Bora and Aurora-A continue to activate Plk1 in mitosis

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Abstract

Polo-like kinase-1 (Plk1) is required for proper cell division. Activation of Plk1 requires phosphorylation on a conserved threonine in the T-loop of the kinase domain (T210). Plk1 is first phosphorylated on T210 in G2 phase by the kinase Aurora-A, in concert with its cofactor Bora. However, Bora was shown to be degraded prior to entry into mitosis, and it is currently unclear how Plk1 activity is sustained in mitosis. Here we show that the Bora/Aurora-A complex remains the major activator of Plk1 in mitosis. We show that a small amount of Aurora-A activity is sufficient to phosphorylate and activate Plk1 in mitosis. In addition, a fraction of Bora is retained in mitosis, which is essential for continued Aurora-A dependent T210 phosphorylation of Plk1. We find that once Plk1 is activated, minimal amounts of the Bora/Aurora-A complex are sufficient to sustain Plk1 activity. Thus, the activation of Plk1 by Aurora-A may function as a bistable switch; highly sensitive to inhibition of Aurora-A in its initial activation, but refractory to fluctuations in Aurora-A activity once Plk1 is fully activated. This provides a cell with robust Plk1 activity once it has committed to mitosis.
Introduction

Polo-like kinase 1 (Plk1) is a key regulator of several important cell cycle-associated processes, such as centrosome maturation, spindle assembly, sister chromatid cohesion, cytokinesis and recovery from a DNA damage-induced arrest (Bruinsma et al., 2012). The distinct functions of Plk1 are controlled at multiple levels, through control of its expression, localization and activity (Archambault and Glover, 2009; Bruinsma et al., 2012). Expression of Plk1 is highly cell cycle-regulated; its expression is first induced in G2 and peaks during the early stages of mitosis (Uchiumi et al., 1997). Upon exit from mitosis, Plk1 is degraded by the anaphase promoting complex/cyclosome (APC/C) (Fang et al., 1998; Lindon and Pines, 2004). Plk1 has been described to be recruited to the centrosomes in G2, as well as to spindle poles and kinetochores during mitosis (Arnaud et al., 1998; Golsteyn et al., 1995; Kang et al., 2006). Spatial control of Plk1 function is in part mediated by the conserved C-terminal polo-box domain (PBD). This domain can recognize phosphorylated threonine or serine residues which are created by other kinases such as Cdk1 (Elia et al., 2003a; Elia et al., 2003b). As such, spatiotemporal control of Plk1 functions is under the influence of other kinases that can prime distinct Plk1 substrates for recognition and eventual phosphorylation by Plk1. Besides controlling substrate specificity, the PBD directs recruitment of Plk1 to distinct subcellular sites, such as the kinetochores in early mitosis and the spindle midzone in late mitosis (Bruinsma et al., 2012). This level of spatio-temporal control is essential to allow Plk1 to execute multiple roles during cell division in an orderly manner.

Part of this intricate regulation is the activation of Plk1. This event is dependent on phosphorylation of a conserved threonine residue (T210) in the T-loop of its kinase
domain (Jang et al., 2002). Phosphorylation of this residue starts in G2, when Plk1 activity gradually increases until it reaches its full activity when cells enter mitosis. The initial phosphorylation of T210 in G2 is mediated by the kinase Aurora-A (Macurek et al., 2008; Seki et al., 2008b). Efficient phosphorylation of T210 in G2 critically depends on Bora, a co-activator of Aurora A, known to bind to Plk1 (Hutterer et al., 2006; Macurek et al., 2008). Once cells enter mitosis, Bora is degraded in a Plk1-dependent manner and Aurora-A can subsequently interact with other co-activators, such as TPX2, which directs Aurora-A to the mitotic spindle (Chan et al., 2008; Eyers et al., 2003; Seki et al., 2008a). Mitotic activity of Plk1 is strictly dependent on continued phosphorylation of T210 (Jang et al., 2002; Macurek et al., 2008; Seki et al., 2008b). However, it is unclear how T210-phosphorylation is maintained once Bora is degraded and which kinase is responsible for the continued T210-phosphorylation of Plk1 in mitosis. In yeast, Cdk1 has been shown to activate Cdc5 (the sole Polo homolog in budding yeast) through phosphorylation of a T-loop residue corresponding to human T214 (Mortensen et al., 2005). However, it has recently been shown that another member of the Aurora kinase family, Aurora B can phosphorylate Polo in Drosophila and can also contribute to T210 phosphorylation during mitosis in mammalian cells (Carmena et al., 2012). Aurora B is part of the chromosomal passenger complex and has several roles during mitosis, including error correction of microtubule attachments at the kinetochores and cytokinesis (van der Waal et al., 2012). Its localization and similarity to Aurora A makes this kinase a very likely suspect in the controlling Plk1 activity during mitosis. Here we have investigated the control of T210 phosphorylation of Plk1 during mitosis. We find that Aurora-B does not have a major contribution to overall mitotic T210
phosphorylation in human cells. In contrast, we provide evidence that phosphorylation of T210 and activation of Plk1 in mitosis is still dependent on Bora/Aurora-A. We show that a small amount of residual activity of Bora/Aurora-A is sufficient to keep Plk1 active. Depletion of both Bora and Aurora-A is required to inactivate Plk1 in mitosis. These data show that in contrast to G2, mitotic activation of Plk1 is extremely robust that can be sustained at very low levels with severely reduced levels and activity of the Bora/Aurora-A complex. This suggests that the activation of Plk1 by Bora/Aurora-A might function as a bistable switch, a feature described for the mitotic kinase Cyclin B/Cdk1 and an integral mode of cell cycle control at the onset of mitosis (Medema and Lindqvist, 2011).

Results

Analysis of T210-phosphorylation in mitosis

Plk1 is first activated in G2 and reaches maximal activity in mitosis, coincident with the kinetics of T210 phosphorylation (Macurek et al., 2009). To further characterize the spatio-temporal pattern of T210 phosphorylation, we made use of two phospho-specific antibodies raised against a phosphorylated peptide encompassing the T210 region of Plk1; ab39068 from Abcam and bd558400 from BD Biosciences (Carmena et al., 2012; Macurek et al., 2009). To obtain mitotic cells, we subjected synchronized cultures of U2OS osteosarcoma cells to a mitotic shake-off, which resulted in samples that contained well over 90% mitotic cells (Fig.1A). We identified a clear band at approximately 70 kDa with both antibodies that migrated at the same height as total Plk1 and disappeared after depletion of Plk1 by RNAi (Fig.1B). In addition, the same band was also recognized by both antibodies in immunoprecipitates of endogenous Plk1. Further, we immunopurified
myc-tagged wild-type and T210A-mutated Plk1 from mitotic cells and tested the reactivity of the pT210 antibodies. As expected both antibodies did recognize the wild-type Plk1 but failed to recognize the T210A mutant, thus confirming the specificity towards phosphorylated T210 of Plk1 on western blot (figure 1C). We next proceeded to look at the specificity of phospho-T210 staining at centrosomes and kinetochores, where Plk1 is located in mitosis. Consistent with our previous report, the bd558400 antibody showed a clear signal at the centrosomes in mitotic cells and depletion of Plk1 by siRNA resulted in a clear reduction of the signal (Fig.1D,E). In contrast, the centrosomal staining was very weak or even absent with ab39068. In addition, both antibodies clearly stained the kinetochores (Fig.1D). However, kinetochore staining with both pT210 antibodies persisted after depletion of Plk1 by RNAi, while the total level of Plk1 at kinetochores was clearly reduced (Fig.1D,F and Supplementary Fig.1A,B). In the case of ab39068 we even observed an increase in the signal at kinetochores when Plk1 was depleted. These observations demonstrate that both antibodies recognize the appropriate epitope on western blot and that bd558400 specifically recognizes T210-phosphorylated Plk1 at mitotic centrosomes. However, our data suggests that the kinetochore staining seen in human cells with both antibodies is possibly an off-target signal and hence demands caution when studying this phosphorylation event in mitosis.

**Aurora-A regulates T210 phosphorylation of Plk1 in mitosis**

We and others have previously shown that the initial activation of Plk1 in G2 is dependent on Aurora-A and Bora (Macurek et al., 2008; Seki et al., 2008b). Nonetheless, Plk1 activity in mitosis appears to be relatively refractory to inhibition of Aurora-A
(Macurek et al., 2008). These observations suggest that Plk1 activation is not exclusively dependent on Aurora-A, implying that another kinase can possibly phosphorylate T210 at later stages of the cell cycle.

Aurora-A and Plk1 are recruited to the centrosomes in G2, and it has been suggested that the centrosomes might be the site where Plk1 is first activated during G2 (Bruinsma et al., 2012). In addition, Plk1 is also recruited to kinetochores (Arnaud et al., 1998; Kang et al., 2006) and therefore we reasoned that a kinetochore-localized kinase might promote the second phase of T210 phosphorylation around the time of mitotic entry. A logical candidate is the Aurora kinase family member Aurora-B, known to phosphorylate several proteins at kinetochores in mitosis (Ruchaud et al., 2007). Interestingly, a recent report indeed showed that Aurora-B is the kinase responsible for T-loop phosphorylation of Polo kinase at kinetochores in Drosophila and that Aurora-B could also contribute to T210 phosphorylation in human cells (Carmena et al., 2012). Therefore we investigated the possibility of sequential activation of Plk1 by Aurora-A and Aurora-B.

To study the requirements for T210 phosphorylation in mitotic cells, we first synchronized cells in mitosis and subsequently treated them with inhibitors of the different Aurora kinases with distinct selectivity for Aurora-A versus Aurora-B. When Aurora-A activity was selectively inhibited using the pharmacological inhibitor MLN 8054 (Manfredi et al., 2007) we observed a partial inhibition of T210 phosphorylation in mitotic cells, while Plk1 levels were not affected (Fig.2A,C). Interestingly, inhibition of Aurora-B with the pharmacological inhibitor ZM 447439 did not result in a significant reduction in the phosphorylation of T210 in mitosis. Nonetheless, both inhibitors did inhibit their respective targets, as demonstrated by a reduction in the autophosphorylation
site at T288 on Aurora-A and by a reduction in Aurora-B kinase substrate phosphorylation on histone H3 at S10 (Fig.2A). Moreover, addition of ZM 447439 caused a complete override of a taxol-induced mitotic arrest (Supplementary Fig.3A), indicating that Aurora-B function was effectively inhibited in these cells. Dual inhibition of both Aurora-A and Aurora-B in mitotic cells reduced T210-phosphorylation to a level similar as seen after single inhibition of Aurora-A, providing further evidence that Aurora-B does not play a major role in T210-phosphorylation in human mitotic cells. To confirm the effects we observed with the Aurora kinase inhibitors we next depleted Aurora-A, Aurora-B or a combination of both by RNAi and examined the contribution of Aurora-A and Aurora-B to T210 phosphorylation in mitosis. Consistent with the data obtained with the selective small molecule inhibitors, we observed a decrease in phospho-T210 upon depletion of Aurora-A (Fig.2B,C). Depletion of Aurora-B did not seem to selectively reduce T210-phosphorylation. These observations imply that the contribution of Aurora-B to overall T210 phosphorylation of Plk1 in mitosis in human cells is very minimal. This is not in line with previous observations that Aurora-B depletion can lead to a reduction in T210-phosphorylated Plk1 at kinetochores in mitosis in human cells (Carmena et al., 2012). However, it should be noted that ab39068 was used to determine the level of T210-phosphorylated Plk1 at kinetochores, which we find to recognize an epitope that might not correspond to T210-phosphorylated Plk1 (see Fig.1). We therefore wondered if we could confirm that the presence of this signal is sensitive to inhibition of Aurora-B. Indeed, upon immunofluorescence staining we could clearly observe a signal at the kinetochores that overlapped with CREST-staining when using an anti-Plk1 or any one of the two anti-phospho-T210 antibodies (Supplementary Fig.1C-F). Treatment with
the Aurora kinase inhibitors clearly reduced the signal seen with the two anti-phospho-T210 antibodies (Supplementary Fig.1C-F). This, taken together with our observations shown in Fig.1F, indicates that these antibodies recognize an epitope at the kinetochores that is sensitive to Aurora kinase inhibition, but which might not correspond to T210-phosphorylated Plk1. In line with this we do not see a reduction in Plk1 T210-phosphorylation on western blot. Since bd558400 does recognize T210-phosphorylated Plk1 at the centrosomes, we quantified the centrosomal signal to see how this was affected by inhibition of different Aurora kinases. In accordance with our results on western blot we observed that inhibition of Aurora-A led to a decrease of phospho-T210 at centrosomes while the level of total Plk1 remained equal (Fig.2D). We conclude that overall mitotic T210-phosphorylation in human cells primarily depends on the activity of Aurora-A, but not Aurora B. Nonetheless, a significant amount of Plk1 remains phosphorylated on the T210 site when Aurora-A is inhibited with MLN 8054 in mitotic cells.

**Aurora-A dependent regulation of Plk1 activity in mitosis**

Although it has been well established that phosphorylation of T210 is associated with activation of Plk1 it is not a direct readout of Plk1 activity itself. Based on the data obtained thus far, we cannot exclude that Aurora-B can affect Plk1 activity in mitosis through a phospho-T210-independent mechanism. To address this issue we directly monitored global Plk1 activity in living cells using a Plk1-specific FRET-based biosensor (Macurek et al., 2008). Phosphorylation of this biosensor by Plk1 causes a change in the CFP/YFP emission ratio, which can be monitored by time-lapse imaging of U2OS cells.
stably expressing the biosensor. Plk1 activation starts approximately 5 to 6 hours before mitosis (Macurek et al., 2008) and inhibition of Plk1 with the selective inhibitor BI 2536 (Lénárt et al., 2007) completely abolishes the shift in FRET-ratio, except for a small change that occurs when cells enter mitosis (Fig.3A). Using the selective Mps1 kinase inhibitor Reversine (Santaguida et al., 2010), we were able to demonstrate that this latter change depends on Mps1, consistent with observations that Mps1 can phosphorylate targets that contain a consensus site for phosphorylation that overlaps the Plk1 consensus (Supplementary Fig.2) (Dou et al., 2011). In order to exclude that inhibition of Aurora-B has an effect on Plk1 activation during mitotic entry we quantified the CFP/YFP ratios of cells entering mitosis in the presence of the indicated inhibitors. As was shown before, the initial activation of Plk1 is dependent on Aurora-A (Macurek et al., 2008). However, when Aurora-B is inhibited, the activation curve of Plk1 completely follows the DMSO control (Fig.3B). The combination of Aurora-A and Aurora-B inhibition shows a similar effect on Plk1 activity as Aurora-A inhibition alone. These results show that Aurora-B does not influence Plk1 activation at the G2/M transition. Next we depleted Aurora-A and Aurora-B and monitored the activation curve (Fig.3C). Again we found that Aurora-A depletion led to a delay of initial Plk1 activation, an effect we do not observe when Aurora-B is depleted. Overall these results show that it is Aurora-A and not Aurora-B that controls the initial activation of Plk1 during G2.

To monitor the contribution of Aurora A and/or B to Plk1 activation in mitosis, we synchronized cells in mitosis with nocodazole. CFP/YFP emission ratios were determined by time-lapse imaging. Similar to monitoring Plk1 activity during mitotic entry, we could decrease the CFP/YFP-ratio by adding BI 2536, showing that we can also use the FRET-
based biosensor to monitor Plk1 activity in mitotic cells (Fig.3D). Next we tested the contribution of different kinases to Plk1 activation in mitosis by adding the Aurora-A and Aurora-B specific inhibitors MLN 8054 and ZM 447439 during imaging (Fig.3E). In addition, the proteasomal inhibitor MG-132 was added to prevent premature mitotic exit that can be induced by inhibition of Aurora activity (Ditchfield et al., 2003). The CFP/YFP emission ratio in DMSO-treated cells remained constant for the duration of the experiment (Fig.3E). In contrast, inhibition of Aurora-A led to a small reduction in Plk1 activity in mitosis (Fig.3E). This partial decrease in Plk1 activity induced by inhibition of Aurora kinases was highly reproducible, although the extent of inhibition was somewhat variable, ranging from 10-30% of the initial activity. However, we never observed inhibition of more than 30% of the total Plk1 activity. In accordance with our earlier results, Aurora-B inhibition did not induce any change in Plk1 activity. In addition, when cells were depleted of Aurora kinases we also did not observe complete inhibition of Plk1 activation in any condition (Fig.3F). These results show that Aurora-A is not only responsible for initial activation of Plk1 but also contributes to Plk1 activation in mitosis. In addition, although our data do not exclude a role for Aurora-B, they clearly indicate that Aurora-B does not provide a major contribution to global Plk1 activation in mitosis.

**Complete inhibition of Aurora-A prevents Plk1 activation**

It has been well established that positive feedback loops can lead to very rapid activation of mitotic kinases and induce a huge increase in protein phosphorylation in mitosis (Medema and Lindqvist, 2011). Since RNAi-mediated depletion or pharmacological inhibition of kinases is never 100% complete we reasoned that a small pool of active
Aurora-A might be enough to sustain Plk1 activation after entry into mitosis. Indeed, when depleting Aurora-A we detected a small amount of residual Aurora-A (Fig.4A). In an attempt to achieve complete inhibition of Aurora-A activity we first depleted the protein through RNAi and then added MLN 8054 to inhibit the remaining protein and monitored Plk1 activity in mitosis. Indeed, while we did detect residual T210-phosphorylated Plk1 in cells depleted of Aurora-A or treated with MLN 8054, we could not detect the band corresponding to T210-phosphorylated Plk1 in mitotic cells depleted of Aurora-A and simultaneously treated with MLN 8054 (Fig.4A). Total levels of Plk1 were largely unaffected in all samples (Fig.4A). Next we monitored the CFP/YFP emission ratio in these conditions to see if full inhibition of Aurora-A affects Plk1 activity to a greater extent. As seen before, inhibition or depletion of Aurora-A caused a delay in Plk1 activation in G2. However, combination of depletion and inhibition resulted in full inhibition of Plk1 activation, similar to what we observed after depletion or inhibition of Plk1 itself (Fig.4B). Next, we tested if we could fully inhibit Plk1 activation in mitotic cells already depleted of Aurora-A by the addition of MLN8054. As we observed before, depletion of Aurora-A or inhibition with MLN 8054 produced a reduction of mitotic Plk1 activity of approximately 10-30% (Fig.4C). Interestingly, addition of MLN8054 to cells already depleted of most of Aurora-A showed a marked decrease of Plk1 activity. After 4 hours of MLN 8054 treatment Plk1 activity was reduced to levels close to that in Plk1-depleted or inhibited cells, indicating a near complete shut-down of Plk1 activity in mitosis when depletion and inhibition of Aurora-A are combined. These observations indicate that effective inhibition of Aurora-A can fully prevent activation of Plk1. In addition, inactivation of Plk1 in mitosis can be
achieved by a similar strategy, indicating that the major kinase responsible for Plk1 activation in mitosis is Aurora-A.

**Aurora-A and Bora regulate activity in mitosis**

Since we find that Aurora-A is the main activator of Plk1 and Aurora-A requires several different co-activators to exert its functions (Kufer et al., 2002; Macurek et al., 2008; Seki et al., 2008b), we wondered which co-activator would mediate the phosphorylation of Plk1 by Aurora-A. The best characterized co-activators are Bora and TPX2 so we decided to take a closer look at the role of these two proteins. In order to study Bora we generated an antibody to detect the endogenous protein. This antibody clearly recognized Bora in G2 and mitosis (Fig.5A). Consistent with previous literature we observed a clear mobility shift in Bora retrieved from mitotic cells, as well as a reduction of total levels as Bora is being degraded in a β-TrCP-dependent manner (Chan et al., 2008; Seki et al., 2008a). However, there is still a small amount of Bora present in mitotic cells, and depletion of Bora through RNAi shows a further reduction of mitotic Bora (Fig.5A). We next looked at phosphorylated T210 in mitotic cells after Bora depletion. We observed a marked decrease in T210-phosphorylated Plk1 which we could even further reduce by treatment with MLN 8054 indicating that interfering with the Bora/Aurora-A complex severely perturbs mitotic T210 phosphorylation (Fig.5B). When co-depleting Aurora-A and Bora we saw a similar effect, as phosphorylated-T210 was undetectable in the double knock down (Fig.5C). Interestingly, Bora levels were increased in the Aurora-A depleted samples, possibly as a consequence of reduced Plk1 activity during G2. This could provide a mechanism to cope with reduced Aurora-A activity. Since T210
phosphorylation leads to activation of Plk1 we monitored activity with our FRET-based bio-sensor. Depletion of Bora led to partial inhibition of Plk1 activation (Fig.5D). However, when Bora depletion was combined with Aurora-A inhibition through MLN 8054 the activation curve completely mimicked that of Plk1-depleted cells. Cells in which Aurora-A and Bora were co-depleted showed a similar effect on Plk1 activity (Fig.5E). Next we monitored if we could also shut down Plk1 activity in mitotic cells. Indeed, we observed that mitotic Plk1 activity in Bora-depleted cells was also reduced when compared to control cells (Fig.5F). When adding MLN 8054 to these Bora-depleted cells, Plk1 activity was reduced to levels almost overlapping with Plk1-depleted mitotic cells. In addition, cells entering mitosis after co-depletion of Aurora-A and Bora also displayed a clear reduction of Plk1 activity, albeit not complete. To test if Aurora-B is responsible for this small pool of remaining Plk1 activity we inhibited both Aurora-A and Aurora-B in Bora-depleted cells. However further inhibition of Aurora-A or -B did not lower the activity any further (Supplementary Fig.3). Also, depletion of the other Aurora-A co-factor TPX2 did not result in loss of T210-phosphorylated Plk1 or a substantial reduction in Plk1 activity (Supplementary Fig.4). Together these results suggest that the small pool of Bora that is left in mitosis is sufficient to mediate the Aurora-A-dependent phosphorylation and activation of Plk1. It is only when this small pool of Bora is also absent that mitotic Plk1 T210 phosphorylation and activity is lost.

**Aurora-A and Bora depletion produce T210-dependent phenotypes of Plk1**

As we observed that co-depletion of Aurora-A and Bora lead to inactivation of Plk1 and loss of phosphorylated T210 we reasoned we should also be able to observe phenotypes
associated with inactive Plk1. The most prominent phenotype of Plk1 depletion is a mitotic arrest due to the formation of monopolar spindles (Sunkel and Glover, 1988). We therefore determined the amount of mitotic cells 48 hours after transfection. While asynchronously growing control cells have around 2% of mitotic cells, depletion of Plk1 elevates this amount to approximately 30% (Fig.6A). Single depletions of Aurora-A and Bora did lead to an increase in mitotic indices, but this was not more than 5%. However, co-depletion resulted in 20% mitotic cells, indicating that co-depletion of Aurora-A and Bora leads to a prominent mitotic arrest. We next scored monopolar spindles among the mitotic cells present in the different conditions (Fig.6B). While monopolar spindles only occurred occasionally in the control population, Plk1 depletion results in 60% of the mitotic cells displaying this phenotype (Fig.6C). Single depletions mildly elevated the amount of monopolar spindles, but co-depletion of Aurora-A and Bora resulted in a similar fraction of mitotic cells arrested with a monopolar spindle when compared to Plk1. To test if monopolar spindle formation was indeed due to the loss of T210 phosphorylation we proceeded to rescue this monopolar spindle phenotype with a phospho-mimicking constitutive active Plk1-T210D mutant. We made use of a tetracyclin-inducible cell line resistant to Plk1 siRNA (Macurek et al., 2009). In this way we are able to deplete endogenous Plk1, Aurora-A and Bora and reconstitute a Plk1 mutant that is active regardless of its upstream activator (Fig.6D). We then scored the fraction of mitotic cells which display a monopolar spindle phenotype in both the absence and presence of this constitutive active Plk1 mutant. In our control samples we saw a slight increase in monopolar spindles upon expression of the T210D mutant. This might be attributed to the fact that a constitutive active mutant of Plk1 lacks its refined
regulation of activity in mitosis and displays a mitotic delay by itself (van de Weerdt et al., 2005). However, we observed that we could reduce the fraction of monopolar spindles in both the Plk1-depleted and Aurora-A/Bora-depleted cells (Fig. 6E). We were unable to completely rescue the phenotype, but this might be due to additional functions of Bora/Aurora-A. However, this observation shows that the monopolar spindles observed in Bora/Aurora-A deficient cells are at least in part the result of aberrant Plk1 phosphorylation at T210. Together these results demonstrate that Aurora-A and Bora act as the predominant activators of Plk1 in mitosis.
Discussion

Plk1 is activated during the G2 phase of the cell cycle by Aurora-A together with its cofactor Bora (Macurek et al., 2008; Seki et al., 2008b). However, Bora was shown to be degraded upon entry into mitosis (Chan et al., 2008; Seki et al., 2008a), raising the question how Plk1 activation is sustained once a cell has entered mitosis. Here, we show that Bora and Aurora-A are required for the maintenance of Plk1 activity in mitosis. We show that a fraction of the total pool of Bora is retained in mitotic cells, and that this fraction is crucial for continued Plk1 activation. Also, we show that very little Aurora-A activity is sufficient to maintain Plk1 in its active state in mitotic cells, and that Plk1 is rather refractory to dephosphorylation and inactivation once cells have entered mitosis. Taken together, these data point to a complex of Bora/Aurora-A as the primary activator of Plk1, both in interphase, as well as in mitosis.

Our data show that the contribution of Aurora B to T210-phosphorylation of Plk1 is very limited at best. Our data cannot rule out the possibility that Aurora B can phosphorylate a small pool of Plk1 in mitosis at specific locations or during a limited period of time. The recent work by Carmena et al. indeed suggests that Aurora-B-dependent activation of Polo in Drosophila is restricted to a very short period in mitosis. However, unlike Carmena et al., we find that the kinetochore signal in human cells does not disappear when Plk1 is depleted, indicating that this signal corresponds to a different epitope. It is of course possible that a small amount of Plk1 that remains at kinetochores after RNAi is highly phosphorylated, but this is not confirmed by immunoblots. These contrasting observations could stem from the use of different batches of phospho-specific antibody. Indeed, Abcam has temporarily discontinued its antibody in the past, claiming variability
between batches (V. Archambault, personal communication). Thus, it seems most plausible that Aurora-A is the primary kinase responsible for Plk1 activation in mitosis. But is the Bora/Aurora-A complex the sole activator of Plk1? While our data cannot unequivocally prove this, we feel there are good reasons to conclude that it is responsible for the majority, if not all, of the T210-phosphorylation in mitosis. For one, siRNA-mediated depletion of Bora combined with depletion or inhibition of Aurora-A leads to complete loss of Plk1-T210-phosphorylation. Similarly, depletion of Aurora-A, combined with pharmacological inhibition of the residual Aurora-A, leads to an almost complete loss of Plk1-T210-phosphorylation. But if Bora/Aurora-A is the major activator of Plk1, also in mitosis, then why is mitotic Plk1 activity refractory to the addition of the selective Aurora-A inhibitor MLN 8054. We envision two, not mutually exclusive scenarios. First, the Bora/Aurora-A complex might be in direct contact with Plk1, since Bora is known to bind Plk1 directly (Chan et al., 2008; Seki et al., 2008a). In this way it would be very difficult, if not impossible, for an ATP-competitive compound such as MLN 8054, to completely inhibit target phosphorylation. Whenever MLN 8054 is exchanged for ATP in this complex, T210-phosphorylation could occur very rapidly, before the ATP is exchanged again for MLN 8054. Secondly, T210-dephosphorylation might be inhibited in mitotic cells. As a result, mitotic Plk1 activity would be relatively refractory to inhibition of its upstream kinase. Indeed, we find that it takes much more time to shut down Plk1 activity when Bora/Aurora-A are inhibited (approximately 4 hours) than when we directly inhibit Plk1 (approximately 30 minutes, Fig.4C). This suggests that the phosphatase responsible for T210-dephosphorylation is not fully active in mitosis. PP1 and its regulatory subunit MYPT1 have been shown to dephosphorylate T210 in mitotic
cells (Yamashiro et al., 2008). Plk1 was recently shown to affect MYPT1 activity through optineurin, uncovering a potential positive feedback loop (Kachaner et al., 2012). Further work will be required to resolve how T210 dephosphorylation is controlled at the different stages of mitosis.

Several other kinases have been suggested to phosphorylate T210 on Plk1, such as the Ste20-like kinases SLK and LOK (Ellinger-Ziegelbauer et al., 2000; Walter et al., 2003). Both these kinases are homologs of the Xenopus xPlkk1, which is known to function in a positive feedback loop with Plx1, the Xenopus homolog of Plk1 (Erikson et al., 2004). siRNA-mediated depletion of SLK and LOK did not result in loss of T210-phosphorylation, either in interphase or mitosis (data not shown). This might indicate that their effect might be marginal or only exerted in specific situation such as the cell cycle of lymphocytes (Walter et al., 2003). More recently, Aurora-B was implicated in the activation of Polo/Plk1 in mitotic cells, both in Drosophila as well as in human cells (Carmena et al., 2012). From the data presented it seems very plausible that Aurora-B is at least partially responsible for the activation of Polo in Drosophila. Interestingly, we do not find any support for a role for Aurora-B in global Plk1 phosphorylation and activation in human mitotic cells. However, Carmena et al postulate that phosphorylation of Polo by Aurora-B occurs at a very distinctive time window in early mitosis at the kinetochores. Our FRET-based biosensor can rapidly diffuse in cells and this might therefore not be the right tool to pick up such restricted spatio-temporal regulation. Thus it remains a distinct possibility that Aurora-B has a very specific localized role in controlling Plk1 activation at the kinetochores while the majority of Plk1 is controlled by Aurora-A.
Our findings that sustained Plk1 activation in mitosis relies on small amounts of Aurora-A/Bora and that Plk1 inactivation occurs very slowly suggest that the Aurora-A/Bora-mediated Plk1 activation could act as a bistable system. Bistable systems consist of interlinked feedback loops that render cell cycle transition irreversible as switching on occurs at a high threshold, but once switched on, these systems enforce their own activation making it difficult to switch them off (Lindqvist et al., 2009). This type of regulation is known to play a role in regulating Cyclin B/Cdk1 activation, a process in which Plk1 itself is also involved (Lindqvist et al., 2009). Such a mode of action for the Aurora-A-Bora-Plk1 activation loop could provide an important contribution to the all-or-nothing decision of cells on the brink of mitosis.

**Materials and Methods**

**Antibodies, siRNAs and reagents.** Phosphospecific Plk1-pT210 was obtained from Abcam and BD biosciences. Anti-Plk1 was previously described (Macurek et al., 2008). Anti-Plk1 (F8), anti-TPX2 (H300), γ-tubulin (H183) and anti-Actin (I-19) were from Santa Cruz. Anti-Aurora-A and anti-Aurora-A-pT288 were from Cell Signaling. MPM-2 and H3-pS10 were from Millipore. Anti-α-tubulin was from Sigma, CREST from Cortex Biochem, anti-Aurora-B from (transduction) and anti-Myc (clone 9E10) from Covance. The rabbit anti-Bora antibody was raised against recombinant His-tagged human Bora comprising amino acid residues 79 to 559. The corresponding cDNA fragment was cloned in pET-28a vector (Novagen) and the recombinant protein fused to a histidine tag was purified using Ni-NTA resin (Qiagen) following the manufacturer's instructions before immunization. Secondary antibodies Alexa-488, Alexa-568 and Alexa-633 from
Molecular Probes and horseradish peroxidase coupled secondary antibodies from Dako. ON-TARGETplus smart pools targeting luciferase or GAPDH (as a negative controls) and Aurora-A and Bora were from Dharmacon. Short interfering RNA's targeting TPX2 were described previously (Macurek et al., 2008), Oligo's targeting Plk1 were obtained from Ambion, GCUCUGUGAUACACACUGUG and an siRNA for which the Plk1-T210D mutant is insensitive was based on the pSuper described previously (van Vugt et al., 2004) CGGCAGCGUGCGATCAAC and siAurora-B was obtained from Dharmacon, GGAAGAAGGGACAUCCCUAA. The following drugs were used: BI 2536 (100nM, Boehringer Ingelheim Pharma), MLN8054 (1 µM, Millennium Pharmaceuticals), ZM447439 (2 µM, AstraZeneca), MG132 (1 µM, Sigma), Nocodazole (250 ng/ml, Sigma), Reversine (250 nM, Roche) and Tetracyclin (1 µg/ml, Sigma).

**Cell culture and transfections.** Human osteosarcoma U2OS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% FCS (Lonza), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cell lines expressing myc tagged PLK1 wildtype, Plk1-T210A and Plk1-T210D mutants under the control of tetracycline-inducible promoter were described previously (Macurek et al., 2008). U2OS cells stably expressing the FRET-based biosensor were generated by transfection, selection of stable clones by zeocin (400 mg/ml, Invitrogen) treatment followed by clonal selection. Stable clones were grown in media containing Tet system approved fetal bovine serum (Lonza). For induction of expression, cells were treated for indicated times with tetracycline (1 mg/ml). Transfections of siRNA's were done using Lipofectamine RNAiMAX reagent (Life Technologies) using the manufacturers instructions.
**Cell synchronization.** Mitotic cells were obtained through synchronization by thymidine (2.5 mM, 24 h) treatment followed by a 16 hour release into Nocodazole (250ng/ml). For reconstitution assays, expression of PLK1-T210D was induced by addition of tetracycline (1 mg/ml). To determine the mitotic index, cells were harvested and fixed in ice-cold ethanol (70%). Cells were stained with MPM-2 and alexa488-conjugated secondary antibodies and counterstained with propidium-iodide. Cell cycle distribution was determined by flow cytometry counting $10^4$ events as described.

**Immunoprecipitations and western blotting.** Cells were extracted in lysis buffer (50 mM HEPES, pH 7.4, 1 mM MgCl2, 1 mM EGTA, 1% NP-40, 1 mM NaF, 1 mM Na3VO4, protease inhibitors), normalized for total protein content and incubated overnight (15 h) at 4°C with polyclonal anti-PLK1 antibody immobilized on protein A (BioRad). Immunocomplexes were extensively washed and analysed by immunoblotting. Samples for Western blotting were either prepared in lysis buffer or Laemmli sample buffer and analyzed by immunoblotting.

**Immunofluorescence and FRET analysis.** Fixation and antibody staining for immunofluorescence were performed as described (Macurek et al., 2008). Double staining of Plk1 and phosphorylated T210 was done sequentially with the phosphorylated T210 antibody that was prelabeled with alexa 488. Images show maximum intensity projections of deconvolved Z-stacks, acquired on a Deltavision RT imaging system using 0.95NA 40x objectives. Quantification of immunofluorescence was performed as described, measuring the centrosomal and kinetochoral maximum intensity. The FRET-based probe for monitoring PLK1 activity has been described previously (Macurek et al., 2008). The CFP/YFP emission ratio after CFP excitation of U2OS cells stably
expressing the FRET-based biosensor, was monitored on a Deltavision Elite imaging system, using a 20x 0.75NA objective. Images were acquired every 10 or 20 min. The images were processed with ImageJ using the Ratio Plus plug-in (http://rsb.info.nih.gov/ij/).

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References


Figure 1. Phosphorylation of T210 on Plk1 in mitosis.

A) U2OS cells were treated for 24 hours with thymidine and subsequently released for 16 hours in the presence of nocodazole. Mitotic cells were harvested by mitotic shake-off. B) Plk1 was immunoprecipitated out of U2OS cell lysates treated as in A and blotted for total levels of Plk1 and for phosphorylated T210. C) Myc-tagged Plk1 wildtype and T210A were immunoprecipitated form lysates synchronized with thymidine and nocodazole and blotted with the indicated antibodies. D) Cells were treated with siRNA's targeting Plk1. After synchronization by thymidine and nocodazole, samples were stained for total levels of Plk1 and phosphorylated T210. Scale bars: 5 μm. E) Quantification of centrosomal stainings of cells depicted in D. The fluorescence intensity is depicted relative to the γ-tubulin staining to correct for centrosome size. F) Quantification of kinetochore stainings of cells depicted in D. The fluorescence intensity is depicted relative to CREST staining to correct for kinetochore size.

Figure 2. Aurora-A affects T210 phosphorylation in mitosis.

A) Cells were treated as in 1A. The indicated inhibitors were added for 90 minutes together with the proteasomal inhibitor MG-132 to prevent premature mitotic exit. The concentrations used were 1 μM MLN 8054 and 2μM ZM 447439. B) Cells were treated with siRNA's targeting the indicated genes then treated as in 1A. C) Bands of T210-phosphorylated Plk1 and total Plk1 from figures 1A,B were quantified. A ratio of phosphorylated T210 over total Plk1 was calculated and an average of three independent experiments was plotted. Error bars represent the SD of three independent experiments.
D) Quantification of centrosomal stainings of cells treated as in A. The fluorescence intensity is depicted relative to the $\gamma$-tubulin staining to correct for centrosome size.

**Figure 3. Aurora-A affects Plk1 activity in mitosis.**

A) Stills from a movie showing false color-coded CFP/YFP emission ratios. The stills show control and BI 2536-treated cells expressing the FRET-based biosensor for Plk1 activity entering mitosis. BI2536 was used at a concentration of 100 nM. Scale bars: 10 $\mu$m. B) Quantifications of CFP/YFP emission ratios of cells entering mitosis in the presence of the indicated inhibitors. Error bars indicate the standard deviation of 10 individual cells. C) Quantifications of CFP/YFP emission ratios of cells entering mitosis treated with siRNA's targeting the indicated genes. D) Stills from a movie showing false color-coded CFP/YFP emission ratios. The stills show control and BI 2536-treated cells expressing the FRET-based biosensor for Plk1 activity while synchronized in mitosis by subsequent thymidine and nocodazole treatment. MG-132 was added to prevent premature mitotic exit. E) Quantifications of CFP/YFP emission ratios of cells synchronized in mitosis by nocodazole. The arrow indicates addition of the inhibitors and MG-132. F) Quantifications of CFP/YFP emission ratios of cells treated with siRNA's targeting the indicated genes and then synchronized in mitosis by nocodazole. The arrow indicates addition of BI 2536.

**Figure 4. Complete inhibition of Aurora-A abolishes T210 phosphorylation and Plk1 activation.**
A) Cells were treated with either control siRNA's or siRNA's targeting Aurora-A. After cells were synchronized in mitosis as in 2A, cells were treated with 1 µM MLN 8054 in the presence of MG-132 and harvested by mitotic shake-off. B) Quantifications of CFP/YFP emission ratios of cells entering mitosis treated with siRNA's targeting the indicated genes and indicated inhibitors. C) Quantifications of CFP/YFP emission ratios of cells treated with siRNA's targeting the indicated genes and then synchronized in mitosis by nocodazole. The arrow indicates addition of the indicated inhibitors.

**Figure 5. Aurora-A and Bora regulate activity in mitosis.** A) U2OS cells were transfected for 48 hours with siRNA's. For G2 samples U2OS cells were harvested 7 hours after thymidine release. Mitotic samples (M) were obtained by mitotic shake-off after overnight treatment with nocodazole. B) Cells were treated as in 4A. Asterisk indicates aspecific band. C) Cells were treated as in 2B. D) Quantifications of CFP/YFP emission ratios of cells entering mitosis transfected with the indicated siRNA's and in the presence of the indicated inhibitors. E) Quantifications of CFP/YFP emission ratios of cells entering mitosis transfected with the indicated siRNA's. F) Quantifications of CFP/YFP emission ratios of cells synchronized in mitosis by nocodazole. F) Quantifications of CFP/YFP emission ratios of cells treated with siRNA's targeting the indicated genes and then synchronized in mitosis by thymidine and nocodazole. The arrow indicates addition of the inhibitors and MG-132.

**Figure 6. Aurora-A and Bora depletion mediate T210 dependent phenotypes of Plk1.** A) U2OS cells were harvested 48 hours after transfection with siRNA's and
analyzed by FACS to determine the percentage mitotic cells based on MPM-2 staining. Error bars represent the standard deviation of 3 independent experiments. B) Examples of mitotic cells showing a bipolar spindle (upper panel) and a monopolar spindle (lower panel). Stainings are indicated in the lower panel. Scale bars: 5 µm. C) Cells were fixed and stained as in B, 48 hours after transfection with siRNA's. The percentage of mitotic cells displaying monopolar spindles was determined through microscopy. Error bars represent the standard deviation of 3 independent experiments. D) Inducible myc-Plk1-T210D U2TR cells were transfected with siRNA's for 48 hours and treated with or without tetracyclin to induce expression of the exogenous myc-tagged Plk1-T210D. For the last 16 hours cells were treated in nocodazole and finally harvested through mitotic shake-off. E) Inducible myc-Plk1-T210D U2TR cells were treated and fixed as in C. Error bars represent the standard deviation of 3 independent experiments.

**Supplementary figure 1. Kinetochore staining of antibodies targeted against phosphorylated Plk1 at T210 respond to Aurora inhibition.** A,B) Cells were treated as in 1A and double-labeled for Plk1 and phosphorylated-T210. The ratio of phosphorylated T210 over Plk1 signal at individual kinetochores was plotted. Scale bars: 5 µm. D-F) Cells were synchronized in mitosis by nocodazole and treated with the indicated inhibited for 90 minutes hours. After fixation the cells were stained for total Plk1 and phosphorylated T210. Scale bars: 5 µm.

**Supplementary figure 2. Mps1 is responsible for the aspecific FRET-based biosensor signal in mitosis.** Quantifications of CFP/YFP emission ratios of cells entering
mitosis in the presence of the indicated inhibitors. BI 2536 was used at a concentration of 100 nM and Reversine at a concentration of 250 nM). Error bars represent standard deviations of 10 individual cells.

**Supplementary figure 3. Aurora-B inhibition in mitotic cells**

A) U2OS cells were treated for 24 hours with thymidine and subsequently released for 16 hours in the presence of paclitaxel. Cells were subsequently treated for 2 hours with either DMSO as a control or ZM 447439 and harvested for FACS. B-C) Quantifications of CFP/YFP emission ratios of cells treated with siRNA's targeting the indicated genes and then synchronized in mitosis by thymidine and nocodazole. The arrow indicates addition of the inhibitors and MG-132.

**Supplementary figure 4. TPX2 has no effect Plk1-T210 phosphorylation and activity of Plk1.** A) U2OS cells were transfected with the indicated siRNA's, synchronized in mitosis by thymidine and nocodazole treatment. Mitotic cells were treated with 1 µM MLN 8054 for 90 minutes and harvested by mitotic shake-off. B) Quantifications of CFP/YFP emission ratios of cells entering mitosis transfected with the indicated siRNA's and in the presence or absence of 1 µM MLN 8054. C) Quantifications of CFP/YFP emission ratios of cells treated with siRNA's targeting the indicated genes and then synchronized in mitosis by thymidine and nocodazole. The arrow indicates addition of the inhibitors and MG-132.
Figure 2

(A) Western blot analysis of various proteins under different conditions.

(B) Western blot showing different targets under control and treated conditions.

(C) Graph showing the ratio of T210 phosphorylated Plk1 under different conditions.

(D) Bar graph and dot plot showing fluorescence intensity of Plk1 and Plk1-pT210 (bd558400) under various treatments.
Figure 4

A. Western blot images showing protein expression levels of pT210 (bd558400), Plk1, Aurora A-pT288, Aurora A, and Tubulin under different conditions.

B. Nuclear FRET-ratio during mitotic entry.

C. FRET-ratio during mitosis, showing the effect of different treatments over time relative to inhibitors (hours).