

Perspective

BubR1 is an effector of multiple mitotic kinases that specifies kinetochore

Microtubule attachments and checkpoint

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BubR1 is a critical component of the mitotic checkpoint but has also been shown to play an essential role in establishing kinetochore: microtubule attachments. BubR1 is hyperphosphorylated in mitosis and recent studies in human and *Xenopus* have identified 9 phosphorylation sites. Plk1-dependent phosphorylations (T792, T1008 and S676) were reported to stimulate BubR1 kinase activity, promote kinetochore microtubule attachments, monitor kinetochore tension, as well as the recruitment of Mad2 checkpoint protein to kinetochores. Plk1-independent sites (S435, S543, S670 and S1043) were also identified and some of these were found to be sensitive to the loss of microtubule attachment but not tension. Functional studies showed that phosphorylation of S670 is critical for correcting aberrant attachments. Once end-on attachments are established, dephosphorylation of S670 appeared to be important for generating tension to signal anaphase onset. The collective data when combined with early EM studies that showed BubR1 is present at both the inner and outer kinetochore plates suggest that BubR1 maybe an effector of multiple kinases that specifies its roles in microtubule attachments and checkpoint functions.

The kinetochore is assembled at the centromere of a chromosome where it specifies attachments with microtubules that are essential for accurate chromosome segregation. Nearly a hundred proteins have been identified to reside at kinetochores and they can be loosely categorized to play roles in structure, microtubule binding, and quality and checkpoint control.^{1,2,34} Understanding how different components of the kinetochore contribute towards its ability to capture and stably associate with the highly dynamic plus-end of the microtubule remains a tremendous challenge. Of

equal significance is the issue of how kinetochore attachments provide cues that ensure even a single unaligned chromosome is capable of delaying the cell from prematurely exiting mitosis to form aneuploid cells. Our current understanding is that upon establishing bipolar attachments, the opposing poleward forces generate tension between the sister kinetochores. Once an appropriate level of tension is achieved, the “wait anaphase signal” that is generated from kinetochores is silenced, the inhibition of the APC/C E3 ubiquitin ligase is relieved and cells undergo the metaphase to anaphase transition.³⁻⁷ The checkpoint output of a kinetochore is believed to be regulated by a delicate balance of phosphorylation and dephosphorylation events that are controlled by kinases and the phosphatases that monitor the various aspects of kinetochore: microtubule attachments.⁵⁻¹²

Early studies of insect cells showed that the expression of the 3F3/2 phosphoepitope at kinetochores was directly regulated by tension as opposed to microtubule attachments.^{6,7} These studies along with those in mammalian cells⁵ showed that tension-sensitive phosphorylations such as those represented by the 3F3/2 phosphoepitope helped to maintain cells in a checkpoint activated state. Thus, phosphorylation of kinetochore components activates and sustains checkpoint signaling from kinetochores with defective attachments. Once end-on attachments are established and tension is generated, dephosphorylation of these proteins silences the generation of the “wait for anaphase” signal.

BubR1 is one of numerous kinases (Plk1, Aurora B, Mps1 and Bub1) that are localized to kinetochores. As with many components of the kinetochore, the level of BubR1 is highest at kinetochores that lack microtubule attachments and is reduced but not eliminated upon microtubule attachment.¹³ Early studies showed that BubR1 was an essential component of the mitotic checkpoint and one of its roles at the kinetochore is postulated to act as a mechanosensor to monitor the plus-ended kinesin-like motor CENP-E.¹⁴⁻¹⁷ In this capacity, BubR1 is believed to generate the “wait for anaphase” signal from kinetochores with defective attachments. In addition, it is part of the Mitotic Checkpoint Complex (MCC) that can inhibit the APC/C.^{18,19} Aside from its role in the mitotic checkpoint, BubR1 has also been

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shown to be important for kinetochores to establish microtubule attachments.²⁰ HeLa cells depleted of BubR1 when blocked from exiting mitosis with a proteasome inhibitor exhibited severe attachment defects. Recently, BubR1 was reported to associate with adenomatous polyposis coli protein, and its kinase activity regulated the adenomatous polyposis coli—EB1 complex to establish kinetochore microtubule attachments.^{21,22}

How BubR1 can specify microtubule attachment and checkpoint functions is clearly a question at the forefront and efforts to understand its regulation may shed some light into these functions. It has been long known that BubR1 is hyperphosphorylated during mitosis and early studies suggested it maybe preferentially phosphorylated at unattached kinetochores.^{13,15} The importance of mitotic phosphorylations to BubR1 has remained obscure until very recently. Studies in human and *Xenopus laevis* showed that BubR1 is phosphorylated by Plk1 (Fig. 1). A search for consensus sites in human BubR1 revealed that T792 and T1008 could be phosphorylated by Plk1 in vitro.²³ A T792, 1008E phosphomimic mutant exhibited stronger autokinase in vitro than wild type, and like wild type, rescued the attachment defects of cells depleted of BubR1. Rescue activity was absent if the phosphomimic mutations were combined with a kinase-dead mutation, or if the sites were mutated (T792, 1008A) to prevent phosphorylation. As all of the mutants could localize to kinetochores, the data suggested that Plk1 phosphorylation enhanced BubR1 kinase activity, which in turn promoted kinetochore attachments.

In a separate study, Elowe et al. identified S676 to be phosphorylated by Plk1 in vivo.²⁴ As with other Plk1 substrates,^{25,26} phosphorylation of S676 depended on a priming phosphorylation at T620 by Cdk1. Quantitative immunofluorescence staining with a phospho-S676 antibody showed enhanced phosphorylation of BubR1 at unattached kinetochores. Taxol, which is used to pharmacologically reduce kinetochore tension without disrupting microtubule attachments, was found to increase phospho S676 staining at kinetochores. Functionally, cells expressing a T620A phosphomutant that prevented Plk1 dependent phosphorylation of S676 failed to establish stable kinetochore attachments. Phosphorylation of S676 was not essential for checkpoint functions as time-lapse microscopy revealed that cells transfected with the T620A mutant were delayed in mitosis in response to the presence of unaligned chromosomes. The importance of S676 phosphorylation to BubR1 kinase activity was not investigated. However, their analysis of a KD mutant did not reveal attachment defects as reported by Matsumura et al. or Huang et al.

Phosphorylation of BubR1 by Plk1 maybe conserved, at least amongst vertebrates, as BubR1 was found to be phosphorylated by Plx1 in *Xenopus* egg extracts and directly in vitro.²⁷ Although the sites phosphorylated by Plx1 were not identified, one of them forms the 3F3/2 phosphoepitope^{28,29} that required priming phosphorylation of T605 by Cdk1. Immunofluorescence staining showed that the 3F3/2 signal present at kinetochores likely reflected the phosphorylation of BubR1 as the signal was lost when the endogenous BubR1 was replaced with either a T605A or E phosphomutant. Functionally, phosphorylation of BubR1 stimulated its kinase activity. Furthermore, phosphorylation was

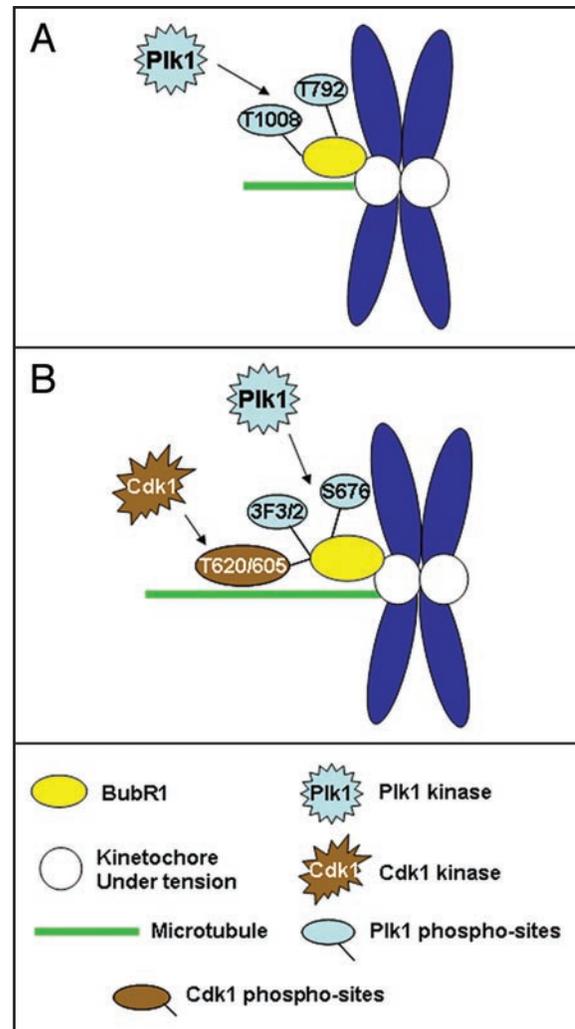


Figure 1. Plk1 dependent phosphorylation of BubR1. (A) Phosphorylation at T792 and T1008 in BubR1 by Plk1 facilitates kinetochore: microtubule attachment (Matsumura et al., 2007); (B) Cdk1 mediated priming phosphorylation at T620/T605 enables Plk1 to phosphorylate human BubR1 at S676 and create 3F3/2 phosphoepitopes in *Xenopus* BubR1 (Elowe et al., 2007; Wong and Fang, 2007).

important for the checkpoint as it was required by kinetochores to recruit Mad2. The reason for why the analogous phosphorylation in human BubR1 was not essential for the checkpoint is not clear. One possibility maybe the inherent differences in the two experimental systems. For example, phosphorylation of T620 in human BubR1 was not required for recruitment of Plk1 to kinetochores in human cells. Even though the effects of the T620 phosphomutant on the localization of Mad2 and other checkpoint proteins at kinetochores were not examined, the cells were delayed in mitosis in response to defective kinetochore attachments.²⁴

In addition to the phosphorylation sites described above, four additional mitosis-specific sites (S435, S543, S670 and S1043) that were independent of Plk1 were reported in human BubR1,³⁰ (Fig. 2). Phospho-antibodies to each site were used to verify their existence in vivo. S435 and S543 lie within a region that was reported to be a Cdc20 binding site,³¹ but the effects

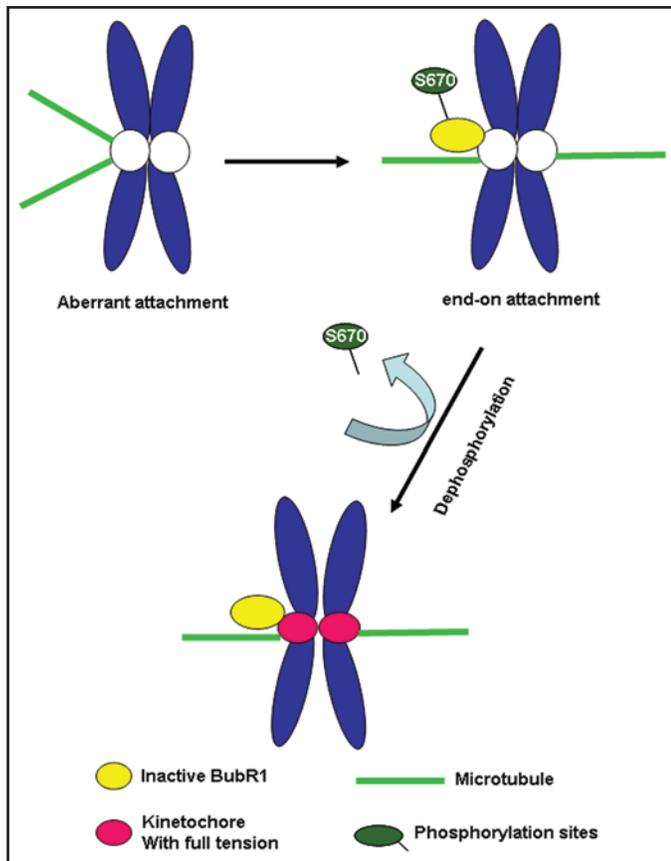


Figure 2. BubR1 phosphorylations independent of Plk1. Phosphorylation at S676 and S1043 in BubR1 is important for correction of bad attachments. Once the end-on attachments are established, dephosphorylation of these sites facilitates generation of tension (Huang et al., 2008).

of phosphorylation on Cdc20 binding was untested. Phospho-antibodies to S670 and S1043 stained kinetochores (the other two antibodies did not work for IF) only after nuclear envelope breakdown (NEB), and clearly after BubR1 had already assembled onto kinetochores. Phosphorylation of S670 and S1043 signals was strongest at kinetochores of prometaphase cells and was reduced by metaphase when kinetochores were saturated with microtubules as determined by the absence of Mad1 staining. A small fraction of metaphase cells did not exhibit detectable P-S670 and P-S1043 signals at their kinetochores. This pattern suggested that dephosphorylation of S670 and S1043 occurred after microtubule attachment and perhaps reflected late stage events just prior to the onset of anaphase. Indeed, phospho-signal was never detected at kinetochores of anaphase cells. Closer examination of S670, which is conserved amongst all species examined, showed that it was preferentially phosphorylated in response to loss of microtubule attachment but not inter-kinetochore tension. This differed from the phospho-S676 site which was reported to be sensitive to the loss of tension.²⁴ Thus, BubR1 may respond to two different inputs via separate phosphorylation sites (S670 and S676). Consistent with this possibility, phosphorylation of S670 and the other three sites was not dependent on Plk1.

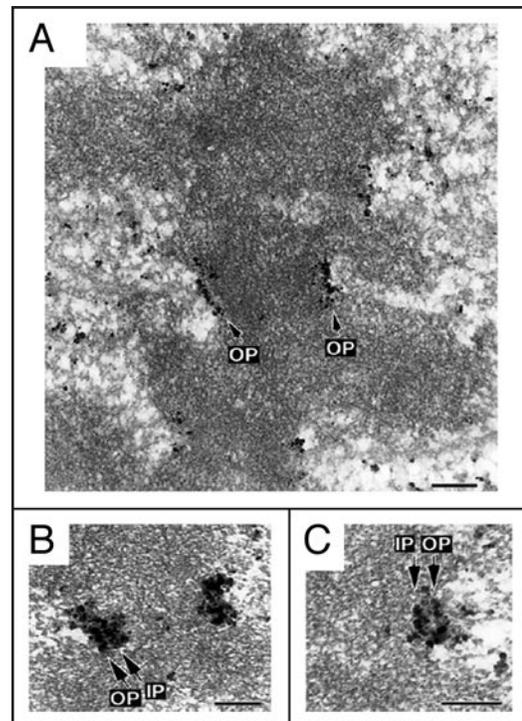


Figure 3. Immunogold EM localization of hBubR1 at kinetochores. (A) hBubR1 is localized in the outer kinetochore plate (OP) in this metaphase cell; (B and C) In prometaphase cells, hBubR1 appears as two parallel lines that correspond to the inner (IP) and outer (OP) kinetochore plates (Jablonski et al. 1998).

Functional studies using phospho-defective and mimic BubR1 mutants showed that these sites were not essential for the checkpoint. In fact, cells expressing S670A and S670D mutants exhibited a transient metaphase delay after chromosomes were aligned at the spindle equator. Detailed analysis showed that the S670A mutant exhibited a higher frequency of aberrant kinetochore attachments (syntelic, merotelic) than those expressing wild type BubR1. The failure to resolve these defective attachments explains the increased frequency of lagging chromosomes in anaphase cells that expressed the S670A mutant.^{32,33} The attachment defects of S670A mutant were similar to cells that expressed the kinase-dead mutant. Whether phosphorylation of S670 affects BubR1 kinase activity as was reported for the Plk1 sites remain to be tested. Regardless, these data along with Matsumura et al. show that BubR1 kinase activity is important for establishing microtubule attachments.

The analysis of the S670D phosphomimic mutant showed kinetochores were able to establish end-on attachments but failed to generate inter-kinetochore tension. This suggests that once end-on attachments are established, dephosphorylation of BubR1 is necessary for generating tension. In this model, BubR1 is not so much a sensor of tension (as is the case for S676 phosphorylation) but is contributing towards the mechanochemical processes that generate tension at bipolar attached kinetochores. Thus, the small fraction of metaphase cells that did not exhibit P-S670 staining at kinetochores may reflect a final check of the mechanical integrity of

the microtubule attachments before cells proceed into anaphase.

It is interesting that early EM studies showed that BubR1 was present at both the inner and outer kinetochore plates in prometaphase cells, but was only detectable at the outer plate by metaphase¹³ (Fig. 3). This difference may reflect the 3–5 fold decrease in BubR1 staining that is seen at the light level when kinetochores achieve alignment at the spindle equator.³⁵ In the light of the current findings, we speculate that the localization of BubR1 to different subdomains within the kinetochore may reflect its different roles in error correction and tension generation. Collectively, the recent studies of BubR1 phosphorylation in human and *Xenopus* suggest that it may be an effector of multiple upstream kinases that minimally include Cdk1, Plk1 and Mps1 kinases. These phosphorylations collectively contribute towards microtubule attachment activities that generate and monitor tension. These activities are likely mediated by multiple downstream targets whose access to BubR1 kinase may also be spatially regulated within the kinetochore.

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