Structural analysis reveals features of the spindle checkpoint kinase Bub1–kinetochore subunit Knl1 interaction

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The function of the essential checkpoint kinases Bub1 and BubR1 requires their recruitment to mitotic kinetochores. Kinetochore recruitment of Bub1 and BubR1 is proposed to rely on the interaction of the tetra-tricopeptide repeats (TPRs) of Bub1 and BubR1 with two KI motifs in the outer kinetochore protein Knl1. We determined the crystal structure of the Bub1 TPRs in complex with the cognate Knl1 KI motif and compared it with the structure of the equivalent BubR1TPR–KI motif complex. The interaction developed along the convex surface of the TPR assembly. Point mutations on this surface impaired the interaction of Bub1 and BubR1 with Knl1 in vitro and in vivo but did not cause significant displacement of Bub1 and BubR1 from kinetochores. Conversely, a 62-residue segment of Bub1 that includes a binding domain for the checkpoint protein Bub3 and is C terminal to the TPRs was necessary and largely sufficient for kinetochore recruitment of Bub1. These results shed light on the determinants of kinetochore recruitment of Bub1.

Introduction

The spindle assembly checkpoint, or mitotic checkpoint, coordinates mitotic timing with chromosome–spindle interactions during mitosis, restricting mitotic exit to cells that have achieved bi-orientation of all their chromosomes (Musacchio and Salmon, 2007). Cells in which the checkpoint is artificially inactivated undergo precocious mitotic exit in the presence of unattached or incorrectly attached chromosomes. Alterations of checkpoint function might be relevant for tumor development, possibly by rendering cells more susceptible to the development of aneuploidies and to consequent genetic instability (Kolodner et al., 2011).

Bub1 (budding uninhibited by benzimidazole 1) was originally characterized as a conserved component of the spindle assembly checkpoint (Hoyt et al., 1991; Taylor and McKeon, 1997; Musacchio and Salmon, 2007). More recently, Bub1 was also shown to play a function in chromosome alignment (Johnson et al., 2004; Meraldi and Sorger, 2005; Windecker et al., 2009).

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Abbreviations used in this paper: GLEBS, Gle2 binding site; IRES, internal ribosomal entry site; TPR, tetra-tricopeptide repeat.

Precisely how Bub1 performs these functions at the molecular level is unclear (Bolanos-Garcia and Blundell, 2011; Elowe, 2011). Bub1 localization at kinetochores, in which it displays slow exchange dynamics during mitosis (Howell et al., 2004; Shah et al., 2004), might be important or even essential for its functions. Bub1 phosphorylates Cdc20, the target of the checkpoint, on several sites, promoting its ability to engage in an inhibitory complex with other checkpoint proteins (Tang et al., 2004a). Moreover, Bub1 promotes kinetochore recruitment of other checkpoint proteins, including Mad1, Mad2, Mad3/BubR1 (Bub1 related), and Bub3. Such recruitment is in turn believed to be important for the activity of these proteins (Sharp-Baker and Chen, 2001; Chen, 2002; Johnson et al., 2004; Vignon et al., 2004; Meraldi and Sorger, 2005; Boyarchuk et al., 2007; Rischitor et al., 2007; Klebig et al., 2009; Storchová et al., 2011). Bub1 also phosphorylates H2A (histone 2A), promoting the recruitment of Sgo1 and Aurora B to the centromere (Kitajima et al., 2004, 2005; Tang et al., 2004b; Vaur et al., 2005; Fernius and Hardwick, 2007; Perera et al., 2007; Yamagishi et al., 2010; Kawashima et al., 2010; Wang et al., 2011).
BubR1, whose overall domain organization is very similar to that of Bub1 (Fig. 1 A), is also implicated both in the spindle checkpoint and in chromosome alignment (Li and Murray, 1991; Taylor et al., 1998; Chan et al., 1999; Johnson et al., 2004; Lampson and Kapoor, 2005). Unlike Bub1, BubR1 is incorporated together with Bub3, Mad2, and Cdc20 in the checkpoint effector, the so-called mitotic checkpoint complex, which inactivates the anaphase-promoting complex/cyclosome to prevent mitotic exit (Musacchio and Salmon, 2007).

Kinetochore recruitment of Bub1 and BubR1 may be strongly intertwined with their activation and functions there. For instance, kinetochore localization of Bub1 and BubR1 might be important for their phosphorylation, which in turn contributes to the functions of these kinases (Yamaguchi et al., 2003; Elowe et al., 2010). The exact mechanism of kinetochore recruitment of Bub1 and BubR1, however, remains unclear. It was originally shown that ∼300 residues in the N-terminal region of murine Bub1 (shown schematically in Fig. 1 A) are sufficient for kinetochore localization (Taylor and McKeon, 1997; Taylor et al., 1998). This region of Bub1 includes an array of three tetratricopeptide repeats (TPRs; D’Arcy et al., 2010; Bolanos-Garcia et al., 2011, 2009) followed by a motif, also present in BubR1, which binds to the checkpoint protein Bub3 (Taylor et al., 1998). Bub3 is a β-propeller structure that uses its top surface to interact directly with the Bub3-binding motifs of Bub1 and BubR1/Mad3 (Larsen et al., 2007). The Bub3-binding motif is now also often referred to as the Gle2 binding site (GLEBS) motif (Wang et al., 2001). Here, however, we prefer to use the name Bub3-binding domain (abbreviated as Bub3-BD) because there is no evidence, to our knowledge, that Bub1 and BubR1 interact with the Gle2 protein (also known as Rae1).

Further deletion mapping of Bub1 demonstrated that the TPR region is dispensable for kinetochore localization and that a segment containing the Bub3-BD might be sufficient for kinetochore localization (Taylor et al., 1998). Mutations in the Bub3-BD prevent kinetochore localization of Bub1 and BubR1 and impair BubR1’s function in checkpoint and chromosome congression (Taylor et al., 1998; Klebig et al., 2009; Malureanu et al., 2009; Elowe et al., 2010). As the only known function of the Bub3-BD of Bub1 and BubR1 is Bub3 binding, these data argue that the interaction of Bub1 and BubR1 with Bub3 might be necessary and sufficient for their kinetochore localization. Paradoxically contradicting this idea, however, depletion of Bub3 does not affect Bub1 localization, whereas it might affect the localization of BubR1 (Meraldi et al., 2004; Logarinho et al., 2008). Conversely, depletion of Bub1 or BubR1 was found to reduce kinetochore recruitment of Bub3, suggesting that these proteins are not simply recruited by Bub3 (Sharp-Baker and Chen, 2001; Chen, 2002).

More recently, insight into the mechanism of kinetochore recruitment of Bub1 and BubR1 developed around the discovery that their TPRs interact with the outer kinetochore protein Knl1 (also known as Blinkin, CASC5, and AF15q14 in human cells, Spc105 in Saccharomyces cerevisiae, and Spc7 in Schizosaccharomyces pombe; Kiymitsu et al., 2007, 2011; Schittenhelm et al., 2009). Knl1 is a subunit of the KMN network, a 10-subunit assembly of three complexes, the Knl1 complex (KNL1-C, comprised of Knl1 and Zwint-1), the MIS12 complex (MIS12-C, comprised of Mis12/Mtw1, Dsn1, Nuf1, and Nsl1), and the NDC80 complex (NDC80-C, comprised of Ndc80/HeC1, Nuf2, Spc24, and Spc25). The KMN network mediates microtubule attachment through microtubule-binding domains located in the Ndc80 and Knl1 subunits (Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009). It has also been implicated in the recruitment of all known checkpoint proteins, suggesting that it plays a crucial role in relaying microtubule attachment status to the spindle checkpoint response.

The TPRs of Bub1 and BubR1 interact with distinct, but related, 12-residue motifs in the N-terminal region of Knl1, the KI motifs (from the first two residues of their consensus sequence, KI(D/N)XXXF(L/I)XXLK, in which X’s are non-conserved residues; Fig. 1 A; Bolanos-Garcia et al., 2011; Kiymitsu et al., 2011). The two consecutive motifs are herewith indicated as KI1 and KI2. Although it was originally hypothesized that these interactions might engage residues on the concave surface of the superhelically twisted TPR repeat assemblies of Bub1 and BubR1 (D’Arcy et al., 2010; Kiymitsu et al., 2011), a very recent structural analysis of the BubR1–K12 complex revealed that the K12 motif of Knl1 engages the convex surface of the BubR1 TPR region (Bolanos-Garcia et al., 2011).

The potential importance of the interaction of the KI motif of Knl1 with the Bub1 TPRs is underpinned by the observation that a point mutant in the TPRs prevents kinetochore recruitment of Bub1 (Kiymitsu et al., 2007). Furthermore, depletion of Knl1 by RNAi prevents kinetochore recruitment of Bub1 and BubR1 (Kiymitsu et al., 2007). Finally, a deletion mutant lacking the TPRs of Bub1 failed to localize to kinetochores, reinvigorating the previously dismissed idea that this region of Bub1 participates in kinetochore recruitment (Klebig et al., 2009). Thus, both the N-terminal TPRs and the Bub3-BD, which bind to Knl1 and Bub3, are thought to contribute to kinetochore recruitment of Bub1 and BubR1, but there is no unifying view of the relative importance of their contributions. We have therefore set out to clarify this important question.

**Results**

**Role of the TPRs of Bub1 and BubR1 in kinetochore recruitment**

We tested whether the TPR domain of human Bub1 (included in two constructs encompassing residues 1–150 or residues 1–190) is sufficient for kinetochore binding in HeLa cells. Expression of EGFP fusions of wild-type full-length Bub1 (Bub1(FL)) resulted in bright kinetochore staining (Fig. 1 B and Fig. S1 for expression levels of the transgens). On the other hand, EGFP fusions of Bub1(1–150) or Bub1(1–190) failed to localize to kinetochores (Fig. 1 B). These results suggest that the TPR region of Bub1 is not sufficient for kinetochore localization. We next tested whether this region is necessary for kinetochore binding. Bub1 mutants lacking either 150 or 189 residues from their N terminus (Bub1(Δ150) or Bub1(Δ189)) localized normally to kinetochores (Fig. 1 B). It is unlikely that these results were...
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Overall, these results indicate that the TPR region of Bub1 and BubR1 is neither sufficient, nor strictly necessary, for kinetochore recruitment. The TPR domains of Bub1 and BubR1 bind Knl1 directly

The TPR regions of Bub1 and BubR1 have been previously shown to mediate an interaction with the kinetochore protein Knl1 and have been suggested to promote the recruitment of Bub1 and BubR1 to kinetochores via this interaction (Kiyomitsu et al., 2007, 2011; Klebig et al., 2009; D’Arcy et al., 2010). Our results in Fig. 1, however, indicate that the TPR region of Bub1 is neither sufficient nor strictly necessary, for kinetochore recruitment. Overall, these results indicate that the TPR region of Bub1 and BubR1 is neither sufficient, nor strictly necessary, for kinetochore recruitment.
the TPR region is predicted not only to remove the Knl1 binding site but also to relieve an intramolecular inhibitory function, with the consequent constitutive exposure of the high-affinity binding site even in the absence of Knl1 binding.

secondary kinetochore-binding domain in Bub1 and BubR1. This secondary site would provide the bulk of the kinetochore-binding affinity but should only become exposed after the initial binding of Knl1 to the TPR motifs. In such a model, deletion of the TPR region is predicted not only to remove the Knl1 binding site but also to relieve an intramolecular inhibitory function, with the consequent constitutive exposure of the high-affinity binding site even in the absence of Knl1 binding.
An implication of the model is that if one were able to selectively perturb the interaction of Bub1 with Knl1, without disrupting the hypothetical intramolecular switch, the resulting mutant impaired in Knl1 binding might also be impaired in kinetochore binding as a consequence of constitutive inhibition. Testing this model required a better understanding of the structural basis of the interaction of the Bub1 and BubR1 TPRs with Knl1, so as to allow the creation of separation of function mutants. Thus, we attempted to unveil the biochemical and structural basis of this interaction. Because it is formally undemonstrated that the interaction of Bub1 and BubR1 with Knl1 is direct, we expressed and purified recombinant versions of Bub1(1–150) and BubR1(1–204) and tested their ability to bind to Knl1 constructs encompassing the previously identified KI motifs (depicted in Fig. 1 A; Kiyomitsu et al., 2011). When analyzed by size-exclusion chromatography (a technique that allows the separation of macromolecules based on their size and shape), Bub1(1–150) and BubR1(1–204) were shown to form stoichiometric complexes with Knl1(150–250), a construct that encompasses both the KI1 and the KI2 motif (Fig. 2, A and B). Conversely, Bub1(1–150) bound to Knl1(150–200), which contains the KI1 motif (Fig. 2 C), but was unable to bind to Knl1 (201–250), which contains the KI2 motif. Precisely the opposite result was obtained with BubR1(1–204). The latter bound Knl1 (201–250), which contains the KI2 motif, but not Knl1(150–200), which contains the KI1 motif (Fig. 2 D). Thus, the TPR motifs of Bub1 and BubR1 bind directly and specifically to the KI1 and KI2 motif, respectively.

Crystall structure of the Bub1(1–150)–Knl1(150–200) complex

Next, we determined the crystal structure of the Bub1(1–150)–Knl1(150–200) complex. Single crystals of the complex diffracted to a maximal resolution of 2.6 Å in the space group P2₁ (Table 1). The four Bub1(1–150)–Knl1(150–200) complexes in the asymmetric unit are very similar, with the only exceptions (fractured to a maximal resolution of 2.6 Å in the space group P2₁) allowing the separation of macromolecules based on their size and a mutant carrying the four alanine mutations characterized in Fig. 4 A). Additionally, there are hydrogen bonds between Thr179(Knl1) and Thr180(Knl1) with Gln84(Bub1). These observations are consistent with a previous analysis of the effects of mutations in the KI1 motif of Knl1 on Bub1 binding (Kiyomitsu et al., 2011). On Bub1, the A-type helix α4 contributes the side chains of Phe75 and Asn79, thus participating from the bottom to the creation of the Knl1-binding ridge. The B-type α3 and α5 helices surround the ridge, with α5 contributing the side chains of Gln84, Phe85, and Phe88 (Fig. 4 A).

In vitro and in vivo validation of the Bub1–Knl1 interaction

We individually mutated Phe75, Asn79, Gln84, and Phe85 to alanine and evaluated the ability of the mutants to bind Knl1(150–250) by size-exclusion chromatography (mutation of Phe88 to alanine rendered the protein insoluble when expressed in Escherichia coli; Fig. 4 C). We observed essentially complete disruption of the Bub1(1–150)–Knl1(150–250) complex in three out of four cases (only the Gln84 to Ala mutant had mild or no effects on binding). The results confirm the role of the interface on the convex surface of Bub1 in Knl1 binding in vitro.

Next, we tested the effects of Bub1 mutations on the ability of Bub1 to bind Knl1 in vivo. For this, we generated stable inducible cell lines expressing EGFP-tagged wild-type Bub1 and a mutant carrying the four alanine mutations characterized in Fig. 4 C (Bub1(4A)). We next evaluated the interaction of

| Variable | Value |
|----------|-------|
| Beamline | ESRF ID14 EH1 |
| α (Å) | 59.3 |
| b (Å) | 131 |
| c (Å) | 75 |
| α and γ (°) | 90 |
| β (°) | 110.2 |
| Space group | P2₁ |
| Wavelength (Å) | 0.9334 |
| Resolution | 65-2.6 (2.74-2.60) |
| Completeness (%) | 98.8 (99.6) |
| Multiplicity | 2.8 (2.8) |
| Mean (I)/σ(I) | 13.7 (3.2) |
| Rmerge | 0.072 (0.50) |

Numbers in parentheses correspond to the highest resolution shell. Rmerge is the multiplicity-weighted merging R factor according to Diederichs and Karplus (1997), I, reflection intensity.
By fluorescence polarization anisotropy, we determined that the binding affinity of BubR1(1–204) for a synthetic fluorescent peptide corresponding to Knl1(210–226) is 0.45 µM, almost 100-fold tighter than the value measured for the interaction of Bub1(1–150) to Knl1(174–190) (Fig. 4 B and Fig. 5 C). The significance of this difference in binding affinity is currently unclear but might be caused by a technical limitation of the assay, as we note that Bub1(1–150) interacts stoichiometrically with Knl1(150–250) or Knl1 (150–200) when the individual proteins are mixed at a concentration of 5 µM, suggesting that the $K_d$ of the interaction may realistically be lower (i.e., higher affinity. See Materials and methods for details on the assay). While this paper was under review, the crystal structure of the complex of human BubR1 TPR region bound to the KI2 motif was published (Protein Data Bank accession no. 3SI5; Bolanos-Garcia et al., 2011). The BubR1 backbone superimposes on the Bub1 TPR structure with a root-mean-square deviation of 0.9 Å. These results strongly support the view, based on the crystal structure, that the convex surface of the Bub1 TPR region contributes to Knl1 binding in cells.

**Interaction of BubR1 with Knl1**

The sequences of the TPR regions of Bub1 and BubR1 are closely related (Fig. 5 A). Similarly, the sequences of the previously identified KI motifs of Knl1 are also closely related (Fig. 5 B). By fluorescence polarization anisotropy, we determined that the binding affinity of BubR1(1–204) for a synthetic fluorescent peptide corresponding to Knl1(210–226) is 0.45 µM, almost 100-fold tighter than the value measured for the interaction of Bub1(1–150) to Knl1(174–190) (Fig. 4 B and Fig. 5 C). The significance of this difference in binding affinity is currently unclear but might be caused by a technical limitation of the assay, as we note that Bub1(1–150) interacts stoichiometrically with Knl1(150–250) or Knl1 (150–200) when the individual proteins are mixed at a concentration of 5 µM, suggesting that the $K_d$ of the interaction may realistically be lower (i.e., higher affinity. See Materials and methods for details on the assay). While this paper was under review, the crystal structure of the complex of human BubR1 TPR region bound to the KI2 motif was published (Protein Data Bank accession no. 3SI5; Bolanos-Garcia et al., 2011). The BubR1 backbone superimposes on the Bub1 TPR structure with a root-mean-square deviation of 0.9 Å. These results strongly support the view, based on the crystal structure, that the convex surface of the Bub1 TPR region contributes to Knl1 binding in cells.

**Figure 3. Crystal structure of the Bub1(1–150)–Knl1(150–200) complex.** (A) Side and top view of a cartoon representation of Bub1(1–150) (gray) and Knl1 peptide (red). (B) Surface representation of the complex, oriented so as to show the convex (left) and concave (right) side. Sequence conservation (limited to Bub1 orthologues) was mapped onto the Bub1 structure using ConSurf (Ashkenazy et al., 2010). The structure was illustrated using PyMOL (Delano Scientific, LLC). (C) Sequence of the Bub1 (gray) and Knl1 (red) fragments used for crystallization. Secondary structure elements are mapped onto the sequence. C, C terminus; N, N terminus.
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  The binding interface of BubR1 and appears to make favorable contacts that are likely to contribute significantly to the interaction (Fig. S2). In KI1, Thr179 substitutes for Phe215 of KI2. Its smaller side chain contributes more modestly to the binding interface.

  Residues Trp125, Leu128, Cys132, Asp137, and Met138 of BubR1 occupy positions that are equivalent to those identified at the Bub1–Knl1 interface (Fig. 5, A [alignment] and E) and which have been recently shown to participate in the interaction with the KI2 region (Bolanos-Garcia et al., 2011). To probe the function of these residues in Knl1 binding, we mutated them individually into alanine and used size-exclusion chromatography to probe the interaction with Knl1(150–250) (Fig. 5 F).

  Individual mutations of Trp125, Leu128, Cys132, or Asp137 deviation of 1.41 Å over 129 atoms. The interactions of the KI2 motif with BubR1 and of the KI1 motif with Bub1 engage equivalent interfaces located between helices α3 and α5 of the TPR assembly. Furthermore, like KI1, KI2 also adopts a helical conformation in complex with the BubR1 TPR (Fig. 5 D). Thus, there are extensive structural similarities between these two interactions (further analyzed in Fig. S2). Nevertheless, two features of the interaction of KI2 with BubR1 might explain its specificity and its apparently higher affinity with respect to the KI1–Bub1 complex. First, Arg221 of KI2 is perfectly positioned to interact with the side chains of Glu103 and Glu107 of BubR1. Asn185 of KI1, equivalent to Arg221 of KI2, does not form equally favorable contacts. Second, the bulky side chain of Phe215 of KI2 points toward the binding interface of BubR1 and appears to make favorable contacts that are likely to contribute significantly to the interaction (Fig. S2). In KI1, Thr179 substitutes for Phe215 of KI2. Its smaller side chain contributes more modestly to the binding interface.

  Residues Trp125, Leu128, Cys132, Asp137, and Met138 of BubR1 occupy positions that are equivalent to those identified at the Bub1–Knl1 interface (Fig. 5, A [alignment] and E) and which have been recently shown to participate in the interaction with the KI2 region (Bolanos-Garcia et al., 2011). To probe the function of these residues in Knl1 binding, we mutated them individually into alanine and used size-exclusion chromatography to probe the interaction with Knl1(150–250) (Fig. 5 F). Individual mutations of Trp125, Leu128, Cys132, or Asp137
to alanine were sufficient to disrupt the interaction with Knl1(150–250), whereas the effect of mutating Met138 was milder. Furthermore, structural analysis of the BubR1–KI2 complex shows that the side chain of this residue is partly buried (Bolanos-Garcia et al., 2011). Overall, these results demonstrate that BubR1 interacts with its cognate KI2 motif of Knl1 using a surface on the TPR that is analogous to that used by Bub1 to bind KI1.

Figure 5. Modeling and validation of the BubR1–Knl1 binding mechanism. (A) Sequence alignment of the human Bub1 and BubR1 TPRs; residues highlighted in yellow occupy similar positions at the Knl1 interface and were mutated as discussed in the section Interaction of BubR1 with Knl1. (B) Alignment of the KI motifs of human Knl1; residues highlighted in red define the conserved binding motifs KI1 and KI2. (C) Fluorescence polarization anisotropy experiments for the interaction of a fluorescent version of Knl1(210–226) with BubR1(1–204). Red line is a fit of the data with the equation discussed in Materials and methods, Fluorescence anisotropy. AU, absorbance units. (D) The structure of the BubR1 TPRs bound to the KI2 region of Knl1 (gray and red, respectively; Protein Data Bank [PDB] accession no. 3SI5; Bolanos-Garcia et al., 2011) is displayed in the same orientation used in Fig. 3 A. The KI2 peptide is positioned on BubR1 on an interface between helices α3 and α5, like the KI1 peptide on Bub1. (E) Close-up of the structure in D from a slightly rotated view emphasizing the role of BubR1 residues implicated in Knl1 binding. (F) Elution profiles for size-exclusion chromatography runs for binding reactions of the indicated BubR1(1–204) mutants with Knl1(150–250). C, C terminus; N, N terminus.
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Knl1 binding and kinetochore recruitment of Bub1 and BubR1

Results in Fig. 1 demonstrate that the N-terminal TPR regions of Bub1 and BubR1 are not sufficient for kinetochore recruitment. More surprisingly, the results also suggest that these regions might not even be strictly necessary for kinetochore recruitment. A possible conclusion from this analysis is that the interaction of the Bub1 and BubR1 TPR with the Knl1 (150–250) region might not be essential for kinetochore recruitment of these checkpoint kinases.

As explained in section The TPR domains of Bub1 and BubR1 bind Knl1 directly, the characterization of Knl1-binding interfaces on Bub1 and BubR1 allowed us to probe the role of Knl1 binding in kinetochore recruitment of these proteins in the context of the full-length protein, i.e., without resorting to deletion mutants that might disrupt hypothetical intramolecular regulatory steps. We therefore tested the ability of EGFP fusions of Bub1 or BubR1 mutants carrying multiple alanine substitutions on their KI1 or KI2 binding sites, respectively (described in the legend of Fig. 6 and abbreviated as Bub1(3A), Bub1(4A), BubR1(4A), and BubR1(4A*) or of individual alanine point mutants (Fig. S3, A and B) to be recruited to kinetochores.

We thus generated stable doxycycline-inducible HeLa cell lines. After a 24-h treatment with doxycycline, EGFP-tagged proteins were expressed at comparable levels that equaled or slightly exceeded the levels of endogenous Bub1 or BubR1 (Fig. 6 A). As for the case of the N-terminal deletion mutants,
kinetochore recruitment of Bub1(3A) or Bub1(4A) was not affected; recruitment of the two variants of BubR1(4A) was increased relative to the wild-type control (Fig. 6, B–D). As a further control, and to exclude any possible effect from endogenous Bub1, we assessed kinetochore recruitment of EGFP-Bub1 proteins in HeLa cells in which we had previously depleted Bub1 by RNAi (Fig. S3, C–E). Also in this case, we observed normal kinetochore recruitment of Bub1(4A) and Bub1(Δ189). Overall, these observations confute the hypothesis that the interaction of the TPR regions of Bub1 and BubR1 with the KI motifs of Knl1 regulates intramolecularly the degree of exposure of a kinetochore-binding region located elsewhere in the sequence of Bub1 or BubR1. On the contrary, the data strongly suggest that the TPR regions of Bub1 and BubR1 play a marginal role in kinetochore recruitment of Bub1 and BubR1.

Effects of the TPR region on the kinase activity of Bub1

To investigate alternative functions of the TPR region, we asked whether it influenced the catalytic activity of Bub1. Various EGFP-Bub1 constructs were expressed in stable doxycycline-inducible HeLa cell lines, partially purified via EGFP immunoprecipitation, and tested in kinase assays with H2A as a substrate. To assess the specificity of Bub1 activity in our immunoprecipitates, we first confirmed that the activity of a recombinant version of Bub1–Bub3 purified from insect cells was inhibited with 5 µM 2OH-BNPP1, a small-molecule inhibitor of Bub1 (Fig. S4 A; Kang et al., 2008). Next, we added 2OH-BNPP1 to a kinase assay reaction with immunoprecipitated Bub1 and found levels of inhibition comparable with those observed with the recombinant kinase (Fig. S4 B). These experiments testify to the specificity of the kinase assay. As an additional control, we tested the effects of mutations in the active site of the recombinant Bub1–Bub3 kinase (Fig. S4 C).

Next, we tested the H2A kinase activity of immunoprecipitates of cycling cells expressing similar amounts of EGFP-Bub1 or its variants, including Bub1(4A), Bub1(Δ189), and a kinase-dead mutant (Bub1(KD); Fig. S4 D). Deletion of the TPR region decreased the kinase activity of Bub1 to levels comparable with those of the kinase-dead mutant (Fig. 7 A). On the other hand, the catalytic activity of Bub1(4A) was unaffected in this assay, suggesting that the ability of this domain to bind Knl1 might not be essential for Bub1 kinase activity and that the determinants required for activity map elsewhere in the TPR region. Essentially identical results were obtained when using H3 (histone 3) as a substrate (unpublished data). In agreement with the idea that the interaction with Knl1 does not modulate the catalytic activity of the Bub1–Bub3 complex, the H3 kinase activity of recombinant Bub1–Bub3 purified from insect cells was not affected by the addition of a recombinant Knl1(150–200) segment (Fig. 7 B).

Identification of the minimal kinetochore-binding domain of Bub1

We tried to identify a minimal kinetochore-binding domain of Bub1. In Fig. 1, we demonstrated that residues 190–436, from which the TPRs of Bub1 are excluded, might be necessary for kinetochore recruitment of Bub1(3A) or Bub1(4A). As a further control, and to exclude any possible effect from endogenous Bub1, we assessed kinetochore recruitment of EGFP-Bub1 proteins in HeLa cells in which we had previously depleted Bub1 by RNAi (Fig. S3, C–E). Also in this case, we observed normal kinetochore recruitment of Bub1(4A) and Bub1(Δ189). Overall, these observations confute the hypothesis that the interaction of the TPR regions of Bub1 and BubR1 with the KI motifs of Knl1 regulates intramolecularly the degree of exposure of a kinetochore-binding region located elsewhere in the sequence of Bub1 or BubR1. On the contrary, the data strongly suggest that the TPR regions of Bub1 and BubR1 play a marginal role in kinetochore recruitment of Bub1 and BubR1.
Next, we refined our analysis of this interaction by expressing additional constructs, including Bub1(209–270) and Bub1(227–270). The latter construct matched almost exactly the segment of budding yeast Bub1 that was previously co-crystallized with Bub3 (Larsen et al., 2007), whereas the former is preceded by an 18-residue N-terminal extension of unknown function (Fig. 8 A, alignment). Although Bub1(209–270) localized robustly to kinetochores, Bub1(227–270) was unable to reach kinetochores (Fig. 8 B and Fig. S5 B for expression levels). These results suggest that efficient kinetochore recruitment requires the Bub3-BD and a short N-terminal extension.

To test the importance of the Bub3-binding region, we mutated Glu248 of Bub1, a residue previously shown to be essential for the interaction of Bub1 with Bub3 (Larsen et al., 2007), into lysine (E248K, referred to as EK mutant) in the context of the Bub1(209–270) construct and expressed it in HeLa cells (Fig. S5 C shows expression levels). As expected, the mutation abolished kinetochore recruitment of the Bub1(209–270) construct, demonstrating that binding to Bub3 is essential for kinetochore localization of Bub1 (Fig. 8 C). Moreover, the E248K mutation did not have additional effects on kinetochore localization in the context of Bub1(227–270) (Fig. S5, D and E).

To assess whether the N-terminal segment of the Bub1 (209–270) construct shown to be necessary for kinetochore recruitment is also important for the interaction with Bub3, we expressed GFP fusion proteins of Bub1(209–270), Bub1 (227–270), and Bub1(227–270-EK) in HeLa cells and quantified the abundance of Bub3 in the resulting anti-GFP immuno-precipitates (Fig. 8 D). We observed a strong correlation between the strength of Bub3 binding by the different constructs and their interaction with kinetochores, in agreement with the results from localization experiments (Fig. 8 B and Fig. S5 D). This result supports the idea that Bub3 binding is essential for the interaction of Bub3 with kinetochores. This idea was further emphasized by robust coprecipitation of at least two kinetochore subunits, Knl1 and Hec1, with Bub1(209–270), a construct that binds Bub3 with high affinity (Fig. 8 D). Similarly, the interaction of Bub1 with BubR1 appeared to correlate with the ability of Bub1 to bind Bub3 (Fig. 8 B). Conversely, Bub1(227–270) and Bub1(227–270-EK), which bind poorly to Bub3, did not interact robustly with kinetochores or BubR1.

**Discussion**

Structural analysis of Bub1, a 1,085-residue multidomain protein, has so far revealed the organization of the kinase domain (Kang et al., 2008), the TPR region (Bolanos-Garcia et al., 2009; D’Arcy et al., 2010), and of the Bub3-BD in complex with Bub3 (Larsen et al., 2007). Here, we extend these previous analyses by elucidating the structure of the complex of the Bub1 TPRs with its cognate KI1 motif of Knl1. The Knl1-binding interface is located on the convex surface of the TPRs. Our structure is equivalent to the crystal structure of the BubR1 TPRs bound to the KI2 region of Knl1, which was reported while this paper was under review (Bolanos-Garcia et al., 2011). Based on current structural knowledge on the interaction of helical repeats with their protein ligands, this is unusual but not unprecedented. For instance, the *S. cerevisiae* protein Caf4 interacts both on the concave and convex surface of the TPRs of Fis1 (Protein Data Bank accession no. 2PQR; Zhang and Chan, 2007).

It is plausible that the regulation of the catalytic output of Bub1 at kinetochores is mediated by complex intramolecular conformational changes triggered by kinetochores. Structural analysis of the kinase domain of Bub1, which revealed an intramolecular inhibitory switch that must be relieved for full kinase activation, lends support to this hypothesis (Kang et al., 2008). Our observations show that the N-terminal region of Bub1 influences the catalytic output of the kinase domain at the opposite end of the primary structure but that this effect might not depend on the KI1-binding interface (Fig. 7 A). In the future, we will try to clarify whether this occurs by releasing the previously identified intramolecular inhibitory mechanism (Kang et al., 2008). Furthermore, we show that the interaction of Bub1 with Bub3, and therefore presumably with kinetochores, is important to mediate the interaction of Bub1 with BubR1. Future studies will have to address the precise molecular mechanism through which these effects take place. Eventually, these studies will illuminate the detailed molecular mechanism of activation of Bub1, and possibly of BubR1, at the kinetochore.

By showing that the Bub3-binding region of Bub1, rather than the TPRs, is essential for kinetochore recruitment, our analysis resolves an open controversy, and it lends support to original studies indicating that the Bub3-BD of Bub1 (Taylor et al., 1998), later renamed (less informatively) as GLEBS motif (Wang et al., 2001), is necessary and sufficient for kinetochore recruitment of Bub1. The previously described deleterious effects on kinetochore localization of the mutations Leu122 to Gly (L122G, already mentioned in the Introduction; Kiyomitsu et al., 2007) might be an unexpected consequence of the destabilization of the hydrophobic core of the TPR region, where the side chain of Leu122 is located.

Impairment of the interaction of the Bub1 TPRs with the KI motif is, in principle, expected to reduce the kinetochore-binding affinity of Bub1. But the extent of this effect, if at all existing, is insufficient to alter the levels of kinetochore Bub1 significantly. With the goal of identifying subtle differences in the dynamics of kinetochore residence of Bub1 or of the Bub1 mutants incapable of binding Knl1, we performed FRAP experiments. These experiments, however, failed to reveal significant differences in recovery rates between Bub1(WT) and the Bub1(4A) mutant impaired in Knl1 binding (unpublished data).

Our analysis of the requirements for recruiting BubR1 to kinetochores is also in line with previous studies showing that BubR1(1–203) or BubR1(1–363) is unable to reach kinetochores, whereas a BubR1(357–1,052) construct localizes apparently normally (Malureau et al., 2009; Elowe et al., 2010). When considered together, therefore, the available evidence supports the unifying theme that the TPR regions of Bub1 and BubR1 are both dispensable for kinetochore recruitment. At least in the case of Bub1, its Bub3-binding region, likely through concomitant interactions of Bub3 with currently unknown...
Figure 8. **Minimal kinetochore-binding domain and the role of the TPR region.** (A) Sequence alignment of the Bub3-binding region of Bub1 (Hs, *Homo sapiens*; Xl, *Xenopus laevis*; Dr, *Danio rerio*; Sc, *S. cerevisiae*). (B) Fluorescence images of mitotic HeLa cells expressing the indicated EGFP-Bub1 constructs and treated with nocodazole. Cells were stained with DAPI (DNA) and CREST sera (kinetochores). Insets show a higher magnification of kinetochore regions (boxes). (C) Immunofluorescence images of mitotic HeLa cells expressing the EGFP-tagged fragments of the Bub3-BD of Bub1 and carrying the E248K mutation (EK). Cells were treated as in B. Note that the E248K mutation abolished the localization of EGFP-Bub1(209–270). The data in B and C derive from a single experiment, and the same image for the Bub1(209–270) construct is shown in B and C. (D) Immunoprecipitation of EGFP-tagged Bub1 proteins. HeLa cells were transfected with the corresponding plasmids and treated with 330 nM nocodazole for 16 h. (top) Coimmunoprecipitating proteins were analyzed by SDS-PAGE and Western blotting. On the bottom, a graph showing the quantification of Bub3 levels at the top normalized to the corresponding GFP levels and normalized to the value of Bub1(209–270). The data in B and C derive from a single experiment, and the same image for the Bub1(209–270) construct is shown in B and C. (E) Models of Bub1 and BubR1 kinetochore recruitment. Bub1 and BubR1 have similar domain structures, and it is plausible that they interact with kinetochores through partly related mechanisms. The interaction of Bubs with the Bub3-BD (yellow boxes) is crucial for kinetochore recruitment. The TPRs of Bub1 and BubR1 interact with the KI1 and KI2 motifs of Knl1, respectively. This interaction is important for the ability of Bub1 to bind BubR1 possibly because Knl1 acts as a scaffold for recruiting both proteins. The PP1-binding motif of Knl1 is negatively regulated through Aurora B phosphorylation. Aurora B, on the other hand, facilitates the interaction of Bub1 and BubR1 with kinetochores. N, N terminus; C, C terminus; WT, wild type. Bars, 5 µm.
targets, contributes the bulk of the binding affinity required for kinetochore binding (Fig. 8, B–D). A previous study making use of a 42-residue deletion in the Bub3-BD of BubR1 indicates that this might be true also for BubR1 (Taylor et al., 1998).

Identification of the kinetochore targets that mediate the interaction of Bub1 and BubR1 with kinetochores is of crucial importance. Knl1 is required for kinetochore recruitment of Bub1 and BubR1 (Kiyomitsu et al., 2007; Pagliuca et al., 2009). Because the interaction of Bub1 with the KI motifs of Knl1 is insufficient for kinetochore recruitment, it is plausible that at least another segment of Knl1 is involved, possibly through an interaction with the Bub3-binding region of Bub1 and with Bub3. This is in line with findings that Bub1 and Bub3 may be reciprocally required for efficient kinetochore recruitment (Taylor et al., 1998; Sharp-Baker and Chen, 2001; Chen, 2002; Meraldi et al., 2004; Vigneron et al., 2004; Logarinho et al., 2008). An important conclusion from our analysis is that the integrity of the Bub3-binding region of Bub1 is important for the interaction of Bub1 with BubR1 and with kinetochores (Fig. 8 D).

The precise molecular mechanism underlying the interaction of Bub1–Bub3 with BubR1–Bub3 and with kinetochores is currently unclear (Fig. 8 E). Kinetochores are very complex and dynamic assemblies, whose most intriguing feature is the ability to regulate checkpoint signaling as a function of the progression of kinetochore–microtubule attachment. The feedback mechanisms that couple the maturation of kinetochore–microtubule attachment to checkpoint control are known only imperfectly. The Mad1–Mad2 complex, which plays an essential role in Mad2 activation at the kinetochore (De Antoni et al., 2005), is progressively removed from kinetochores via a dynein-dependent pathway (Howell et al., 2001; Wojcik et al., 2001; Gassmann et al., 2008). Forced retention of Mad1–Mad2 at kinetochores prevents checkpoint satisfaction despite the formation of a normal metaphase plate (Maldonado and Kapoor, 2011a). Thus, the removal of at least a subset of the checkpoint proteins from the kinetochore is a prerequisite for exit mitosis.

Bub1 is also progressively removed from kinetochores upon microtubule attachment (Taylor et al., 2001; Famulski and Chan, 2007). How this is achieved at the molecular level is not known but worthy of further investigations. A fundamental emerging concept is that the KMN network complex plays a crucial role in relaying kinetochore–microtubule attachment to checkpoint silencing. This idea is corroborated by the observation that the KMN acts not only as the crucial microtubule-binding moiety of the kinetochore but also as a recruitment platform for all known checkpoint proteins, excluding Aurora B (Santaguida and Musacchio, 2009). It is therefore from this platform that the checkpoint proteins have to be removed for checkpoint silencing to be achieved.

The Knl1 subunit is probably a focal point of this dynamic regulation. In the immediate vicinity of the Bub1 KI1 motif, Knl1 contains a bipartite binding region for the PP1 phosphatase (Fig. 1 A and Fig. 8 E; Liu et al., 2010). This region has also been implicated in microtubule binding (Pagliuca et al., 2009; Welburn et al., 2010). The RRVSF motif in the PP1-binding region is a target of the Aurora B kinase, and Aurora B phosphorylation antagonizes PP1 binding, limiting the timing of its kinetochore recruitment to the final phases of kinetochore–microtubule attachment (Wu et al., 2009; Liu et al., 2010). Failure to recruit the PP1 phosphatase results in a metaphase arrest with an active spindle checkpoint (Maldonado and Kapoor, 2011b; Rosenberg et al., 2011). In the future, it will be important to investigate this complex network of interactions and if and how Bub1 recruitment to Knl1 impinges on the opposition of Aurora B and PP1.

Materials and methods

Mammalian plasmids

All the plasmids (except the one used in Fig. S1) were derived from the pcDNA5/FRT/TO vector (Invitrogen). The control plasmid for EGFP expression was created by PCR amplifying the EGFP sequence from pEGFP-C1 (Takara Bio Inc.) and cloning it into the pcDNAs/FRT/TO vector previously modified to carry an internal ribosomal entry site (IRES) sequence to obtain the pcDNAs/FRT/TO EGFP/IRES vector. To create all N-terminal EGFP-Bub1 plasmids, Bub1 sequences were obtained by PCR amplification from a pEGFP-C1 vector containing RNAi-resistant Bub1 (a gift from M. Yanagida, University of Kyoto, Kyoto, Japan) and subcloned into the pcDNAs/FRT/TO EGFP/IRES vector. To create the BUB1(Δ189)-EGFP fusion used in Fig. S1, the Bub1 sequence was PCR amplified and cloned into pEGFP-N1 (Takara Bio Inc.). All Bub1 constructs were RNAi resistant (Kiyomitsu et al., 2007). To create all N-terminal EGFP fusions, BubR1 sequences were amplified by PCR and cloned in frame with the EGFP tag in the pcDNAs/FRT/TO EGFP/IRES vector. Site-directed mutagenesis was performed with a mutagenesis kit (QuickChange; Agilent Technologies) to generate single and multiple mutants in the Bub1 and BubR1 constructs. pcDNAs/FRT/TO-based plasmids were used for both transient transfection and to generate stable inducible cell lines. All plasmids were checked by DNA sequencing.

Cell culture and transfection

HeLa cells were grown in DME (EuroClone) supplemented with 10% FBS (Hyclone) and 2 mM-glutamine. Nocodazole (Sigma-Aldrich) was used at a concentration of 3–3.5 µM unless differently specified. 2 nM thymidine was purchased from Sigma-Aldrich. For all plasmid transfections of HeLa cells, transfection agent (FuGENE 6; Roche) was used at a 3:1 ratio with plasmid DNA. Cells were analyzed 36–68 h after transfection.

Flip-In T-REx HeLa cells used for stable doxycycline-inducible cell lines were a gift from S.S. Taylor (University of Manchester, Manchester, England, UK). Flip-In T-REx HeLa host cell lines were maintained in DME with 10% tetrazycline-free FBS (Invitrogen) supplemented with 50 µg/ml Zeocin (Invitrogen). Flip-In T-REx HeLa expression cell lines were generated as previously described (Screpanti et al., 2011). In brief, Flip-In T-REx HeLa host cells were cotransfected with a ratio of 9:1 (wt/wt) pOG44/pcDNAs/FRT/TO expression plasmid using transfection agent (FuGENE6). 48 h after transfection, Flip-In T-REx HeLa expression cell lines were put under selection for 2 wk in DME with 10% tetracycline-free FBS supplemented with 250 µg/ml hygromycin (Roche) and 5 µg/ml blastocidin (MP Biomedicals). The resulting foci were pooled and tested for expression. Gene expression was induced with 0.5 µg/ml doxycycline (Sigma-Aldrich) for 24 h. To generate mitotic populations of these cells used in 4D, cells were treated with 330 nM nocodazole for 16 h. Mitotic cells were then harvested by shake off.

RNAi

Bub1 siRNA duplexes had the sequence 5′-GGUGGCGAACAAGUGUCUCU-3′ and were purchased from Thermo Fisher Scientific. To perform RNAi, 50 nM Bub1 siRNA duplexes were transfected using Lipofectamine 2000 reagent [Invitrogen] according to the manufacturer’s instructions. After 5 h from transfection of siRNA duplexes, cells were synchronized with a double thymidine arrest. In brief, cells were washed with PBS, treated with thymidine for 16 h, and then released into fresh medium. 3 h after the release, 50 nM siRNA duplexes were transfected again. After 5 h from transfection of siRNA duplexes, cells were treated with thymidine for 16 h and released in fresh medium.

Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer (150 mM KCl, 75 mM Heps, pH 7.5, 1.5 mM EGTA, 1.5 mM MgCl₂, 10% glycerol, and 0.075% NP-40 supplemented with 0.5% Nonidet P-40). Lysates were cleared by centrifugation and subjected to immunoprecipitation (IP) with the appropriate antibodies. The immunoprecipitates were washed and resolved by SDS-PAGE, and proteins were transferred to a nitrocellulose membrane (BioRad). Membranes were blocked in 5% nonfat dry milk and probed with primary antibodies.

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with protease inhibitor cocktail (Proteinase Inhibitor Cocktail Set III; EMD) and phosphatase inhibitors (PhosSTOP; Roche). For immunoprecipitation experiments, extracts were preclarified with a mixture of protein A–Sepharose (CL-4B; GE Healthcare) and protein G–Sepharose (rec-Protein G-Sepharose 4B Conjugate; Invitrogen) for 1 h at 4°C and centrifuged for 4 min at 4,000 rpm. Supernatants were then incubated with GFF-Traps (Chromotek; 3 µl/µg of extract) for 2–4 h at 4°C. Immunoprecipitates were washed with lysis buffer and resuspended in sample buffer, boiled at 95°C for 5 min, and analyzed by SDS-PAGE and Western blotting.

The following antibodies were used for immunoblotting: anti-Actin (mouse monoclonal antibody AC-40; Sigma-Aldrich; working dilution of 1:1,000), anti-GFP (in house made rabbit polyclonal antibody; working dilution of 1:400), anti-GFP (mouse monoclonal B-2, 9996; Roche; working dilution of 1:1,000), anti-Hec1 Tex, Inc.; working dilution of 1:1,000), anti-Mis12 (in house made mouse monoclonal antibody; clone Q015; working dilution of 1:5), anti-KNL1 (in house made rabbit polyclonal SI0788 antibody; working dilution of 1:1,000), anti-Bub1 (rabbit polyclonal Ab3900; Abcam; working dilution of 1:15,000), anti-BubR1 (mouse; BD; working dilution of 1:1,000), mouse anti-Bub3 (mouse; BD; working dilution of 1:1,000), antivinculin (mouse monoclonal antibody; clone hVIN-1; Sigma-Aldrich; working dilution of 1:100,000), anti-phospho–S10-H3 (rabbit polyclonal 605–760; Millipore; working dilution of 1:1,000). For Fig. 4 D, F, and G A, and Fig. 7 A, blots were incubated with anti–mouse and anti–rabbit affinity-purified secondary antibodies with horseradish peroxidase conjugate (Bio-Rad Laboratories; working dilution of 1:10,000) detected with a Western blotting system (ECL; GE Healthcare) with films or digital imaging (Chemiblis 3.2; DNR Bio-Imaging Systems). All other blots were incubated with anti–mouse and anti–rabbit IRDye 680LT or IRDye 800CW secondary antibodies obtained from LI-COR Biosciences (working dilution of 1:10,000) and scanned with Odyssey 3.0 (LI-COR Biosciences).

Immunofluorescence
Hela cells were plated onto coverslips pretreated with 15 µg/ml poly(l)lysine (Sigma-Aldrich). The day after, cells were transfected with the plasmids for 48–68 h and treated with nocadazole for 6–10 h. Immunofluorescence was performed as described previously (Scrapieni et al., 2011). Anti-centromere antibody (working dilution of 1:1,000), anti-BubR1 (rabbit polyclonal 204–229; GeneTex, Inc.; working dilution of 1:1,000), anti–mouse and anti–rabbit IRDye 680LT or IRDye 800CW secondary antibodies were used for immunodetection with appropriate FITC- or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.) and incubated for 1 h at room temperature. Coverslips were then mounted with Mowiol mounting media. Cells were imaged at room temperature using a confocal microscope (TCS SP2; Leica) equipped with a 63×, NA 1.4 objective lens using the LCS 3D software (Leica). Images in Fig. S3 A and B were acquired as z sections at 0.2442 µm and converted into maximal intensity projections using ImageJ (National Institutes of Health). Intensities of kinetochore areas, from at least four cells per condition, were measured with ImageJ software and corrected for the mean intensity of nonkinetochore areas. Measurements were graphed with Excel (Microsoft) software.

In vitro kinase assays
Recombinant His-Bub1–Bub3 wild-type and kinase-dead proteins were expressed and purified from SF9 insect cells infected with recombinant baculoviruses as previously described (Santaguida et al., 2010). In brief, SF9 insect cells infected with recombinant baculoviruses were expressed in E. coli BL21(DE3)pLysS. Expression plasmids for KNL1(150–200), KNL1(201–250), or KNL1(150–250) were created by PCR amplification and subcloned in the first cassette of pGEX-6P-2Brs (Sironi et al., 2001). Site-directed mutagenesis was performed with a mutagenesis kit (QuikChange) and used to generate mutants in the Bub1 and Bub3 expression constructs. All constructs were verified by sequencing.

Bacterial strain E. coli BL21(DE3)pLysS was used for expression of Bub1 and Bub3 proteins. Rosetta strain was used for expression of KNL1 proteins. To express Bub1–[1–150], BL21 cells were grown in Luria-Bertani medium at 37°C until an OD600 of ~0.7. At this point, expression was induced with 0.3 mM IPTG at 20°C and harvested at OD600 of ~0.8 for collection. The Bub1-[1–204] construct was expressed in BL21(DE3)pLysS cells. Expression was induced with 0.2 mM IPTG at an OD600 of ~0.7, and cells were further cultured at 25°C for 16 h. Rosetta cells carrying expression plasmids for KNL1(150–200), KNL1(201–250), or KNL1(150–250) were grown in Luria-Bertani medium at 37°C. Expression was induced with 0.3 mM IPTG at an OD600 of ~0.8, and cells were cultured at 22°C for 16 h before collection by centrifugation.

Cell pellets were resuspended in lysis buffer (50 mM Tris, 0.5% NP-40, 150 mM NaCl, 2 mM EDTA, and 1 mM DTT, pH 7.4) supplemented with protease inhibitor cocktail (Proteinase Inhibitor Set III). After sonication, the cell lysate was cleared by high-speed centrifugation. The supernatant was collected and incubated with glutathione Sepharose 4 Fast Flow beads (GE Healthcare) at room temperature for 2 h. The beads were washed with GST-tagged protein constructs were collected by centrifugation and washed with 20 bed volumes PBS buffer followed by 5 bed volumes protease cleavage buffer (PreScission; 50 mM Tris, 150 mM NaCl, 1 mM EDTA, and 1 mM DTT). The beads were incubated with PreScission protease at a 200:1 ratio (estimated recombinant protein/w protease w) at 4°C overnight. The collected eluate was applied to a column (Superdex 75 10/300; GE Healthcare) equilibrated in size-exclusion chromotography buffer (10 mM Na-phosphate and 150 mM NaCl, pH 7.4). Size-exclusion chromatography was performed at a flow rate of 0.5 ml/min, and the fractions containing target proteins were collected, concentrated, flash frozen in liquid N2, and stored at −80°C.

Analytical size-exclusion chromatography
Analytical size-exclusion chromatography experiments were performed on a column (Superdex 75 10/300). All samples were eluted at 4°C in size-exclusion chromatography buffer at a flow rate of 0.5 ml/min. Elution of proteins was monitored at 280 nm. To detect complex formation, different concentrations of proteins were mixed at a concentration of 5 µM in 600 µl, incubated at 4°C for >1 h, and then subjected to chromatography. Fractions were collected and analyzed by SDS-PAGE and Coomassie staining.

Fluorescence anisotropy
Fluorescence anisotropy measurements were performed with a microplate reader (Infinite F200; Tecan) at 20°C. Fluorescence (5-FAM)-labeled KNL1(174–190) peptide (synthesized by Mimotopes), at a concentration
Table 2. Refinement statistics used in this paper

| Variable                          | Value          |
|----------------------------------|----------------|
| Unique reflections               | 30,887         |
| R factor                         | 18.59          |
| Rfree                            | 24.36          |
| **Refined atoms**                |                |
| Protein non-H atoms              | 5,375          |
| Solvent                          | 120            |
| **r.m.s. deviations**            |                |
| Bond lengths (Å)                 | 0.009          |
| Bond angles                      | 1.173          |
| Ramachandran analysis            |                |
| Most favored (%)                 | 92.8           |
| Additionally allowed             | 6.5            |
| Generously allowed               | 0.3            |
| Disallowed                       | 0.3            |

Rfree is the same for the test set (% of the data). R factor = \( \frac{\sum ||F_o|| - ||kF_c||}{\sum ||F_o||} \), in which k is the scaling factor, Fc is the calculated amplitude of the structure factor, and Fo is the observed amplitude of the structure factor.

ESRF, European Synchrotron Radiation Facility; r.m.s., root-mean-square.

*This number does not include the free R set of reflections (% of total reflections).

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Online supplemental material

Fig. S1 shows the expression levels of several Bub1 and BubR1 constructs discussed in this paper. Fig. S2 reports a detailed structural comparison of the complexes of the Bub1 and BubR1 TPRs with K11 and K12, respectively. Fig. S3 shows the localization pattern of single point mutants in the Bub1 TPR region. Fig. S4 shows additional kinase assays and loading controls for experiments in Fig. 7. Fig. S5 complements Figs. 8 and shows additional localization experiments as well as loading controls. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201110013/DC1.
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