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# Bora and Aurora A Cooperatively Activate Plk1 and Control the Entry into Mitosis

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## Abstract

A central question in cell proliferation is what controls cell cycle transitions. Although classical experiments indicate that accumulation of mitotic cyclins drives the G2/M transition in embryonic cells, the trigger for mitotic entry in somatic cells remains unknown. We report here that synergistic action of Bora and Aurora A controls the G2/M transition. Bora accumulates in G2 and promotes Aurora A-mediated activation of the Plk1 kinase, leading to the Cdk1 activation and mitotic entry. Mechanistically, Bora interacts with Plk1 and controls the accessibility of its activation loop for phosphorylation and activation by Aurora A. Thus, the Aurora A-Bora-Plk1 pathway controls the Cdk1 activation at mitotic entry and defines a mechanism for one of the most important, and yet ill-defined events in the cell cycle.

### Keywords

Aurora A; Bora; Plk1; Cdk1; Mitotic Entry; Protein Phosphorylation

Entry into mitosis is controlled by the activation of the Cdk1 kinase, whose activity, in turn, is regulated directly by mitotic cyclins and the activating Cdc25 phosphatase (1), as well as indirectly by other kinases, such as Plk1 and Aurora A (2–4). Plk1, Cdc25 and Cdk1 form a feedback loop and positively regulate each other's activity (1). A fundamental question in cell cycle regulation is what activates this feedback loop at the G2/M transition. Existing evidence indicates that activation of Plk1 is likely an initiating event (5,6), as Plk1, through phosphorylation, activates Cdc25 (5,7) and down-regulates Wee1 (8), a kinase inhibitory to Cdk1, and as Plk1 can be activated in the absence of the Cdk1 feedback loop (9,10). Thus, precise regulation of Plk1 is critical for the timely G2/M transition.

The kinase activity of Plk1 is tightly controlled in the cell cycle (11). During mitosis, Plk1 is phosphorylated by a unknown upstream kinase on a Threonine residue (Thr210 in Plk1 and Thr201 in Plx1, the *Xenopus* homolog of Plk1) in its activation loop (T-loop) and this phosphorylation is required for its activity (5,11–14). Plk1 consists of an N-terminal kinase domain and a C-terminal Polo-box domain (PBD), which binds to prime-phosphorylated substrates (15,16). The Plk1 kinase activity is also regulated by its unique conformation, as PBD interacts with the kinase domain and suppresses its kinase activity when Thr210 is not phosphorylated in interphase cells (14,17). This inhibitory interaction is absent in mitotic Plk1 phosphorylated on Thr210 (17). Two fundamental questions in Plk1 regulation are which kinase phosphorylates and activates Plk1 and how the auto-inhibition by PBD is relieved at

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the G2/M transition. We report here that Aurora A is the physiological Plk1-activating kinase and that the G2-induced Bora protein interacts with Plk1 to control the accessibility of its T-loop and to relieve auto-inhibition by PBD. Thus, Bora and Aurora A act synergistically to phosphorylate Plk1 on Thr210 and to promote mitotic entry.

We first determined the kinetics of Plk1 accumulation and activation during the G2/M transition in HeLa S3 cells synchronously released from a double-thymidine treatment, which arrests cells at the G1/S boundary (Fig. 1). Although the Plk1 protein accumulated in early G2 (TT6, 6 hours after release from the double-thymidine arrest), active Plk1 phosphorylated on Thr210 (Plk1-T210-P) was only detectable at late G2 (TT9), right before mitosis, consistent with the measurement of the Plk1 kinase activity (Fig. 1A & B). Thus, accumulation of the Plk1 protein is not sufficient for mitotic entry and Plk1 activity is under active regulation. We also noted that active Aurora A phosphorylated on Thr288 appeared slightly ahead of the activation of Plk1 (Fig. 1A), suggesting that Aurora A may act upstream of Plk1.

To identify the trigger for Plk1 activation at the G2/M transition, we focused on genes transcriptionally induced in G2, given that most of the known regulators of mitotic entry, such as Plk1, Aurora A and cyclin B, are induced in G2 (1,18). The human transcriptome has 239 genes induced in G2 (18), among which only 22 genes with good induction profiles are functionally uncharacterized. We initially selected 7 novel genes with the best G2-induction profiles and investigated their potential roles in mitotic entry in knockdown experiments (see online supplementary material). This study led to the identification of a novel regulator for mitotic entry, FLJ22624, whose *Drosophila* homolog was recently reported as dBora involved in asymmetric cell division (19).

Knockdown of FLJ22624/Bora in HeLa cells synchronously released from the G1/S boundary did not affect the progression from G1 to S and then to G2, but substantially delayed the entry into mitosis (Fig. 2A–C and S1A–D), similar to the phenotype observed in Plk1-knockdown cells (Fig. S2) (3, 4, 10). This phenotype is specific due to depletion of Bora, as stable expression of GFP-Bora resistant to siRNA knockdown rescued the delay in mitotic entry (Fig. S1E–I). Analysis of cell cycle markers indicated that depletion of Bora in HeLa cells did not affect the accumulation of cyclin B, Plk1 or Aurora A at the G2/M transition, but prevented the dephosphorylation of Cdk1 on Tyr15 (Fig. 2D), indicating that Bora acts upstream from Cdk1. Indeed, Bora controls the activation of Plk1, as knockdown of Bora substantially delayed the phosphorylation of Plk1 on Thr210 and the activation of its kinase activity (Fig. 2D & E). This effect is specific to Plk1, as depletion of Bora only marginally affected the activation of Aurora A phosphorylated on Thr288 (Fig. 2D). As degradation of Wee1 in mitosis requires Plk1 activity (11, 20), depletion of Bora also prevented the destruction of Wee1.

This lack of phosphorylation on Plk1-T210 is the cause of the cell cycle delay, not an indirect consequence resulting from a change in the cell cycle profile at the time-points analyzed, as knockdown of Bora in cells with a cell cycle profile similar to that of control cells also had a lower level of Plk1-T210-P (Fig. 2F). Similarly, knockdown of Bora in prometaphase cells arrested with nocodazole and collected by mitotic shake-off reduced the level of Plk1-T210-P, but not Aurora A-T288-P (Fig. 2G). On the other hand, ectopic expression of GFP-Bora increased the levels of Plk1-T210-P (Fig. 2H). Again, this increase is not simply caused by a change in the cell cycle distribution, as GFP-Bora-expressing prometaphase cells also had a substantially higher level of Plk1-T210-P than GFP prometaphase cells (Fig. 2I). Thus, Bora controls the activation of Plk1 and the entry into mitosis by promoting Thr210 phosphorylation.

To understand the mechanism of Bora function, we purified Bora-associated proteins from a cell line stably expressing tandem-tagged Bora. Mass spectrometric analysis identified Plk1 as a major Bora-interacting protein (28 independent peptides identified from Plk1 with a

sequence coverage of 31.8%) (Fig. 1D). Next, we examined the interaction of Bora with Plk1 across the cell cycle in synchronized HeLa S3 cells. Consistent with its function in mitotic entry, levels of Bora peaked in G2 (TT8-9), but were gradually degraded once cells entered mitosis (TT10–12) (Fig. 1A) (21). Plk1 and Bora mutually co-precipitated, and the Bora/Plk1 complex peaked in G2 (TT8-9) (Fig. 1B & D). Interestingly, although the peak of Plk1-T210-P and the Plk1 kinase activity in total cell lysates occurred in mitosis (TT10–11) (Fig. 1A & B), Bora already associated with active Plk1-T210-P in G2 cells (TT8-9) (Fig. 1D), suggesting that Bora may be directly involved in the activation of Plk1 prior to mitotic entry. This interaction between Bora and Plk1 is specific, as control IgG precipitated neither Bora nor Plk1 (data not shown) and as recombinant Bora also interacted with the *in vitro* synthesized Plk1 and Plx1 (Fig. 1E and S3A). Bora interacts with Plk1 independent of its phosphorylation, even though Bora is hyperphosphorylated in G2 (Fig. 1F). Bora specifically interacts with Plk1, but not with Aurora A, as assayed by immunoprecipitation-Western blotting and by mass spectrometry analysis (Fig. 1C & D).

The highly conserved sequence around Thr210 in the Plk1 T-loop fits the consensus recognition site for Aurora A (Fig. 3A) (22), a mitotic kinase required for the G2/M transition (2). Furthermore, the centromere protein, CenpA, is phosphorylated by Aurora A at prophase on R-R-R-pS (23), a sequence similar to that in the Plk1 T-loop. Indeed, Aurora A directly phosphorylated recombinant Plx1 *in vitro* and this phosphorylation is enhanced by recombinant Bora (Fig. 3B & S4A–C). This enhancement on Plx1 phosphorylation is not due to a general stimulation of the Aurora A kinase activity by Bora as measured by its reactivity toward Histone H3, a mitotic substrate commonly assayed for the Aurora A activity (2).

Next, we analyzed the *in vitro* effect of Aurora A and Bora on the kinase activity of myc-Plk1 immuno-purified from 293 cells and of inactive recombinant GST-Plk1 puried from asynchronous *Sf9* cells (Fig. 3C, Fig. S4A & S4E). While Bora alone had no effect on Plk1 activation and Aurora A alone enhanced Plk1 activity to some extent, combination of Bora and Aurora A synergistically stimulated Plk1 kinase activity by 7 to 9 fold. Interestingly, whereas Aurora A by itself only weakly phosphorylated Plk1-T210, addition of Bora greatly stimulated phosphorylation of the T-loop, suggesting that binding of Bora may control the accessibility of Plk1-Thr210 by Aurora A.

Given the conservation of Plk1 and Plx1 (81% aa identity and 92% similarity) and the availability of well-characterized Plx1 mutants (6), we also performed biochemical characterization and reconstitution with Plx1. Indeed, Aurora A- and Bora-mediated phosphorylation and activation of Plk1 are conserved, as inactive recombinant Plx1 purified from asynchronous *Sf9* cells was efficiently phosphorylated on Thr201 and became activated, but only in the presence of both Bora and Aurora A (Fig. 3D and S4A, D & F–H). This activation occurred in a manner dependent on the dose of Bora (Fig. S4I). However, active Plx1 purified from mitotic *Sf9* cells already had Thr201 phosphorylated and the presence of Bora and Aurora A neither enhanced the Thr201 phosphorylation nor stimulated its kinase activity (Fig. 3E). Interestingly, dephosphorylation of active Plx1 by λ-phosphatase allowed efficient activation of Plx1 by Bora and Aurora A (Fig. 3E). Thus, Bora and Aurora A only activate inactive Plx1, but does not further enhance the activity of mitotic Plx1.

This activation of Plx1 is through phosphorylation of Thr201, as Bora and Aurora A failed to further activate the kinase activity of the Plx1-T201D mutant, even after its dephosphorylation (Fig. 3F). Consistent with this, Bora did not enhance the phosphorylation of Plx1-T201D by Aurora A (Fig. 3G), indicating that enhancement in the wild-type Plx1 resulted from specific phosphorylation on Thr201 (cf. Fig. 3G vs. 3B). Thus, activation of Plx1 by Bora and Aurora A is mediated through phosphorylation of Plx1-Thr201.

To understand the structure basis of the Bora-mediated activation of Plk1, we further analyzed the interactions between Plk1 and Bora. Both the Plk1 kinase domain (aa13–345, Plk1-K) and the PBD domain (aa 352–603, Plk1-PBD) directly associated with Bora (Fig. 3H–I and S3B–C). PBD interacted with both the N and C terminal domains of Bora (Fig. 3I) and the interactions between PBD and Bora are independent of phosphorylation (Fig. S3C). Consistent with this, the phospho-peptide binding site in PBD is not required for the activation of Plx1 by Bora and Aurora A (Fig. S4J).

We next analyzed the effect of Bora and Aurora A on the Plk1 kinase domain. Myc-Plk1-K was phosphorylated on Thr210 by Aurora A independent of Bora (Fig. 3J), consistent with the fact that Thr210 is solvent accessible in the Plk1-K atomic structure (24). Thus, Bora only controls accessibility of Thr210 in the full-length Plk1, in which PBD interacts with and inhibits the kinase domain (17). Interestingly, Bora still contributed to the activation of myc-Plk1-K to some extent (Fig. 3J), likely through its direct interactions with Plk1-K. Thus, Bora regulates the Plk1 activation through both T210-phosphorylation-dependent and independent mechanisms.

To analyze the role of Aurora A in activation of Plk1 *in vivo*, we depleted Aurora A in double-thymidine-treated cells (Fig. 4). Knockdown of Aurora A delayed mitotic entry as analyzed by mitotic index and Cdk1-Y15 phosphorylation (Fig. 4A–C) (2). Depletion of Aurora A prevented phosphorylation of Plk1 on Thr210, even though both Plk1 and Bora were expressed, indicating that Aurora A controls the activating phosphorylation of Plk1 at the G2/M transition. Consistent with this, ectopic expression of Aurora A stimulated phosphorylation of Plk1-Thr210 and this enhancement is dependent on the kinase activity of Aurora A and on the presence of Bora (Fig. 4D–E). Similarly, ectopic expression of Bora also promoted the Plk1-T210 phosphorylation and this enhancement by Bora is dependent on the Aurora A kinase activity, even though Aurora A kinase activity was not required for the Bora-Plk1 interaction (Fig. 4D & F). Thus, Aurora A controls Plk1 activation through phosphorylation of Thr210 *in vivo*.

Activating phosphorylation of Plk1/Plx1 is required for mitotic entry (6,20). Although the *Xenopus* Polo-like kinase kinase, xPlkk1, phosphorylates and activates Plx1 *in vitro* (25), xPlkk1 acts downstream of Plx1 and is not the trigger kinase for mitotic entry in *Xenopus* extracts (6,26). We report here that Aurora A phosphorylates and activates Plk1 at the G2/M transition and that this activation requires a synergistic action of Bora through its control of Plk1 conformation. Previously, function of Bora has only been characterized in *Drosophila*, in which both dBora and dAurora A act in a genetic pathway involved in asymmetric division of sensory cells (19). Although dBora has been proposed to activate dAurora A in *Drosophila* (19), we showed here that Plk1 is the target of Bora regulation in mammalian cells, as Bora forms a stable complex with Plk1, not with Aurora A (Fig. 1). In addition, when Plx1 and Histone H3 were incubated with Aurora A and Bora, Bora specifically stimulated Aurora A-mediated phosphorylation of Plx1-Thr201, not of Histone H3 or Plx1-T201D (Fig. 3 & Fig. S4). Furthermore, depletion of Bora *in vivo* reduced the activity of Plk1, not Aurora A (Fig. 2D–G).

Data presented here support a model in which Bora and Aurora A act cooperatively to activate Plk1 and to control mitotic entry (Fig. 4G). The accessibility to Thr210 in its unphosphorylated state is blocked by PBD in interphase cells (17). However, induction of Bora in G2 and its subsequent binding to both the kinase and PBD domains changes the Plk1 conformation that allows phosphorylation of Thr210 by Aurora A. Once phosphorylated on Thr210, PBD no longer interacts with the kinase domain (14,17) and active Plk1 initiates the Plk1-Cdc25-Cdk1 positive feedback loop for mitotic entry (Fig. 4G). Consistent with Aurora A and Plk1 act upstream of the Cdk1 kinase, inhibition of Cdk1 in mitosis did not affect the activating

phosphorylation of Aurora A and Plk1, while inhibition of Cdk1 in G2 only weakly reduced the Aurora A and Plk1 activation (Fig. S5). This is in agreement with previous studies showing that Plk1 can be activated in the absence of the Cdk1 feedback loop (9,10). On the other hand, activation of Plk1 and Aurora A are mutually dependent on each other in both G2 and mitosis (Fig. 4C,Fig. S2C & Fig. S5), indicating that these two kinases form a positive feedback loop that initiates the Cdk1 activation and mitotic entry. Our discovery of Aurora A- and Boramediated activation of Plk1 is part of this loop. On the other hand, Aurora A is not a direct substrate of active Plk1 *in vitro* (data not shown) and the feedback pathway from Plk1 to Aurora A likely involves intermediates. In mammalian cells, Aurora A is positively controlled by multiple regulators, such as Ajuba, HEF1 and PAK (2, 27,28), and Plk1 is likely to regulates one of these proteins, which feedbacks into Aurora A. We note that inactivation of Aurora A, Bora, Plk1 and Ajuba all results in a delay in, but not a complete block on, mitotic entry (Fig. 2,Fig. 4 & Fig. S2) (2–4), suggesting the existence of compensatory mechanisms at the G2/M transition. Alternatively, the lack of a stable G2 arrest could simply result from partial inactivation of these regulators under experimental conditions.

Once in mitosis, the activity of Plk1 is enhanced by binding of prime-phosphorylated substrates to PBD (15) and we investigated how this mechanism coordinates with the Bora pathway in Plk1 regulation. Phospho-peptide (P-peptide) bound by PBD enhanced, by three fold, the activity of active Plx1 purified from mitotic *Sf9* cells, but had a minimal or no effect on the inactive Plx1 purified from asynchronous *Sf9* cells that had not been phosphorylated on Thr201 (Fig. S6A–C). When incubated with Aurora A and inactive Plx1, P-peptide neither promoted the phosphorylation of Plx1-Thr201, nor the activation of inactive Plx1 (Fig. S6D), indicating that, in sharp contrast to Bora, P-peptide does not alter the accessibility of Thr201 in inactive Plx1. Incubation of P-peptide with the Plx1−Bora complex that had been activated by Aurora A only slightly affected the Plx1 activity (less than 30%) (Fig. S6D–F), suggesting a possibility that binding of Bora interferes with the stimulation of Plx1 by P-peptide. Indeed, incubation of active Plx1 first with P-peptide and then with Bora prevented the enhancement of the Plx1 activity by P-peptide (Fig S6G–H), even though incubation of active Plx1 with P-peptide alone substantially enhanced the Plx1 activity, while incubation of active Plx1 with Bora alone did not affect its activity. Thus, Bora dominantly competes against the prime-phosphorylated substrates in Plk1 regulation.

Thus, Bora has a dual role on Plk1 regulation in the cell cycle. In G2, it acts as an activator to allow access of Plk1-Thr210 by Aurora A. Once in mitosis, Bora complexed with active Plk1 is likely to interfere with the interaction between PBD and prime-phosphorylated substrates. This inhibitory effect of Bora is relieved by its mitotic destruction, as the majority of Bora is degraded by proteasomes in mitosis (21). Interestingly, upon its activation by Bora and Aurora A, Plk1 actively promotes the degradation of Bora by the β-TrCP/SCF ubiquitin ligase (21). Degradation of Bora after mitotic entry releases Plk1 free to associate with prime-phosphorylated substrates, which enhance its kinase activity and target Plk1 to kinetochores and centrosomes for its mitotic function (Fig. 4G). Expression of a non-degradable Bora variant reduces the association of Plk1 to centrosomes and delays the metaphase to anaphase transition (21), indicating the physiological importance of Bora degradation.

We also noted that mitotic regulation of Bora is complex as low levels of Bora persist in mitosis (Fig. 1A & Fig. 2G) and as mitotic Bora is required for proper dynamics of the mitotic spindle (21). Indeed, Bora also contributes to the Plk1 activation in mitosis (Fig. 2G). However, the fact that knockdown of Bora in prometaphase cells only reduced, but did not completely abolished, the Plk1-T210-P (Fig. 2G) suggest that either the knockdown is incomplete or there may exist a Bora-independent mechanism for the maintenance of Plk1-T210-P in mitosis. As active Plk1 is no longer in an auto-inhibitory conformation during mitosis (17), we speculate that Thr210 is likely accessible in active Plk1 independent of Bora and that mitotic Aurora A

alone may be sufficient for the maintenance of the Plk1-Thr210 phosphorylation. This explains the differential requirement of Bora in the initial establishment of T210-P from inactive Plk1 in G2 vs. the maintenance of T210-P from active Plk1 in mitosis. Alternatively, Plk1-T210-P generated at mitotic entry may be unusually stable and persists through mitosis.

The Aurora A-Bora-Plk1 regulatory module described here may not necessarily control the mitotic entry in all cell types. For example, Bora is not expressed in *Xenopus* egg extracts and thereby not required for the G2/M transition in embryonic cell cycle (data not shown). Thus, in embryonic extracts there exists an alternative mechanism for Plx1 activation, possibly through the rapid and robust accumulation of mitotic cyclins, which directly activates the Plk1-Cdc25-Cdk1 positive feedback loop. The introduction of the Aurora A-Bora-Plk1 pathway in somatic cells affords versatile and precise control of mitotic entry and we propose that this pathway is the target of regulation under different physiological and environmental stresses in the cell cycle. Since Plk1, Bora and Aurora A are all required for asymmetric cell division in *Drosophila* (19,29), we speculate that the modular Aurora A-Bora-Plk1 regulatory circuit discovered here functions beyond the cell cycle regulation in other physiological processes, such as in asymmetric cell division and in self-renewal and differentiation of stem cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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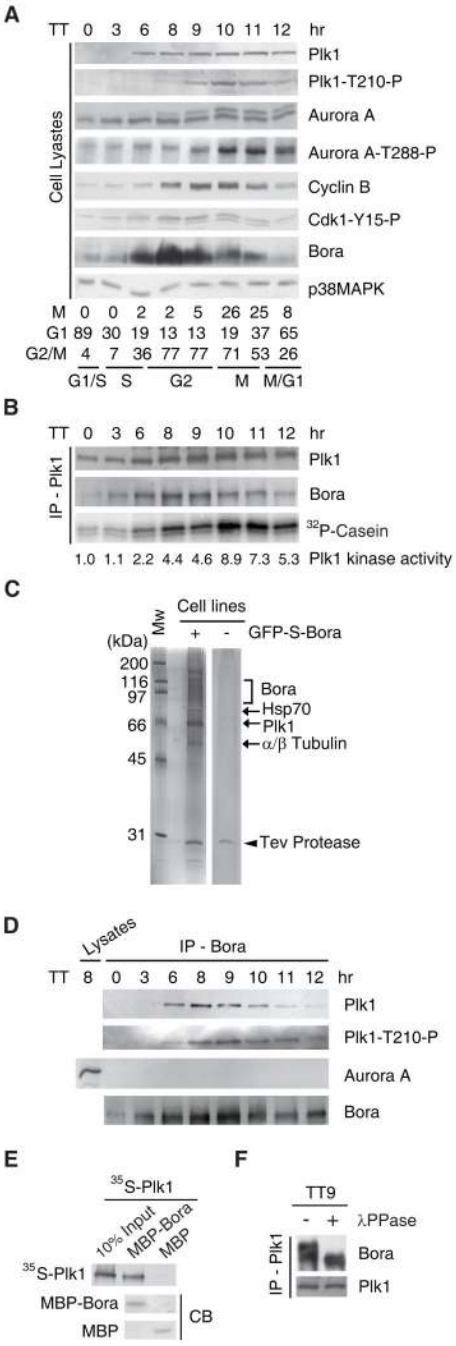


**FIGURE 1. Activation of Plk1 is regulated at the G2/M transition**

**(A, B & D)** HeLa S3 cells were synchronized at the G1/S boundary by a double-thymidine arrest (TT), released into fresh media and harvested at indicated times. Cell cycle stages were assayed by FACS and levels of indicated proteins analyzed by Western blotting of total cell lysates (A). p38MAPK served as a loading control. Plk1 was immunopurified from cell lysates and blotted for Plk1 and Bora or assayed for Plk1 kinase activity using casein as a substrate (B). The Bora complex was immunoprecipitated from cell lysates and blotted for Plk1, Plk1-T210-P, Aurora A and Bora (D). Cdk1-Y15-P in (A) represents the inactive Cdk1 phosphorylated on Tyr15. (**C**) The Bora complex was purified from G2 cells stably expressing the GFP and S-peptide tagged Bora. Eluates of the tandem-affinity purified Bora complexes

were separated by SDS–PAGE and visualized by silver staining. In addition to the proteins labeled in this panel, other interacting proteins identified include β-TrCP1, β-TrCP2, Cul1 and Skp1, which are subunits of the β-TrCP/SCF ubiquitin ligase responsible for degradation of Bora in mitosis (21). These proteins were not detected by silver-staining due to their low abundance. The identity of the band between 116 kDa and 200 kDa remains to be determined. Mw, molecular weight markers. (**E**) Recombinant MBP-Bora and MBP were incubated with *in vitro* synthesized $^{35}$S-Plk1 and then purified by anti-MBP antibody beads. Bora and associated Plk1 were analyzed by SDS-PAGE. CB, Coomassie blue staining. (**F**) Plk1 complexes immunoprecipitated from TT9 cell lysates were treated with λ-phosphatase (λPPase) and washed. Materials remained on Plk1 beads were blotted for Bora and Plk1.

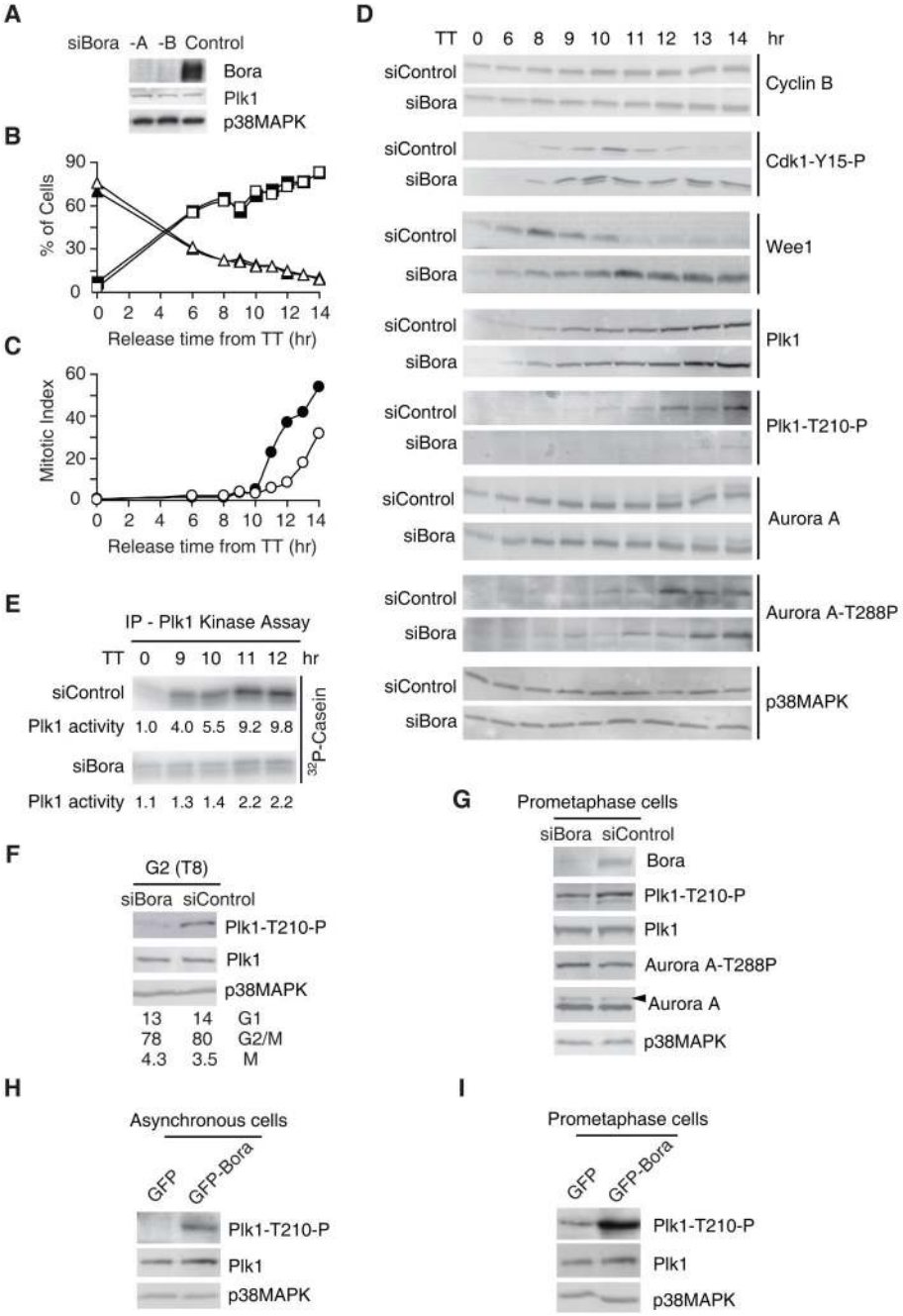


**FIGURE 2. Bora activates Plk1 and controls mitotic entry**

**(A)** HeLa cells were controltransfected or transfected with siRNAs against Bora (siBora-A & −B). Knockdown efficiency was determined by Western blotting for Bora, Plk1 and p38MAPK (a loading control). **(B–E)** HeLa cells were synchronized by a double-thymidine treatment and transfected with a control siRNA or siBora-A during the 2nd thymidine arrest. Cells were released into fresh media and Taxol added at 9 hours post-release to prevent mitotic exit. Cell cycle profile was analyzed by FACS (B–C) and by Western blotting (D). Plk1 from selected timepoints was immunopurified and assayed for its kinase activity using casein as a substrate (E). The Plk1 activity in (E) was normalized to the TT0 sample in siControl. Filled symbols, siControl; Open symbols, siBora. Triangles, G1; Squares, G2/M; Circles, M. Knockdown by

siBora-B gave a similar phenotype. **(F–G)** HeLa cells were transfected with siBora-A, synchronized by a single thymidine arrest and harvested 8 hr post-release (T8) (F) or synchronized by a thymidine-nocodazole arrest and harvested by mitotic shake-off (TN0) (G). Cell cycle profile was determined by FACS (F) and indicated proteins assayed by Western blotting of total cell lysates (F–G). Partial synchronization in (F) enriched G2/M cells and generated cells of similar cell cycle distribution between control and Bora knockdown. **(H–I)** HeLa cells were transfected with GFP or GFP-Bora. Asynchronous (H) or prometaphase (I) cells were collected at 34 and 48 hours post-transfection, respectively. Prometaphase cells were enriched by a thymidine-nocodazole arrest and harvested by mitotic shake-off. Indicated proteins were analyzed by Western blotting.

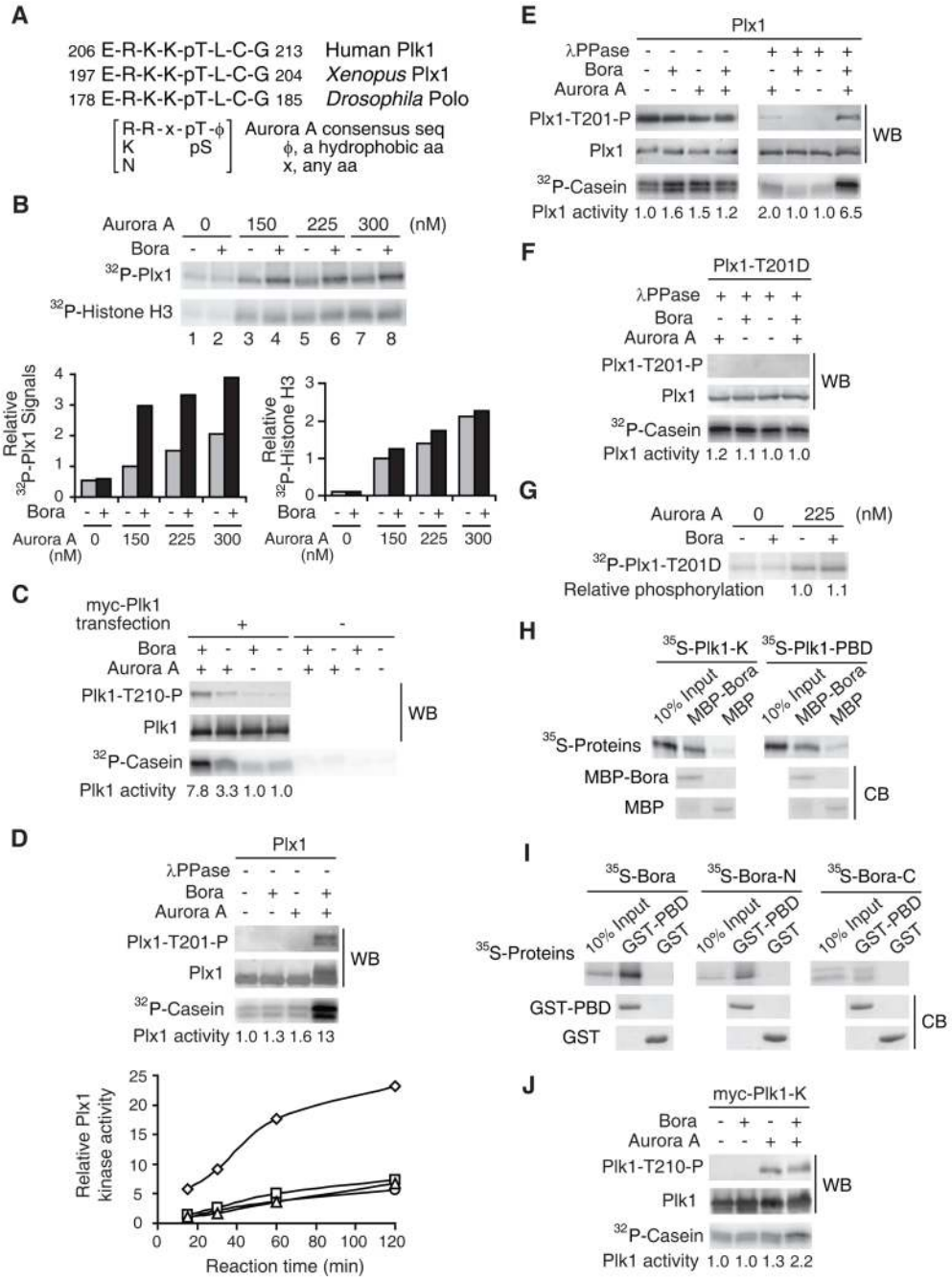


**FIGURE 3. Bora and Aurora A synergistically activate Plk1**

**(A)** Plk1 T-loop and Aurora A recognition sequences. **(B)** Recombinant Plx1 (purified from asynchronous *Sf9* cells) and Histone H3 were mixed together and then incubated with Aurora A and MBP-Bora/MBP for 60 min in the presence of radioactive ATP and BI 2536, a Plx1 inhibitor (3). The relative $^{32}P$ incorporation into Plx1 and H3 were plotted after normalizing to their respective samples in lane 3. **(C & J)** Myc-Plk1 and myc-Plk1-K were expressed in 293 cells by transient transfection, immunopurified and incubated with or without recombinant Aurora A and MBP-Bora/MBP. Myc-Plk1/myc-Plk1-K beads were then washed to remove Aurora A and assayed for Plk1 kinase activity or by Western blotting (WB). 293 cells without transfection were used as a negative control in (C). **(D–F)** Recombinant Plx1 and Plx1-T201D

were sequentially incubated with or without λPPase, with EGTA (to stop the λPPase reaction), and then with or without Aurora A and MBP-Bora/MBP. Plx1/Plx1-T201D were then analyzed by Western blotting or immunopurified away from Aurora A to assay for Plx1 kinase activity. Inactive Plx1 (D) and Plx1-T201D (F) were purified from asynchronous *Sf9* cells without okadaic acid treatment, whereas active mitotic Plx1 (E) was purified from okadaic-acid-treated mitotic *Sf9* cells (7). In a separate experiment in (D), the kinetics of the Plx1 kinase activity was analyzed and plotted after normalizing all the samples to that of −Bora−Aurora A at the 15 min timepoint. Diamonds, +Bora+Aurora A; Squares, −Bora+Aurora A; Circles, +Bora −Aurora A; Triangles, −Bora−Aurora A. **(G)** Phosphorylation of Plx1-T201D by Aurora A, as described in (B). **(H–I)** Recombinant MBP-Bora/MBP/GST-PBD/GST were incubated with *in vitro* synthesized $^{35}$S-proteins and then purified with anti-MBP antibody beads or Glutathione Sepharose. Associated proteins were analyzed by SDS-PAGE. CB, Coomassie blue staining.

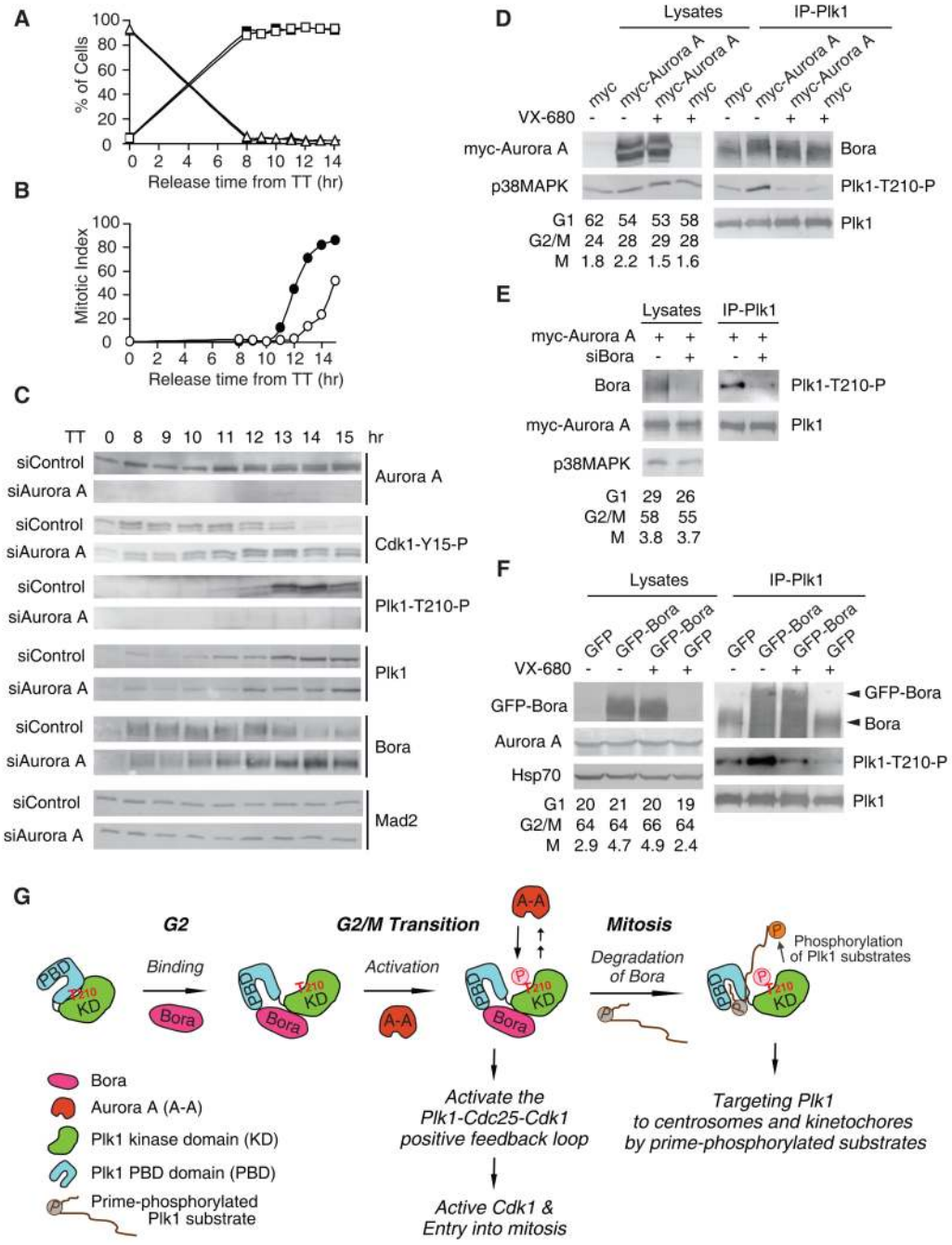


**FIGURE 4. Aurora A activates Plk1 and controls mitotic entry**

**(A–C)** HeLa cells were synchronized by a double-thymidine treatment and transfected with a control siRNA or siRNA against Aurora A (siAurora A) during the 2nd thymidine arrest. Cells were released into fresh media and Taxol added at 9 hours post-release to prevent mitotic exit. Cell cycle profile was analyzed by FACS (A–B) and by Western blotting (C). Mad2 served as a loading control. Filled symbols, siControl; Open symbols, siAurora A. Triangles, G1; Squares, G2/M; Circles, M. Inhibition of Aurora A by VX680 (30) in G2 cells released from the double-thymidine arrest resulted in similar phenotypes (data not shown). **(D, F)** HeLa cells were transfected with myc-Aurora A/myc (D) or with GFP-Bora/GFP (F) and synchronized by a single thymidine arrest. Seven (F) or eight and half (D) hours post-release, cells were

treated with 1 μM VX-680 or DMSO and harvested one hour later. Indicated proteins were assayed by Western blotting of either total cell lysates or the Plk1 immunoprecipitates. Cell cycle profile was determined by FACS to ensure that any change in the Plk1-T210-P level was not due to an altered cell cycle distribution among samples. **(E)** HeLa cells were transfected first with a control siRNA or siBora-A and then with myc-Aurora A. Cells were synchronized by a single thymidine arrest and harvested eight hours post-release. Cell cycle profile was determined by FACS and indicated proteins assayed by Western blotting of either total cell lysates or the Plk1 immunoprecipitates. **(G)** Summary on the regulation of Plk1 by Bora and Aurora A in the cell cycle.