Plk1 regulates mitotic Aurora A function through βTrCP-dependent degradation of hBora

Eunice H. Y. Chan · Anna Santamaria · Herman H. W. Silljé · Erich A. Nigg

Abstract Polo-like kinase 1 (Plk1) and Aurora A play key roles in centrosome maturation, spindle assembly, and chromosome segregation during cell division. Here we show that the functions of these kinases during early mitosis are coordinated through Bora, a partner of Aurora A first identified in Drosophila. Depletion of human Bora (hBora) results in spindle defects, accompanied by increased spindle recruitment of Aurora A and its partner TPX2. Conversely, hBora overexpression induces mislocalization of Aurora A and monopolar spindle formation, reminiscent of the phenotype seen in Plk1-depleted cells. Indeed, Plk1 regulates hBora. Following Cdk1-dependent recruitment, Plk1 triggers hBora destruction by phosphorylating a recognition site for SCFβ-TrCP. Plk1 depletion or inhibition results in a massive accumulation of hBora, concomitant with displacement of Aurora A from spindle poles and impaired centrosome maturation, but remarkably, co-depletion of hBora partially restores Aurora A localization and bipolar spindle formation. This suggests that Plk1 controls Aurora A localization and function by regulating cellular levels of hBora.

Introduction

The precise regulation of the spindle apparatus, a bipolar array of highly dynamic microtubules (MTs), is indispensable for accurate sister chromatid segregation and genome stability. In somatic animal cells, centrosomes control spindle assembly in time and space. At the G2 to M transition, centrosomes undergo a maturation process that is reflected in the enhanced recruitment of β-tubulin ring complexes (γ-TuRCs) and other MT regulatory factors. Concomitantly, the two centrosomes separate from each other to form the spindle poles. These crucial events are controlled by three highly conserved serine/threonine kinases, cyclin-dependent kinase 1 (Cdk1), polo-like kinase 1 (Plk1), and Aurora A (Barr et al. 2004; Blagden and Glover 2003; Nigg 2001; Vagnarelli and Earnshaw 2004). Cdk1 is initially activated at the centrosome (Jackman et al. 2003) and required for centrosome separation (Blangy et al. 1995; Sawin and Mitchison 1995). Likewise, Plk1 and Aurora A localize to centrosomes and are activated at the G2/M transition, but the coordination of their activities is not presently understood. When Plk1 activity is impaired by antibody injection (Lane and Nigg 1996), RNAi-mediated Plk1 depletion (Hanisch et al. 2006; Sumara et al. 2004; van Vugt et al. 2004), or small molecule inhibitors (Lenart et al. 2007; McInnes et al. 2006; Peters et al. 2006;
Santamaria et al. 2007), cells fail to recruit γ-TuRCs and often form monoastral spindles. Similarly, RNAi-mediated depletion of Aurora A results in the formation of monopolar spindles, decreased accumulation of γ-tubulin, and reduced density of centrosomal MTs (Ducat and Zheng 2004). Thus, both Plk1 and Aurora A clearly play important roles in centrosome maturation and bipolar spindle formation, and so one would expect that the functions of these kinases should be coordinated. However, although Plk1 is required for centrosomal localization of Aurora A (De Luca et al. 2006; Hanisch et al. 2006), we presently lack a mechanistic understanding of the links between these kinases.

Plk1 and Aurora A are activated through phosphorylation within their respective activation loops (T210 in human Plk1, T288 in human Aurora A; Jang et al. 2002; Littlepage et al. 2002), and both kinases are also controlled by additional mechanisms. In the case of Plk1, the C-terminal end domain (the so-called polo-box domain; PBD) functions as a phospho-peptide-binding module that mediates the binding of Plk1 to proteins pre-phosphorylated by ‘priming’ kinases (Cheng et al. 2003; Elia et al. 2003a, b). In the case of Aurora A, regulation appears to be conferred primarily through interactions with binding partners. Of several Aurora A interactors (Chen et al. 2002; Farruggio et al. 1999; Hirota et al. 2003; Mori et al. 2007; Ouchi et al. 2004), the role of the MT-binding protein TPX2 (targeting protein for XKlp2) is understood best (Eyers et al. 2003; Bayliss et al. 2003; Eyers et al. 2003). Moreover, TPX2 and Plk1 partially restores Aurora A localization to the centrosome and bipolar spindle formation, suggesting that the monoastral spindles typically seen upon interference with Plk1 activity could be due, at least in part, to the upregulation of hBora. Taken together, our data indicate that Plk1 regulates Aurora A localization and function through its ability to adjust cellular levels of hBora. This implies that hBora contributes to integrate the functions of three major mitotic kinases, Cdk1, Plk1, and Aurora A.

Results

Cell-cycle expression of hBora

To study the cell-cycle regulation of hBora expression, lysates from synchronized HeLa S3 cells were analyzed by Western blotting using an antibody raised against full-length hBora. For comparison, levels of Wee1, Plk1, Cyclin B1, Aurora A, and α-tubulin were determined in parallel. After release from a thymidine block, hBora levels increased gradually as cells approached mitosis but began to decrease before the onset of cyclin B degradation (Fig. 1). Moreover, several hBora forms with retarded electrophoretic mobility could be seen (see also ESM Fig. S1A left panel). These largely collapsed upon treatment of samples with alkaline phosphatase, indicating that they partly reflect phosphorylation (Fig. 1). Indeed, when tested as a substrate for mitotic kinases in vitro, hBora could be phosphorylated by Cdk1/cyclin B, Plk1, and Aurora A but not Aurora B (see also Hutterer et al. 2006; ESM Fig. S1B). Both Cdk1/cyclin B and Plk1 retarded the electrophoretic mobility of hBora, albeit to different extents (ESM Fig. S1C).

Depletion of hBora causes aberrant spindle formation

Two siRNA duplexes targeting hBora were used to explore the consequences of hBora depletion. Western blots revealed extensive depletion of hBora after 72 h of siRNA treatment, whereas levels of Aurora A and Plk1 remained unchanged (Fig. 2a). Compared to a GL2 control duplex (Elbashir et al. 2001), the two hBora siRNA duplexes produced similar increases in mitotic indices (ESM Fig. S2A) and a range of spindle abnormalities (Fig. 2b and ESM Fig. S2B). Specifically, many cells displayed bipolar spindles that were larger and denser than normal spindles (Fig. 2c). In addition, we observed cells with long, wavy spindles and lagging chromosomes and, as reported previously (Hutterer et al. 2006), occasional multipolar spindles (Fig. 2b and ESM Fig. S2B). Long, wavy spindles occasionally displayed fragmented poles (not shown), suggesting that they evolved to form multipolar spindles.
a conclusion supported by live cell microscopy (data not shown).

In line with the original identification of Bora as a partner of Aurora A (Hutterer et al. 2006), co-immunoprecipitation between the N terminus of hBora and endogenous Aurora A could readily be confirmed (ESM Fig. S3A). Additionally, we found that phosphorylation of hBora was required for binding to Aurora A (ESM Fig. S3A) and that alkaline phosphatase treatment of lysates abolished the interaction (ESM Fig. S3B). Having confirmed the Aurora A–hBora interaction, we next asked whether hBora depletion would affect Aurora A localization. To facilitate comparisons with controls, we focused on those hBora-depleted cells that showed relatively normal bipolar spindles. Even though such cells may not reflect the most severe phenotype of hBora depletion, immunofluorescence microscopy readily revealed an enrichment of both Aurora A and TPX2 on the spindles (Fig. 2c). In addition, the density of spindle MT was increased in hBora-depleted cells (Fig. 2c), whereas other proteins, notably pericentrin or Plk1, were not detectably affected (data not shown).

In view of evidence implicating the Aurora A–TPX2 complex in the stabilization of kinetochore MTs (K-fibers) in Caenorhabditis elegans (Ozlu et al. 2005), we asked, using cold treatment (Rieder and Borisy 1981), whether the increase in MT density in the spindles of hBora-depleted cells was accompanied by an increased stability of K-fibers. After 1 h of cold treatment, only centrosomal tubulin remained visible in control cells, as expected. In stark contrast, K-fibers remained largely intact in hBora-depleted cells (Fig. 2d and ESM Fig. S2C). Taken together, the above results indicate that hBora depletion results in an increased association of Aurora A with the spindle apparatus, which probably contributes to explain the observed spindle aberrations. Independently, a role for hBora in the stabilization of spindle microtubules was also observed by Seki et al. (2008).

Excess hBora causes Aurora A mislocalization and monoastral spindle formation

Staining of mitotic HeLa S3 cells with anti-hBora antibodies failed to highlight any specific structures (not shown), and immunostaining of ectopically expressed Myc-tagged hBora confirmed a diffuse localization of hBora (Fig. 3a). Remarkably, nearly all mitotic cells overexpressing hBora showed monoastral spindles (Fig. 3a), indicating that excess hBora interferes with bipolar spindle formation. The same phenotype was also observed upon overexpression of the N terminus of hBora (1–379; hBoraN), whereas the C terminus (377–599; hBoraC) produced no effect (Fig. 3a). Since it is the N terminus of hBora that binds Aurora A, this result suggested that the induction of monoastral spindles by excess hBora could reflect interference with Aurora A function. Indeed, overexpression of either hBora or hBoraN resulted in displacement of Aurora A from the spindle, whereas the kinase localized normally to the bipolar spindles of untransfected cells or cells expressing hBoraC (Fig. 3b). The most straightforward interpretation of these
results is that excess cytoplasmic hBora interferes with bipolar spindle formation through the sequestration of Aurora A away from the spindle.

To corroborate the above conclusion, we generated a tetracycline (tet)-inducible HEK293T cell line that allows the controlled expression of Myc-tagged hBoraN. As shown by Western blotting, tet induction of hBoraN for 48 h resulted in two Myc-immunoreactive bands (Fig. 3c). The upper band was sensitive to alkaline phosphatase treatment, confirming that it represents a phosphorylated form of Myc-hBoraN (Fig. 3c). Strikingly, more than 80% of the cells expressing hBoraN were arrested in mitosis and mostly displayed monoastral spindles (Fig. 3d), and as seen before (Fig. 3a,b), Aurora A was displaced from these spindles (data not shown). As expected, in view of the role of Aurora A in centrosome maturation, the recruitment of γ-tubulin was also drastically impaired, whereas TPX2 and Aurora B analyzed for control were not displaced (data not shown).

hBora interacts with Plk1 during mitosis

Intrigued by the remarkable similarity between the phenotype induced by excess hBora and that displayed by cells that lack Plk1 protein or activity (De Luca et al. 2006; Hanisch et al. 2006), we explored a potential link between
hBora and Plk1. First, we asked whether hBora overexpression would perturb Plk1 localization. We found that induction of Myc-hBoraN led to Plk1 displacement from both the spindle poles and the kinetochores (Fig. 4a), and as described above for Aurora A, this effect was induced by hBora and hBoraN but not hBoraC (Fig. 4b). Next, we asked whether Plk1 and hBora might interact with each other in vivo. When Myc-hBoraN was immunoprecipitated from the tet-inducible cell line, both Plk1 and Aurora A could readily be detected in the immunoprecipitates, whereas TPX2 was absent (Fig. 4c). Plk1 was also precipitated when isolating the Aurora A/hBoraN complex with anti-Aurora A antibodies (data not shown). Thus, hBoraN and TPX2 do not bind to the same pool of Aurora A, but Plk1 and Aurora A are able to form a ternary complex with hBoraN. Interestingly, when these samples were probed with an antibody recognizing phosphorylated Threonine 288 (pT288) within the activation loop of Aurora A, the corresponding signal was clearly reduced in the lysate harboring excess hBoraN. Furthermore, whereas the Aurora A co-precipitating with TPX2 was readily recognized by the anti-pT288 antibody, the Aurora A co-precipitating with hBoraN did not react. Taken at face value, these results argue that the Aurora A in the TPX2 complex is more active than the kinase associated with hBoraN (Fig. 4c).
To determine which domain of Plk1 interacts with hBora, co-immunoprecipitation experiments were performed on HEK293T cells after co-transfection with Myc-hBora and various green fluorescent protein (GFP)-Plk1 constructs. As shown in Fig. 4d, phosphorylated forms of hBora were co-precipitated with both wild-type (WT) and catalytically inactive (KD) versions of Plk1, indicating that Plk1 activity is not required for this interaction. However,
the hBora proteins co-precipitated by active and inactive versions of Plk1 displayed different electrophoretic mobilities, suggesting that they differed in phosphorylation state. Most importantly, the GFP-tagged C-terminal end domain of Plk1 (GFP-Plk1 PBD) also bound to hBora, whereas the catalytic domain (GFP-Plk1Cat) did not (Fig. 4d). Taken together, the above results suggested that Plk1 interacts via its PBD with a phosphorylated form of hBora and that this binding prompts hBora phosphorylation by Plk1. Given that both Drosophila and human Bora can be phosphorylated by Cdk1 (Hutterer et al. 2006; ESM Fig. S1B and S1C) and a potential Cdk1-induced PBD-docking site in hBora (S252) has been conserved in evolution (Fig. 4e), we tested the functionality of this candidate PBD docking site in a far-Western ligand-binding assay (Neef et al. 2003). Without pre-phosphorylation by Cdk1, wild-type hBora showed very little binding to the glutathione-S-transferase (GST)-PBD, but strong binding was seen after phosphorylation (Fig. 4f, central panel). PBD binding to a S252A mutant of hBora was markedly reduced, albeit not completely abolished, even after pre-phosphorylation by Cdk1 (Fig. 4f, central panel). Virtually no binding to any hBora protein was seen with a PBD mutant (GST-PBD AA) that is unable to bind to phosphopeptides (Elia et al. 2003a, b; Fig. 4f, right panel). Coomassie Blue staining confirmed the presence of equal amounts of hBora (Fig. 4, left panel). These results identify the motif centered on S252 as a major Cdk1-dependent Plk1-PBD binding site in hBora.

Plk1 triggers the SCFβ-TrCP-mediated degradation of hBora

Having shown that hBora is a binding partner (Fig. 4) and potential substrate of Plk1 (ESM Fig. S1B and S1C), we next explored the physiological significance of this interaction. We found that siRNA-mediated depletion of Plk1 led to a striking accumulation of hBora (Fig. 5a), and a similar increase in hBora levels was seen upon inhibition of
Plk1 by the small molecule inhibitor ZK–thiazolidinone (TAL) (Santamaria et al. 2007; data not shown). Plk1 is well known to trigger the degradation of several cell-cycle regulators through phosphorylation of a phoshodegron (DpSgxpxT), which then leads to the recruitment of the β-transducin repeat-containing protein (β-TrCP) component of a Skp1-Cul1-F-box-protein ubiquitin ligase, followed by polyubiquitination and proteasomal degradation (Liu and Mallar 2005; Mamely et al. 2006; Moshe et al. 2004; Rauh et al. 2005; Tung et al. 2005; Watanabe et al. 2004). Since examination of the hBora sequence revealed an evolutionarily conserved potential β-TrCP-binding motif within the C terminus (ESM Fig. S4A), we asked whether Plk1 might act through this putative phoshodegron to control cellular levels of hBora. First, we established that Plk1 phosphorylated full-length hBora, as well as N- and C-terminal fragments, but that phosphorylation was reduced upon mutation of the putative phoshodegron (S497A/T501A; data not shown). Next, we tested the ability of Plk1 KD to interfere with hBora degradation by acting as a dominant negative mutant. In response to co-expression of full-length Myc-hBora with Plk1 KD, a significant increase in hBora (phospho-) protein levels could be seen, whereas the co-expression of WT Plk1 had no obvious effect (ESM Fig. S4B). Mutation of the phoshodegron (S497/T501) or the PBD-docking site (S252A) both abolished responsiveness of the mutant hBora proteins to alterations in Plk1 activity (ESM Fig. S4B). Finally, the phosphorylated form of wild-type hBora could readily be co-immunoprecipitated with β-TrCP, whereas both the phoshodegron and the PBD-docking site mutants failed to interact (ESM Fig. S4C). Moreover, siRNA-mediated depletion of β-TrCP-1/2 resulted in the concomitant upregulation of both hBora and Wee1, a known β-TrCP target (ESM Fig. S1A). Taken together, these results demonstrate that the phoshodegron identified in hBora is functional and that Plk1 binding is essential for triggering the β-TrCP-mediated degradation of hBora. As a consequence, interference with Plk1 activity results in the accumulation of hBora (phospho-) protein. These conclusions are in excellent agreement with a recent independent study (Seki et al. 2008), in which human Bora was also identified as a Plk1-dependent substrate for β-TrCP-mediated proteolysis.

Could Plk1 regulate Aurora A by controlling hBora levels?

Considering that hBora interacts with both Aurora A and Plk1 but is itself controlled by Plk1, it follows that hBora could contribute to coordinate the functions of these two key regulators of mitotic progression. One possibility is that Plk1 may determine Aurora A localization and/or activity through its ability to regulate the intracellular levels of hBora. Several experiments were carried out to test this possibility. First, when Aurora A was immunoprecipitated from Plk1-depleted cells, most Aurora A was complexed to hBora, reflecting the increased levels of hBora in such cells (Fig. 5a). In contrast, the amounts of TPX2 co-precipitating with Aurora A were substantially reduced, although total levels of TPX2 were not changed (Fig. 5a). Second, HeLa S3 cells were depleted of TPX2, Aurora A, hBora, or Plk1, and lysates were then probed with the activation-state specific anti-pT288 antibody (Fig. 5b). Depletion of either TPX2 or Aurora A caused a clear reduction in the level of active Aurora A, as reported by this antibody. A similar reduction was also seen when hBora levels were increased through depletion of Plk1, whereas depletion of hBora did not significantly affect Aurora A activity, when compared to a GL2-control (Fig. 5b). Together, these results indicate that hBora competes with TPX2 for Aurora A binding. As a consequence, levels of hBora are expected to determine how much Aurora A is available for binding to TPX2 and perhaps other activating partners.

Our findings suggest a plausible explanation for the observation that Plk1 is required for Aurora A localization to spindle poles (De Luca et al. 2006; Hanisch et al. 2006). Specifically, the requirement for Plk1 during centrosome maturation and spindle formation could in principle reflect its role in lowering hBora levels below a threshold, such as to allow Aurora A to exert its functions on the centrosome and spindle. This model predicts that a reduction of hBora levels might alleviate at least some of the early mitotic defects that are typically seen upon depletion of Plk1 or inhibition of Plk1 activity. To explore this possibility, we treated cells with siRNA duplexes targeting Plk1 and hBora, either in combination or singly (paired with GL2 for control), and asked whether co-depletion of hBora and Plk1 would restore Aurora A localization to the centrosome and spindle bipolarity. Co-depletion of hBora with Plk1 suppressed the accumulation of hBora that normally results from Plk1 depletion, as expected (Fig. 6a). Yet, Plk1 depletion and Plk1/hBora co-depletion both led to marked increases in mitotic indices (Fig. 6b), indicating that not all early mitotic functions of Plk1 can be attributed to its interaction with hBora. Interestingly, the two cell populations displayed remarkably different phenotypes. Whereas the Plk1-depleted cells displayed mostly monopolar spindles, as expected, the Plk1/hBora co-depleted cells showed mostly bipolar spindles, albeit with uncongressed chromosomes (Fig. 6c,d). Moreover, Aurora A was displaced from spindle poles in Plk1-depleted cells, consistent with previous data (De Luca et al. 2006; Hanisch et al. 2006), but largely restored to these structures in Plk1/hBora co-depleted cells (Fig. 7a lower panel). To facilitate the comparison with Plk1-depleted cells, Plk1/hBora co-depleted cells were also treated with monastrol to induce monopolar spindles (Fig. 7a middle panel), and furthermore, both cell
populations were exposed to 4°C in order to depolymerize spindle MTs and better visualize centrosome-associated Aurora A (Fig. 7b). A similar, albeit partial, rescue of spindle bipolarity could also be observed upon hBora depletion from cells treated with the Plk1 inhibitor TAL (data not shown). Taken together, these results demonstrate that co-depletion of hBora partially rescued the defects in bipolar spindle formation and Aurora A localization that normally result from Plk1 depletion.

Discussion

The results reported here indicate that precise levels of hBora are critical for correct Aurora A localization, spindle assembly, and accurate chromosome segregation. In particular, we demonstrate that hBora binds not only to Aurora A but also to Plk1 and that Plk1 regulates hBora levels through SCFβ-TrCP-mediated degradation. While these results are in excellent agreement with findings recently reported by Seki et al. (2008), our data additionally identify the Cdk1 site S252 as critical for the recruitment of Plk1 to hBora. It is most interesting to note that the interference with Plk1 activity resulted not only in a drastic upregulation of hBora but in a concomitant sequestration of Aurora A within the cytosol. So, by virtue of its ability to regulate the abundance of a cytoplasmic hBora–Aurora A complex, Plk1 controls the availability of Aurora A for interactions with spindle-associated partners such as TPX2. Collectively, our findings lead us to propose that hBora contributes to integrate the functions of three major mitotic kinases, Cdk1, Plk1, and Aurora A.

hBora levels are critical for proper spindle assembly

In response to siRNA-mediated depletion of hBora, we observed the formation of long and wavy spindles, which eventually progressed to form multipolar spindles. In
addition, we observed unusually ‘fat’ spindles that were characterized by increased MT density, increased amounts of spindle-associated Aurora A, and increased cold stability of K-fibers. The exact mechanisms underlying these spindle defects remain to be unraveled, but since hBora and TPX2 do not bind simultaneously to Aurora A, any reduction in hBora levels is expected to favor complex formation between Aurora A and spindle-associated activators such as TPX2 (Eyers et al. 2003; Kufer et al. 2002; Tsai et al. 2003). In turn, the increased abundance of Aurora A on the spindle is likely to cause enhanced activity of downstream effectors, notably chTOG, the human homolog of *Xenopus* XMAP215, and *Drosophila* minispindles (Barros et al. 2005; Giet et al. 2002; Kinoshita et al. 2005; Peset et al. 2005). Consistent with the above interpretation, the phenotypes seen in hBora-depleted cells resemble those of cells overexpressing Aurora A (Anand et al. 2003; Meraldi et al. 2002). Although modest overexpression of hBora did not produce a significant phenotype (data not shown; Seki et al. 2008), striking spindle defects were observed upon expression of hBora to high levels. In particular, excess hBora severely impaired the recruitment of both Aurora A and γ-tubulin to centrosomes, so that centrosome maturation and separation failed, resulting in the formation of monopolar spindles. Importantly, this phenotype was dependent on the ability of hBora to bind Aurora A. While excess hBora caused the sequestration of Aurora A into a diffusely localized pool, TPX2 remained on the spindle apparatus, suggesting that cytoplasmic hBora determines the size of the Aurora A pool that is available for interactions with spindle-associated binding partners. In support of this conclusion, a rapid exchange of Aurora A between the spindle and the cytosol has previously been observed in photobleaching experiments (Stenoien et al. 2003). Considering that Aurora A displays activity when bound to hBora (Hutterer et al. 2006; this study), it is possible that this diffusely localized complex carries out important functions by acting on cytosolic substrates, perhaps regulating cell-cycle progression. However, when hBora is deregulated, it perturbs Aurora A functions that are important for spindle assembly. Thus, the phenotypic consequences of hBora depletion and overexpression can be explained, at least in part, by deregulation of Aurora A complexes on mitotic structures. In future, it will be interesting to investigate whether hBora also regulates the function of other interaction partners, notably the activity of Plk1.

**Plk1 regulates hBora stability**

As shown here and elsewhere (Seki et al. 2008), hBora interacts not only with Aurora A but also with Plk1, in both cases through its N-terminal domain. The interaction between hBora and Plk1 requires the Plk1 PBD and prior phosphorylation of hBora on a Cdk1 site, S252, in line with a well-established docking model (Elia et al. 2003a, b). After its recruitment to hBora, Plk1 phosphorylates a conserved phosphodegron, which then serves as a recognition motif for the ubiquitin ligase SCFβ-TrCP, leading to the proteasomal degradation of hBora. We consistently observed significant amounts of hBora in nocodazole-arrested cells. This indicates that not all hBora gets...
degraded when Cdk1 and Plk1 are activated at the G2/M transition, suggesting that a population of hBora is protected against β-TrCP-mediated degradation during early mitosis, perhaps through phosphorylation at particular sites and/or the binding of interaction partners (perhaps Aurora A itself). Furthermore, phosphatases counteracting Cdk1 and/or Plk1 are likely to contribute to the establishment of a steady-state level of hBora. Complex regulation of protein stability is not without precedent (Mailand et al. 2002), and so we anticipate the existence of multiple mechanisms to ensure physiological levels of hBora and appropriate timing of hBora degradation. As an extension of this view, we also note that hBora carries within its N terminus potential destruction motifs for yet another ubiquitin ligase, the anaphase-promoting complex/cyclosome. So, although our data point to SCFβ-TrCP as the ubiquitin ligase responsible for Plk1-induced hBora degradation, other mechanisms are likely to contribute to the regulation of hBora levels, particularly during later stages of mitosis and the subsequent G1 phase.

Does hBora confer Aurora A regulation through Plk1?

Both Plk1 and Aurora A regulate key events during late G2 and early mitosis, including centrosome maturation and spindle assembly (see “Introduction”). Although no direct interactions between these kinases have so far been established, Plk1 was found to be required for Aurora A localization to centrosomes (De Luca et al. 2006; Hanisch et al. 2006). Our present data, centered on hBora, suggest an attractive explanation for this observation. Specifically, Plk1 activity might be required to keep hBora levels below a threshold, such as to limit the extent of sequestration of Aurora A into cytoplasmic complexes with hBora. In support of this view, several of the early mitotic defects typically seen in cells deprived of Plk1 protein and/or activity could be rescued by hBora co-depletion. It is possible, therefore, that the centrosome maturation and separation defects seen in Plk1-depleted cells may stem, at least in part, from the hBora-mediated impairment of Aurora A function. Alternatively (or in addition), hBora might negatively regulate Plk1. We emphasize that not all early mitotic functions of Plk1 can be explained through hBora-mediated regulation of Aurora A. In particular, co-depletion of hBora did not rescue the chromosome congression defect or the mitotic arrest typically seen in Plk1-depleted cells. On the other hand, we note that high levels of hBora produced a more severe effect on Aurora A localization (displacement from the entire spindle) than Plk1 inhibition or depletion (displacement primarily from the spindle poles). This notwithstanding, our studies on hBora have uncovered an important mechanistic relationship between Plk1 and Aurora A. Thus, it is interesting to consider the possible implications of this relationship for the proposed roles of mitotic kinases in tumorigenesis. Both Plk1 and Aurora A are often overexpressed in tumors (Knecht et al. 1999; Sen et al. 1997), and Aurora A is considered a cancer susceptibility gene (Ewart-Toland et al. 2003; Meraldi et al. 2004). It is intriguing, therefore, that hBora also maps to a chromosomal region (13q21) that is often altered in tumors (Rozenblum et al. 2002). In consideration of the data reported here, one would predict that deregulation of hBora should lead to similar cellular phenotypes as the aberrant expression of either Plk1 or Aurora A.

**Experimental procedures**

Plasmids, cells, and immunofluorescence microscopy

Plasmid constructions, site-directed mutagenesis, transfections, cell-cycle synchronization, and production of full-length hBora are described in Electronic Supplementary Material. HEK293T Tet-on inducible cell lines for expression of full-length Myc-hBora and Myc-hBoraN were generated as described (Chalamalasetty et al. 2006), and transgene expression was induced by addition of 1 μg/ml tetracycline for 48 h. Immunofluorescence microscopy was carried out as described in Electronic Supplementary Material.

Transient transfections and siRNA

Plasmid transfections were performed using Fugene 6 reagent (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions. siRNA duplexes were transfected using Oligofectamine (Invitrogen, Carlsbad, CA, USA), using the GL2 duplex for control (Elbashir et al. 2001). hBora siRNA duplexes (sihBora1: 5′-CCGGTTGA TAATGGCAGTTTA-3′ and sihBora2 5′-TACTAGTCC TTCGCCATT5′-3′) and β-TrCP1/2 siRNA duplex (5′-AAGTGAATTTGGAACATC-3′) were purchased from Qiagen (Hilden, Germany). Plk1 and TPX2 were depleted using previously published siRNA duplexes (Hanisch et al. 2006; Kufer et al. 2002). For co-depletion experiment, HeLa S3 cells were treated with GL2 or hBora siRNA for 36 h. Subsequently, fresh media were replaced containing combinations of Plk1/GL2 or Plk1/hBora siRNA duplexes for another 36 h.

Biochemical assays

Immunoprecipitations, Western blots, and kinase assays are described in Electronic Supplementary Material. Far-Western ligand binding assays were carried out as described.
(Neat et al. 2003), using GST-tagged PBD (1 μg/ml) for 2 h at 4°C, followed by detection of bound protein with monoclonal anti-Plk1 antibody.

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