with α-INCENP and α-APC2.

Discussion

Polo-box- and phosphorylation-dependent binding of Plk1 to Bub1 in mitosis

Ever since Yaffe and coworkers discovered the phosphopeptide-binding activity of the polo-box domain (PBD) of Plk1 (Elia *et al*., 2003a), numerous studies have established a role of the Plk1 PBD in targeting Plk1 to its binding partners and substrates (Litvak *et al*., 2004; Yoo *et al*., 2004; Fabbro *et al*., 2005; Preisinger *et al*., 2005). The prevailing view that emerged from these studies is that the Plk1-binding protein is first phosphorylated by a "priming" kinase, which creates a docking site for the PBD of Plk1. Binding of the phosphopeptide to PBD recruits Plk1 to its binding partner and allosterically activates the kinase activity of Plk1, thus facilitating the phosphorylation of Plk1 substrates. In addition to being Plk1 substrates, certain Plk1-binding proteins also target Plk1 to various mitotic structures. Our results presented herein suggest that binding of Plk1 to Bub1 appears to exploit a similar mechanism and targets Plk1 to kinetochores.

Because the optimal PBD-binding motif is S-pS/pT-P, the priming kinase for Plk1-binding proteins is likely a proline-directed protein kinase. Not surprisingly, the master mitotic kinase, Cdk1, has been shown to be the priming kinase for the majority of Plk1-

binding proteins, including Cdc25C, GRASP65, Cep55 and Nir2, although the MAP kinase, Erk2, has also been implicated in the priming phosphorylation of Cep55 (Elia *et al*., 2003a; Litvak *et al*., 2004; Fabbro *et al*., 2005; Preisinger *et al*., 2005). In the case of Bub1, we have shown that Cdk1 is sufficient to phosphorylate T609 and facilitates the binding of Plk1 to Bub1 and the subsequent phosphorylation of Bub1 by Plk1. We have also shown that Bub1 is phosphorylated at T609 in mitotic HeLa cells. Inhibition of Cdk1 by roscovitine in cells causes the dephosphorylation of Bub1 and disrupts its interaction with Plk1. The fission yeast Bub1 is also phosphorylated by Cdk1 (Yamaguchi *et al*., 2003). These results strongly suggest that Cdk1 is the priming kinase for Bub1. However, because Cdk1 is necessary and sufficient for maintaining cells in mitosis, it is exceedingly difficult to prove that Cdk1 is the actual kinase that phosphorylates Bub1 at T609 *in vivo*. Other kinase(s) may also be involved in phosphorylating this site on Bub1. For example, the *Xenopus* Bub1 protein is phosphorylated by MAPK at multiple S/T-P sites (Chen, 2004), one of which corresponds to T609 in human Bub1. It remains to be determined whether human Bub1 can be phosphorylated by MAP kinases at T609 *in vitro* and *in vivo*.

Bub1 is rapidly phosphorylated in mitotic mammalian cells that are briefly treated with nocodazole or taxol (Taylor *et al*., 2001). Furthermore, Bub1 becomes hyperphosphorylated when bound to chromatin (Chen, 2004). The kinase activity of Bub1 toward Cdc20 is also enhanced in mitosis (Tang *et al*., 2004a). Plk1 efficiently phosphorylates Bub1 that had been phosphorylated by Cdk1 *in vitro* (this study). Future experiments are needed to test whether Plk1 phosphorylates Bub1 *in vivo* and to determine the functional consequences of these phosphorylation events.

Requirement for Bub1 in the kinetochore localization of Plk1

It has become increasingly clear that Plk1 has important functions at the kinetochores during mitosis (Ahonen *et al*., 2005; Wong and Fang, 2005). It is thus critical to understand how Plk1 itself is recruited to kinetochores. Numerous elegant studies in *Xenopus* egg extracts and mammalian cells have established the interdependency and hierarchy of a large collection of mitotic regulatory proteins with respect to their localization at the kinetochores (Sharp-Baker and Chen, 2001; Johnson *et al*., 2004; Vigneron *et al*., 2004). Our results presented herein have established a requirement for Bub1 in the kinetochore localization of Plk1. Our findings are consistent with the notion that the kinetochore localization of Plk1 is facilitated by its polo-box- and phosphorylation-dependent binding to Bub1.

Intriguingly, although Plk1 also binds to BubR1 in mitosis, the kinetochore localization of Plk1 is not significantly affected by BubR1-depletion. There are several possible explanations for this observation. First, depletion of Bub1 might have caused the loss of other yet unidentified Plk1-binding proteins at the kinetochores. For the first possibility to be correct, the kinetochore localization of this putative Plk1-binding protein would also have to require T609 of Bub1 because expression of Bub1-T609A fails to restore the kinetochore localization of Plk1. Second, the concentration of BubR1 at the kinetochores might be lower than that of Bub1. Thus, loss of the BubR1-bound pool of Plk1 in BubR1-RNAi cells does not significantly alter the concentration of Plk1 at the kinetochores. Third, our results showed that the kinetochore localization of BubR1 requires Plk1 (Figure 8). Studies in yeast have also revealed that the mitotic phosphorylation of Mad3, the yeast

ortholog of BubR1, requires Plk1 (Rancati *et al*., 2005). It is possible that Plk1 also mediates the mitotic phosphorylation of BubR1 in mammalian cells. Binding between Plk1 and BubR1 might simply be a result of a kinase-substrate relationship and only occur following the recruitment of Plk1 to the kinetochores by Bub1. Consistent with this notion, the central spindle protein, Nir2, binds to Plk1 and is a Plk1 substrate, but Nir2 is not required for the localization of Plk1 at the central spindle (Litvak *et al*., 2004). Regardless of which possibility is correct, our results clearly indicate that Bub1, but not BubR1, is upstream of Plk1 with respect to kinetochore localization.

Relationship between Bub1 and INCENP in the kinetochore targeting of Plk1

In a recent paper, Goto *et al.* show that Plk1 binds directly to INCENP and the Plk1–INCENP interaction depends on the PBD of Plk1 and Cdk1-mediated phosphorylation of INCENP at T388 (Goto *et al*., 2006). Depletion of INCENP by RNAi resulted in inefficient kinetochore targeting of Plk1, which can be rescued by ectopic expression of the wild-type INCENP, but not the T388A mutant of INCENP. Contrary to published reports (Honda *et al*., 2003), Goto *et al.* further show that depletion of Aurora B by RNAi does not affect the kinetochore localization of INCENP and thus does not affect the kinetochore localization of Plk1. These results led Goto *et al.* to propose that INCENP directly recruits Plk1 to the kinetochores in an Aurora B-independent manner (Goto *et al*., 2006).

However, the model by Goto *et al.* is inconsistent with the following observations. First and foremost, as mentioned earlier, INCENP localizes to the inner kinetochores whereas Plk1 localizes to outer kinetochores (Figure 9A), suggesting that the bulk of kinetochore-

bound pools of these two proteins does not associate with each other. Bub1 and Plk1 co-localize to outer kinetochores, consistent with their direct physical interaction. Second, the mechanisms of kinetochore targeting of many kinetochore components are conserved between mammalian cells and *Xenopus* egg extracts. T388 of INCENP is not conserved in *Xenopus* INCENP. Third, the kinetochore localization of INCENP and Aurora B is interdependent (Honda *et al*., 2003). We have confirmed that the kinetochore localization of INCENP is indeed diminished in Aurora B-RNAi cells (Figure 11). Rather expectedly, we also show that the kinetochore localization of Plk1 is diminished in Aurora B-RNAi cells (Figure 11). These observations contradict the findings of Goto *et al.* that INCENP and Plk1 localize normally to kinetochores in Aurora B-RNAi cells and challenge their conclusion that the kinetochore localization of Plk1 is independent of Aurora B. One possible explanation is that the siRNAs against Aurora B used by Goto *et al.* did not deplete the levels of Aurora B as efficiently. Finally, numerous studies have shown that the Aurora B-INCENP complex is one of the most upstream components in the signaling cascades that control the kinetochore targeting of many kinetochore proteins. In particular, the Aurora B-INCENP complex is required for the localization of other spindle checkpoint proteins, such as Bub1, BubR1 and Mad2 (Johnson *et al*., 2004; Vigneron *et al*., 2004). Given that the kinetochore localization of Plk1 also requires Bub1 (this study), we favor the notion that the Aurora B-INCENP complex targets Bub1 to kinetochores, which in turn helps to recruit Plk1 to kinetochores. However, it remains possible that the Aurora B-INCENP complex is required to recruit Plk1 from the cytoplasm to the kinetochores initially, which enables Plk1 to associate with Bub1 and other PBD-docking proteins at kinetochores and is maintained at the kinetochores by

Bub1. This latter model explains the defective kinetochore localization of Plk1 in INCENP-T388A-expressing cells.

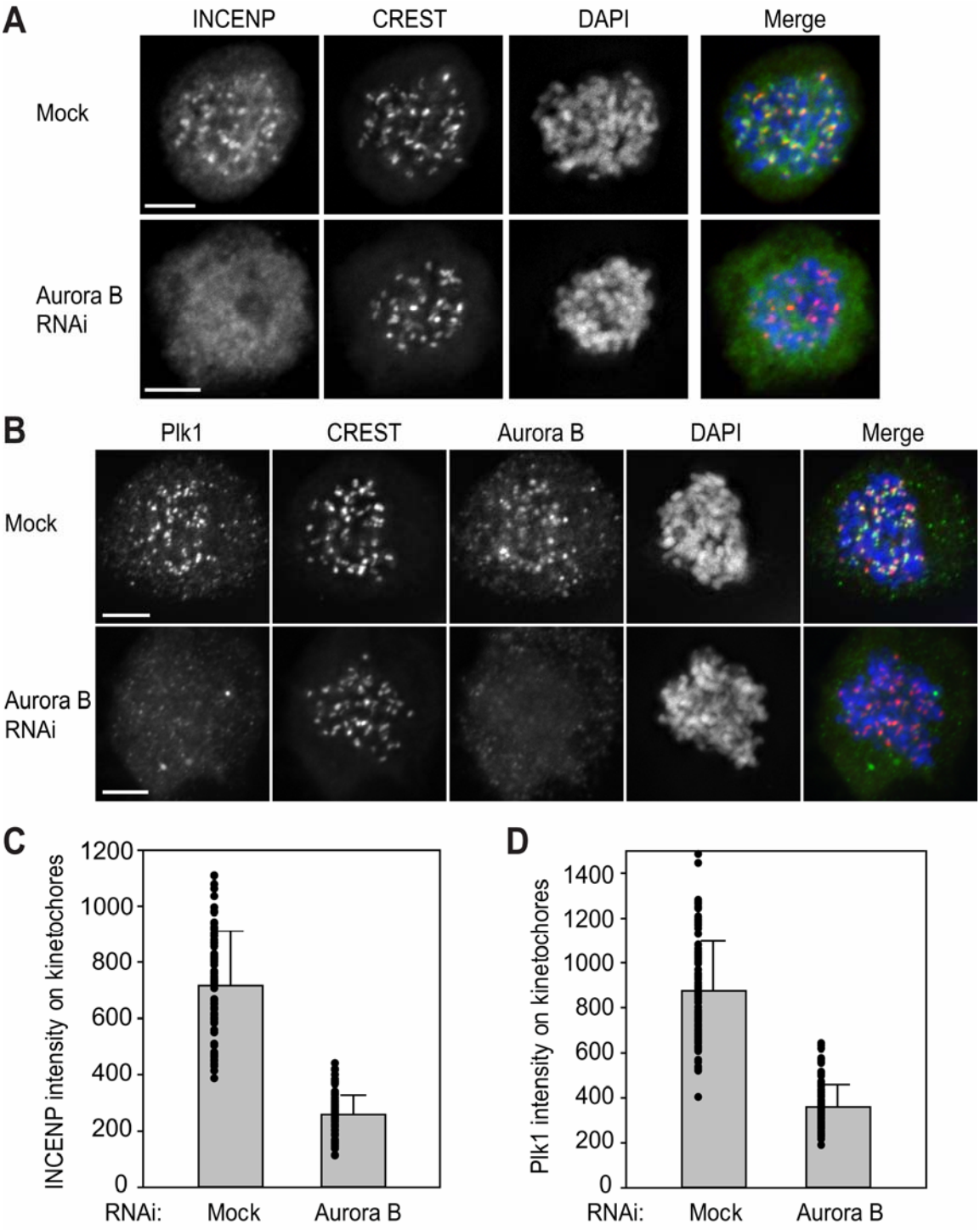


Figure 11. The kinetochore localization of INCENP and Bub1 is diminished in Aurora B-RNAi cells. (A) HeLa cells that were either mock transfected or transfected with siRNA against Aurora B were stained α-INCENP, CREST, and DAPI. In the merge, INCENP-staining is shown in green, CREST in red, and DAPI in blue. (B) HeLa cells that were either mock transfected or transfected with siRNA against Aurora B were stained α-Plk1, α-Aurora B, CREST, and DAPI. In the merge, Plk1-staining is shown in green, CREST in red, and DAPI in blue. (C) Quantification of INCENP immunofluorescence signals at the kinetochores in cells described in (A). (D) Quantification of Plk1 immunofluorescence signals at the kinetochores in cells described in (B).

Functions of Plk1 at kinetochores and in the spindle checkpoint

Recently, two independent studies in *Xenopus* egg extracts and mammalian cells have demonstrated that Plk1/Plx1 creates the tension-sensing 3F3/2 phosphoepitope at the kinetochores in response to the lack of mechanical tension across sister kinetochores (Ahonen *et al*., 2005; Wong and Fang, 2005). Loss of proper Plk1/Plx1 function not only reduces the 3F3/2 signal at the kinetochores, but also decreased the concentrations of other proteins at the kinetochores, including Hec1/Ndc80, Spc24, Mad2, Cdc20, CENP-E, and possibly BubR1 (Ahonen *et al*., 2005; Wong and Fang, 2005). These findings strongly suggest that Plk1 is crucial for spindle checkpoint signaling, at least in response to the lack of tension at kinetochores. Our study establishes a role of Bub1 upstream of Plk1 in the hierarchy of kinetochore localization and, quite possibly, in the tension-sensing pathway. The fact that Bub1, a well-established spindle checkpoint protein, controls the kinetochore localization of Plk1 further supports a role of Plk1 in the spindle checkpoint. Paradoxically, RNAi-mediated depletion of Plk1 in mammalian cells causes abnormal mitotic spindles and a spindle checkpoint-dependent mitotic arrest (Sumara *et al*., 2004; van Vugt *et al*., 2004). We

have so far failed to detect spindle checkpoint defects in Bub1-T609A expression cells that are treated with Bub1 RNAi. For example, expression of Bub1-T609A restores nocodazole/taxol-dependent mitotic arrest of Bub1-RNAi cells (unpublished data). There are two possible explanations for this finding. Loss of Bub1-dependent kinetochore targeting of Plk1may not completely deplete Plk1 at kinetochores. Alternatively, the kinetochore localization of Plk1 might not be absolutely required for the spindle checkpoint. Soluble, cytoplasmic pools of Plk1 might be sufficient to generate the 3F3/2 phosphoepitope.

In conclusion, we have shown that the Bub1–Plk1 interaction is required for efficient kinetochore targeting of Plk1 in mitosis. It has been shown previously that Bub1 and Plk1 are required for the kinetochore localization of Mad2 and other down-stream checkpoint proteins in a manner that does not require the kinase activity of Bub1 (Sharp-Baker and Chen, 2001; Johnson *et al*., 2004; Ahonen *et al*., 2005; Wong and Fang, 2005). Taken together, these findings are consistent with the following model: phosphorylation- and polo-box-dependent binding of Plk1 to Bub1 recruits Plk1 to kinetochores. The kinetochore-bound Plk1 then facilitates the kinetochore localization of BubR1, Mad2 and other checkpoint components.

REFERENCES

Ahonen, L.J., Kallio, M.J., Daum, J.R., Bolton, M., Manke, I.A., Yaffe, M.B., Stukenberg, P.T., and Gorbsky, G.J. (2005). Polo-like kinase 1 creates the tension-sensing 3F3/2 phosphoepitope and modulates the association of spindle-checkpoint proteins at kinetochores. Curr. Biol. *15*, 1078-1089.

Arnaud, L., Pines, J., and Nigg, E.A. (1998). GFP tagging reveals human Polo-like kinase 1 at the kinetochore/centromere region of mitotic chromosomes. Chromosoma *107*, 424-429.

Barr, F.A., Sillje, H.H., and Nigg, E.A. (2004). Polo-like kinases and the orchestration of cell division. Nat. Rev. Mol. Cell Biol. *5*, 429-440.

Bharadwaj, R., Qi, W., and Yu, H. (2004). Identification of two novel components of the human NDC80 kinetochore complex. J. Biol. Chem. *279*, 13076-13085.

Bharadwaj, R., and Yu, H. (2004). The spindle checkpoint, aneuploidy, and cancer. Oncogene *23*, 2016-2027.

Casenghi, M., Meraldi, P., Weinhart, U., Duncan, P.I., Korner, R., and Nigg, E.A. (2003). Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. Dev. Cell *5*, 113-125.

Chen, R.H. (2004). Phosphorylation and activation of Bub1 on unattached chromosomes facilitate the spindle checkpoint. EMBO J. *23*, 3113-3121.

Clarke, A.S., Tang, T.T., Ooi, D.L., and Orr-Weaver, T.L. (2005). POLO kinase regulates the Drosophila centromere cohesion protein MEI-S332. Dev. Cell *8*, 53-64.

Cleveland, D.W., Mao, Y., and Sullivan, K.F. (2003). Centromeres and kinetochores: from epigenetics to mitotic checkpoint signaling. Cell *112*, 407-421.

Cooke, C.A., Heck, M.M., and Earnshaw, W.C. (1987). The inner centromere protein (INCENP) antigens: movement from inner centromere to midbody during mitosis. J. Cell Biol. *105*, 2053-2067.

Cyert, M.S., Scherson, T., and Kirschner, M.W. (1988). Monoclonal antibodies specific for thiophosphorylated proteins recognize Xenopus MPF. Dev. Biol. *129*, 209-216.

DeLuca, J.G., Howell, B.J., Canman, J.C., Hickey, J.M., Fang, G., and Salmon, E.D. (2003). Nuf2 and Hec1 are required for retention of the checkpoint proteins Mad1 and Mad2 to kinetochores. Curr. Biol. *13*, 2103-2109.

Ditchfield, C., Johnson, V.L., Tighe, A., Ellston, R., Haworth, C., Johnson, T., Mortlock, A., Keen, N., and Taylor, S.S. (2003). Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. J. Cell Biol. *161*, 267-280.

Elia, A.E., Cantley, L.C., and Yaffe, M.B. (2003a). Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. Science *299*, 1228-1231.

Elia, A.E., Rellos, P., Haire, L.F., Chao, J.W., Ivins, F.J., Hoepker, K., Mohammad, D., Cantley, L.C., Smerdon, S.J., and Yaffe, M.B. (2003b). The molecular basis for phosphodependent substrate targeting and regulation of Plks by the Polo-box domain. Cell *115*, 83-95.

Fabbro, M., Zhou, B.B., Takahashi, M., Sarcevic, B., Lal, P., Graham, M.E., Gabrielli, B.G., Robinson, P.J., Nigg, E.A., Ono, Y., and Khanna, K.K. (2005). Cdk1/Erk2- and plk1-

dependent phosphorylation of a centrosome protein, cep55, is required for its recruitment to midbody and cytokinesis. Dev. Cell *9*, 477-488.

Fang, G., Yu, H., and Kirschner, M.W. (1998). Direct binding of CDC20 protein family members activates the anaphase-promoting complex in mitosis and G1. Mol. Cell *2*, 163-171.

Fisk, H.A., Mattison, C.P., and Winey, M. (2004). A field guide to the Mps1 family of protein kinases. Cell Cycle *3*, 439-442.

Glover, D.M. (2005). Polo kinase and progression through M phase in Drosophila: a perspective from the spindle poles. Oncogene *24*, 230-237.

Golsteyn, R.M., Mundt, K.E., Fry, A.M., and Nigg, E.A. (1995). Cell cycle regulation of the activity and subcellular localization of Plk1, a human protein kinase implicated in mitotic spindle function. J. Cell Biol. *129*, 1617-1628.

Gorbsky, G.J., and Ricketts, W.A. (1993). Differential expression of a phosphoepitope at the kinetochores of moving chromosomes. J. Cell Biol. *122*, 1311-1321.

Goto, H., Kiyono, T., Tomono, Y., Kawajiri, A., Urano, T., Furukawa, K., Nigg, E.A., and Inagaki, M. (2006). Complex formation of Plk1 and INCENP required for metaphase-anaphase transition. Nat. Cell Biol. *8*, 180-187.

Hansen, D.V., Loktev, A.V., Ban, K.H., and Jackson, P.K. (2004). Plk1 regulates activation of the anaphase promoting complex by phosphorylating and triggering SCFbetaTrCP-dependent destruction of the APC Inhibitor Emi1. Mol. Biol. Cell *15*, 5623-5634.

Hauf, S., Roitinger, E., Koch, B., Dittrich, C.M., Mechtler, K., and Peters, J.M. (2005). Dissociation of cohesin from chromosome arms and loss of arm cohesion during early mitosis depends on phosphorylation of SA2. PLoS Biol. *3*, e69.

Honda, R., Korner, R., and Nigg, E.A. (2003). Exploring the functional interactions between Aurora B, INCENP, and survivin in mitosis. Mol. Biol. Cell *14*, 3325-3341.

Hoyt, M.A., Totis, L., and Roberts, B.T. (1991). S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. Cell *66*, 507-517.

Johnson, V.L., Scott, M.I., Holt, S.V., Hussein, D., and Taylor, S.S. (2004). Bub1 is required for kinetochore localization of BubR1, Cenp-E, Cenp-F and Mad2, and chromosome congression. J. Cell Sci. *117*, 1577-1589.

Kitajima, T.S., Hauf, S., Ohsugi, M., Yamamoto, T., and Watanabe, Y. (2005). Human Bub1 defines the persistent cohesion site along the mitotic chromosome by affecting Shugoshin localization. Curr. Biol. *15*, 353-359.

Kumagai, A., and Dunphy, W.G. (1996). Purification and molecular cloning of Plx1, a Cdc25-regulatory kinase from Xenopus egg extracts. Science *273*, 1377-1380.

Lee, K.S., Park, J.E., Asano, S., and Park, C.J. (2005). Yeast polo-like kinases: functionally conserved multitask mitotic regulators. Oncogene *24*, 217-229.

Litvak, V., Argov, R., Dahan, N., Ramachandran, S., Amarilio, R., Shainskaya, A., and Lev, S. (2004). Mitotic phosphorylation of the peripheral Golgi protein Nir2 by Cdk1 provides a docking mechanism for Plk1 and affects cytokinesis completion. Mol. Cell *14*, 319-330.

Liu, J., and Maller, J.L. (2005). Xenopus Polo-like kinase Plx1: a multifunctional mitotic kinase. Oncogene *24*, 238-247.

Liu, X., Zhou, T., Kuriyama, R., and Erikson, R.L. (2004). Molecular interactions of Polo-like-kinase 1 with the mitotic kinesin-like protein CHO1/MKLP-1. J. Cell Sci. *117*, 3233-3246.

Lowery, D.M., Lim, D., and Yaffe, M.B. (2005). Structure and function of Polo-like kinases. Oncogene *24*, 248-259.

Martin-Lluesma, S., Stucke, V.M., and Nigg, E.A. (2002). Role of Hec1 in spindle checkpoint signaling and kinetochore recruitment of Mad1/Mad2. Science *297*, 2267-2270.

Meraldi, P., and Sorger, P.K. (2005). A dual role for Bub1 in the spindle checkpoint and chromosome congression. EMBO J. *24*, 1621-1633.

Mishima, M., Pavicic, V., Gruneberg, U., Nigg, E.A., and Glotzer, M. (2004). Cell cycle regulation of central spindle assembly. Nature *430*, 908-913.

Moshe, Y., Boulaire, J., Pagano, M., and Hershko, A. (2004). Role of Polo-like kinase in the degradation of early mitotic inhibitor 1, a regulator of the anaphase promoting complex/cyclosome. Proc. Natl. Acad. Sci. U. S. A. *101*, 7937-7942.

Neef, R., Preisinger, C., Sutcliffe, J., Kopajtich, R., Nigg, E.A., Mayer, T.U., and Barr, F.A. (2003). Phosphorylation of mitotic kinesin-like protein 2 by polo-like kinase 1 is required for cytokinesis. J. Cell Biol. *162*, 863-875.

Nicklas, R.B., Ward, S.C., and Gorbsky, G.J. (1995). Kinetochore chemistry is sensitive to tension and may link mitotic forces to a cell cycle checkpoint. J. Cell Biol. *130*, 929-939.

Pinsky, B.A., and Biggins, S. (2005). The spindle checkpoint: tension versus attachment. Trends Cell Biol. *15*, 486-493.

Preisinger, C., Korner, R., Wind, M., Lehmann, W.D., Kopajtich, R., and Barr, F.A. (2005). Plk1 docking to GRASP65 phosphorylated by Cdk1 suggests a mechanism for Golgi checkpoint signalling. EMBO J. *24*, 753-765.

Qian, Y.W., Erikson, E., and Maller, J.L. (1999). Mitotic effects of a constitutively active mutant of the Xenopus polo-like kinase Plx1. Mol. Cell. Biol. *19*, 8625-8632.

Rancati, G., Crispo, V., Lucchini, G., and Piatti, S. (2005). Mad3/BubR1 phosphorylation during spindle checkpoint activation depends on both Polo and Aurora kinases in budding yeast. Cell Cycle *4*, 972-980.

Sharp-Baker, H., and Chen, R.H. (2001). Spindle checkpoint protein Bub1 is required for kinetochore localization of Mad1, Mad2, Bub3, and CENP-E, independently of its kinase activity. J. Cell Biol. *153*, 1239-1250.

Sumara, I., Gimenez-Abian, J.F., Gerlich, D., Hirota, T., Kraft, C., de la Torre, C., Ellenberg, J., and Peters, J.M. (2004). Roles of polo-like kinase 1 in the assembly of functional mitotic spindles. Curr. Biol. *14*, 1712-1722.

Sumara, I., Vorlaufer, E., Stukenberg, P.T., Kelm, O., Redemann, N., Nigg, E.A., and Peters, J.M. (2002). The dissociation of cohesin from chromosomes in prophase is regulated by Polo-like kinase. Mol. Cell *9*, 515-525.

Tang, Z., Bharadwaj, R., Li, B., and Yu, H. (2001). Mad2-Independent inhibition of APCCdc20 by the mitotic checkpoint protein BubR1. Dev. Cell *1*, 227-237.

Tang, Z., Shu, H., Oncel, D., Chen, S., and Yu, H. (2004a). Phosphorylation of Cdc20 by Bub1 provides a catalytic mechanism for APC/C inhibition by the spindle checkpoint. Mol. Cell *16*, 387-397.

Tang, Z., Shu, H., Qi, W., Mahmood, N.A., Mumby, M.C., and Yu, H. (2006). PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. Dev. Cell *10*, 575-585.

Tang, Z., Sun, Y., Harley, S.E., Zou, H., and Yu, H. (2004b). Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis. Proc. Natl. Acad. Sci. U. S. A. *101*, 18012-18017.

Tang, Z., and Yu, H. (2004). Functional analysis of the spindle-checkpoint proteins using an in vitro ubiquitination assay. Methods Mol. Biol. *281*, 227-242.

Taylor, S.S., Hussein, D., Wang, Y., Elderkin, S., and Morrow, C.J. (2001). Kinetochore localisation and phosphorylation of the mitotic checkpoint components Bub1 and BubR1 are differentially regulated by spindle events in human cells. J. Cell Sci. *114*, 4385-4395.

Taylor, S.S., and McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. Cell *89*, 727-735.

Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A., and Nishida, E. (2001). Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. Nature *410*, 215-220.

van Vugt, M.A., and Medema, R.H. (2005). Getting in and out of mitosis with Polo-like kinase-1. Oncogene *24*, 2844-2859.

van Vugt, M.A., van de Weerdt, B.C., Vader, G., Janssen, H., Calafat, J., Klompmaker, R., Wolthuis, R.M., and Medema, R.H. (2004). Polo-like kinase-1 is required for bipolar spindle formation but is dispensable for anaphase promoting complex/Cdc20 activation and initiation of cytokinesis. J. Biol. Chem. *279*, 36841-36854.

Vigneron, S., Prieto, S., Bernis, C., Labbe, J.C., Castro, A., and Lorca, T. (2004). Kinetochore localization of spindle checkpoint proteins: who controls whom? Mol. Biol. Cell *15*, 4584-4596.

Watanabe, N., Arai, H., Nishihara, Y., Taniguchi, M., Hunter, T., and Osada, H. (2004). M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. Proc. Natl. Acad. Sci. U. S. A. *101*, 4419-4424.

Wong, O.K., and Fang, G. (2005). Plx1 is the 3F3/2 kinase responsible for targeting spindle checkpoint proteins to kinetochores. J. Cell Biol. *170*, 709-719.

Xie, S., Xie, B., Lee, M.Y., and Dai, W. (2005). Regulation of cell cycle checkpoints by polo-like kinases. Oncogene *24*, 277-286.

Yaffe, M.B., Schutkowski, M., Shen, M., Zhou, X.Z., Stukenberg, P.T., Rahfeld, J.U., Xu, J., Kuang, J., Kirschner, M.W., Fischer, G., Cantley, L.C., and Lu, K.P. (1997). Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. Science *278*, 1957-1960.

Yamaguchi, S., Decottignies, A., and Nurse, P. (2003). Function of Cdc2p-dependent Bub1p phosphorylation and Bub1p kinase activity in the mitotic and meiotic spindle checkpoint. EMBO J. *22*, 1075-1087.

Yoo, H.Y., Kumagai, A., Shevchenko, A., and Dunphy, W.G. (2004). Adaptation of a DNA replication checkpoint response depends upon inactivation of Claspin by the Polo-like kinase. Cell *117*, 575-588.

Yu, H. (2002). Regulation of APC-Cdc20 by the spindle checkpoint. Curr. Opin. Cell Biol. *14*, 706-714.

Yu, H., and Tang, Z. (2005). Bub1 multitasking in mitosis. Cell Cycle *4*, 262-265.

Zhou, T., Aumais, J.P., Liu, X., Yu-Lee, L.Y., and Erikson, R.L. (2003). A role for Plk1 phosphorylation of NudC in cytokinesis. Dev. Cell *5*, 127-138.

CHAPTER FIVE

PERSPECTIVES AND FUTURE DIRECTIONS

Bub1 is an essencial component of the spindle checkpoint and is conserved from yeast to man. The functions of Bub1 in spindle checkpoint signaling have been established in the last five years. First, Bub1 is required for the kinetochore localization of other checkpoint proteins, including BubR1, Bub3, Mad1 and Mad2. Second, work from our group showed that Bub1 phosphorylates Cdc20 and inhibits APC/C directly. This establishes a second function of Bub1 in the spindle checkpoint that is distinct from its function in the MCC branch of the spindle checkpoint.

My work presented in this dissertation further dissects the mechanisms of Bub1 signaling. I showed that Bub1 on unattached kinetochores is hyperphosphorylated and highly active toward Cdc20, suggesting that Bub1 is likely a catalyst of the wait-anaphase signals. That the kinase activity of Bub1 is required for proper spindle checkpoint signaling in cells underscores the importance of the Bub1-Cdc20 connection. Furthermore, Plk1 is identified as a key downstream component of Bub1 that regulates the kinetochore localization of BubR1 and Mad2. Bub1 directly interacts with Plk1 through Cdk1 phosphorylation and recruits Plk1 to kinetochores in mitosis. Plk1 in turn regulates the kinetochore localization of BubR1 and Mad2, transducing checkpoint signals (Figure 1).

APC/C^{Cdc20} promotes metaphase-anaphase transition and is the target of the spindle checkpoint. After all chromosomes are aligned at the metaphase plate, the spindle checkpoint is satisfied, leading to chromosome segregation and mitotic exit. Bub1, an APC/C inhibitor, is itself degraded by APC/C ^{Cdh1} during mitotic exit (Figure 1). Several other spindle checkpoint proteins are also APC/C^{Cdh1} substrates, such as Aurora B, Plk1, and Mps1. This feed forward mechanism likely prevents the reactivation of spindle checkpoint during mitotic exit and early G1.

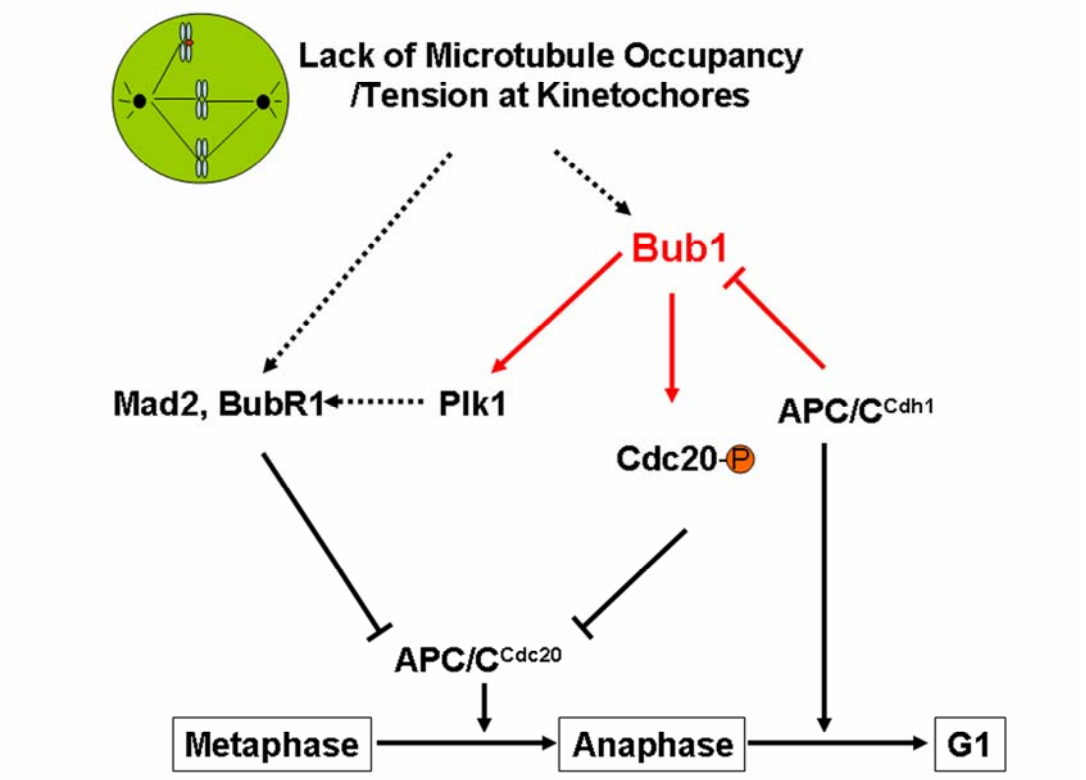


Figure 1. Summary of the dissertation. The connections studied in this dissertation are indicated by red arrows.

A fundamental question in the spindle checkpoint field is how the checkpoint proteins are recruited to kinetochores. Earlier studies using immunodepletion and RNAi methods demonstrated the hierarchy of the checkpoint proteins on kinetochores in *Xenopus* egg extracts and mammalian cells. However, the kinetochore component that directly recruits Bub1 has no been identified in mammalian cells. We have attempted to study this problem by anti-Bub1 immunopurification using mitotic HeLa cell lysate. The mitotic chromosome lysate described in Chapter 3 may be a better input in IP experiments to identify proteins that recruit Bub1 to kinetochores. Bub1, Mps1 and BubR1 are highly phosphorylated on kinetochores and these phosphorylation events may be required for the recruitment of these and other spindle checkpoint proteins to kinetochores. A large scale anti-Bub1 immunopurification using metaphase chromosome lysate will be valuable to identify the kinetochore components that recruit Bub1 and to systematically map phosphorylation sites on Bub1.

Apart from the spindle checkpoint functions of Bub1, evidence from our lab and other groups indicates that Bub1 has additional functions in mitosis. One of these functions is the protection of centromeric cohesion. Bub1 is required for proper chromosome segregation during meiosis in yeast (Bernard et al., 2001). Watanabe and colleagues identified Shugoshin (Sgo) family of proteins that is required for the sister-chromatid non-disjunction during meiosis I and early mitosis in yeast (Kitajima et al., 2004). Very recently, Bub1 in mammalian cells has been shown to target Sgo-PP2A complexes to kinetochores (Kitajima et al., 2005; Kitajima et al., 2006; Tang et al., 2006; Tang et al., 2004). These complexes counteract the phosphorylation of cohesin by Plk1 at centromeres, thereby preserving

centromeric cohesion. Another function of Bub1 proposed by Meraldi and colleagues is that Bub1 promotes the formation of stable bipolar kinetochore-microtubule attachments (Meraldi and Sorger, 2005). They showed by elegant microscopy analysis that depletion of Bub1 by RNAi causes side-on (instead of head-on) attachment of microtubule and congression defect (Meraldi and Sorger, 2005). One apparent question is how Bub1 performs its multiple functions at kinetochores. It will be useful to test whether the kinase activity of Bub1 is required for all its functions by rescue experiments after Bub1 RNAi. If it is required, Bub1 likely has different substrates that are involved in different functions. We have demonstrated the requirement of the kinase activity of Bub1 in spindle checkpoint signaling and identified Cdc20 as the key substrate in this process. Identifying the substrates of Bub1 for other functions by candidate approaches or proteomic methods will provide insights into the mechanisms of chromosome segregation.

In summary, there are exciting challenges in understanding the multiple functions and complex regulations of Bub1 kinase. Studies on Bub1 will provide further insights into the spindle checkpoint and chromosome segregation.

References

Bernard, P., Maure, J. F., and Javerzat, J. P. (2001). Fission yeast Bub1 is essential in setting up the meiotic pattern of chromosome segregation. Nat Cell Biol *3*, 522-526.

D'Angiolella, V., Mari, C., Nocera, D., Rametti, L., and Grieco, D. (2003). The spindle checkpoint requires cyclin-dependent kinase activity. Genes Dev *17*, 2520-2525.

Kitajima, T. S., Hauf, S., Ohsugi, M., Yamamoto, T., and Watanabe, Y. (2005). Human Bub1 defines the persistent cohesion site along the mitotic chromosome by affecting Shugoshin localization. Curr Biol *15*, 353-359.

Kitajima, T. S., Kawashima, S. A., and Watanabe, Y. (2004). The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis. Nature *427*, 510-517.

Kitajima, T. S., Sakuno, T., Ishiguro, K., Iemura, S., Natsume, T., Kawashima, S. A., and Watanabe, Y. (2006). Shugoshin collaborates with protein phosphatase 2A to protect cohesin. Nature *441*, 46-52.

Meraldi, P., and Sorger, P. K. (2005). A dual role for Bub1 in the spindle checkpoint and chromosome congression. Embo J *24*, 1621-1633.

Tang, Z., Shu, H., Qi, W., Mahmood, N. A., Mumby, M. C., and Yu, H. (2006). PP2A is required for centromeric localization of Sgo1 and proper chromosome segregation. Dev Cell *10*, 575-585.

Tang, Z., Sun, Y., Harley, S. E., Zou, H., and Yu, H. (2004). Human Bub1 protects centromeric sister-chromatid cohesion through Shugoshin during mitosis. Proc Natl Acad Sci U S A *101*, 18012-18017.

VITAE

Wei Qi was born in Shandong province, China on February 22, 1977, the second of two daughters of Jixiang Qi and Lizhen Zhao. After completing her work at the Number One High School (Dezhou, China) in 1995, she entered Nanjing University (Nanjing, China) and received the degree of Bachelor of Science with a major in Biology in June 1999 and the degree of Master of Science in June 2001. In August 2002 she entered the Graduate School of Biomedical Sciences at the University of Texas Health Science Center at Houston. In June 2003, she transferred to the University of Texas Southwestern Medical Center at Dallas, where she did her work under the supervision of Dr. Hongtao Yu. In May 2001, she married Baoliang Song, Ph.D. Their daughter, Siyuan Song was born in April 2002.

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