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Mitotic Activation of the Kinase Aurora-A Requires Its Binding Partner Bora

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Summary

The protein kinase Aurora-A is required for centrosome maturation, spindle assembly, and asymmetric protein localization during mitosis. Here, we describe the identification of Bora, a conserved protein that is required for the activation of Aurora-A at the onset of mitosis. In the *Drosophila* peripheral nervous system, *bora* mutants have defects during asymmetric cell division identical to those observed in *aurora-A*. Furthermore, overexpression of *bora* can rescue defects caused by mutations in *aurora-A*. Bora is conserved in vertebrates, and both *Drosophila* and human Bora can bind to Aurora-A and activate the kinase in vitro. In interphase cells, Bora is a nuclear protein, but upon entry into mitosis, Bora is excluded from the nucleus and translocates into the cytoplasm in a Cdc2-dependent manner. We propose a model in which activation of Cdc2 initiates the release of Bora into the cytoplasm where it can bind and activate Aurora-A.

Introduction

Cell division involves the coordinated execution of several distinct steps. First, chromosomes condense and the nuclear envelope breaks down. Then, the mitotic spindle forms, sister chromatids separate, and chromosomes segregate into the two daughter cells. Finally, mitosis finishes with cytokinesis, the actual division of the cell into two separate daughter cells. Mitosis involves the sequential activation of several protein kinases that are required for all or a subset of these mitotic events: while Cdc2 is a master regulator of mitosis and is required for the initiation of mitosis, kinases of the Aurora and Polo families are responsible for distinct subsets of mitotic events (Nigg, 2001). How these kinases are activated and how they regulate individual mitotic events is not very well understood.

Aurora kinases were originally identified in *Drosophila* (Glover et al., 1995), but homologs were later found in all eukaryotic organisms. While yeast contains only a single Aurora kinase called Ipl1p (Chan and Botstein, 1993), at least two families with distinct functions and subcellular localizations can be distinguished in multicellular organisms: Aurora-A is concentrated on the spindle and on centrosomes and is required for centrosome maturation and spindle assembly, while Aurora-B is localized on chromosomes and on the central spindle and is involved in chromosome condensation, kinetochore-microtubule attachment and cytokinesis (Carmena and Earnshaw, 2003). Aurora-B is part of a multimeric complex containing the so-called chromosome passenger proteins INCENP, surviving, and borealin (Adams et al., 2001; Vagnarelli and Earnshaw, 2004). The individual members of that complex are codependent for their subcellular localization, and their role is to direct Aurora-B to its correct localization within the cell (Gassmann et al., 2004; Romano et al., 2003). Consistent with the conserved function and localization of Aurora-B, all members of the complex are conserved in evolution (Vagnarelli and Earnshaw, 2004) (in *C. elegans*, they are called ICP-1, BIR-1, and CSC-1 [Romano et al., 2003], respectively). Binding partners have also been identified for Aurora-A, but in this case, their evolutionary conservation is less clear (Kufer et al., 2003). TPX2 is a microtubule binding protein required for spindle assembly (Gruss et al., 2001). It can bind Aurora-A and activate the kinase via an N-terminal domain (Bayliss et al., 2003; Eyers et al., 2003; Kufer et al., 2002; Tsai et al., 2003). Upon TPX2 RNAi, Aurora-A fails to localize to the spindle whereas its centrosome localization is unaffected (Kufer et al., 2002). Since the interaction of TPX2 with Aurora-A is stimulated by the small GTPase Ran, a model was proposed in which activated Ran is generated by condensed chromatin and locally activates Aurora-A, thereby stabilizing microtubules. Although a putative *C. elegans* TPX2 homolog was identified (Ozlu et al., 2005), the homology does not extend over the whole protein (Karsenti, 2005) and no homologs are present in other invertebrates, including *Drosophila*. Another Aurora-A binding partner is the LIM domain protein Ajuba (Hirota et al., 2003). Like TPX2, Ajuba can activate Aurora-A, but again, no homologs have been identified in invertebrates.

Besides its role in centrosome maturation and spindle assembly, Aurora-A has a special function during asymmetric cell division (Berdnik and Knoblich, 2002). To divide asymmetrically, some cells are capable of segregating cell fate determinants into one of their two daughter cells (Betschinger and Knoblich, 2004). Asymmetric cell divisions are particularly well understood in *Drosophila* external sensory (ES) organs where they contribute to the formation of four different cell types from a single sensory organ precursor (SOP) cell (Bardin et al., 2004; Jan and Jan, 2001). The SOP cell divides into a pIIa and a pIIb cell. Later, pIIa gives rise to the two outer cells, while pIIb generates the two inner cells of the organ. During each division, the cell fate determinant Numb localizes asymmetrically and segregates into one of the two daughter cells where it regulates cell fate by repressing Notch signaling (Guo et al., 1996; Rhyu

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et al., 1994). In *numb* mutants, Notch is not repressed and abnormal ES organs with too many outer and no inner cells are formed. A similar phenotype is observed in *aurora-A* mutants. In these mutants, Numb does not localize asymmetrically and is not segregated into one of the two daughter cells (Berdnik and Knoblich, 2002). Since asymmetric Numb localization requires actin (Knoblich et al., 1997), but not microtubules (Berdnik and Knoblich, 2002; Knoblich et al., 1995), this phenotype is not an indirect consequence of the centrosome maturation and spindle assembly defects that are also observed in *aurora-A*. Thus, besides its role in regulating microtubules, Aurora-A also regulates actin-dependent mitotic processes.

Despite its functional conservation, a conserved pathway for the activation of Aurora-A is not known. Here, we describe the identification of Bora, an interaction partner of Aurora-A that is conserved from *C. elegans* to humans. We identify Bora due to its phenotypic similarity to *aurora-A* and show that *bora* overexpression can partially rescue *aurora-A* mutants. Bora binds to Aurora-A and can activate the kinase in vitro. Bora is a nuclear protein that translocates into the cytoplasm upon activation of Cdc2, suggesting that its subcellular localization might contribute to the regulation of Aurora-A. Our results describe a regulator of Aurora-A that is conserved from *Drosophila* to humans and suggest a potential mechanism for the sequential activation of Cdc2 and Aurora-A.

Results

Bora Is Required for Asymmetric Cell Division

In a genetic screen for mutations affecting the development of *Drosophila* external sensory (ES) organs, we identified mutations in *aurora-A* (Berdnik and Knoblich, 2002). In these mutants, Numb fails to localize asymmetrically and the proteins γ-Tubulin and Centrosomin are not recruited to centrosomes during mitosis, leading to spindle abnormalities. Two other mutations from the same screen caused similar phenotypes but are not allelic to *aurora-A*. Both alleles affect the same gene, which we named *bora* (for aurora borealis) to indicate its similarity with *aurora-A*. Flies that are homozygous for *bora* on the head and eye were generated by the ey-Flp/FRT system (Newsome et al., 2000). These flies frequently show duplicated hairs and sockets, a phenotype indicative of defects in asymmetric cell division (Figures 1A and 1B). To determine whether this morphological defect results from cell-fate transformations, we analyzed the SOP cell progeny by using different molecular markers. The socket cell expresses the transcription factor Suppressor of Hairless (Su(H)), whereas the sheath cell can be recognized by expression of Prospero. All four cells express the transcription factor Cut, and the hair cell can be distinguished from the neuron based on its larger size (Figure 1C). In *bora* mutant ES organs, four equally sized Cut-positive cells are found, two of which express Su(H), while no Prospero-positive cell can be detected (Figure 1D). Thus in *bora* mutants, inner cells are transformed into additional outer cells, which is a phenotype characteristic of a defect in Numb localization (Berdnik and Knoblich, 2002; Bhalerao et al., 2005). Indeed, whereas in wild-type SOP cells Numb localizes asymmetrically into a crescent in mitosis and segregates into one of the two daughter cells (Figures 1E and 1F), in *bora* mutant SOP cells, the protein is uniformly cortical in metaphase and equally distributed into both daughter cells (Figures 1G and 1H). Defects in asymmetric localization (although at lower frequency) are also observed for the Numb binding partner Pon (Partner of Numb), but localization of Gαi and Pins is normal (data not shown). Gαi and Pins are required for Numb localization and can act as markers for the polarization of SOP cells, which already occurs in interphase (Bellaiche et al., 2001b; Schaefer et al., 2001). Thus, *bora* is required for the asymmetric localization of cell fate determinants during mitosis but is not essential for polarization of SOP cells in general.

To further explore the phenotypic similarity with *aurora-A*, we analyzed centrosome maturation in *bora* mutants. In wild-type SOP cells, several proteins including γ-Tubulin and Centrosomin are recruited to centrosomes during mitosis (Figure 1I). In *bora* mutant SOP cells (n = 28), however, Centrosomin recruitment is either weak (21%) (Figure 1J) or not detected at all (14%) (Figure 1K). Frequently, we also observe only one (57%) (Figure 1L) or two closely spaced (7%) (Figure 1M) centrosomin dots, indicating defects in centrosome separation. Thus, *bora* mutants recapitulate all aspects of the *aurora-A* mutant phenotype in SOP cells.

To test whether Aurora-A is active in *bora* mutants, we used phosphospecific antibodies against D-TACC, a substrate of Aurora-A (Barros et al., 2005; Giet et al., 2002). In wild-type cells, phosphorylated D-TACC is found at centrosomes and on the mitotic spindle (Figure 1N). In both $aurA^{37}$ and *bora* mutants, however, P-D-TACC staining is significantly reduced and not enriched on any intracellular structures (Figures 1O and 1P). These results suggest that Bora is required for the activation of Aurora-A during mitosis.

Bora Is a Conserved Protein

To determine which gene is affected in *bora* mutants, we narrowed down the mutation to the cytological interval 75B-C by P-element and deficiency mapping. Single-nucleotide polymorphism (SNP) mapping was used for further refinement and sequencing of candidate genes in the respective region revealed that both mutants carry lesions in a transcript that has been annotated as CG6897 by the *Drosophila* sequencing consortium (Myers et al., 2000). $bora^{15}$ is a 14 base-pair out-of-frame deletion in the coding region, which introduces a stop codon after amino acid 162, while $bora^{18}$ is a G-to-A transition that affects a splice-acceptor site (Figure 2A). Both alleles are lethal during pupal stages when homozygous, transheterozygous, or hemizygous over Df(3L)Cat, suggesting that they are either null or strong hypomorphic alleles. Flies carrying large $bora^{15}$ or $bora^{18}$ mutant clones frequently show duplication of hairs and sockets (Figure 2C). These defects can be rescued by expression of a Bora-GFP fusion-protein under the control of *scabrous*-Gal4, indicating that CG6897 is indeed responsible for the *bora* mutant phenotype (Figure 2D).

Bora has no obvious protein domains of known function or structure. Blast searches reveal homologs in other insect species and a bioinformatics analysis identifies sequence homologs in all vertebrate species,

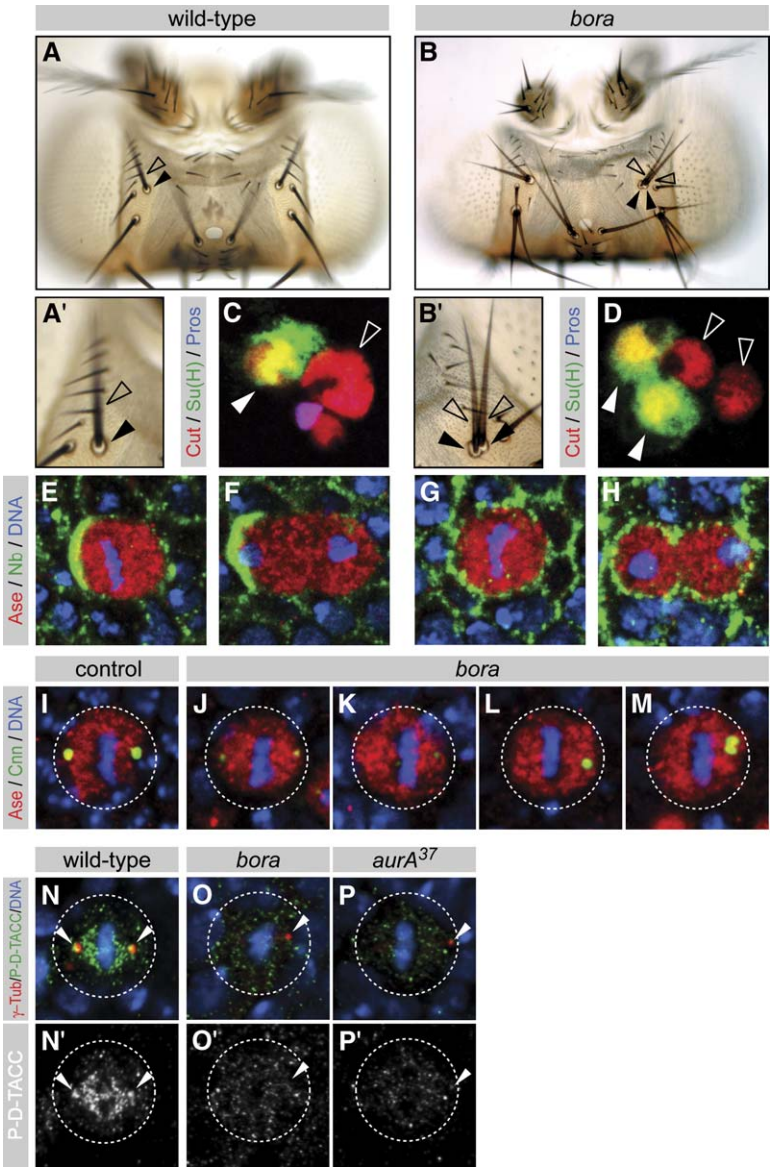

Figure 1. Bora Is Required for Asymmetric Cell Division and Centrosome Maturation

(A and B) Wild-type head (A) and head carrying large *bora* mutant clones (B) generated by the eyFlp/FRT/cell-lethal system. In wild-type flies (A and A′), each bristle contains one hair (open arrowhead) and one socket (arrowhead), while in *bora* mutants (B and B′), most bristles contain two hairs and two sockets.

(C and D) Cut (red) stains all four cells of an ES organ, Suppressor of Hairless (Su(H), green) only socket cells, and Prospero (Pros, blue) marks sheath cells. Neuron and hair (both only Cut-positive) can be distinguished by size. In wild-type (C), one of each cell type is found. *bora* mutant ES organs (D) consist of two sockets and two hairs, but no inner cells.

(E–H) In wild-type SOP cells (E and F), Numb localizes to the anterior cell cortex in metaphase (E) and telophase (F). In *bora* mutant SOP cells (G and H) Numb is found uniformly around the cell cortex throughout mitosis.

(I–M) Wild-type (I) or *bora* mutant (J–M) mitotic SOP cells stained with centrosomin (Cnn, green). In wild-type (I) one centrosomin-positive centrosome is found at each spindle pole. In *bora* mutant cells, centrosomin is found only weakly at the centrosomes (J) or dispersed in the cytoplasm (K). Frequently, only one (L) or two closely spaced (M) centrosomin-positive dots are found. Asense (E–M, red) is used as SOP cell marker.

(N–P) Wild-type (N), *bora* (O), or $aurA^{37}$ (P) mutant mitotic SOP cells stained with P-D-TACC (N–P, green; N′–P′, gray) and γ-tubulin (red). In wild-type cells, P-D-TACC is found on the two centrosomes and on the mitotic spindle (N and N′). In both *bora* (O and O′) and $aurA^{37}$ (P and P′) mutant SOP cells, P-D-TACC staining is greatly reduced and not enriched at intracellular structures. (Arrowheads mark position of centrosomes.)

including humans (Figure 2B and Figure S1B, see the Supplemental Data available with this article online). Conservation of Bora is highest in an N-terminal domain extending approximately from aa 65 to aa 247 of the *Drosophila* protein, while the rest of the protein is less conserved (Figure 2B and Figure S1A). Mouse Bora has been annotated as BAE24669, and human Bora is annotated as LOC79866 and located at 13q22.1. Bora is also conserved in *C. elegans*, where it is encoded by gene F57C2.6, but no homologs were detected in unicellular organisms.

Bora Interacts with Aurora-A

The phenotypic similarity suggests a close connection between Bora and Aurora-A. To test whether *bora* and *aurora-A* interact genetically, we performed rescue experiments with the hypomorphic *aurora-A* allele $aurA^{37}$ (Berdnik and Knoblich, 2002). Overexpression of Bora-GFP with *scabrous*-Gal4 does not cause a phenotype by itself (data not shown) but can rescue the bristle duplications, which are observed in $aurA^{37}$ mutants (Figures 2E and 2F). Antibody staining reveals that both the defects in Numb localization (Figures 2G and 2I) and the centrosome defects (Figures 2H and 2J) are rescued by Bora-GFP. While in $aurA^{37}$ animals Numb is mislocalized and centrosome maturation is impaired in all SOP cells, asymmetric Numb localization is rescued to 77% (n = 26) in metaphase SOP cells and centrosome maturation to 35% (n = 26) upon overexpression of Bora-GFP. In contrast to $aurA^{37}$ clones, eyFlp/FRT clones of *aurora-A* null mutants die early after clone induction. Overexpression of Bora-GFP cannot inhibit this cell lethal effect (data not shown) suggesting that Bora can increase the activity of Aurora-A but not compensate for the complete loss of kinase activity. Taken together, these results suggest that Bora is a rate-limiting regulator of Aurora-A activity.

To test whether the genetic interaction reflects a physical interaction between Bora and Aurora-A, we performed binding assays in *Drosophila* tissue culture cells. *Drosophila* S2 cells were transfected with Aurora-A and Bora-GFP, and protein lysates were subjected to immunoprecipitation by anti-GFP. Since Aurora-A is

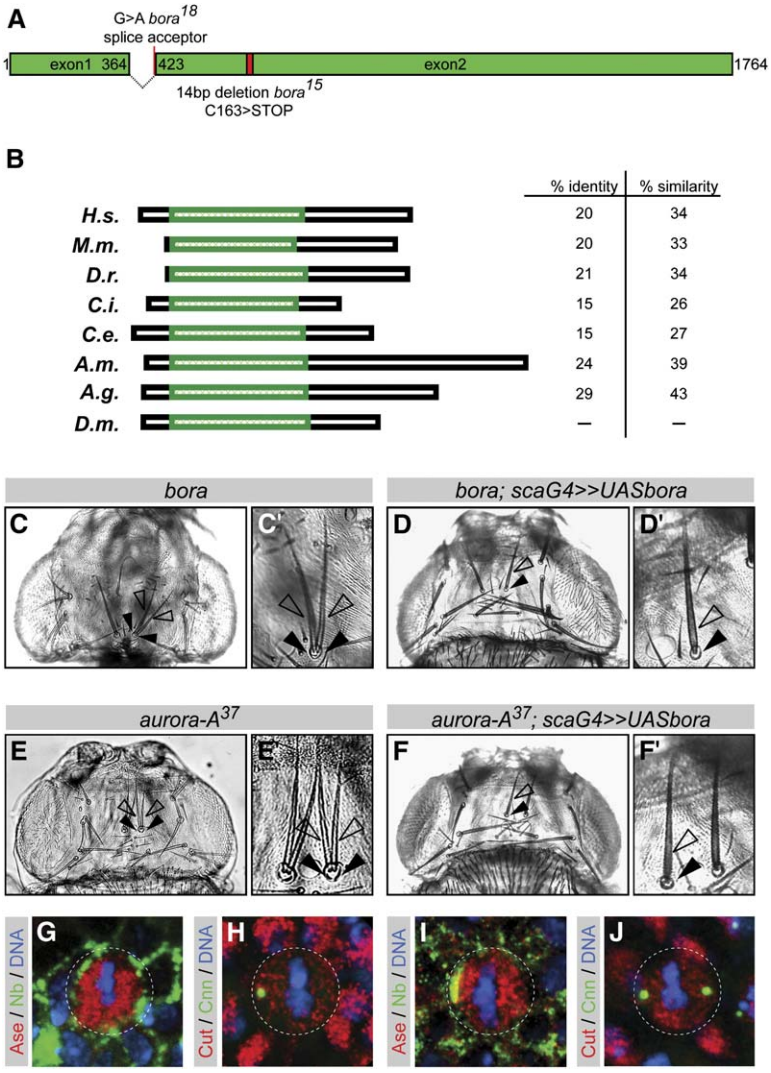

Figure 2. Bora Is a Conserved Protein and Genetically Interacts with *aurora-A*

(A) The molecular lesion in the *bora*^{15} allele is a 14 base-pair deletion in the open-reading frame leading to a premature STOP codon after amino acid 162. The mutation in *bora*^{18} changes a guanine to an adenosine in the splice acceptor sequence.

(B) Protein sequence conservation of the Bora protein in human (*H.s.*), mouse (*M.m.*), zebrafish (*D.r.*), ascidan (*C.i.*), worm (*C.e.*), bee (*A.m.*), mosquito (*A.g.*). The green box corresponds to the region of homology identified by the NCBI-blastp searches. The percentage of identical or similar residues between *Drosophila* Bora and the respective other organisms is indicated on the right. The GENEDOC program (http://www.psc.edu/biomed/genedoc/) was used for calculation. Similar residues were grouped according to the BLOSUM62 matrix (Henikoff and Henikoff, 1992).

(C and D) Bristles on the heads of flies carrying large *bora* mutant clones (C and C′) frequently contain two sockets (arrowheads) and two hairs (open arrowheads). Overexpression of UAS-Bora-GFP with *scabrous*-Gal4 in such a mutant background (D and D′) can suppress this phenotype, and bristles contain one socket and one hair.

(E–F) *Aurora-A*^{37} mutant bristles (E and E′) contain two sockets and two hairs. Overexpression of UAS-Bora-GFP with *scabrous*-Gal4 in *aurora-A*^{37} mutant flies (F and F′) can suppress this phenotype, and bristles contain one socket and one hair. (Note that in contrast to Figures 1A and 1B, flies in Figures 2C–2F were cut out of the pupal case prior to eclosion.)

(G–J) *aurora-A*^{37} mutant mitotic SOP cells have defects in Numb localization (G, green) and centrosome maturation (H, shown by staining for Cnn, green). Upon overexpression of UAS-Bora-GFP, Numb localizes asymmetrically in 77% (n = 26) of all metaphase SOP cells (I), and mature centrosomes (J) are found at each spindle pole in 35% of all metaphase SOP cells (n = 26). Asense (G and I, red) and Cut (H and J, red) are used as SOP cell markers.

specifically detected in the immunoprecipitate, we conclude that Bora can bind to Aurora-A in vivo (Figure 3A). To test whether this is due to a direct interaction, we performed in vitro binding experiments. In vitro translated Aurora-A binds to a GST-Bora fusion-protein (Figure 3B) but not to GST alone. While the nonconserved C terminus of Bora is dispensible for Aurora-A binding, the interaction is abrogated by deleting the conserved region (BoraΔ2) or a region N-terminal to the conserved part (BoraΔ1) (Figures 3B and 3C). Interestingly, the interaction is also observed between in vitro translated human Aurora-A and MBP-HsBora (Figure 3D). Human Aurora-A can even bind to *Drosophila* MBP-Bora in vitro. The interaction with Aurora-A seems to be essential for Bora function since the N-terminal 404 amino acids of Bora (almost identical to BoraΔ3) can rescue the *bora* and *aurA*^{37} mutant phenotypes, while the C terminus (amino acids 404–539) does not (data not shown). Thus, Bora and its homologs act as binding partners of Aurora-A.

Bora Is a Substrate for Aurora-A

Several Aurora-A regulators—like TPX2 (Eyers and Maller, 2004; Kufer et al., 2002)—were shown to also act as substrates for the kinase. To test whether Bora can be phosphorylated by Aurora-A, we performed in vitro kinase assays. *Drosophila* Aurora-A expressed and purified from *E. coli* can phosphorylate bacterially expressed MBP-Bora but not MBP alone (Figure 4A). Interestingly, the kinase activity of Aurora-A toward Bora is as potent as toward myelin basic protein, which is often used as a model substrate. Similarly, human Aurora-A can phosphorylate the human Bora homolog (Figure 4B). To test which region of Bora is phosphorylated, we used Bora deletions in the kinase assay (Figure 4C). Deletion of 125 amino acids from the N terminus of Bora (BoraΔ2) eliminates phosphorylation by Aurora-A, while deletion of the C terminus from amino acid 209 onward (BoraΔ5) does not affect it. Interestingly, Bora is still phosphorylated when the N-terminal 67 amino acids are deleted

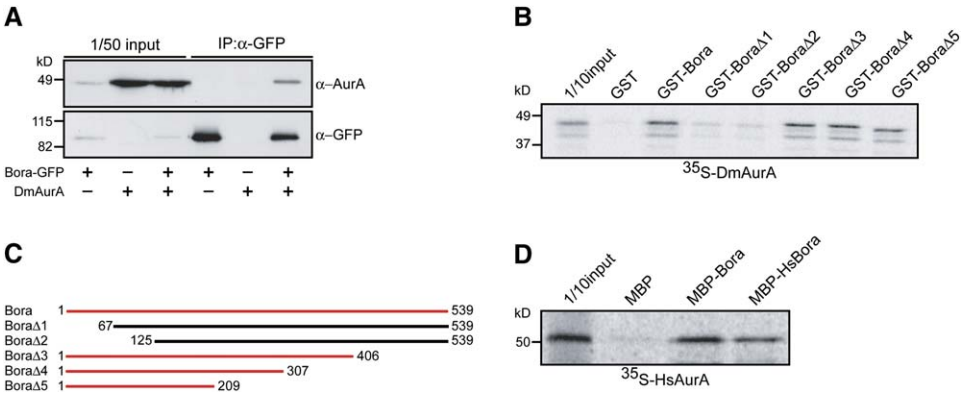

Figure 3. Physical Interaction between Bora and Aurora-A

(A) *Drosophila* S2 cells were cotransfected with Bora-GFP and Aurora-A. Bora-GFP can coprecipitate Aurora-A from cell lysate. (IP: immunoprecipitation.)

(B) GST-Bora coupled to glutathione Sepharose beads can coprecipitate Aurora-A, which was in vitro translated in the presence of ^{35}S-methionine. Deletions of the N terminus of Bora (BoraΔ1 and BoraΔ2) abolish this interaction, whereas C-terminal deletions (BoraΔ3–BoraΔ5) are still capable of coprecipitating Aurora-A.

(C) Schematic representation of the different Bora deletion constructs used in (B). (Numbers indicate amino acid positions.)

(D) MBP, MBP-Bora, and MBP-HsBora were coupled to amylose beads and incubated with ^{35}S-labeled in vitro translated human Aurora-A. Both MBP-Bora and MBP-HsBora, but not MBP alone, can coprecipitate Aurora-A.

(BoraΔ1), suggesting that direct binding to Aurora-A is not necessary for Bora to act as a substrate (Figure 4D). These experiments suggest that the N terminus of Bora is phosphorylated by Aurora-A.

Bora Activates Aurora-A

To test whether Bora can influence the kinase activity of Aurora-A, we used recombinant human Bora in an in vitro kinase assay with myelin basic protein as a

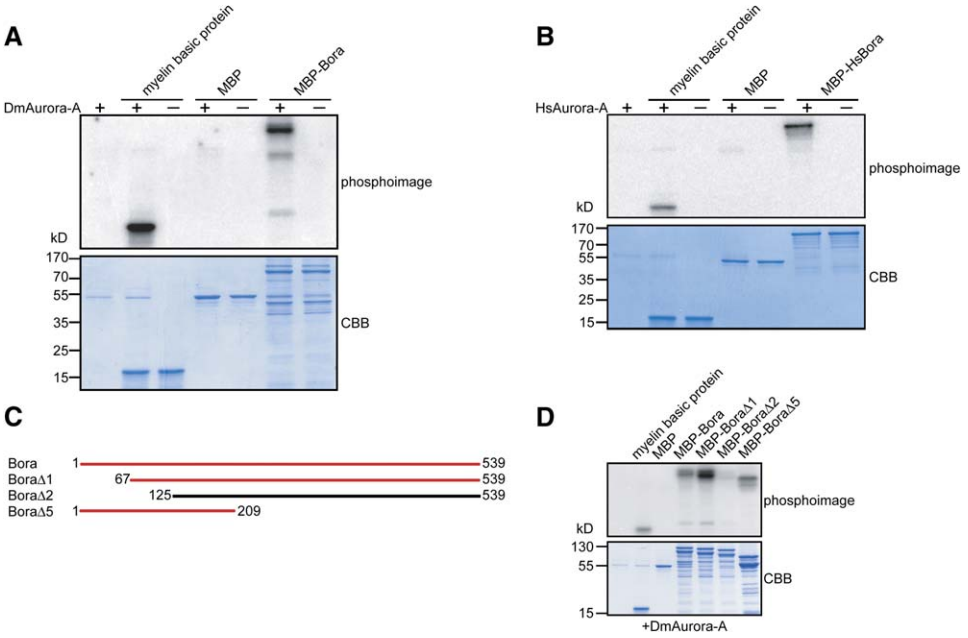

Figure 4. Aurora-A Phosphorylates Bora In Vitro

(A) Equal amounts of bacterially produced recombinant MBP-Bora and MBP as well as myelin basic protein (control substrate) were tested in an in vitro kinase assay for phosphorylation by *Drosophila* Aurora-A (purified from *E. coli*). MBP-Bora and myelin basic protein, but not MBP alone, are strongly phosphorylated by Aurora-A.

(B) Equal amounts of bacterially produced recombinant MBP-HsBora and MBP as well as myelin basic protein (control substrate) were tested in an in vitro kinase assay for phosphorylation by human Aurora-A (purified from *E. coli*). MBP-HsBora and myelin basic protein, but not MBP alone, are strongly phosphorylated by Aurora-A.

(C) Schematic representation of the different Bora deletion constructs used in (D) (numbers indicate amino acid positions).

(D) Different recombinant N-terminally MBP-tagged Bora truncations were tested for phosphorylation by *Drosophila* Aurora-A in vitro. Deletion of the first 67 amino acids of Bora (BoraΔ1) has no effect on phosphorylation, whereas further deletions (BoraΔ2) are no longer phosphorylated by Aurora-A in vitro. C-terminal deletions of Bora (BoraΔ5) do not abolish phosphorylation. Myelin basic protein was used as a control substrate and kinase only and the MBP-tag alone served as negative controls. (CBB: Coomassie brilliant blue.)

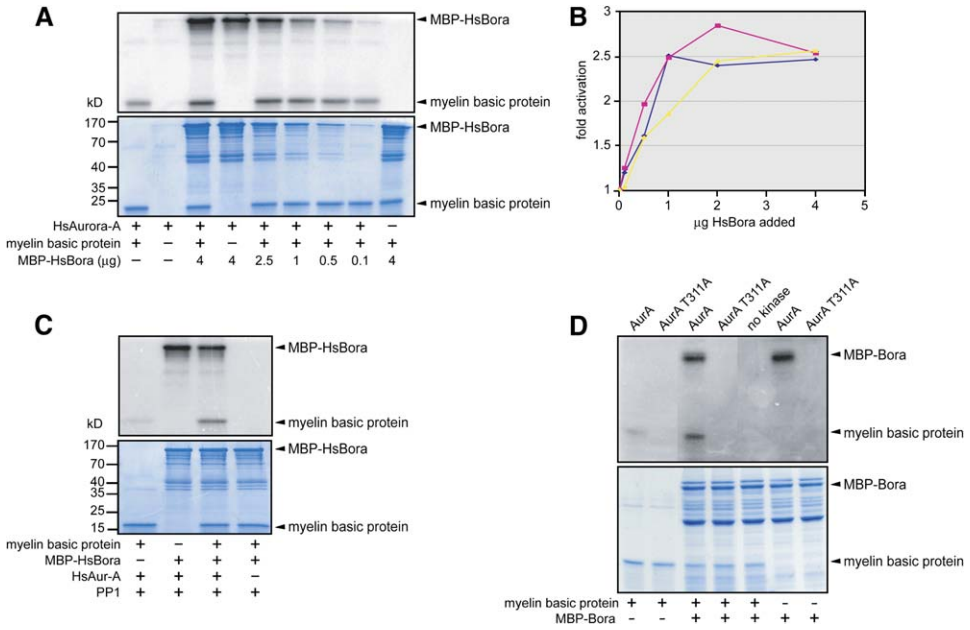

Figure 5. Bora Activates Aurora-A

(A) Addition of MBP-HsBora can increase the kinase activity of Aurora-A toward myelin basic protein.

(B) Quantification of Aurora-A kinase assays as performed in (A). The experiment represented in (A) and two independent experiments are shown. Kinase reaction was performed for 10 min (blue and yellow curve) or 25 min (red curve).

(C) Addition of MBP-HsBora to PP1 inactivated Aurora-A leads to a 7.6- ± 0.4-fold increase in phosphorylation of myelin basic protein (n = 3).

(D) Addition of MBP-Bora can increase the kinase activity of wild-type *Drosophila* Aurora-A toward myelin basic protein but cannot restore kinase activity of Aurora-AT311A, a mutant inactive kinase in which the autophosphorylation site T311 is mutated to alanine. (CBB: Coomassie brilliant blue.)

substrate. Addition of Bora increases Aurora-A activity in a dose-dependent manner (Figure 5A), and a 2.5-fold maximum increase in kinase activity was observed (Figure 5B). Aurora-A is regulated by phosphorylation in the activation loop of the kinase. Since Aurora-A can autophosphorylate, any kinase preparation may be partially active, and this might explain the modest degree of activation by recombinant Bora. Consistent with this, when Aurora-A is inactivated by pretreatment with protein phosphatase 1 (PP1), addition of Bora induces an over 7-fold increase in kinase activity (7.6 ± 0.4, n = 3 independent experiments) (Figure 5C). Analogous experiments with the *Drosophila* homologs reveal that *Drosophila* Bora similarly activates the *Drosophila* kinase (Figure 5D), showing that it acts as a kinase activator as well. Taken together, these results demonstrate that Bora is an activator of Aurora-A.

Mutation of the autophosphorylation site of Aurora-A to alanine renders the kinase inactive (Figure 5D) (Littlepage et al., 2002), and an interesting question is if the stimulation of Aurora-A by Bora bypasses the need for autophosphorylation. We find that addition of Bora does not restore activity to the mutant kinase (Figure 5D), suggesting that activation by Bora requires autophosphorylation of Aurora-A.

Bora Shuttles between Nucleus and Cytoplasm

To determine the subcellular localization of Bora in SOP cells, we performed live imaging of a Bora-GFP fusion protein, which can rescue both *bora* and *aurA*^{37} mutant phenotypes. Histone-RFP is used to label chromosomes and indicates the cell-cycle stage. Constructs were specifically expressed by *neuralized*-Gal4 in SOP cells and dividing cells were imaged in whole living pupae. In interphase, Bora is a nuclear protein (Figure 6A). When chromosomes condense, however, Bora is released from the nucleus (Figure 6B). It is completely excluded from the nucleus by late prophase (Figure 6C) and is uniformly distributed in the cytoplasm after nuclear envelope breakdown (Figures 6D and 6E). In telophase, Bora enters both daughter cells where it relocates into the nucleus (Figure 6F). Bora does not have an obvious nuclear localization signal. However, we find that the first 125 amino acids of the protein are sufficient for nuclear retention (Figure S2A), suggesting that they contain the sequence that mediates nuclear import. Live imaging of GFP-Aurora-A together with Histone-RFP allows us to correlate the localization of Aurora-A with Bora (Figures 6G–6L). In interphase, the two proteins are in distinct compartments. Nuclear release of Bora coincides with centrosome separation and strong recruitment of Aurora-A to the maturing centrosomes. Since both centrosome separation and maturation defects are observed in *aurora-A* mutants, these results suggest that release of Bora coincides with Aurora-A activation.

While Aurora-A is required for a subset of mitotic events, Cdc2 is essential for all steps of mitosis. How Cdc2 activates Aurora-A is unclear (Marumoto et al., 2002; Maton et al., 2003). To test whether Cdc2 regulates the release of Bora into the cytoplasm, we analyzed Bora localization in *string* mutants. String is the *Drosophila* homolog of the Cdc25 phosphatase, and in *string* mutants, Cdc2 is not activated (Edgar et al., 1994; Edgar and O'Farrell, 1989). Antibody staining of

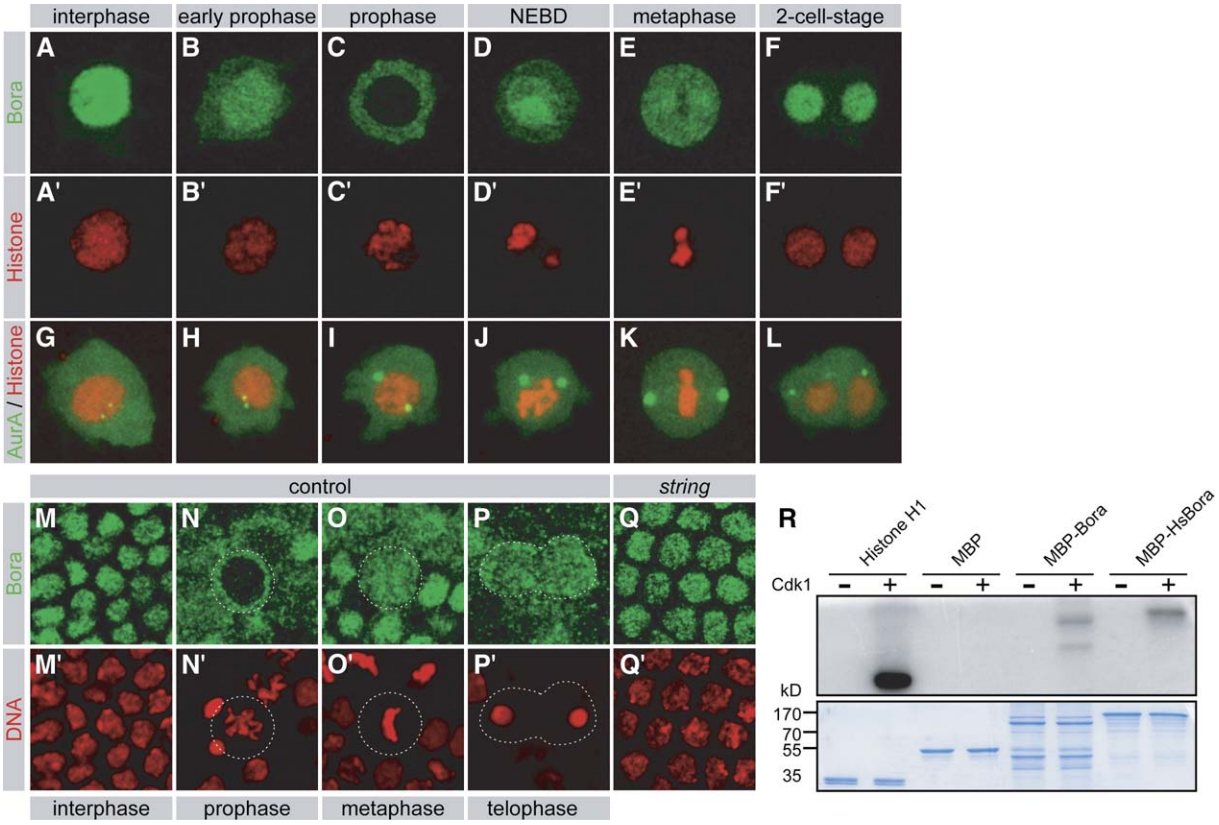

Figure 6. Bora Shuttles between the Nucleus and the Cytoplasm in a Cell-Cycle-Regulated Manner

(A–F) UAS-Bora-GFP (A–F, green) and UAS-Histone-RFP (A′–F′, red) were expressed in SOP cells with *neuralized*-Gal4. Shown are stills from a time-lapse movie. During interphase (A), Bora is a nuclear protein, but upon entry into mitosis (B), Bora is released into the cytoplasm and fully excluded from the nucleus (C) until nuclear envelope breakdown (D). During the rest of mitosis, Bora is uniformly cytoplasmic for the remainder of mitosis (E, metaphase) and eventually reenters the nucleus after exit from mitosis (F) (see Movie S1).

(G–L) UAS-GFP-Aurora-A (green) and UAS-Histone-RFP (red) were expressed in SOP cells with *neuralized*-Gal4. Aurora-A localizes to the cytoplasm throughout the cell cycle and is enriched at centrosomes. At the beginning of mitosis (H–J), centrosomes enlarge and separate, which coincides with nuclear release of Bora. Shown are maximum projections of several optical slices recorded at each time point of the time-lapse movie.

(M–Q) Shown are surface views of *Drosophila* stage 4 embryos. In control cells, Bora is released from the nucleus into the cytoplasm at the entry into mitosis (M–P, green), whereas in *string* mutants, which arrest at the G2-M transition, Bora does not leave the nucleus (Q). Corresponding DNA images are shown in the bottom panel (M′–Q′, red). (R) Equal amounts of bacterially produced MBP-Bora, MBP-HsBora, and MBP, as well as Histone H1 (control substrate), were assayed for phosphorylation by Cdk1 in vitro. MBP-Bora and MBP-HsBora, as well as Histone H1 but not MBP alone, are phosphorylated by Cdk1. (Upper panel shows phosphoimage, and lower panel shows CBB [Coomassie brilliant blue] staining.)

Drosophila embryos reveals that endogenous Bora shows the same dynamic localization during the cell cycle as the functional GFP fusion protein (Figures 6M–6P). In *string* mutant embryos, however, we never observed Bora in the cytoplasm, indicating that Cdc2 activation is required for the release of Bora from the nucleus (Figure 6Q). To test whether Cdc2 might directly phosphorylate Bora, we performed in vitro kinase assays. Both Bora and HsBora are phosphorylated by recombinant Cdk1 kinase (Figure 6R). Although the in vivo relevance of Cdk1 phosphorylation remains to be tested, these experiments show that Bora is released into the cytoplasm at the onset of mitosis in a Cdc2-dependent manner.

Human Bora Is Required for Mitosis

To determine whether the requirement for activation of Aurora-A by Bora is conserved between flies and vertebrates, we tested whether loss of human Bora leads to mitotic defects. We silenced the gene in mammalian U2OS cells by siRNA (Elbashir et al., 2001) and detect a significant reduction of HsBora mRNA 48 hr after siRNA transfection (Figure 7A). In contrast to cells treated with a control siRNA, cells treated with siRNAs against Bora frequently displayed multipolar spindles in mitosis (Figures 7B–7E), a phenotype that is also observed upon TPX2 RNAi and after injection of antibodies blocking Aurora-A function (Garrett et al., 2002; Marumoto et al., 2003). Taken together, our experiments suggest that Bora is a key activator of Aurora-A that is functionally conserved between *Drosophila* and vertebrates.

Discussion

A Conserved Control Mechanism for Aurora-A Activation?

Aurora-A is involved in centrosome maturation, spindle assembly, and asymmetric protein localization during mitosis. We show here that the conserved binding partner Bora is essential for Aurora-A to perform these

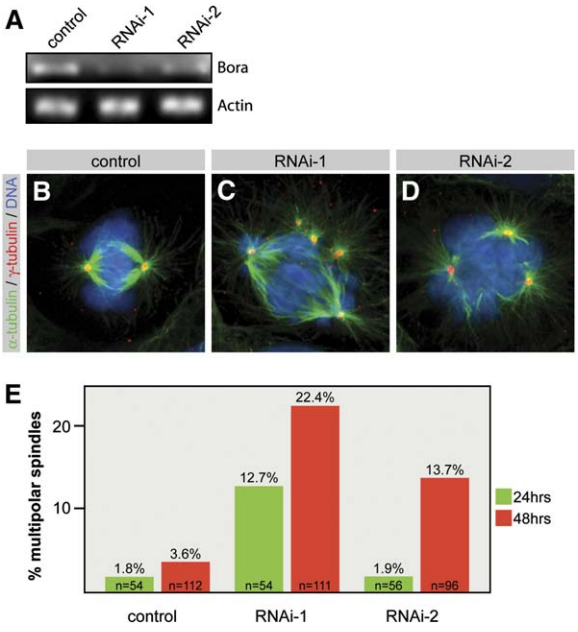

Figure 7. Loss of Human Bora Leads to Mitotic Defects

(A) RT-PCR was performed to determine RNAi efficiency. Actin is used as loading control.

(B–D) To visualize centrosomes and the mitotic spindle U2OS cells were stained with γ-tubulin (red) and α-tubulin (green) 2 days after transfection with siRNA. In mitotic control cells (B), a bipolar spindle is formed between the two opposing centrosomes. Upon transfection with two different Bora siRNAs defective, multipolar spindles with three to five (C and D) centrosomes are frequently observed.

(E) The siRNA experiment was performed twice in duplicate, and the number of cells with multipolar spindles was determined. Twenty-four hours after siRNA transfection, we observe a clear increase in the number of multipolar spindles with RNAi-1. After 48 hr, both RNAi-1 and RNAi-2 show an increase of cells with multipolar spindles. The difference between the two siRNAs might be due to a difference in the efficiency of protein knockdown.

functions in *Drosophila*. Bora can activate Aurora-A in vitro. Bora is a nuclear protein that is excluded from the nucleus during prophase in a Cdc2-dependent manner. Nuclear retention of Bora might help to keep Aurora-A inactive during interphase. When Cdc2 becomes activated, Bora is released into the cytoplasm where it can bind and activate Aurora-A. This hypothesis could provide a molecular explanation for previous results demonstrating that Cdc2 is crucial for the activation of Aurora-A (Marumoto et al., 2002; Maton et al., 2003). Since Bora is a substrate for Cdc2 in vitro and—at least in vertebrates—a fraction of Cdc2 has been reported to be nuclear (Bailly et al., 1989; Riabowol et al., 1989), it is conceivable that direct phosphorylation of Bora might facilitate its exclusion from the nucleus. However, nuclear release of Bora is not the only mechanism by which its activation of Aurora-A is regulated since the *bora* mutant phenotype can also be rescued by Bora fused to a myristylation signal, which keeps the protein in the cytoplasm, or fused to a nuclear localization signal, which retains the protein in the nucleus until nuclear envelope breakdown (data not shown).

Although in *Drosophila*, Bora so far is the only known activator of Aurora-A, several in vitro activators of Aurora-A have been identified in other organisms. In vertebrates, TPX2 prevents PP1-dependent dephosphorylation and thereby locks the kinase in its active conformation (Bayliss et al., 2003). The activation of Aurora-A by Cdc2 is PP1 independent (Maton et al., 2005), and, therefore, TPX2 is unlikely to participate in this particular event. Furthermore, TPX2 is only required for a subset of Aurora-A-dependent processes: TPX2 inactivation by RNAi causes spindle defects and loss of Aurora-A from the mitotic spindle, but centrosome maturation is normal (Garrett et al., 2002), and the centrosome pool of the kinase is unaffected (Kufer et al., 2002). TPX2/Aurora-A binding is stimulated by the small GTPase Ran (Tsai et al., 2003), which in turn is activated by RCC1, an exchange factor that is located on condensed chromatin and is involved in microtubule nucleation and spindle formation (Moore et al., 2002). Thus, unlike Bora, TPX2 seems to be specifically responsible for the spindle assembly function of Aurora-A. So far, no TPX2 homolog has been identified in *Drosophila*. Whether this is due to a low level of sequence similarity that escapes standard homology searches or whether it reflects a fundamental difference in Aurora-A function between organisms is currently unclear.

One protein that might be generally required for Aurora-A activation is Ajuba (Hirota et al., 2003). Upon Ajuba RNAi, Aurora-A fails to be activated. In HeLa cells, this leads to a cell-cycle block in G2 and prevents entry into mitosis. However, since *ajuba* null mutant mice are completely viable (Pratt et al., 2005) and keratinocytes from these mice have no cell-cycle block (Marie et al., 2003), the significance of these RNAi experiments is unclear. Furthermore, no Ajuba homologs are found in *C. elegans* or *Drosophila*, suggesting that a functional connection between Ajuba and Bora is unlikely.

More recently, two other activation pathways for Aurora-A have been described. The focal adhesion protein HEF1 binds to Aurora-A and is required and sufficient for Aurora-A activation (Pugacheva and Golemis, 2005). The protein kinase PAK relocalizes to centrosomes during mitosis where it is activated and in turn phosphorylates and activates Aurora-A (Zhao et al., 2005). Since PAK is a part of focal adhesion complexes, both pathways might be part of a mechanism establishing crosstalk between cell adhesion and the mitotic apparatus (Cotteret and Chernoff, 2005). However, PAK inhibition only delays centrosome maturation, suggesting that this pathway is not a crucial regulator of the G2/M functions of Aurora-A. In *Drosophila*, both PAK and HEF1 are conserved, but the PAK mutant phenotype does not suggest any requirement of the kinase for mitosis. Taken together, these observations suggest that Bora does not participate in any of the known pathways but is more globally involved in the activation of Aurora-A.

Bora and Asymmetric Cell Division

Like Aurora-A, Bora is required for actin-dependent asymmetric protein localization during mitosis (Berdnik and Knoblich, 2002). It is thought that the polarized localization of the kinase aPKC leads to asymmetric phosphorylation of the cytoskeletal protein Lgl (Betschinger and Knoblich, 2004). Since phosphorylation inactivates Lgl (Betschinger et al., 2003) and Lgl is essential for establishing a cortical binding site for cell fate determinants (Mayer et al., 2005), those determinants

accumulate exclusively on the side of the cortex that is free of aPKC. Aurora-A could act at several points in this pathway: either the cortical binding site could already be polarized in interphase and activation of Aurora-A could establish its affinity for cell-fate determinants, or alternatively, Aurora-A could regulate the activity of aPKC. In this case, aPKC would be asymmetric but inactive in interphase and its activation in prophase would initiate asymmetric localization of cell-fate determinants. At the moment, we cannot distinguish between these possibilities, but identification of the Aurora-A substrates relevant for asymmetric protein localization should clarify its mode of action. In any case, the observation that Cdc2 is essential for asymmetric determinant localization as well (Tio et al., 2001) is consistent with a model where Cdc2 is required for the Bora-dependent activation of Aurora-A.

Aurora-A has been implicated in carcinogenesis. It is overexpressed in a number of cancers and its overexpression results in polyploidy or cells containing multiple centrosomes (Giet et al., 2005; Meraldi et al., 2004). Aurora-A has therefore been used as a drug target for cancer therapy (Matthews et al., 2006), and the identification of Bora offers an alternative route for the discovery of Aurora-A selective inhibitors. The human Bora homolog (annotated as FLJ22624; Strausberg et al., 2002) is located on chromosome 13 in a region that contains a breast cancer susceptibility gene and has been implicated in a variety of malignant tumors (Rozenblum et al., 2002). Future studies will reveal whether it is involved in carcinogenesis as well.

Experimental Procedures

Identification of *bora*

bora was identified in a genetic screen of chromosome arm 3L, carried out essentially as described previously (Berdnik and Knoblich, 2002). Random mutations were generated on an isogenized *FRT [80B]* chromosome by EMS treatment and analyzed in large mitotic clones induced by *eyFlp* over a cell-lethal chromosome (Newsome et al., 2000). Among approximately 52,000 flies, we identified three complementation groups and six individual alleles that cause visible cell fate transformations in ES organs on the head. One complementation group contained $bora^{15}$ and $bora^{18}$. The mutations were mapped between cytological region 75B and 75C based on lethality over deficiency Df(3L)Cat and recombination mapping with P-elements (Berdnik et al., 2002). To further narrow down the region containing the mutations, we employed a similar recombination strategy with single-nucleotide polymorphisms (SNPs) between the paternal strain, which was used for mutagenesis, and a strain carrying the dominant marker *Wrinkled*, which was further used to generate recombinants for mapping. We performed sequence analysis of the coding regions of candidate genes within the mapped region with DNA isolated from homozygous mutant larvae. Mutations $bora^{15}$ and $bora^{18}$ were the only sequence differences to the paternal chromosome.

Identification of Bora Homologs

An NCBI-blastp (Altschul et al., 1990) search in the nonredundant database of the NCBI with the *Drosophila* Bora sequence identified a region (aa 65–377) with significant homology (E values < 1e-04) to other insects as well as to worms and vertebrates. Reciprocal NCBI-blastp searches with the conserved region of the collected Bora proteins significantly hit back to *Drosophila* Bora und thus confirm the phylogenetic relationship. The Bora conserved domain has no detectable homology to sequences with known function or structure.

Constructs

The *bora* coding region was obtained from the EST LD27847. Full-length Bora and the various truncations were cloned into a vector containing a β-globin leader and one copy of GFPS65T (M. Schaefer and J.A.K., unpublished data). The resulting Bora-GFP fusions were cloned into pUAST (Brand and Perrimon, 1993), and transgenic flies were generated following standard procedures. GST-Bora fusions were generated in pGEX4T1 (Amersham Pharmacia), MBP-Bora fusions in pMAL-c2x (NEB). The coding region of human Bora (HsBora) was obtained from the EST IMGCLO4098541 and fused to MBP at the N terminus.

Flies and Time-Lapse Microscopy

Bora clones were generated by the *ey-Flp*/FRT/cell-lethal system (Newsome et al., 2000), while $aurora\text{-}A^{37}$ mutants were analyzed as homozygotes. For the rescue experiments, transgenes were expressed under the control of *scabrous*-Gal4 (Brand and Perrimon, 1993). For live imaging, Bora-GFP, GFP-Aur-A (Berdnik and Knoblich, 2002), and Histone-RFP (Emery et al., 2005) were expressed with *neuralized*-Gal4, and time-lapse microscopy was performed essentially as described (Bellaiche et al., 2001a). *String7b* mutant embryos (gift from Ch. Lehner) were used for analyzing the cell-cycle dependency of Bora localization.

Immunofluorescence and Antibodies

Immunofluorescence experiments were carried out essentially as described (Hutterer and Knoblich, 2005). Antibodies used were: rabbit anti-Prospero (1:1000) (Vaessin et al., 1991), rat anti-Su(H) (1:2000; gift from F. Schweisguth), mouse anti-Cut (1:500, mAB2B10, DSHB), guinea-pig anti-Asense (1:2000, gift from Y.N. Jan), rabbit anti-Numb (1:100) (Schober et al., 1999), rabbit anti-Centrosomin (1:500) (Li and Kaufman, 1996), rabbit anti-γ-Tubulin (1:3000, Sigma), mouse anti-γ-Tubulin (1:1000, Sigma), mouse anti-α-Tubulin (1:1500, Invitrogen), rabbit anti-P-D-TACC (1:200) (Barros et al., 2005), rabbit anti-GFP (affinity purified, 1:500, Abcam). Mouse anti-Aurora-A was generated against an N-terminal His_6 Aurora-A fusion-protein and used 1:300. Rabbit anti-Bora was generated against an N-terminal His_6 fusion of aa 1–432 and used 1:100. Hoechst 33258 (Sigma) or Propidium Iodide were used to visualize DNA. Images were recorded on a Zeiss LSM510 confocal microscope and processed with Adobe Photoshop.

Cell Culture and Immunoprecipitations

Drosophila S2 cells were propagated in Schneider's medium (Gibco) containing 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. UAS constructs were expressed by cotransfection with actin-Gal4 (gift from T. Volk) with Cellfectin (Invitrogen). Immunoprecipitations were carried out essentially as described (Betschinger et al., 2003).

siRNA Experiments

U2OS cells were propagated under standard conditions, plated onto eight-chamber well slides and allowed to attach overnight. For siRNA transfection, Lipofectamine 2000 (Invitrogen) was used together with Optimem (Invitrogen). The following Silencer predesigned siRNAs (Ambion) have been used: siRNA ID number 140887 (RNAi-1) and 140886 (RNAi-2). Firefly Luciferase siRNA was used as negative control. Twenty-four and 48 hr after transfection, cells were fixed and stained by standard protocols. Experiments were performed twice in duplicate each. For RT-PCR, total RNA was isolated with the RNeasy kit (Qiagen) 48 hr after transfection. Primers used were: 5′-AAGCATCTCCGACTCCTTACCTTC-3′, 5′-GAAAACTGCCCCGAACTTGG-3′ for human Bora; and for human β-Actin: 5′-AAAGCCATGCCAATCTCATC-3′; 5′-AAGTACTCCGTG TGGATCGG-3′.

In Vitro Binding and Kinase Assays

In vitro binding assays were performed as previously described (Hutterer et al., 2004). Full-length *Drosophila* Aurora-A was translated from the EST LD19783. Human Aurora-A was translated from a plasmid containing a β-globin leader and two N-terminal myc tags. In vitro kinase assays were carried out essentially as described (Betschinger et al., 2003). His_{6x} Aurora-AT311A was generated by site-directed mutagenesis.

Bacterially produced *Drosophila* or human His_{6x}-Aurora-A (kind gift of T. Hirota) or Cdk1/CyclinB (Calbiochem) were incubated with MBP-Bora for 20 min at 30°C (HsAur-A, Cdk1) or 25°C (*Dm*Aur-A). Myelin basic protein (Sigma Aldrich) or Histone H1 (Upstate) were used as control substrates. For activation assays, human

Aurora-A was incubated with MBP-HsBora in the presence of myelin basic protein for 10 min at 30°C. For reactivation assays, human Aurora-A (300 ng) was treated with 0.4 units PP1 (NEB) for 1 hr at 30°C and subsequently assayed for phosphorylation of myelin basic protein in the absence or presence of MBP-Hora. Reactivation assays were carried out at 30°C for 5 min. ImageQuant Software was used for quantification of increase in phosphorylation.

Supplemental Data

Supplemental data include bioinformatical methods, two figures, and a movie and are available at http://www.developmentalcell.com/cgi/content/full/11/2/147/DC1/.

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