Bora phosphorylation substitutes in trans for T-loop phosphorylation in Aurora A to promote mitotic entry

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Polo-like kinase 1 (Plk1) is instrumental for mitotic entry and progression. Plk1 is activated by phosphorylation on a conserved residue Thr210 in its activation segment by the Aurora A kinase (AURKA), a reaction that critically requires the co-factor Bora phosphorylated by a CyclinA/B-Cdk1 kinase. Here we show that phospho-Bora is a direct activator of AURKA kinase activity. We localize the key determinants of phospho-Bora function to a 100 amino acid region encompassing two short Tpx2-like motifs and a phosphoSerine-Proline motif at Serine 112, through which Bora binds AURKA. The latter substitutes in trans for the Thr288 phospho-regulatory site of AURKA, which is essential for an active conformation of the kinase domain. We demonstrate the importance of these determinants for Bora function in mitotic entry both in Xenopus egg extracts and in human cells. Our findings unveil the activation mechanism of AURKA that is critical for mitotic entry.
t onset of mitosis, eukaryotic cells undergo a drastic cellular reorganization. The chromatin condenses into individual chromosomes and the nuclear envelope breaks down to permit the capture of chromosomes by the microtubules emanating from the centrosomes, leading to the assembly of the mitotic spindle. This choreography is orchestrated in space and time by evolutionarily conserved mitotic kinases including Aurora kinases (AURK), CyclinA/B-Cdk1, and the Polo-like kinase 1 (Plk1) (for review, see refs. 2-4). In particular, Plk1 promotes chromosome condensation, nuclear envelope breakdown (NEBD), centrosome maturation, and kinetochore-microtubule attachments (for review, see refs. 5-7). In addition to a role in this cellular reorganization, Plk1 triggers the G2/M transition8,9 and is essential for the cell cycle restart after recovery from DNA damage10. To coordinate these different functions, Plk1 is dynamically recruited to very specific subcellular sites where it phosphorylates a plethora of substrates.

Plk1 is composed of two functional domains (Fig. 1a); an amino-terminal kinase domain (KD) and a C-terminal polo-box domain (PBD) critical for Plk1 subcellular localization and substrate targeting11-13. The PBD binds to phosphorylated motifs known as Polo-docking sites, generated by other kinases, notably by Cyclin-Cdk or Plk1 itself14. The PBD also regulates Plk1 kinase activity through repressive intramolecular interactions with the KD15. The binding of phosphopeptides to the PBD relieves these auto-inhibitory interactions thereby tightly coupling localization/substrate recognition with catalytic activation16-18.

In its auto-inhibitory (closed) conformation, Plk1 is cytoplasmic. Upon activation at mitotic entry, Plk1 transitions to an open conformation that also exposes a nuclear localization sequence (NLS) within the KD to binding by importins16,17. Thus, Plk1 activation at mitotic entry is further coupled to its nuclear import17-19. Plk1 activation also depends on trans-phosphorylation of a conserved residue (Thr210) in its activation segment (T-loop)20 by the Aurora A kinase (AURKA)21,22, a member of the larger Aurora family that includes AURKB and AURKC (Fig. 1a and Supplementary Fig. S1). T-loop phosphorylation critically requires the evolutionarily conserved protein Bora21-23 phosphorylated by Cyclin-Cdk24-27. Bora contains two evolutionarily conserved Cyclin-binding motifs (Cy)25 (Fig. 1a) and its phosphorylation by Cyclin-Cdk is essential for Plk1 function in mitotic entry in Caenorhabditis elegans embryos24, in Xenopus egg extracts27, and for cell cycle restart after checkpoint recovery from DNA damage in human cells25,26. CyclinA-Cdk1, which coordinates DNA replication and mitotic entry, is a positive upstream regulator of Bora in Xenopus27 and most likely in human cells where CyclinA appears to translocate into the cytoplasm in G2 phase to interact with and initiate Plk1 activation9,28. As Bora is exclusively cytoplasmic in human cells28-30, it is likely responsible for activating the cytoplasmic pool of Plk1 at the G2-M transition thereby triggering Plk1 nuclear import.

While Bora and its phosphorylation by CyclinA-Cdk1 are essential for Plk1 activation by AURKA at mitotic entry, the precise mechanism by which Bora contributes to T-loop phosphorylation of Plk1 is not understood. Cyclin-Cdk phosphorylates Bora at multiple sites including notably a polo-docking site (S252), which primes Bora binding to the Plk1 PBD22,31. This binding event was proposed to increase the accessibility of T210, thereby supporting T-loop phosphorylation by AURKA22,32. However, a Bora21-224 fragment, lacking the polo-docking site S252, can still promote AURKA-dependent phosphorylation of Plk1 on the T-loop25 and support mitotic entry in Xenopus egg extracts27, suggesting that Bora harbors additional functions beyond opening of the Plk1 structure.

In one possible model, Bora could act by interacting with and promoting the activation of AURKA. Indeed, precedents for the activation of Aurora family kinases by trans-acting factors are well documented33-35. For example, the microtubule and mitotic spindle regulator Tpx2 (Targeting Protein for Xenopus kinesin-like protein 2) allosterically binds the N-lobe of the AURKA KD to promote an active conformation competent for microtubule nucleation and mitotic spindle assembly36-40. In addition to triggering auto-phosphorylation of AURKA on activation segment residues Thr287 or Thr288 and by preventing T-loop dephosphorylation by phosphatases40, Tpx2 may also allosterically activate AURKA independent of a need for T-loop phosphorylation41,42.

To decipher the mechanism by which phospho-Bora (hereafter pBora) promotes T-loop phosphorylation on Plk1 by AURKA, we use a combination of biochemical and structural approaches along with functional studies in Xenopus and in human cells. We show that Bora contains three critical elements required to maximally activate AURKA kinase activity, namely two linear Tpx2-like motifs and a phosphorylated motif on Ser112 generated by Cyclin-Cdk activity. These elements act synergistically to stabilize AURKA in an active conformation, allowing AURKA to phosphorylate and activate Plk1. In particular, the Tpx2-like motifs position the Ser112 phospho-motif toward the kinase active site to substitute in trans for phosphorylation of the T-loop on AURKA.

Results

The KD of Plk1 is a sufficient substrate for pBora potentiated phosphorylation by AURKA. Previous works established that an amino terminal fragment of Bora (residues 1–224) is sufficient to stimulate phosphorylation of Plk1 on the critical T-loop regulatory site T210 (henceforth Plk1 phosphorylation) by AURKA in vitro23 and in Xenopus egg extracts27. To investigate the minimal determinants for this phosphorylation event, we reconstituted the reaction in vitro using purified components (Fig. 1a, b). As shown in Fig. 1c, in the absence of Bora, no phosphorylation of Plk1 was observed (lanes 3, 8, and 13). However, upon addition of Bora1-224, weak phosphorylation of Plk1 was detected (lanes 2, 7, and 12) that could be potentiated greatly by pre-phosphorylation of Bora1-224 by CyclinA2-Cdk2 (lanes 1, 6, and 11), consistent with a previous report25. Deletion of the PBD of Plk1 (ΔPBD) (lanes 6–10), or both the PBD and interdomain linker (i.e. the kinase domain-only construct (KD)) (lanes 11–15), had no effect on Plk1 phosphorylation by AURKA indicating that the critical determinants for efficient phosphorylation of Plk1 lie within the KD of Plk1. Likewise, a catalytically inactive mutant (K82R) of the Plk1 KD was also efficiently phosphorylated by AURKA, ruling out a role for auto priming by Plk1 in the process. Furthermore, by removing AURKA from the reaction, we excluded a direct role for Cyclin-Cdk in phosphorylating the T-loop of Plk1 (lanes 5, 10, 15). These results indicate that the KD of Plk1 is a minimal substrate for T-loop phosphorylation by AURKA in the presence of phosphorylated Bora.

Bora is an intrinsically disordered protein that directly binds AURKA. We next investigated the binding properties of Bora to AURKA and the Plk1 KD. To this end, we employed Heteronuclear Single Quantum Coherence (HSQC) Nuclear Magnetic Resonance (NMR) analysis with 15N isotopically labeled Bora1-224. Initial HSQC analysis of non-phosphorylated 15N-Bora1-224 in isolation revealed a pattern indicative of an intrinsically disordered protein (Supplementary Fig. 2a, left panel). Specifically, all resonance peaks clustered between 8.5 and 7.5 ppm on the 1H axis. Such a narrow dispersion reflects the similar
environment sampled by residues in a disordered state relative to the greater diversity of environments sampled by residues in a folded state. Phosphorylation of Bora$^{1-224}$ by Cyclin-Cdk on an average of 8 to 10 S/T-P sites (Supplementary Fig. 2a, right panel), led to the expected down-field shift of resonance peaks corresponding to phosphorylated amino acids. The majority of resonance peaks remained clustered between 8.5 and 7.5 ppm on the $^1$H axis suggesting that phosphorylation of Bora did not induce a transition to an ordered tertiary structure. Addition of unlabeled Plk1 KD to non-phosphorylated Bora $^{1-224}$ or to Cyclin-Cdk-phosphorylated Bora $^{1-224}$ revealed little peak shifting or line broadening (Fig. 2a, b, left panels respectively). In contrast,
Fig. 1 In vitro reconstitution of a minimal system recapitulating T-loop phosphorylation of Plk1 by Bora and AURKA. a Domain architecture of Homo sapiens AURKA, Bora, and Plk1. The positions of the T-loop phospho-regulatory sites of AURKA (T288) and Plk1 (T210) are indicated. Cy motifs in Bora correspond to Cyclin-binding motifs that mediate substrate targeting. b Two-step in vitro reconstitution of T-loop phosphorylation on T210 of Plk1 (pT210) by AURKA and Bora1–224. In step 1, Bora is phosphorylated by CyclinA2-Cdk2 kinase (yielding pBora). In step 2, phosphorylated Bora from the reaction mix in step 1 is incubated with AURKA and the catalytically dead mutant Plk1ΔPBD in full-length (FL), polo-box domain deleted (ΔPBD), or kinase domain-only (KD) forms. During step 2, AURKA phosphorylates Plk1 (noted pT210 Plk1) and Bora or pBora (noted pBora). c Western blot analysis of kinase reactions carried out with Bora1–224 phosphorylated (+) or not (−) by CyclinA2-Cdk2 (step 1) in the presence of Plk1ΔPBD (FL or ΔPBD or KD) and AURKA (step 2). Schematics of the Plk1 constructs used as substrates are shown at bottom. Blots were probed with antibodies to Bora, AURKA, and phosphoT210 was quantified to a sensor chip with Bora1 – 224 (Fig. 2c, left panel) but not Plk1 KD (Fig. 2c, center panel). This finding is consistent with previous observations showing that Bora does not co-immunoprecipitate with the Plk1 KD in human cells31. Furthermore, addition of AURKA to the Plk1 KD binding reaction did not assist binding of Plk1 KD to Bora1–224 (Fig. 2c, right panel).

To further examine binding between Bora1–224 and AURKA, we employed Surface Plasmon Resonance (SPR). AURKA bound to biotinylated Bora1–224 and biotinylated pBora1–224 immobilized to a sensor chip with K₆ values of ~52 μM and ~18 μM, respectively (Fig. 2d, e and Supplementary Fig. 2d, e; see also Supplementary Table 1 for quantified values described in this manuscript). Together, our NMR, pull-down and SPR binding studies indicated that Bora1–224 can directly bind to the KD of AURKA, and not appreciably to the KD of Plk1. Furthermore, the phosphorylation of Bora1–224 by Cyclin-Cdk appeared to enhance its affinity for AURKA to some degree (greater than 2.5-fold). These observations support a model in which pBora1–224 promotes the phosphorylation of the Plk1 T-loop in part by directly binding to AURKA.

pBora can activate AURKA lacking T-loop phosphorylation. Auto-kinase/ATPase on T-loop residue Thr288 (corresponding to T295 in Xenopus AURKA, Supplementary Fig. 1) is essential for the catalytic activation of AURKA23,44. Indeed, similar to the catalytically dead mutant AURKAD274N, dephospho-AURKA WT and the AURKA T288V mutant, which all lack T-loop phosphorylation (Supplementary Fig. 3a), were devoid of catalytic activity toward Plk1 KD ΔK82R even in the presence of Bora1–224 (Fig. 3a, b—compare lane 2 with 5, 8, and 11). Likewise, in contrast to AURKA WT, the AURKA T288V mutant could not phosphorylate Bora1–224 (Fig. 3b, compare the electrophoretic shift of Bora1–224 in lanes 2 and 5 in step 2). Strikingly though, in the presence of pBora1–224, both AURKA T288V and dephospho-AURKA WT, but not the catalytically dead mutant AURKAD274N, displayed robust activity against Plk1 KD ΔK82R (Fig. 3b, compare lanes 1, 4, 7, and 10). This potentiating activity of Bora on AURKA T288V was completely dependent on CyclinA2-Cdk2 activity as Bora1–224 phosphorylated solely by AURKA (lane 6) did not stimulate AURKA activity toward Plk1 (Supplementary Fig. 3b). Thus, the presence of phosphates on Cyclin-Cdk S/T-P consensus sites in Bora appears to compensate for the absence of essential T288 T-loop phosphorylation in AURKA.

To determine if the potentiating effect of Bora1–224 on AURKA was substrate specific, we tested the unrelated Kemptide substrate by monitoring ATPase activity using the ADP-Glo assay, which quantifies the amount of ADP produced during the kinase reaction. AURKA WT in the presence (Fig. 3c) and absence of Kemptide (Supplementary Fig. 3c) displayed very low basal ATPase activity. Titration of either Bora or pBora potentiated the ATPase activity of AURKA WT multifold. The EC₅₀ value for potentiation was superior for pBora relative to Bora (0.19 ± 0.1 μM vs 1.2 ± 0.7 μM, respectively) (Fig. 3c), consistent with the tighter binding affinity of pBora for AURKA discerned by SPR (Fig. 3d, e).

AURKA T288V displayed negligible ATPase activity alone (Supplementary Fig. 3d) or in the presence of Kemptide substrate (Fig. 3d) and titration of Bora had no discernable activating effect on AURKA T288V. Strikingly, however, pBora greatly potentiated the ATPase activity of AURKA T288V in both conditions (+/− Kemptide substrate) (0.04 ± 0.001 μM vs 0.02 ± 0.003 μM, respectively). As reflected by a lower EC₅₀ value, this potentiation was even superior to that achieved by pBora on the AURKA WT (Fig. 3c), suggesting enhanced binding affinity of pBora for AURKA T288V. This inference was supported by SPR as pBora bound AURKA T288V more tightly compared to non-phosphorylated Bora (K₆ = 6 vs 11 μM, respectively) (Fig. 3e, f and Supplementary Fig. 3e, f). Thus, phosphate(s) on Bora enable binding affinity for AURKA WT and AURKA T288V and contribute to a more pronounced ability to stimulate AURKA kinase/ATPase activity.

To corroborate these findings against another known AURKA substrate, we examined AURKA phosphorylation of Histone 3 on Ser10. Consistent with our findings using Plk1 KD and Kemptide as substrates, pBora1–224 but not its non-phosphorylated form greatly potentiated AURKA T288V phosphorylation of Histone 3 (Supplementary Fig. 4a, lanes 4 and 5). Interestingly, however, while pBora1–224 was also able to promote AURKA WT phosphorylation of Histone 3 (to a small degree), non-phosphorylated Bora1–224 was even more effective (Supplementary Fig. 4a, lanes 1 and 2). This result suggests that in some contexts, the presence of phosphate on both Bora and AURKA can lead to antagonistic interactions.

As the phosphomimetic AURKA T288D variant is often used as a proxy for the constitutively active enzyme, we decided to test whether this variant behaved more similar to wild-type or its AURKA T288V counterpart in response to Bora. Strikingly, the T-loop phospho-mimic mutant AURKA T288D behaved more similar to AURKA T288V with no ability to phosphorylate Plk1 on its T-loop alone or in the presence of Bora1–224 but a strong ability to phosphorylate Plk1 in the presence of pBora1–224 (Supplementary Fig. 4b). Thus, the AURKA T288D variant is not a reliable proxy for the activated form of AURKA achieved by pBora binding. We conclude that pBora is a potent activator of AURKA catalytic activity against multiple substrates, particularly when AURKA is not phosphorylated on its activation segment at T288 as exemplified by the T288V and T288D AURKA mutants.
AURKAT288V is functional in the AURKA–Bora–Plk1 pathway in vivo. The surprising observation that Cyclin-Cdk-phosphorylated Bora can activate AURKA even in the absence of T288 phosphorylation, led us to investigate whether pBora can similarly activate the AURKAT288V mutant in vivo. To this end, we used Xenopus egg extracts prepared from oocytes arrested in metaphase of meiosis II (CSF: cytostatic factor arrested-extracts). CSF extracts are typically arrested in a mitotic state with high CyclinB-Cdk1 (MPF) activity. At this stage, the Greatwall kinase (Gwl) is active and maintains the mitotic state by inhibiting dephosphorylation of key cell cycle regulatory factors by the protein phosphatase 2A PP2A-B55 (complexed with its B55 regulatory subunit) (Fig. 4a). However, Gwl...
immunodepletion releases active PP2A-B55, which can then dephosphorylate CyclinB-Cdk1 substrates and consequently cause exit from the mitotic state\(^4\). Complementation of the extracts with hyperactive recombinant Gwl\(^{K72M}\) can restore the mitotic state if the extracts contain an active AURKA, Bora, and Plk1 pathway (Fig. 4a, b).

Taking advantage of this system, we tested whether human (hs) AURKA\(^{T288V}\) can replace Xenopus (Xe) AURKA function in CSF extracts. To this end, endogenous Gwl and AURKA were sequentially immunodepleted, and then the extract was supplemented with recombinant hyperactive Gwl\(^{K72M}\) and either hs AURKA\(^{WT}\), AURKA\(^{T288V}\), or the catalytically dead mutant Plk1\(^{K82R}\), CyclinA2-Cdk2

\[ \text{Step 1} \]

\[ + \text{CyclinA2-Cdk2} + \text{Mg/ATP} \]

\[ \text{Bora}^{1-224} \rightarrow \text{pBora}^{1-224} \]

\[ \text{AURKA}^{WT} \text{Plk1}^{KD_{K82R}}, \text{CyclinA2-Cdk2} \]

\[ \text{Step 2} \]

\[ + \text{Mg/ATP} \]

\[ \text{ppBora}^{1-224} \]

\[ \text{T288V, WTDephos} \text{D274N (Kinase dead)} \]

Lane 1 5 4326 7 891 0 14131211

Bora1-224>25++Plk1 KDK82R

\[ \text{Step 1} \]

\[ + \text{CyclinA2-Cdk2} + \text{Mg/ATP} \]

\[ \text{Bora}^{1-224} \rightarrow \text{pBora}^{1-224} \]

\[ \text{AURKA}^{WT} \text{Plk1}^{KD_{K82R}}, \text{CyclinA2-Cdk2} \]

\[ \text{ppBora}^{1-224} \]

\[ \text{T288V, WTDephos} \text{D274N (Kinase dead)} \]

Lane 1 5 4326 7 891 0 14131211

Bora1-224>25++Plk1 KDK82R

\[ \text{Step 2} \]

\[ + \text{Mg/ATP} \]

\[ \text{ppBora}^{1-224} \]

\[ \text{T288V, WTDephos} \text{D274N (Kinase dead)} \]
AURKA.D274N (Fig. 4c). As shown in Fig. 4d (lane 3), CSF-arrested extracts exited the mitotic state upon Gwl immunodepletion, as reflected by the dephosphorylated state of the mitotic substrates Cdc25 and the protein phosphatase 1 (PP1) and the accumulation of inactive Cdk phosphorylated on the inhibitory site tyrosine 15 (pTyr). Similarly, Plx1 (Xenopus Plk1) was also rapidly dephosphorylated on its T-loop site T201 (denoted pPlx1) upon Gwl immunodepletion (ΔGwl) (Fig. 4d, lanes 3, 9, and 17). Complementation of the extract with recombinant Gwl.K72M and AURKA.WT (lanes 3–8), but not the catalytically dead AURKA.D274N mutant (lanes 17–22), within minutes restored the mitotic state. Active Plx1 phosphorylated on its T-loop (pPlx1) accumulated 60–90 min after complementation of the extracts with recombinant Gwl.K72M and AURKA.WT. This was accompanied by the accumulation of phosphorylated mitotic substrates Cdc25 and PP1 and the dephosphorylation of Cdk on tyrosine 15 (Fig. 4d, lanes 7 and 8). Remarkably, the downregulated mutant AURKA.T288V, similar to AURKA.WT, promoted Plx1 T-loop phosphorylation and high Cdk-Cdk activity in these extracts with comparable kinetics (Fig. 4d, lanes 9–14). Taken together, these results indicate that human AURKA.T288V can support AURKA function in Xenopus oocyte extracts. We conclude that in this context, and similarly to the situation in vitro, pBora can activate AURKA independently of T-loop phosphorylation on T288 and can thus compensate for the lack of T-loop phosphorylation on AURKA.

A minimal fragment of Bora encompassing residues 18–120 supports AURKA-dependent phosphorylation of Plk1 in vitro. To identify the minimal region of Bora that can recapitulate Cyclin-Cdk-dependent activation of AURKA against Plk1, we performed a deletion analysis on the minimally active Bora1–224 fragment (see Fig. 5a for schematic). Deletion of Cy motifs (Fig. 5a) in Bora adversely affected our ability to phosphorylate Bora with Cyclin-Cdk25 (see Supplementary Fig. 5a for example). Therefore, we employed the proline-directed MAP kinase ERK in place of Cyclin-Cdk in our pre-phosphorylation reactions. Validating this approach, Bora phosphorylated by ERK and Bora phosphorylated by CyclinA2-Cdk2 both efficiently stimulated Plk1 T210 phosphorylation in our reconstitution assay (Supplementary Fig. 5b, compare lanes 1 and 6). Importantly, ERK itself was not able to directly phosphorylate Plk1 on the T-loop (Supplementary Fig. 5b, lane 5). Mass spectrometry analysis confirmed that ERK was able to phosphorylate all tested Bora fragments in vitro (Supplementary Fig. 6a–i).

We then tested the ability of the Bora deletion fragments to support Plk1 phosphorylation by AURKA.WT or AURKA.T288V. pBora1–224 supported the most efficient phosphorylation of Plk1 by both AURKA.WT and AURKA.T288V (Fig. 5b, lanes 1–4). N- and C-terminal deletions to Bora1–224 in the form of the Bora35–157 fragment abolished all stimulatory functions (lanes 5–8). Given the N-terminal part of Bora is highly conserved25, we extended the N-terminal part of Bora35–157 to recover activity. Whereas the Bora35–157 fragment was totally inactive, Bora18–120 was able to stimulate AURKA.WT and AURKA.T288V function similar to Bora1–224 (lanes 9–12). Likewise, Bora18–120 was able to stimulate AURKA.WT and AURKA.T288V function (lanes 13–16). Collectively, these results indicate that the Bora18–120 fragment is sufficient to stimulate the catalytic function of AURKA toward Plk1.

As phosphorylation of Bora18–120 by a proline-directed kinase contributed greatly to its ability to stimulate the catalytic activity of AURKA (especially the AURKA.T288V mutant), we used site-directed mutagenesis to probe which specific phospho-sites in Bora were responsible (Fig. 5b, right panel). Bora18–120 contains four S- and T-P sites, S27, S41, T52, and S112 that match the Cyclin-Cdk consensus sequence. Mutation of S27 and T52 together had no impact on the AURKA stimulating activity of Bora18–120 (lanes 17–24) but the addition of a third mutation to either S41 (lanes 25–28) or S112 (lanes 29–32) impaired its function to some degree. Specifically, the triple mutant Bora18–120 [S27A S41A T52A], phosphorylated or not by ERK, lost all ability to activate AURKA.WT but retained the ability to activate AURKA.T288V when pre-phosphorylated by ERK (i.e. phosphorylated on S112 only) (lane 27). In contrast, the triple mutant Bora18–120 [S27A T52A S112A], phosphorylated or not by ERK (on S41 only), not only lost all ability to activate AURKA.WT but also AURKA.T288V (lanes 29–32). To corroborate the greater importance of the S112 phosphoregulatory site, we analyzed both Bora18–120 [S41A] and Bora18–120 [S112A] single site mutants. Consistent with expectations, while Bora18–120 [S41A] lost the ability to activate AURKA.WT, it still retained the ability to activate AURKA.T288V when pre-phosphorylated by ERK. In contrast Bora18–120 [S112A], phosphorylated or not by ERK, lost the ability to activate both AURKA.WT and AURKA.T288V (Supplementary Fig. S5c). Together, these results indicate that Bora18–120, phosphorylated on the Cyclin-Cdk site S112, is minimally sufficient to potentiate the catalytic activity of AURKA.T288V toward Plk1. These results also reveal that Bora phosphorylation on S112 can compensate for the absence of T-loop phosphorylation on AURKA. Since mutation of Ser41 adversely affected the ability of both Bora and pBora to activate AURKA, we reason that this residue may play an important structural and non-phosphorylation-dependent role in supporting the Bora–AURKA interaction.

Bora contains two short Tpx2-like motifs required for AURKA activation. The microtubule-associated protein Tpx2 is a well-characterized allosteric activator of AURKA. The crystal structure of a Tpx2/AURKA complex (PDB: 1OL5) revealed how Tpx2...
uses its first 43 amino acids to bind the catalytic domain of AURKA\textsuperscript{40,41,46}. Sequence comparison of Bora\textsuperscript{18–120} with the first 43 residues of Tpx2 revealed two regions of similarity, denoted motif 1 (M1; residues 25–34 in Bora) and motif 2 (M2; residues 101–110 in Bora) (Fig. 5c). Motif 1 of Tpx2 (residues 7–21) adopts an extended conformation that binds the N-terminal lobe of AURKA while motif 2 (residues 30–43) adopts an alpha helical conformation that binds between the N- and C-terminal lobes, adjacent to helix $\alpha C$ and the activation segment\textsuperscript{40}.

Remarkably, Tyr10, Phe16, Phe19, Trp34, and Phe35 in Tpx2 are crucial for binding to AURKA\textsuperscript{47–49}. Within the two Tpx2 motifs, Tyr8, Tyr10, Phe16, Phe19, Trp34, and Phe35 are crucial for binding to AURKA\textsuperscript{47–49}. Remarkably, Tyr10, Phe16, Phe19, Trp34, and Phe35 in Tpx2 are...
also conserved in two analogous motifs in Bora albeit with greater motif separation (Fig. 5c). Furthermore, the general pattern of hydrobobicity and polarity across the two motifs appear conserved suggesting that Bora might engage AURKA in a similar fashion to Tpx2.

To test this hypothesis, we mutagenized Bora\(^{18-120}\) (harboring the benign S27A/T52A double mutation) by substituting key hydrophobic residues with negatively charged residues in M1 (F25D/Y31D) and in M2 (F103D/F104D). Testing of each construct in our in vitro reactions revealed that mutations in M1 or M2 abolished the stimulatory activity of Bora\(^{18-120}\) toward AURKA (Fig. 5d, lanes 5–12). Together, these results uncovered three essential determinants in Bora for promoting AURKA phosphorylation of Plk1, namely two Tpx2-like motifs M1 and M2 in addition to a Cyclin-Cdk phosphorylation site at Ser112 (denoted M3).

**Bora and Tpx2 compete for AURKA binding and display different abilities to activate AURKA.** The finding that Bora and Tpx2 share similar motifs for binding to AURKA suggested that the two proteins may bind to AURKA in a competitive manner. To test this hypothesis, we established a competitive displacement binding assay using fluorescein-labeled Tpx2\(^{1-43}\) (FITC-Tpx2\(^{1-43}\)) as a probe (see Fig. 6a for schematic). As assessed by fluorescence polarization measurement, FITC-Tpx2\(^{1-43}\) bound to AURKA\(^{WT}\) and AURKA\(^{T288V}\) with \(K_d\) values of 0.009 \(\mu M\) and 0.015 \(\mu M\), respectively (Fig. 6b, d). Supporting the notion of similar binding modes, Tpx2\(^{1-43}\), Bora\(^{1-224}\), and pBora\(^{1-224}\) could all competitively displace FITC-Tpx2\(^{1-43}\) from binding to AURKA\(^{WT}\) (Fig. 6c) and to AURKA\(^{T288V}\) (Fig. 6e). Notably, Tpx2\(^{1-43}\) appeared more efficient than pBora\(^{1-224}\) and Bora\(^{1-224}\) at competitive binding to AURKA\(^{WT}\) (IC\(_{50}\) = 0.1 \(\mu M\), 2.7 \(\mu M\), and ND, respectively), whereas pBora\(^{1-224}\) appeared more efficient than Tpx2\(^{1-43}\) or Bora\(^{1-224}\) at competitive binding to AURKA\(^{T288V}\) (IC\(_{50}\) = 0.032, 0.11, and 0.73 \(\mu M\), respectively). We note that while the general trends of binding preference observed by fluorescence polarization (Fig. 6) and SPR (Figs. 2 and 3) are similar, the binding affinities appear under-estimated in the SPR experiment possibly due to Bora immobilization via its N-terminus.

Consistent with expectation, the shorter pBora\(^{18-120}\) construct behaved similar to pBora\(^{1-224}\) in its ability to displace FITC-Tpx2\(^{1-43}\) from AURKA\(^{T288V}\), albeit with \(\approx 20\)-fold less efficiency (Fig. 6f). Introduction of aspartic acid substitutions in the hydrophobic M1 and M2 motifs drastically reduced Tpx2\(^{1-43}\) displacement function (Fig. 6f), consistent with our findings that these mutations abrogate the ability of pBora\(^{18-120}\) to activate AURKA\(^{T288V}\) phosphorylation of Plk1 (Fig. 5d). Together, these results confirm the prediction that Bora and Tpx2 share similar binding features to AURKA. Furthermore, they demonstrate an optimal binding interaction arises when Bora is phosphorylated and when AURKA is not phosphorylated.

The different apparent binding affinities of Tpx2\(^{1-43}\), Bora\(^{1-224}\), and pBora\(^{1-224}\) toward AURKA\(^{WT}\) and AURKA\(^{T288V}\) led us to compare the relative regulatory effects of these proteins on AURKA activity using the ADP-Glo assay. Consistent with the competitive binding studies, Tpx2\(^{1-43}\) displayed superior activating potential relative to pBora\(^{1-224}\) and Bora\(^{1-224}\) on AURKA\(^{WT}\) (Fig. 6g). Surprisingly, despite its ability to bind AURKA\(^{T288V}\), Tpx2\(^{1-43}\) (like Bora\(^{1-224}\) and unlike pBora\(^{1-224}\)) displayed no ability to activate AURKA\(^{T288V}\) (Fig. 6h). Furthermore, consistent with their ability to bind but not activate AURKA\(^{T288V}\), both Tpx2\(^{1-43}\) and Bora\(^{1-224}\) acted as competitive inhibitors of pBora\(^{1-224}\) activation of AURKA\(^{T288V}\) (Fig. 6i). Taken at face value, these results demonstrate unequivocally that Bora and Tpx2 compete for binding to AURKA with affinities and activation capabilities dependent on the phosphorylation status of both Bora and AURKA. These results echo previous observations showing that Bora depletion in human cells increases the association of Tpx2 with AURKA on the mitotic spindle while overexpression of an N-terminal fragment of Bora (Bora N-ter\([1-379]\)) prevents co-immunoprecipitation of Tpx2 with AURKA\(^{31}\).

**Mutational analysis of putative phospho-binding sites on AURKA.** Our in vivo and in vitro results indicate that pBora can compensate for the absence of T-loop phosphorylation in AURKA. This raises the question of how structurally this is achieved. One possibility is that the phosphoSer112 moiety of Bora binds the same site on AURKA that normally engages the phosphorylated T-loop residue T288 of AURKA in \(cis\). This would involve coordination by Arg255, Arg180, and Arg286 (Fig. 7a and Supplementary Fig. 1). To our knowledge, no precedent for a kinase regulator acting in this manner has been described. An alternate possibility is that the phosphoSer112 motif binds to a different site on AURKA from the phosphorylated T-loop. For example, a Tpx2-AURKA co-structure (PDB: 10L5) reveals an adjacent and partially overlapping binding site involving His176, Arg180, and Arg286 that coordinates a free sulfate ion (Fig. 7a). A precedent for this site in coordinating phosphate was established for AURKC, which is regulated by binding to the centromere and mitotic regulator INCENP\(^{30}\). One of two phospho-sites in INCENP (within a TSS motif) binds to the corresponding sulfate binding site conserved with AURKA (see Supplementary Fig. 7a, b). Of the four residues involved in the two adjacent binding sites, only Arg255 is unique to
coordinating the phosphorylated T-loop residue T288 whereas only His176 is unique to coordinating the sulfate ion (Fig. 7a and Supplementary Fig. 1).

To probe the involvement of the two aforementioned AURKA sites in binding to a phosphate moiety in Bora, we individually substituted residues His176, Arg180, Arg255, and Arg286 for alanine in AURKA T288V and tested for responsiveness to pBora18–120 in protein kinase assays. All four AURKA mutants (AURKA[T288H176A], AURKA[T288V R180A], AURKA[T288V R255A], and AURKA[T288V R286A]), which retained abilities to bind Tpx21–43 (ref. 47 and Fig. 7b), displayed reduced responsiveness to pBora18–120 (Fig. 7c). These observations supported a role for AURKA residues His176, Arg180, Arg255, and Arg286 in coordinating a phosphate moiety in Bora. To examine this issue further, we tested the ability of the four AURKA mutants to bind pBora1–224. Taking advantage that all four AURKA mutants bind
with similar affinity to FITC-Tpx2

-120 mutants (S27A and T52A plus/minus mutations within motifs M1 or M2) phosphorylated (+) or not (−) by the ERK kinase (step 1) in the presence of Plk1K82R KD and AURKAWT or AURKAT288V (step 2). Blots were probed with antibodies to Bora, AURKA, and phosphoT210 Plk1 or pan Plk1 as indicated (from top to bottom). c Alignments of Bora motifs M1 and M2 with Tpx2 motifs responsible for binding to AURKA. Essential residue numbers in Tpx2 responsible for binding AURKA are highlighted in red below the alignments. The corresponding residue numbers in Bora are highlighted in red above the alignments. Also shown is a phospho-motif M3 in Bora not conserved in Tpx2. d Western blot analysis of kinase reactions carried out with the indicated Bora8–120 constructs, harboring S27A and T52A mutations at two Cyclin-Cdk phospho-acceptor sites, were fully competent to support mitotic entry of interphase extracts depleted of Bora. In both cases, addition of GwlK72M was accompanied by activation of Plk1 and entry into mitosis as evidenced by the phosphorylation of PP1 and Plk1 of Bora18–120 (S27A T52A F103D F104D) mutant was not recognized by our Bora antibody but Coomasie staining of the step 1 reaction (bottom panel of step 1) revealed the presence of the protein at the expected size.

Bora determinants are critical for mitotic entry in Xenopus egg extracts. Having determined that the Tpx2-like motifs (M1 and M2) and the phosphorylated serine Ser112 (M3) are essential in the context of the minimal Bora8–120 fragment for function in vitro, we next investigated the relevance of these determinants for Bora function in vivo. We first investigated Bora function at mitotic entry in Xenopus egg extracts using a variation of the experimental setup described earlier, whereby interphase egg extracts are forced to enter into mitosis using the Gwl kinase27,45. Experimental setup described earlier, whereby interphase egg extracts are forced to enter into mitosis using the Gwl kinase27,45. In both cases, addition of GwlK72M was accompanied by activation of Plk1 and entry into mitosis as evidenced by the phosphorylation of PP1 and PhosphoT210 Plk1 on Tyr15 (Fig. 8b, lanes 15–18 and lanes 19–22). We next tested whether the Tpx2-like motifs M1 and M2 of Bora were similarly required for Bora’s function in mitotic entry. As shown in the Fig. 8c, Bora18–120 harboring the benign S27A and T52A mutations together with mutations in either of the motifs M1 (F25D Y31D) or M2 (F103D F104D) failed to support mitotic entry in Xenopus egg extracts. In both cases, Plx1 was not phosphorylated/activated by addition of active GwlK72M and the extracts did not reach the mitotic state as evidence by the maintenance of inhibitory Tyr15 phosphorylation on CyclinB-Cdk1 and the lack of PP1 phosphorylation (Fig. 8c). Taken together, these results validate our in vitro observations that Bora18–120 is minimally sufficient for stimulating the phosphorylation of Plk1 by AURKA and for promoting mitotic entry. These observations further indicate that the two Tpx2-like motifs M1 and M2, together with pre-phosphorylation of Cyclin-Cdk regulatory sites, are critical for Bora function in mitotic entry in Xenopus egg extracts.

S112 residue and Tpx2-like motifs are essential for Bora function during mitotic entry in human cells. Having determined that the Tpx2-like motifs (M1 and M2) and the phosphorylated serine Ser112 (M3) are essential in the context of the minimal Bora8–120 fragment for function in vitro and in Xenopus egg extracts, we next investigated whether these determinants are also required for full-length Bora function in mitotic entry during the human cell cycle. To this end, we used CRISPR/Cas9 technology to C-terminally tag endogenous Bora with NeonGreen (NG) and the Auxin-inducible degron (AID) motif (denoted Bora-NG-AID) in DLD1 cells constitutively expressing the F-box protein TIR1 (Fig. 9a). Following treatment of the Bora-NG-AID DLD1 cell line with Auxin (A), the SCF3TIR-1 E3-ligase targets Bora-NG-AID for degradation by the 26S proteasome resulting in Bora knockdown31 (Supplementary Fig. 8a–c). Using CRISPR/Cas9, we also introduced at the AAVS1 locus either a wild-type or a mutant variant of Bora under the control of a doxycycline-inducible promoter (Fig. 9a). Bora variants included the phospho-

Fig. 5 Bora contains Tpx2-like motifs and a unique phosphosite required for its activation function on AURKA in vitro. a Schematic of Bora1–224 highlighting ten S/T-P consensus sites that are phosphorylatable by the proline-directed kinases Cyclin-Cdk or ERK. Residue positions of the phospho-sites are indicated and highlighted by the letter P (above). Also highlighted are two Cyclin-binding motifs (Cy, violet) and two Tpx2-like motifs 1 and 2 (green). The boundaries of the different Bora fragments analyzed in b–d, are indicated. The most essential phospho-site S112 is highlighted by a large P. b Western blot analysis of 2-step kinase reactions carried out with the indicated Bora fragments phosphorylated (+) or not (−) by the ERK kinase (step 1) in the presence of Plk1K82R KD substrate and AURKAWT or AURKAT288V (step 2). Blots were probed with antibodies to Bora, AURKA, and phosphoT210 Plk1 or pan Plk1 as indicated (from top to bottom). c Alignments of Bora motifs M1 and M2 with Tpx2 motifs responsible for binding to AURKA. Essential residue numbers in Tpx2 responsible for binding AURKA are highlighted in red below the alignments. The corresponding residue numbers in Bora are highlighted in red above the alignments. Also shown is a phospho-motif M3 in Bora not conserved in Tpx2. d Western blot analysis of kinase reactions carried out with the indicated Bora8–120 mutants (S27A and T52A plus/minus mutations within motifs M1 or M2) phosphorylated (+) or not (−) by the ERK kinase (step 1) in the presence of Plk1K82R KD and AURKAWT or AURKAT288V (step 2). Blots were probed with antibodies to Bora, AURKA, and phosphoT210 Plk1 or pan Plk1 as indicated (from top to bottom). Note that the Bora18–120 (S27A T52A F103D F104D) mutant was not recognized by our Bora antibody but Coomasie staining of the step 1 reaction (bottom panel of step 1) revealed the presence of the protein at the expected size.
site mutants Bora\textsuperscript{S41A}, Bora\textsuperscript{S112A} (disruption of motif M3), Bora\textsuperscript{S137A}, Bora\textsuperscript{A35} and the mutated forms Bora\textsuperscript{F25D Y31D} and Bora\textsuperscript{F103D F104D} with disruptions to the TPX2-like motifs M1 and M2, respectively. We specifically tested the triple Bora\textsuperscript{3A} [S41A S112A S137A] mutant as well as individual Bora phosphosite mutants because we previously showed that Bora\textsuperscript{3A} is defective in mitotic entry after checkpoint recovery from DNA damage\textsuperscript{25,26}. Using this experimental setup, we examined mutant Bora function during the human cell cycle by combining endogenous Bora depletion upon Auxin addition with untagged Bora induction under Doxycyclin (D) (Fig. 9a and Supplementary Fig. 8a–c).
Fig. 6 Bora and Tpx2Δ43 compete for AURKA binding. a Schematics of a competitive displacement binding assay using fluorescein-labeled Tpx2Δ43 (FITC-Tpx2Δ43) as a probe. Complex formation between FITC-labeled Tpx2Δ43 polypeptide and AURKA followed by the disassembly of the FITC-Tpx2Δ43/AURKA complex by increasing amount of competitor (cold Tpx2Δ43, Bora, or pBora) was monitored by fluorescence polarization. b Binding of fluorescein-labeled Tpx2Δ43 polypeptide to AURKAWT assessed by monitoring the fluorescence polarization signal in the presence of increasing concentrations of AURKAWT. Kd value represents mean value with standard deviations of the mean as error bars (∼3 independent experiment samples). c Competitive binding assay where fluorescein-labeled Tpx2Δ43 polypeptide in complex with AURKAWT is displaced by increasing amount of competitor (cold Tpx2Δ43, BoraΔ224, or pBoraΔ224) and monitored by fluorescence polarization signal. Displayed data points and the half maximal inhibitory concentration (IC50) value represent the average fluorescence polarization for each reaction conditions with standard deviations of the mean as error bars (∼3 independent experiment samples). ND: not determined. d Binding of fluorescein-labeled Tpx2Δ43 polypeptide to AURKAT288V assessed by monitoring the fluorescence polarization signal in the presence of increasing concentrations of AURKAT288V. Kd value represents mean value with standard deviations of the mean as error bars (∼3 independent experiment samples). e, f Competitive binding assay where fluorescein-labeled Tpx2Δ43 polypeptide in complex with AURKAWT288V is displaced by increasing amount of cold Tpx2, BoraΔ224, or pBoraΔ224 competitor (e), or by increasing amounts of cold pBoraΔ224, pBoraΔ120, or the indicated pBoraΔ43 mutants in the M1 and M2 motifs. Data presented as in c, g Activation of AURKAWT ATPase activity by BoraΔ224, pBoraΔ224, and Tpx2Δ43 as assessed using the ADP-Glo assay with Kemptide substrate. Displayed data points and EC50 values represent the average luminescence for each reaction condition with standard deviations of the mean as error bars (∼3 independent experiment samples). RLU: relative light unit. h Activation of AURKAT288V ATPase activity by BoraΔ224, pBoraΔ224, and Tpx2Δ43 as assessed using the ADP-Glo assay with Kemptide substrate. Data presented as in g. i Activity of the AURKAT288V/pBoraΔ224 complex in the presence of increasing concentration of competitor Tpx2Δ43 or BoraΔ224 assessed using the ADP-Glo assay in the presence of Kemptide substrate. Displayed data points and the half maximal inhibitory concentration (IC50) value represent the average fluorescence luminescence for each reaction conditions with standard deviations of the mean as error bars (∼3 independent experiment samples). ND: not determined.

first examined the effect of Bora depletion by Auxin treatment in DLD1 cells expressing Bora-NG-AID. Within 2 h of Auxin treatment, we observed rapid degradation of Bora-NG-AID, which was accompanied by a severe reduction in Plk1 phosphorylation on T210 in unsynchronized cells (Supplementary Fig. 8d). To rigorously evaluate the impact of Bora-NG-AID depletion on cell cycle progression, we synchronized the cells in G1–S phase using a double thymidine block (DTB), and then released the cells by washing out thymidine. We monitored progression through mitosis by FACS and Western blot analysis of cell cycle markers (Fig. 9b) and Supplementary Figs. 8e, f and 9). Consistent with previous RNAi experiments22, Bora-NG-AID depletion severely delayed mitotic entry. While untreated cells accumulated the mitotic marker Histone H3 8 h after release from the DTB, cells treated with Auxin accumulated this marker only after 12 h of release from DTB (Supplementary Fig. 8e, f). FACS analysis confirmed these results and showed that cells depleted of Bora-NG-AID entered mitosis more slowly with a delay of 4 h (Supplementary Fig. 8f).

We next examined the ability of Bora mutants to reverse the observed effects upon Auxin-induced depletion of Bora-NG-AID. We calculated a rescue index (RI), which reflects the capacity of untagged Bora to rescue the delay in mitotic entry resulting from endogenous Bora-NG-AID degradation (Fig. 9c, d). Briefly, for each Bora mutant cell line, we performed a DTB and release regime, and determined from FACS the percentage of cells in the G2 and M phases, at both 10 h and 13 h time points post-release (Fig. 9b). These experiments were performed under three conditions: cells were either untreated for use as reference controls (Ctrl), treated with Auxin (A) to degrade Bora-NG-AID, or treated with Auxin plus doxycycline (A/D) to degrade Bora-NG-AID and simultaneously induce expression of WT Bora or its mutants (Fig. 9b). RI was calculated as a function of cells in G2–M under the three different conditions: [A – A/D] / [A – Ctrl] (Fig. 9d). As shown in Fig. 9c, the percentage of cells in the G2 and M phases increased in cells depleted of Bora-NG-AID, as compared to untreated control cells, due to mitotic entry delay (Supplementary Fig. 8e, f). If re-expression of a Bora variant was to rescue this phenotype to some degree, the percentage of cells in G2–M should decrease relative to Auxin treatment alone. If the rescue was complete, the percentage of cells at G2–M should equal that observed for untreated control cells (RI = 1). If no rescue was taking place, the percentage of cells

at G2–M should equal that observed for Auxin-treated cells (RI = 0).

As shown in Fig. 9d, a RI value of 0.5 was observed upon induction of wild-type Bora expression at 10 and 13 h after release from the second thymidine block. This indicated that wild-type Bora partially rescued the mitotic delay resulting from Bora-NG-AID depletion possibly due to its lower expression level compared to endogenous Bora (Fig. 9e, compare lanes 1 and 3). BoraS41A and BoraS137A displayed very similar abilities to rescue the cell cycle delay resulting from endogenous Bora-NG-AID depletion (Fig. 9d). In contrast, BoraS41A S112A S137A, BoraS112A, BoraF25D Y31D, BoraF103D F104D, and BoraS43 displayed no ability to rescue the G2–M delay, despite all proteins being efficiently expressed upon addition of doxycycline (Fig. 9e). We corroborated these observations by monitoring T210 phosphorylation on Plk1 10 h after release from DTB (Fig. 9e). The T210 phosphorylation signal was severely reduced upon Auxin addition and this was restored by doxycycline-induced expression of BoraWT (Fig. 9e, compare lanes 1, 2, and 3). Doxycycline-induced expression of BoraS41A and BoraS137A similarly restored high levels of T210 phosphorylation (Fig. 9e, lanes 9 and 15) while the other Bora mutants BoraS41A S112A S137A, BoraS112A, BoraF25D Y31D, BoraF103D F104D, and BoraS43 failed to do so (lanes 6, 12, 18, 21, and 24).

To avoid potential complications resulting from thymidine treatment, we monitored mitotic entry by time-lapse video microscopy in asynchronous DLD1 cell populations expressing Bora-NG-AID and doxycycline inducible BoraWT or BoraS112A, using fluorescent-tagged RCC1-1FP2 as a reporter of NEBD (Supplementary Fig. 10a, b). More than 1000 cells were analyzed per condition. Bora depletion upon Auxin addition severely delayed mitotic entry for at least 4 h. This phenotype was partially rescued by the induction of BoraWT (+ Doxycycline 5 h before Auxin addition) (Supplementary Fig. 10b). Conversely, the expression of BoraS112A mutant form was totally unable to rescue the kinetics of entry into mitosis (Supplementary Fig. 10c), confirming our results obtained with cells synchronized by a DTB-release regime. Collectively, these results indicate that the Tpx2-like motifs (M1, M2) and S112 phosphorylation (M3) are essential determinants for Bora function in Plk1 activation and mitotic entry during the human cell cycle. These results are also fully consistent with our in vitro data and results obtained in Xenopus egg extracts.
Fig. 7 Bora binds AURKA through two Tpx2-like motifs that position a phospho-motif in the kinase active site. 

a Zoom-in view of the binding interface between Tpx2 and AURKA (PDB: 1OL5). The T-loop phospho-residue Thr288, sulfate ion (yellow), and the phospho-T-loop and sulfate ion coordinating residues of AURKA (H176, R180, R255, and R286) are highlighted in stick representation. 

b Binding of fluorescein-labeled Tpx21–43 polypeptide to AURKA mutants assessed by monitoring the fluorescence polarization signal in the presence of increasing concentrations of AURKA mutants. Kd values represent mean value with standard deviations of the mean as error bars (n = 3 independent experiment samples). 

c Western blot analysis of 2-step kinase reactions carried out with Borain–224 phosphorylated (+) or not (−) by the ERK kinase (step 1) in the presence of Plk1K82R KD (substrate) and AURKAWT or mutated versions as indicated (step 2). Blots were probed with antibodies to Bora, AURKA, and phosphoT210 Plk1 or pan Plk1 as indicated (from top to bottom). 

d Competitive displacement of fluorescein-labeled Tpx21–43 from the indicated AURKA mutants by increasing concentrations of pBora1–224 as monitored by fluorescence polarization. Displayed data points and the half maximal inhibitory concentration (IC50) value represent the average normalized fluorescence polarization signal for each reaction conditions (starting binding signal = 100%, end titration binding signal = 0%) (n = 3 independent experiment samples). 

e Theoretical/illustrative model of pBora binding to the kinase domain of AURKA. AURKA is shown in gray surface with helix αC highlighted in yellow. Bora motifs M1 (blue) and M2 (purple) were modeled by two corresponding motifs from Tpx2 and the phospho-motif M3 (pink) was modeled by the TSS motif from phospho-INCENP.
Discussion
Here, we identify the molecular mechanism by which Bora, phosphorylated minimally on Ser112 by a Cyclin-Cdk activity, acts to promote the AURKA-dependent phosphorylation of Plk1 on the T-loop (residue T210) to trigger timely mitotic entry. We demonstrate that Bora binds directly to AURKA to activate its intrinsic catalytic function. Our data are in line with seminal observations indicating that Bora is a co-factor of AURKA in *Drosophila melanogaster*\(^{23}\). Importantly, our mutational studies have uncovered three critical elements in Bora required for AURKA activation in vitro and in vivo. Included are two conserved ~15 residue linear motifs (denoted M1 and M2) separated...
by an 85 residue linker and an evolutionarily conserved Pro/phospho-Ser112/Pro motif (denoted M3) generated by a proline-directed Cyclin-Cdk kinase. Phosphorylation of motif M3 is likely initiated by CyclinA2-Cdk1 (ref. 27) and sustained by CyclinB1-Cdk1 (refs. 25,52).

Based on similarity to Tpx2, we speculate that Bora motif M1 binds in an extended manner parallel to the top surface of helix αC whereas motif M2 adopts a helical conformation and binds parallel to the bottom surface of helix αC (Fig. 7a). As Bora motif M3 is located immediately C-terminal to motif M2, this binding model is attractive because it orients the Ser112 phosphate-moiety in motif M3 in close proximity to two possible phosphate coordination sites in AURKA. Included are the binding sites for the phosphorylated T-loop of AURKA (T288) and a sulfonated ion-binding site that is conserved with AURKB/C and used by AURKB/C to bind a phospho-motif in its corresponding regulator INCENP (Fig. 7c). Mutation of residues in both sites abrogates the responsiveness of AURKA T288V to pBora and diminishes binding of pBora to AURKA suggesting that the Ser112 phosphate moiety on Bora might engage both binding sites. Final confirmation of the underlying binding mechanism awaits a detailed atomic structure of the AURKA-pBora complex. Importantly, we demonstrate that motifs M1, M2, and M3 are all essential for Bora function in vitro and also in vivo to promote mitotic entry in Xenopus egg extracts and in human cells.

X-ray crystallographic, enzymatic, and spectroscopic studies have established that Tpx2 binding and T-loop phosphorylation activate AURKA catalytic function through distinct but complementary mechanisms (also reviewed in refs. 46,55). Two key structural elements of the KD that are recurring targets for regulation include the DFG (aspartate, phenylalanine, glycine) motif and helix αC, both of which function to productively coordinate ATP binding and catalytic elements.

Tpx2 binding drives activation primarily by promoting a productive inward conformation of the DFG motif through the anchoring of an inherently flexible helix αC. This is sufficient to promote a 10-fold increase in AURKA catalytic function in vitro. In contrast, T-loop phosphorylation serves primarily to promote a productive conformation of the activation segment, resulting in a more pronounced 100-fold increase in catalytic function. Consistent with the two regulatory arms acting by independent rather than redundant mechanisms, the combination of Tpx2 binding and T-loop phosphorylation results in a synergistic 1000-fold boost in catalytic function.

Interestingly, Bora contains not only two Tpx2-like motifs (M1 and M2) that mediate binding to the N-lobe of AURKA, but also a highly conserved and essential phosphosite S112 within motif M3. What does this design portend for how Bora mechanistically regulates AURKA kinase activity? Based on mutational and competition binding experiments, we expect that the two Tpx2-like motifs M1 and M2 will bind and act similar to Tpx2 by anchoring the position of helix αC to enforce a productive inward conformation of the DGF motif. Based on mutational analysis, the phospho-motif of M3 binds to either or both of two likely sites on AURKA. If the phospho-motif of M3 binds to the site normally occupied by the phosphorylated (pT288) T-loop, we expect that M3 would influence AURKA catalytic function (as observed for phosphorylation of the T-loop) by stabilizing a productive conformation of the activation segment. If instead the phospho-motif of M3 binds to the sulfonate ion-binding site, M3 could influence AURKA catalytic function either by stabilizing a productive conformation of the activation segment or by anchoring the position of helix αC and in turn by modulating the DFG motif. Either binding mode could in principle account for the observed behavior of Bora on AURKA and both appear feasible based on geometric constraints.

AURKA is instrumental for mitotic entry and progression. Indeed, AURKA not only regulates commitment to mitosis, but also centrosome maturation, mitotic spindle formation and positioning, spindle assembly checkpoint (SAC), central spindle assembly, and cytokinesis. Our results, along with previous observations, indicate that AURKA regulation in these processes occurs typically at two levels: firstly by allostery through binding to a growing list of trans acting factors and secondly by phosphorylation of the T-loop of AURKA. Our current results reveal a twist in AURKA regulation, whereby pBora activates AURKA both by allosteric binding and by providing a phosphate group in trans that compensates for the lack of AURKA T-loop phosphorylation.

Importantly, we show that this “one stop shopping” mode of AURKA regulation is essential for maintaining the mitotic state of CSF-arrested Xenopus egg extracts (Fig. 4). In this context, the mechanism of action of pBora is ideally suited to sustain AURKA catalytic activity against a prevailing complement of counter-acting phosphatases. It also provides a conduit whereby CyclinA2-Cdk activity can regulate the activation of AURKA through the Pkl1-CyclinB-Cdk1 cascade that drives mitotic entry. AURKA is degraded at the end of mitosis and thus must be resynthesized during interphase of the next cell cycle. Once translated, AURKA auto-phosphorylates on its own T-loop but this activity is counteracted by the PP1 and PP2A phosphatases in interphase and by PP6 while bound to Tpx2 during mitosis. As dephosphorylation maintains AURKA in an inactive state, the crucial question arises as to how AURKA overcomes the repressive effect of PPases to drive mitotic commitment. Our observations show that pBora provides a
mechanism to activate AURKA activation in spite of counter-acting phosphatases. Once activated by pBora, AURKA phosphorylates and activates Plk1, which in turn activates CyclinB-Cdk1 thereby transitioning the cell from a phosphatase-dominant to a mitotic kinase-dominant state. How precisely the Ser112 phospho-epitope of Bora escapes the action of the phosphatases that counteract AURKA auto-phosphorylation, remains an open and intriguing question.

Our results also show that the phosphomimetic AURKAT288D mutant commonly used as a proxy for the activated form of AURKA actually behaves in our in vitro kinase assay more like the constitutive inactive mutant AURKAT288V. This once again
Fig. 9 ST12 residue and Tpx2-like motifs are essential for Bora function during the mammalian cell cycle. a Schematic for the structure-function analysis of Bora during the mammalian cell cycle. Endogenous Bora located on chromosome (Chr) XIII was C-terminally tagged with Neogreen (NG) and the Auxin-Inducible degron (AID) in DLD1 cells constitutively expressing the SCFTRI E3-ligase. A wild-type or a mutant copy of untagged Bora driven by the doxycycline promoter (pTRE3G) was inserted on Chr XIX of the same cells using CRISPR/Cas9 genome editing. Doxycycline promotes expression of untagged wild-type or mutant Bora, while Auxin induces SCFTRI-mediated protein degradation of Bora-NG-AID resulting in a Bora knockdown. b Workflow used to follow the progression of the cells with the cell cycle. Expression of BoraWT or the indicated mutants were induced with doxycycline during the second thymidine block. Bora-NG-AID was then depleted at the time of release from DBT using Auxin. Cells were collected 10 h and 13 h after DBT release for Western blot and FACS analyses. c Histogram plots showing the cell cycle distributions (percentage of cells in G1, S, and G2/M) for DLD1-Bora-NG-AID cell lines, expressing either BoraWT or Bora341A 512A A37T, and as treated as outlined in the b. As: Asynchronous cells; Ctrl: non-treated; A: Auxin-treated cells; A/D: Doxycyclin + Auxin-treated cells. d Box plots indicate median (middle line) and min max (Whiskers) showing the quantified rescue index (RI) for each DLD1 Bora-NG-AID cell line expressing BoraWT (in dark green) or the indicated mutants: Bora341A 512A A37T (in red), Bora341A (in blue), Bora512A (in orange), Bora512A 37T (in green), Bora341A 512A 37T (in magenta), Bora341A 512A 37T (in pink), and Bora512A 37T (in purple) assayed 10 (first column) and 13 (second column) hours after DBT release; n indicates the number of replicates. e Western blot analysis of protein lysates from a DLD1-Bora-NG-AID cell line expressing BoraWT or the indicated mutants 10 h after DBT release as depicted in b, c, and d. B. pT210-Pik1, and panPik1 antibodies were used for these analyses (from top to bottom). (→) Asynchronous cells.

highlights the need for caution in the use and interpretation of phospho-mimetic mutations to probe protein kinase functions in biological contexts.

In conclusion, we demonstrate here that Bora is a direct regulator of AURKA that acts by a dual mechanism involving allosterly and by providing a phosphate in trans that compensates for the lack of AURKA T-loop phosphorylation. As AURKA and Bora are overexpressed in several cancers and act as oncogenes, deciphering how AURKA is mechanistically activated by Bora in time and space may pave the way for the development of innovative therapeutic approaches that target only a subset of AURKA’s multiple biological functions.

Methods
Cell cultures. DLD1 (ATCC CCL-221) were grown in Gibco DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% Gibco FBS (Fetal Bovine Serum), 2 ml 1-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies), and 50 μg/ml Normocin (Invivogen). All the cell lines used in the study were regularly tested for contamination.

Xenopus laevis induction and husbandry. Regulations for the use of Xenopus laevis, as outlined in the Animals Scientific Procedures Act (ASPA) and implemented by the Director General de la Recherche et Innovation, Ministère de l’Enseignement Supérieur de la Innovation, France were followed. Frogs were obtained from Centre de Ressources Biologiques Xénopes (CRB) of Rennes, France, and kept in a Xenopus research facility at the CRBM (Facility Center approved by the French Government Approval no. B34-172-39). Females were injected with 500 U of Chorulon (Human Chorionic Gonadotrophin) and oocytes laid 18 h later were used for experiments. Adult females were exclusively used to obtain eggs. All procedures were approved by the Director General de la Recherche et Innovation, Ministère de l’Enseignement Supérieur de l’Innovation de France (Approval no. APAFIS 4971-20160414151777154).

Molecular biology and DNA manipulation. All the DNA constructs were generated by Gateway or with restriction enzymes. All DNA constructs were verified by sequencing. Oligonucleotides used for site-directed mutagenesis were purchased from Eurofins or MerckMillipore and are detailed in the Supplementary Table 3. The list of plasmids used in this study is provided in Supplementary Table 2. AAVS1 T2 CRIPR in pX300 was a gift from Masato Kanemaki (Addgene plasmid # 72833; http://n2t.net/addgene:72833; RRID:Addgene:72833), pX300-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230; http://n2t.net/addgene:42230; RRID:Addgene:42230), pMK245 (Tet-OsTIR1-Puro) was a gift from Masato Kanemaki (Addgene plasmid # 72835; http://n2t.net/addgene:72835; RRID:Addgene:72835), pE13H-His-ERK2-MEK1, R4F, coexpression was a gift from Melanie Cobb (Addgene plasmid # 39212; http://n2t.net/addgene:39212; RRID:Addgene:39212).

Recombinant protein expression. Full-length human AURKAWT, all AURKA mutants, all human Bora fragments, full-length human CyclinA2-G2d, and the fragment 10-360 of human ERK were expressed in Escherichia coli BL21 DE3 pLysS RIL (Thermo Fisher Scientific), Full-length human Pik1, K82R, and delta Pik1, K82R, 112A, 37A, or 112A 37A were expressed in E. coli Rosetta DE3 (MerckMillipore). Transformed bacteria were incubated in LB media at 37 °C under agitation and the cultures were shifted at 18 °C at OD = 0.8. Protein expression was induced by addition of IPTG (final concentration 500 μM) and the cultures were incubated overnight at 18 °C under agitated.

Recombinant protein purification. AURKA and Pik1 were cloned as a TEV cleavable 6His-GST fusion, which leaves no two non-native residues (GA and KK) at the N-terminus (respectively). The N-terminal fragments were then expressed in Escherichia coli Rosetta DE3 (MerckMillipore) and were expressed separately. GST-Cdk2 was coexpressed with GST-Cak from a DLD1-Bora-NG-AID cell line expressing BoraWT or the indicated mutants 10 h after DBT release as depicted in b, c, and d. B. pT210-Pik1, and panPik1 antibodies were used for these analyses (from top to bottom). (→) Asynchronous cells.
pelleted, mixed, and resuspended in lysis buffer (25 mM HEPES pH 7.5, 250 mM NaCl, 5 mM beta-mercaptoethanol) and lysed in a homogenizer. Total extract was clarified by centrifugation (30,000 × g, 30 min) and the resulting soluble fraction was washed over a 0.45-µm filter and loaded onto a Superdex 200 pg sizing column (Cytiva) equilibrated in 25 mM HEPES pH 7.5, 250 mM NaCl, 1 mM TCEP. The fractions containing the CyclinA2-Cdk2 complex were finally resolved on a HiLoad 16/600 Superdex 200 pg sizing column (Cytiva) equilibrated in 25 mM HEPES pH 7.5, 250 mM NaCl, 1 mM TCEP. The fractions containing the CyclinA2-Cdk2 complex were pooled and concentrated using an ultracentrifugal centrifugal protein concentrator. The purified protein was aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C. The phosphorylation efficiency was controlled by intact mass-spectrometry.

Preparative phosphorylation of 15N-Bora1-224 and biotinylated AIV-Bora1-224 by Cyclin A2-Cdk2. Five milligrams of 15N-Bora1-224 or biotinylated AIV-Bora1-224 were mixed with recombinant CyclinA2-Cdk2 (molar ratio 20:1) in 1 mM kinase buffer (25 mM Tris pH 7.5, 150 mM NaCl, 1 mM MgCl2, 1 mM DTT, 2 mM ATP) and incubated for 1 h at 30 °C. The mixture was then loaded on a HiLoad 16/600 Superdex 200 pg sizing column (Cytiva) equilibrated in 25 mM Tris pH 7.5, 150 mM NaCl, 1 mM TCEP. The fractions containing the protein were pooled and concentrated using a centrifugal filter (Cytiva). The purified protein was aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C. The phosphorylation efficiency was controlled by intact mass-spectrometry.

Pull-down assay. Five-hundred nanograms of biotinylated AIV-Bora1-224 phosphorylated or not by CyclinA2-Cdk2 are mixed with AURKAT288V and/or Plk1 KDWT (molar ratio 1:1) in 100 µL of binding buffer (25 mM Tris pH 7.5, 150 mM NaCl, 20 mM MgCl2, 1 mM DTT, 0.05% Tween 20) and incubated on ice for 30 min. Then, the mixture was loaded on 5 µL of packed streptavidin sepharose (Cytiva) beads and incubated on ice for 30 min with intermittent gentle shaking. The beads were washed five times with the binding buffer and proteins were eluted by addition of Laemmli buffer on beads. Proteins were then detected by immunoblotting and visualized by treating the blots with ECL (MerckMillipore).

SFR experiments. Biotinylated AIV-Bora1-224 (7.95 mg/mL; i.e. 296 µM; MW 26889.26) and biotinylated phosphorylated AIV-Bora1-224 (12.26 mg/mL; i.e. 457 µM; MW 27440.55) were concentrated using an ultrafiltration centrifugal protein concentrator (MerckMillipore). The protein was finally resolved on a HiLoad 16/600 Superdex 200 pg sizing column (Cytiva) equilibrated in 25 mM HEPES pH 7.5, 200 mM NaCl, 20 mM MgCl2, 1 mM DTT, 0.01% Brij 35) and immobilized to streptavidin sensor chip (Series S Sensor Chip SA from Cytiva) by biacore S200 in running buffer at 25 °C. 7806 RU of biotinylated AVI-Bora1-224 were immobilized to flow cell 2 (flow cell 1 was used as a reference for flow cell 2). Based on AURKAT288V Rmax response, 13% of Bora was active, and based on AURKAT288V Rmax response, 12% of Bora was active; 6296 RU of biotinylated AVI-Pora1-224 were immobilized to flow cell 4 (flow cell 3 was used as a reference for flow cell 4). Based on AURKAT288V Rmax response, 25% of the phosphorylated AIV-Bora1-224 was active, and based on AURKAT288V Rmax response, 20% of phosphorylated AIV-Bora1-224 was active. Theoretical Rmax for AURKAT288V (MW = 45937.49) interaction with Bora was 13379 RU whereas measured Rmax for AURKAT288V interaction with Bora was 1761 RU; percent active = 1761/13379 = 13%. Theoretical Rmax for AURKAT288V (MW = 45935.52) interaction with phosphorylated Bora1-224 was 13379 RU, whereas measured Rmax for AURKAT288V interaction with phosphorylated Bora1-224 was 13,379 RU; percent active = 1585/13379 = 12%. Theoretical Rmax for AURKAT288V interaction with phosphorylated Bora1-224 was 10,540 RU, measured Rmax for AURKAT288V interaction with phosphorylated Bora1-224 was 10,540 RU; percent active = 2639/10540 = 25%. Theoretical Rmax for AURKAT288V interaction with phosphorylated Bora1-224 was 10,540 RU, measured Rmax for AURKAT288V interaction with phosphorylated Bora1-224 was 10,540 RU; percent active = 2639/10540 = 25%. All protein–protein interactions were tested at 25 °C, flow rate 10 µL/min; injection time 120 s; dissociation time 600 s. A KR224 peptide (GWEGKGAEVKRAAPAPLSLLEREPRPPEAKRSP) was immobilized to flow cell 3; it was performed under a cycle using 30 s injection of 10 mM glycine pH 3 at increased flow rate of 30 µL/min. After regeneration, the sensorsgrams returned to baseline. Raw sensograms data were analyzed and fit using Biacore S200 analysis software (Cytiva).

AVI-Bora1-224 biotinylation. One milligram of AVI-Bora1-224 was mixed with recombinant 6xHis-AURKAT288V (molar ratio 40:1) in 1 mL of phosphorylation buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 1 mM MnCl2, 1 mM TCEP, 0.01% Brij 35) and incubated for 1 h at 30 °C. The mixture was further incubated on a 1 mL Ni++ Hitrap Chelating HP (Cytiva) to remove the 6xHis-Lambda. The flow-through containing dephosphorylated AURKAT288V was concentrated using an ultracentrifugal centrifugal protein concentrator (MerckMillipore). Dephosphorylated AURKAT288V was finally resolved on a Superdex 200 10/300 GL sizing column (Cytiva) equilibrated in 25 mM Tris pH 7.5, 150 mM NaCl, 1 mM TCEP. The fractions containing dephosphorylated AURKAT288V were pooled and concentrated using an ultracentrifugal centrifugal protein concentrator. The purified protein was aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C. The dephosphorylation efficiency was determined by intact mass-spectrometry.

Preparative phosphorylation of Bora1-224 and 6xHis-AURKAT288V. One milligram of Bora1-224 was mixed with recombinant His-AURKAT288V (molar ratio 10:1) in 1 mL of phosphorylation buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 20 mM MnCl2, 1 mM DTT, 2 mM TCEP, 2 mM ATP) and incubated for 1 h at 30 °C. The mixture was then loaded on a 1 mL Ni++ Hitrap Chelating HP (Cytiva) to remove the 6xHis-AURKAT288V. The flow-through containing phosphorylated Bora1-224 was concentrated using an ultracentrifugal centrifugal protein concentrator (MerckMillipore). Phosphorylated Bora1-224 was finally resolved on a Superdex 200 10/300 GL sizing column (Cytiva) equilibrated in 25 mM Tris pH 7.5, 150 mM NaCl, 1 mM TCEP. The fractions containing biotinylated phosphorylated Bora1-224 were pooled and concentrated using an ultracentrifugal centrifugal protein concentrator. The purified protein was aliquoted, flash frozen in liquid nitrogen, and stored at −80 °C. The biotinylation efficiency was determined by intact mass-spectrometry.
in each figure. Each data point was derived from a technical duplicate and each binding curve was performed in triplicate. For the competitive displacement assay, FITC-conjugated (5 nM) and AURKA protein concentrations (35 nM) were kept constant throughout experiments, and unlabeled competitor (either Bora or Tpx2) was added to final concentrations indicated in each figure. Fluorescence polarization was measured on a BioTek Synergy Neo plate reader (BioTek) using Gen5 v2.0.5 software with excitation and emission at 485/528 nm, respectively. Binding graphs and the derived binding constants were generated using GraphPad Prism v8.1.2 and v8.3 (GraphPad).

**Intact protein mass spectrometry.** The liquid chromatography mass spectrometry (LC-MS) system consisted of a 1260 series HPLC connected to a 6538 UHD series electrospray ionization quadrupole time-of-flight mass spectrometer (Agilent Technologies, Santa Clara, CA). Approximately 2.5 µL aliquots of protein samples (~0.1 µM) dissolved in 1.0% formic acid and B: acetonitrile (Fisher Scientific Company, Ottawa, ON). The flow rate was 0.25 mL/min and proteins were eluted rapidly using a linear gradient of 5–80% B over 5 min. Mass spectrometry data were collected up to 2500 m/z and raw spectra were deconvoluted using the maximum entropy algorithm of the Agilent MassHunter software version B.06.01. The peak numbering corresponds to the molecular weight of the protein plus the indicated number of phosphate groups (+7.99 Da/phosphate group).

**NMR spectroscopy.** NMR-HSQC spectroscopy spectra (8 scans, 20 min acquisition time) were recorded at 15 °C on a 600-MHz Bruker AVANCE III spectrometers equipped with a 1.7-mm TCI CryoProbe. All NMR samples contained 180 µM of 15N-Bora-1 (in C4 column (Higgins Analytical Inc., Mountain View, CA). The mobile phase was composed of A: 0.1% aqueous formic acid and B: acetonitrile (Thermo Scientific, Santa Clara, CA). Approximately 2.5 µL aliquots of protein samples (~0.1 µM) dissolved in a 0.1% formic acid buffer were separated on a 2 mm × 150 mm, 3 µm C18 column (Spherisorb, Thermo). The pump flow was 0.3 mL/min and raw spectra were deconvoluted using the maximum entropy algorithm of the Agilent MassHunter software version B.06.01. The peak numbering corresponds to the molecular weight of the protein plus the indicated number of phosphate groups (+7.99 Da/phosphate group).

**Preparation of Xenopus extracts.** CSF extracts were obtained from metaphase II-arrested oocytes. Laid MII-arrested oocytes are first dejellied with 2% cysteine solution, extensively rinsed with XB buffer (100 mM KCl, 0.1 mM MgCl2, 50 mM Sucrose, 5 mM NaEGTA, 10 mM HEPES at pH 7.7) and subsequently centrifuged twice for 20 min at 10,000 × g and the cytoplasmic fractions recovered.

**Interphase egg extracts were obtained from dejellied unfertilized eggs transferred to 25 mM NaCl, 0.25 mM MgCl2, 0.025 mM NaEGTA, 1.25 mM HEPES–NaOH pH 7.7) supplemented with Ca2+–ionophore addition (final concentration 2 µg/mL) and 35 min later recovered and treated as CSF extracts17; 1 µL of Interphase or CSF egg extracts was loaded on a polycrylamide gel and transferred onto N Immobilon-P membranes.

**Immunodepletion Xenopus extracts.** To deplete Xenopus egg extracts, immunoprecipitations were carried out for 20 min at room temperature using 2 µg of affinity-purified antibodies immobilized on 20 µL protein G-Dynabeads (Dynal) and 20 µL of Xenopus egg extracts. When supernatant was used, beads were removed by magnetic racks and supernatants recovered. Two consecutive immunoprecipitations were performed to completely remove endogenous Bora, AURKA, and Gwl proteins. Bora immunoprecipitation was performed using antibodies raised against GST-fusion length Xenopus Bora whereas Western blot was performed with antibodies against Xenopus-Nter-Bora27.

**Generation of knock-in cell lines using Cas9/CRISPR method.** To introduce the AID cassette (with a Neon-Green fluorescent tag) at the Bora locus, the guide RNA sequences targeting exon 12 in Bora were as written in the Supplementary Table 3 (Guide #1 and Guide #2).

- Each guide was phosphorylated and annealed in a thermocycler then sub-cloned into pX330-U6-HspCas9 using Bst1 (Addgene #42230) using the primers listed in the Supplementary Table 3 for the Guide #1, and the Guide #2.
- The vector pRS75 containing the Bora repair template (7501 bp) was generated by Gibson cloning27, the repair template contains the two homology arms flanking the Neon-green and the AID cassette followed by a Hygromycin resistance cassette (separated by P2A). The constructs were transfected using ViAFect into DLD1 stably transfected cell line; 48 h after transfection, the cells were split 1:4 and treated with Puromycin for selection. Individual clones were isolated, amplified, and screened for homozygosity using PCR and sequencing.

**Auxin-mediated Bora degradation.** To induce the degradation of Bora-AID, DLD1-Bora-Neon-Green-AID cell line was treated with 500 µM of Auxin (1 nM of Auxin (MerckMillipore)) or a 280-µM stock solution (50 mg/mL in DMSO). Expression of Bora variants was induced with 0.5 µg/mL Doxycycline (MerckMillipore) for 24 h from a 1 mg/mL stock solution (in DMSO).

**Cell extracts and Western blot analyses.** Whole-cell extracts were prepared from mammalian cells by resuspending cells in 50 mM Tris-HCl at pH 7.5, 1 mM EDTA, 1 mM EGTA, 50 mM sodium fluoride, 0.27 M sucrose, 1% Triton X-100, complete protease inhibitors, and complete phosphatase inhibitor Phostop, incubated on ice for 30 min before clarification by centrifugation. Protein concentrations were measured using Pierce BCA Protein assay kit (Thermo Fisher Scientific) and a Spectramax M2 plate reader (Molecular Devices). To isolate protein in cell lysates, the protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Proteins were detected by Western blot and visualized by treating the blots with ECL (MerckMillipore).

**Immunoprecipitation.** To detect pT210-Plx1 signal in cell extracts, mammalian cells were lysed in 50 mM HEPES pH 7.4, 1 mM MgCl2, 1 mM EGTA, 1% NP40, 1 mM NaF, complete protease inhibitors, and complete phosphatase inhibitor Phostop, incubated on ice for 30 min before clarification by centrifugation. Protein concentrations were measured using Pierce BCA Protein assay kit (Thermo) and a Spectramax M2 plate reader (Molecular Devices). The immunoprecipitation was performed on 1.5 mg of total proteins overnight at 4 °C with 3 µg of anti-pT210 (Abcam #ab17057). The following day, samples were incubated for 2 h with preimmobilized magnetic beads (AIDtech) at 4 °C. Samples were then washed three times with lysis buffer, beads were resuspended in Laemmli buffer and boiled for 10 min. Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes.

**Antibodies.** The antibodies used in this study are the following: anti-human Plx1 (Abcam Cat. #ab17057), anti-human PI3K (Bethyl Cat. #A300-250A), anti-Bora (Cell Signaling Technologies Cat. #D2B9), anti-NeonGreen (ThermoFisher Cat. #sc-393741), anti-Phospho-Histone H3 (Ser10) (Cell Signaling Technologies Cat. #9701), anti-Phospho-Plx1 (Thr210) (Cell Signaling, Technologies Cat. #5412), anti-Actin (MP Biomedical Cat. #AM5101), anti-Plk1 (Abcam #ab17057). The following day, samples were incubated for 2 h with preimmobilized magnetic beads (AIDtech) at 4 °C. Samples were then washed three times with lysis buffer, beads were resuspended in Laemmli buffer and boiled for 10 min. Protein samples were separated by SDS-PAGE and transferred onto nitrocellulose membranes.

**Microscopy.** DLD1 cells were seeded 2 days prior to the experiment (80,000 cells per well) on Krystal 24-well 175 mm glass-bottom microplates (Wildcat Laboratory Sciences) precoated with bovine fibronectin (Promocell) at 1 mg/cm2 and imaged in DMEM without phenol red and supplemented with 5% FCS. Cell and nuclear morphology was performed using an inverted microscope (Nikon Eclipse Ti-E) controlled by Metamorph software and equipped with perfect focus system, fast emission filter wheel (lambda 10-3, Sutter), electron multiplying charge coupled device camera (iXon 3 888 Ultra Andor), Plan Apochromat 20×/NA 0.75 lens, and light-emitting diode (LED)-based illumination system (spectra X-light engine, Luminor). Filters used for IFP2 were ET620/60x, T660lpxr beamsplitter, and ET700/75m from Chroma. Quantifications were performed using ImageJ software.

**Statistics and reproducibility.** All experiments presented in this manuscript have been repeated at least three times with the exception of SPR and NMR analysis. Kd and EC50/IC50 SD values represent deviation between values obtained from three independent experiments.

**Data availability.** The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Source data are provided with this paper.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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Competing interests

The authors declare no competing interests.

Additional information

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