Pin1 acts as a negative regulator of the G2/M transition by interacting with the Aurora-A–Bora complex

Yu-Cheng Lee[1], Jenny Que[2], Yu-Chia Chen[3,4], Jen-Tai Lin[5,6], Yih-Cherng Liou[7], Po-Chi Liao[8], Yu-Peng Liu[6,9], Kuen-Haur Lee[10], Li-Ching Lin[2], Michael Hsiao[9], Liang-Yi Hung[11], Chi-Ying Huang[12] and Pei-Jung Lu[6,*]

[1]Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan
[2]Department of Radiation Oncology, Chi Mei Medical Center, Tainan 710, Taiwan
[3]Graduate Institute of Clinical Medical Sciences, College of Medicine, Chang Gung University, Taiwan
[4]Division of General Surgery, Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan
[5]Division of Urology, Department of Surgery, Kaohsiung Veterans General Hospital, Kaohsiung 813, Taiwan
[6]Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan
[7]Department of Biological Science, National University of Singapore, 117543, Singapore
[8]Department of Environment and Occupational Health, College of Medicine, National Cheng Kung University, Tainan 704, Taiwan
[9]Genomics Research Center, Academia Sinica, Taipei 115, Taiwan
[10]Graduate Institute of Cancer Biology and Drug Discovery, College of Medical Science and Technology, Taipei Medical University, Taipei 115, Taiwan
[11]Institute of Biosignal Transduction, College of Bioscience and Biotechnology, National Cheng Kung University, Tainan 704, Taiwan
[12]Institute of Biopharmaceutical Sciences, National Yang-Ming University, Taipei 115, Taiwan

*Author for correspondence (pjlu2190@mail.ncku.edu.tw)

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Summary

Pin1 was the first prolyl isomerase identified that is involved in cell division. The mechanism by which Pin1 acts as a negative regulator of mitotic activity in G2 phase remains unclear. Here, we found that Aurora A can interact with and phosphorylate Pin1 at Ser16, which suppresses the G2/M function of Pin1 by disrupting its binding ability and mitotic entry. Our results also show that phosphorylation of Bora at Ser274 and Ser278 is crucial for binding of Pin1. Through the interaction, Pin1 can alter the cytoplasmic translocation of Bora and promote premature degradation by β-TrCP, which results in a delay in mitotic entry. Together with the results that Pin1 protein levels do not significantly fluctuate during cell-cycle progression and Aurora A suppresses Pin1 G2/M function, our data demonstrate that a gain of Pin1 function can override the Aurora-A-mediated functional suppression of Pin1. Collectively, these results highlight the physiological significance of Aurora-A-mediated Pin1 Ser16 phosphorylation for mitotic entry and the suppression of Pin1 is functionally linked to the regulation of mitotic entry through the Aurora-A–Bora complex.

Key words: Aurora A, Pin1, G2/M negative regulator, Bora

Introduction

The prolyl isomerase Pin1 acts as pSer/Thr-Pro motif modulator and plays an important post-translational role in many biological processes that involve the 'double-check' mechanism (Lu and Zhou, 2007; Yeh and Means, 2007). Structurally and functionally, Pin1 has a striking substrate specificity for certain pSer/Thr-Pro bonds through its N-terminal WW domain. Pin1 isomerizes specific pSer/Thr-Pro motifs to regulate protein function, including their catalytic activity, interactions with other proteins, protein stability, subcellular localization and phosphorylation status, through conformational control by its C-terminal PPIase domain. Pin1 deregulation has also been implicated in a number of pathological conditions, including aging, immune responses, apoptosis, Alzheimer's disease and cancer (Driver and Lu, 2010; Lee et al., 2011; Liou et al., 2011; Yeh and Means, 2007). However, little is currently known regarding Pin1 regulation.

Pin1 was first identified as a cell-cycle regulator that regulates many cell-cycle-related proteins (Crenshaw et al., 1998; Lu et al., 1996). The temporal expression and degradation of cell-cycle regulatory proteins, such as Cdk–cyclin complexes, are key events in ordered cell-cycle progression (Vermeulen et al., 2003), whereas Pin1 protein levels do not significantly fluctuate during these processes (Shen et al., 1998). Because Pin1 depletion causes premature entry into mitosis and mitotic arrest, and the overexpression of Pin1 inhibits the G2/M transition (Crenshaw et al., 1998; Lu et al., 1996; Shen et al., 1998; Winkler et al., 2000), we hypothesized that post-translational modifications contribute to functional regulation of Pin1 and that Pin1 acts as a negative regulator of the G2/M transition by attenuating the activity of mitosis-promoting factors.

Mitosis is regulated by the activation of the Cyclin-B1–Cdk1 complex, which was first identified as a mitosis-promoting factor (MPF) in *Xenopus* oocytes (Ford, 1985). In G2 phase, Cdk1 is phosphorylated by the Wee1 and Myt1 kinases at Thr14 and Tyr15, thereby maintaining the Cyclin-B1–Cdk1 complex in an inactive state (Atherton-Fessler et al., 1993; Liu et al., 1997). As cells prepare to enter mitosis, Aurora A forms a complex with its cofactor Bora to phosphorylate Plk1 at Thr210. Plk1 activation activates Cdc25 and downregulates Myt1 and Wee1 through phosphorylation events (O'Farrell, 2001), leading to Cdk1 activation at the G2/M transition and promotion of mitotic

entry (Macůrek et al., 2008; Seki et al., 2008b). Bora expression is low during the G1/S boundary but increases in late S phase, accumulates in G2 and is then degraded through β-TrCP-dependent proteasomal degradation in mitosis (Chan et al., 2008; Seki et al., 2008a; Seki et al., 2008b). The physiological degradation of Bora is required for mitotic progression, and a large accumulation of Bora can prolong metaphase and delay anaphase (Seki et al., 2008a). Moreover, Bora degradation can initiate the formation of the Aurora-A–TPX2 complex, which targets Aurora A to the centrosomes and spindle poles, and redirects Aurora A kinase activity toward distinct substrates (Chan et al., 2008). Taken together, these data indicate that the tight regulation of Bora by control of its synthesis and degradation is crucial for entering and exiting mitosis.

We were intrigued by the similar phenomena of delayed mitotic entry in Pin1-overexpressing cells and Bora-depleted cells. In addition, accumulated Bora in mitotic cells can cause mitotic arrest, which is a similar outcome to that seen in Pin1-depleted cells. These results suggested that Pin1 and Bora are both involved in regulating mitotic entry and exit and that a crosstalk exists between Pin1 and Bora in cell cycle regulation. Furthermore, phosphorylation at Ser16 of Pin1 can negatively regulate both its function and localization (Lu et al., 2002). However, the biological significance of this Ser16 phosphorylation event, and the *in vivo* kinase responsible, remains unclear. In our present study, we have identified the mechanism underlying the function of Pin1 as a negative regulator of the G2/M transition through its interplay with the Aurora-A–Bora complex. Our findings reveal that at mitotic entry, the function of Pin1 at the G2/M phase is suppressed by Aurora A, which phosphorylates Pin1 at Ser16. Gain of Pin1 function through overexpression can override this Aurora-A-mediated suppression and lead to binding of Pin1 to phosphorylated Bora. This in turn alters the cytoplasmic translocation and promotes the premature degradation of Bora by β-TrCP. The resulting downregulation of Bora prevents it from forming complexes with Aurora A, which are required to activate Plk1, and thereby delays mitotic entry.

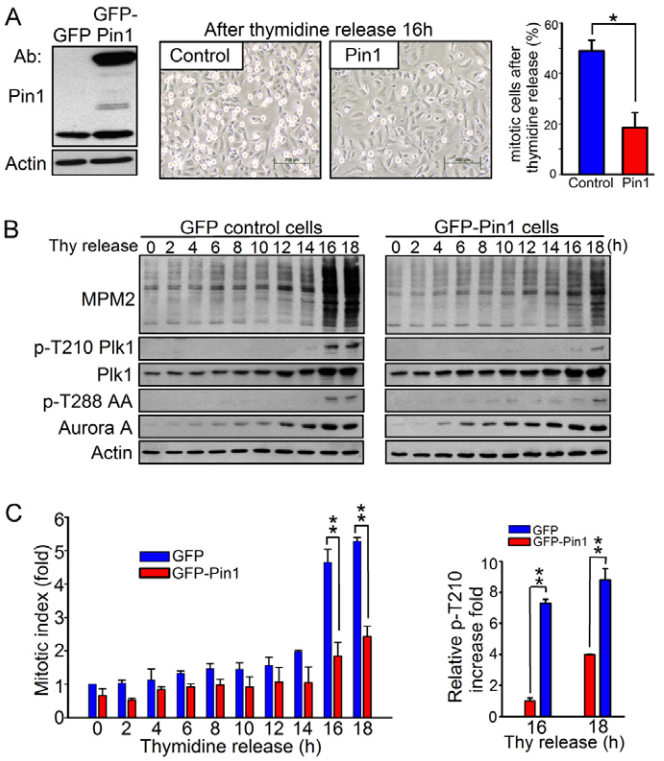

Fig. 1. Gain of Pin1 function delays Plk1 activation and entry into mitosis. (A) Endogenous and exogenous Pin1 expression was analyzed in GFP and GFP-Pin1 U2OS cells by western blotting (left panel). Phase-contrast images of the cells were acquired at 16 hours post release from thymidine (middle panel). Quantification of the mitotic cells is shown on the right. Scale bars: 200 μm. (B) Expression of MPM2 and *P*-T210 Plk1 are examined in thymidine-released cell extracts of stably GFP-Pin1-expressing U2OS cells by western blotting. (C) The mitotic index and Plk1 activity are significantly reduced in stably GFP-Pin1-expressing U2OS cells compared with GFP-control cells. The data are means ± s.d. of three independent experiments. *$P<0.05$; **$P<0.01$.

Results

The negative regulatory function of Pin1 in mitotic entry

Pin1 is believed to be a negative regulator of G2/M. Loss or gain of Pin1 function through its knockdown or overexpression, respectively, can interfere with normal mitotic entry. To further elucidate this role of Pin1, cell extracts of thymidine blocked and then released stable Pin1-knockdown or Pin1-overexpressing U2OS cells were analyzed and subjected to immunoblot analysis. The mitotic index and mitotic entry ability of these cells was investigated by detection of their MPM2 immunoreactivity, which is an indicator of the presence of mitotic phosphoproteins (Tapia et al., 2006). As shown in Fig. 1A, exogenous Pin1 levels were increased up to 4.7-fold compared with endogenous Pin1 in Pin1-overexpressed U2OS cells. Moreover, after a 16 hour thymidine release, the percentage of mitotic cells was found to be markedly reduced in Pin1-overexpressing cells (18.5%) compared with the control cells (48.9%; $P<0.05$; Fig. 1A, middle and right panels), and MPM2 intensity was reduced (Fig. 1B,C; $P<0.01$). Quantitative results revealed that the mitotic index of GFP control cells at 14 hours was similar to that of Pin1-overexpressing cells 18 hours after thymidine release (Fig. 1C, left panel), suggesting that Pin1-overexpressing cells delayed entry into mitosis at least for 4 hours. Furthermore, Thr210 phosphorylation of Plk1, which is an indicator of G2/M, was markedly and significantly delayed and reduced in Pin1-overexpressing cells (Fig. 1B,C; $P<0.01$). These results strongly suggest that a gain of Pin1 function through overexpression can prolong the G2 phase of the cell cycle and delay mitotic entry.

In contrast to Pin1-overexpressing cells, Pin1-knockdown cells exhibited increases in MPM2 intensity (mitotic index) and Plk1 Thr210 phosphorylation, and had a larger mitotic cell population of thymidine-released cells compared with control shRNA cells (supplementary material Fig. S1). This suggests that a loss of Pin1 function can promote premature mitotic entry. Collectively, we have observed that a gain of Pin1 function delays mitotic entry, whereas a loss of Pin1 function promotes premature mitotic entry, indicating that Pin1 plays a negative role in the regulation of mitotic entry.

Elevated Ser16 phosphorylation of Pin1 coincides with increased activity and expression of Aurora A

Protein levels of Pin1 are constant during cell-cycle progression and it acts as a negative regulator of mitotic entry. Therefore, the G2/M function of Pin1 should be suppressed for the normal

regulation of mitotic entry to occur. We next investigated whether the crucial G2/M regulator Aurora A serine/threonine kinase can phosphorylate Pin1 and inhibit its function *in vitro* and *in vivo*. First, Pin1 Ser16 phosphorylation and Aurora A activity in U2OS cells were examined at different times following a thymidine block and release. Aurora A Thr288 phosphorylation was used as an indicator of Aurora A kinase activity (Walter et al., 2000). The results shown in Fig. 2A revealed that the levels of Pin1 Ser16 phosphorylation remain relatively low at the G1/S boundary (time 0) but increase during the G2/M phase (time points from 10 to 18 hours) in parallel with an increase in Aurora A and Cyclin B1 expression levels. This suggests that phospho-Ser16 Pin1 could be used as a marker of G2/M progression. In addition, phosphorylation levels of both Pin1 Ser16 and Aurora A Thr288 gradually increased during the G2/M transition (Fig. 2A, bottom panel, $*P<0.05$), raising the possibility that Aurora A can phosphorylate Pin1 at Ser16.

Aurora A specifically interacts with Pin1 to phosphorylate Ser16

We next examined whether the inhibition of Aurora A activity with a pharmacological inhibitor would reduce the phosphorylation levels of Pin1 Ser16. The results shown in Fig. 2B indicate that Pin1 Ser16 phosphorylation is markedly reduced when U2OS cells are treated with the Aurora A inhibitor MLN8054. A significant decrease in Thr288 phosphorylation inhibited Aurora A activity. Similar results were obtained when using the pan-Aurora kinase inhibitor (supplementary material Fig. S2A). In addition, using both an shRNA to specifically knockdown Aurora A and the mutant Aurora-A-K162M as a competitor of endogenous Aurora A activity, we found a significant reduction in the Pin1 Ser16 phosphorylation levels (Fig. 2C; supplementary material Fig. S2B). Conversely, the transient expression of Aurora A augmented the Ser16

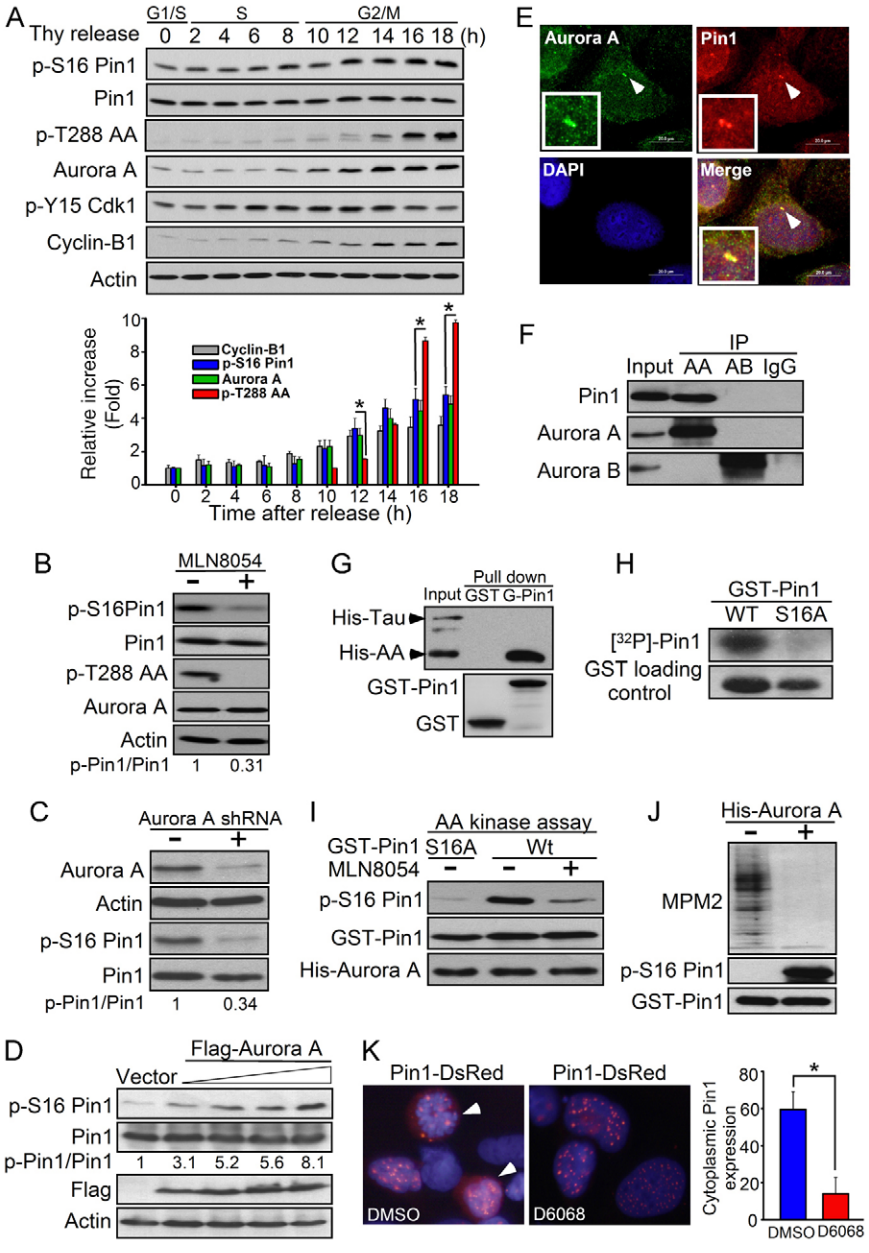

Fig. 2. Aurora A interacts with and phosphorylates Pin1 at Ser16 during the G2/M transition. (A) Thymidine-released U2OS cell lysates were subjected to western blotting using the indicated antibodies (top panel). Quantitative and statistical analyses of the indicated protein expression levels are shown below. (B) Thymidine-released U2OS cells were treated with an Aurora A inhibitor (1 μM MLN8054) for 16 hours and analyzed by western blotting. (C) Aurora A knockdown and shRNA control cells were synchronized with nocodazole. Pin1 Ser16 phosphorylation levels were analyzed by western blotting. (D) U2OS cells were transfected with various dosage of FLAG-Aurora-A and synchronized by nocodazole. (E) U2OS cells were fixed with methanol and stained with anti-Aurora-A (green) and anti-Pin1 (red) antibodies and DAPI (blue). (F) Nocodazole-arrested cell extracts were subjected to a co-immunoprecipitation assay using antibodies against Aurora A, Aurora B, or an IgG negative control. (G) His-Aurora-A and His-tau (negative control) proteins were subjected to a GST pull-down assay. (H) GST-Pin1 and the GST-Pin1-S16A mutant were subjected to *in vitro* Aurora A kinase autoradiography assay. (I) His-Aurora-A was pre-incubated with MLN8054 (5 μM) and then subjected to the kinase assay. Specific anti-phospho-Ser16-Pin1 antibody was used and GST-Pin1-S16A mutant was used as control. (J) GST-Pin1 was pre-phosphorylated or not by His-Aurora-A and then subjected to a GST pull-down assay using mitotic extracts. Anti-phospho-Ser16-Pin1 and MPM2 antibodies were used. (K) Pin1-DsRed-transfected U2OS cells were treated with an Aurora A inhibitor (5 μM) or DMSO to observe the subcellular localization of Pin1 in the G2/M phase. The white arrow indicates the cytoplasmic localization of Pin1 in the control cells. The percentages of cytoplasmic Pin1 expression cells are shown. $*P<0.05$. Scale bars: 20 μm.

phosphorylation of Pin1 in a dose-dependent manner (Fig. 2D). These results suggest a correlation between Aurora A and Pin1 Ser16 phosphorylation levels.

Next, to investigate whether Aurora A phosphorylates Pin1 through a direct interaction, immunofluorescence microscopy analysis along with co-immunoprecipitation, GST pull-down and an *in vitro* kinase assays were performed. The immunofluorescence results shown in Fig. 2E revealed that endogenous Aurora A colocalizes with endogenous Pin1 both in the cytoplasm and nucleus, and appears to be more concentrated at the centrosome in the G2/M phase. In addition, the co-immunoprecipitation analyses confirmed that interactions between Aurora A and Pin1 occur *in vivo*. Anti-Aurora-A (AA) and anti-Aurora-B (AB) antibodies were used in this experiment. Aurora B was used as a control for the specificity of Pin1–Aurora-A interactions. The results indicate that Pin1 co-precipitates with Aurora A but not with Aurora B (Fig. 2F). Taken together with the results using a negative control IgG, these data confirmed that Pin1 specifically interacts with Aurora A.

To next demonstrate that the interaction of Pin1 and Aurora A is through direct binding, a GST pull-down experiment using GST-Pin1 and His-tagged Aurora A was performed. His-tagged tau protein was used as a negative control. As shown in Fig. 2G, His-tagged Aurora A, but not His-tagged tau protein, was pulled down by GST-Pin1. Importantly, the results of an *in vitro* Aurora A kinase assay revealed the incorporation of γ-^{32}P signal in Pin1 wild-type but not in a Pin1-S16A mutant protein, indicating that Ser16 is an essential Aurora A phosphorylation site on Pin1 (Fig. 2H). Consistent results were obtained using specific anti-phospho-Ser16 Pin1 antibody, and GST-Pin1 and GST-Pin1-S16A proteins as substrates to perform *in vitro* Aurora A kinase assay (Fig. 2I). The immunoreactivity of phospho-Ser16 could be detected for the Pin1 wild-type protein, but was abolished for the Pin1-S16A mutant protein. In addition, the immunoreactivity of phospho-Ser16 was found to be markedly reduced when His-Aurora-A was pre-incubated with the Aurora A inhibitor, MLN8054 (Fig. 2I). Collectively, these results clearly demonstrate that Aurora A directly binds and phosphorylates Pin1 at Ser16 in cells.

Aurora-A-mediated phosphorylation of Pin1 Ser16 suppresses the binding ability of Pin1

We examined whether Aurora-A-mediated phosphorylation of Pin1 Ser16 would disrupt Pin1 function. First, GST-Pin1 proteins were pre-phosphorylated by Aurora A *in vitro* and a GST-Pin1 pull-down assay was then performed using abundant MPM2 antigens from mitotic extracts. The results showed that the binding ability of Pin1 was markedly disrupted when GST-Pin1 was pre-phosphorylated by Aurora A (Fig. 2J). In addition, the near complete loss of MPM2 binding was found to be associated with Pin1 Ser16 phosphorylation. Next, nocodazole-synchronized G2/M cells were treated with Aurora A inhibitor to reduce Pin1 Ser16 phosphorylation and were then analyzed by immunofluorescence analysis of Pin1 localization. The results showed that the cytoplasmic translocation of Pin1 was significantly reduced in cells in the G2/M phase treated with Aurora A inhibitor compared with control cells (Fig. 2K, $P<0.05$).

These findings indicate that Aurora A can directly interact with Pin1 and that Aurora-A-mediated Ser16 phosphorylation suppresses the function of Pin1 at the G2/M transition. Moreover, our observation that Pin1 overexpression delays mitotic entry (Fig. 1) suggests that this is caused by gain of Pin1 function, which overrides its Aurora-A-mediated functional suppression.

The subcellular translocation of Bora by Pin1

The Pin1-overexpression-induced delay in mitotic entry is associated with reduced Plk1 activation (Fig. 1), implying a negative role of Pin1 upstream of Plk1-mediated mitotic entry. The Aurora-A–Bora complex also functions upstream of Plk1-mediated mitotic entry by activating Plk1 to control mitotic entry (Macůrek et al., 2008; Seki et al., 2008b). Collectively, it was reasonable to investigate the relationship between Pin1 and Bora in the regulation of mitotic entry.

Previous studies (Seki et al., 2008b) and our present analyses have found that endogenous Bora or GFP-Bora localizes in both the cell cytoplasm and the nucleus (Fig. 3A; supplementary material Fig. S3A). Interestingly, we observed that Pin1-overexpressing cells exhibit a subcellular translocation of Bora. This observation suggested that the Pin1-overexpression-induced delay of mitotic entry is associated with Bora. The results of immunofluorescence analysis in our current study revealed that ~74.2% of the GFP-Bora proteins were distributed both in the nucleus and in the cytoplasm in DsRed-expressing control cells, whereas in DsRed-Pin1-expressing cells, 90.6% of the cells displayed a cytoplasmic expression of GFP-Bora (Fig. 3A). We next used a cell fractionation assay to confirm that Pin1 also altered the cytoplasmic translocation of endogenous Bora. Similar results were also obtained from a transient co-transfection experiment (supplementary material Fig. S3B).

A gain of Pin1 function promotes the premature degradation of Bora through the β-TrCP-mediated ubiquitin–proteasome pathway

The delay in mitotic entry induced by Pin1 overexpression was found to be similar to that observed in Bora-depleted cells; we next examined whether Pin1 overexpression affected the Bora protein levels in cells and whether that effect is involved in a mitotic entry delay. We first analyzed Bora expression after a thymidine block and release in stable GFP-control and GFP-Pin1-expressing U2OS cells. The results showed that Bora levels increase and accumulate from the G1/S to G2/M phase in control cells, whereas the expression of Bora was downregulated in Pin1-overexpressing cells. These reduced Bora levels were observed from the onset of G2 phase to the G2/M transition (Fig. 3C, histogram, 8–16 hours) suggested that a gain of Pin1 function causes the premature degradation of Bora in G2 phase (before mitotic entry) and thereby delays mitotic entry.

We verified this cause–effect hypothesis in which Pin1 mediates the premature degradation of Bora by examining Bora expression in GFP-Pin1 stable cells. As shown in Fig. 3D, these cells exhibited a 43% reduction in endogenous Bora levels compared with GFP-control cells (Fig. 3D, lane 1 versus lane 3). Similar results were obtained in transiently transfected HA-Pin1 cells (supplementary material Fig. S3C). Furthermore, in contrast to the reduction of Bora in Pin1-overexpressing cells, Pin1 knockdown in U2OS cells resulted in an increase in Bora expression (supplementary material Fig. S1C). In addition, the steady state levels of Bora were upregulated 4.3-fold in *Pin1*$^{-/-}$ MEF cells compared with wild-type MEF cells (Fig. 3E). After re-expressing Pin1 (FLAG-Pin1 or GFP-Pin1) in *Pin1*$^{-/-}$ MEF cells to restore Pin1 function, *Pin1*$^{-/-}$ MEF cells exhibited a

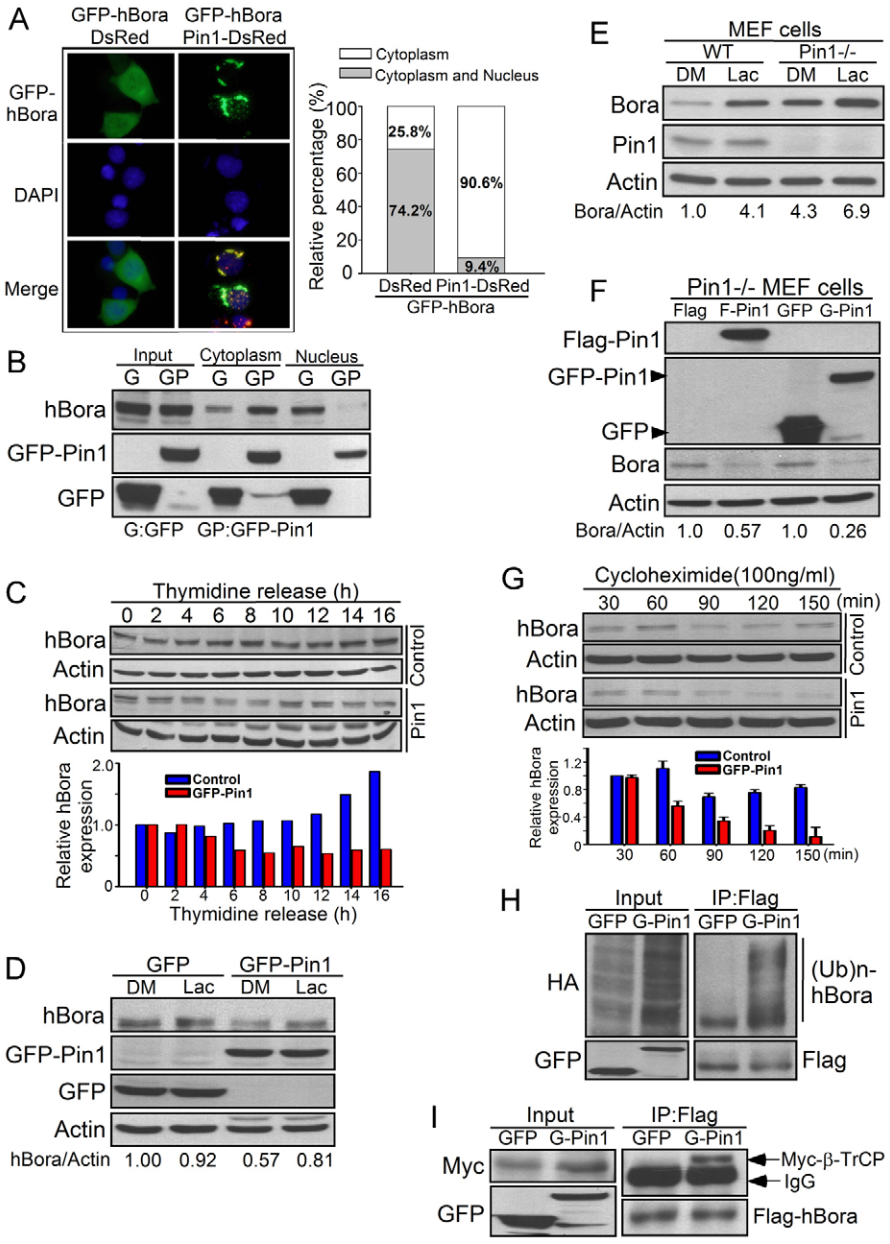

Fig. 3. A gain of Pin1 function alters the cytoplasmic translocation of Bora and promotes its premature degradation by β-TrCP. (A) Immunofluorescence microscopy analysis was performed with U2OS cells expressing GFP-Bora and DsRed-Pin1 or DsRed (left panel). The relative percentages of the GFP-Bora distribution in the cytoplasm or both in the cytoplasm and nucleus of cells were calculated and plotted (right panel). (B) The cytoplasmic and nuclear fractions of GFP-control or GFP-Pin1-expressing U2OS were separated using a cell-fractionation assay to analyze the endogenous Bora subcellular distribution. (C) Bora expression was examined in control and GFP-Pin1 cell extracts at the indicated times after thymidine release. (D) Bora expression in DMSO- and lactacystin-treated control or GFP-Pin1 cells. (E) Expression levels of Bora in DMSO- and lactacystin-treated Pin1-WT or *Pin1*$^{-/-}$ MEFs cell extracts. (F) *Pin1*$^{-/-}$ MEF cells were transfected with FLAG, FLAG-Pin1, GFP and GFP-Pin1, respectively. Bora expression was analyzed. (G) Control and GFP-Pin1 stable U2OS cells were treated with cycloheximide and harvested at the indicated times and the Bora protein levels were analyzed. (H) HEK293T cells were co-transfected with FLAG-Bora, HA-ubiquitin and GFP or GFP-Pin1. After lactacystin treatment (10 μM, 4 hours), the cell lysates were immunoprecipitated with an anti-FLAG antibody and subjected to western blotting. (I) HEK293T cells were transfected with FLAG-Bora, Myc-β-TrCP and GFP or GFP-Pin1. The cell lysates were immunoprecipitated with anti-FLAG antibody, and western blotting was performed using antibodies against FLAG, Myc and GFP.

marked decrease in Bora protein levels (43% and 74% reduction, respectively; Fig. 3F). These results suggested that the gain of Pin1 function through overexpression downregulates Bora expression. Interestingly, when GFP-Pin1 U2OS cells (Fig. 3D) or wild-type MEF cells (Fig. 3E) were treated with the proteasome inhibitor lactacystin, the Pin1-mediated reduction in Bora could be rescued. In addition, when using cycloheximide to examine the protein stability of Bora in GFP-control and GFP-Pin1 U2OS cells, the total Bora protein levels remained stable in GFP-control cells after cycloheximide treatment (Fig. 3G). The half-life of the Bora protein was found to be ~60 minutes in Pin1-overexpressing cells compared with GFP-control cells. The results of *in vivo* ubiquitylation and co-immunoprecipitation assays showed that Pin1 overexpression increases the polyubiquitylation levels of Bora (Fig. 3H) and enhances the interaction of Bora with β-TrCP (Fig. 3I). Collectively, these findings suggest that Pin1 overexpression promotes the premature degradation of Bora through the β-TrCP-mediated ubiquitin–proteasome pathway and thereby delays mitotic entry.

Pin1 is an important regulator of mitotic progression and it exerts this control through its binding of various mitosis-specific phosphoproteins, such as Cdc25, Plk1 and Cep55 (Crenshaw et al., 1998; Shen et al., 1998; van der Horst and Khanna, 2009). In addition, Bora degradation during mitosis is crucial to ensure normal mitotic progression (Chan et al., 2008; Seki et al., 2008a). In contrast to the observed delay in mitotic entry through downregulation of Bora, we next analyzed the role of Pin1 in mitotic progression in Pin1-knockdown or in mitotic cells with inhibited Pin1 PPIase activity (after Juglone treatment) (Hennig et al., 1998). We found from this analysis that a loss of Pin1 function during mitosis causes a striking accumulation of Bora and results in defects in mitotic progression and exit

(supplementary material Fig. S4). This observation suggests that the interaction of Pin1 and Bora has a physiological role in regulating mitotic progression by promoting the degradation of Bora.

Pin1 interacts with Bora in a phosphorylation- and cell-cycle-dependent manner *in vitro* and *in vivo*

We next examined whether Pin1 promotes the premature degradation of Bora through the interaction between Pin1 and Bora. The WW domain of Pin1 has been demonstrated to specifically recognize the pSer/Thr-Pro motifs of phosphoproteins, which can be detected by the MPM2 antibody (Lu et al., 1999). We examined the phosphorylation profiles of Bora during cell cycle progression using a pSer/Thr-Pro antibody. Thymidine-released cell extracts were subjected to an immunoprecipitation assay using an antibody against Bora and then analyzed. As shown in Fig. 4A, the immunoreactivity of the pSer/Thr-Pro motif in Bora was undetectable at the G1/S boundary but gradually appeared in late S phase (8 hours after thymidine release) and peaked in G2 phase (10–14 hours). These results indicate that Bora contains a pSer/Thr-Pro motif in G2 phase, and is therefore a potential Pin1-binding protein at this stage of the cell cycle. In addition, as Bora was demonstrated to be an MPM2 antigen (supplementary material Fig. S5A), it also raised the possibility that Bora is a novel Pin1-binding protein.

The interaction between endogenous Pin1 and Bora was then examined using GST pull-down, co-immunoprecipitation and colocalization analyses. The results showed that Pin1 interacts with Bora in a phosphorylation- and cell-cycle-dependent manner. First, we demonstrated the retarded migration of phosphorylated Bora in G2/M phase using immunoprecipitation and immunoblot analyses (Fig. 4A, time points 10 to 18 hours). Second, to evaluate the interaction of Pin1 with endogenous Bora, cell extracts from non-synchronized interphase cells and nocodazole-synchronized G2/M phase cells were subjected to a GST pull-down assay. As shown in Fig. 4B, Pin1 markedly interacts with the slowly migrating bands of endogenous Bora in nocodazole-synchronized G2/M cells but not in non-synchronous cells, indicating that this interaction is cell-cycle dependent. Third, the *in vivo* interaction between Pin1 and Bora was confirmed using co-immunoprecipitation assay with nocodazole-synchronized G2/M cell lysates. The results showed that endogenous Bora co-precipitates with Pin1 but not with an IgG negative control (Fig. 4C). Similar results were obtained from transient FLAG-Bora and GFP-Pin1 co-expression experiments (supplementary material Fig. S5B). In addition, to examine the colocalization of Pin1 and Bora in cells, U2OS cells were subjected to immunofluorescence analysis using anti-Pin1 and anti-Bora antibodies. The results revealed that Pin1 and Bora expression are both cytoplasmic and nuclear, and clearly evident as nuclear speckles, suggesting the colocalization of endogenous Pin1 and Bora (Fig. 4D). Similar results were obtained upon confocal microscopy analysis of cells transiently co-transfected with GFP-Bora and DsRed-Pin1 (supplementary material Fig. S5C). Collectively, our findings clearly demonstrate that Bora forms a complex with Pin1 *in vitro* and *in vivo* at both endogenous and overexpressed levels.

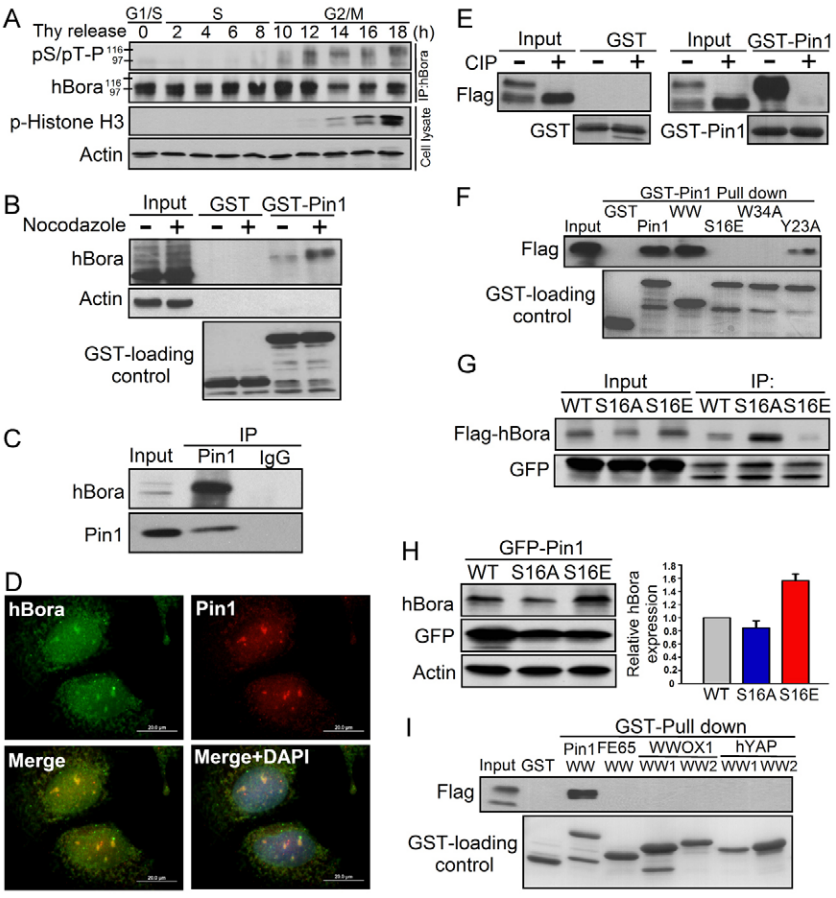

Fig. 4. Bora is phosphorylated in G2 and M phase and interacts with Pin1 in a phosphorylation-dependent manner. (A) Thymidine-released cell extracts were subjected to IP-western blotting using anti-Bora and anti-pSer/Thr-Pro antibodies. The intensity of phospho-histone H3 in the total cell lysate panel was used to represent the mitotic phase. (B) The cell extracts of non-synchronized interphase cells and nocodazole-synchronized G2/M phase U2OS cells were subjected to a GST pull-down assay. (C) Nocodazole-synchronized mitotic U2OS cell extracts were subjected to an immunoprecipitation assay using an anti-Pin1 antibody. (D) Anti-Bora and anti-Pin1 antibodies were used to perform an immunofluorescence assay to localize Bora (green), Pin1 (red) and the nucleus (blue) in U2OS cells. Scale bars: 20 μm. (E) Nocodazole-synchronized FLAG-Bora-expressing HEK293T cell extracts were incubated with or without calf intestinal phosphatase (CIP) before GST pull-down assay. (F) Nocodazole-synchronized FLAG-Bora-expressing HEK293T cell extracts were subjected to a GST pull-down assay using the indicated GST fusion proteins. (G) GFP-tagged wild-type, S16A or S16E mutant Pin1 and FLAG-tagged Bora were co-expressed in stable Pin1-knockdown U2OS cells. Cell extracts were collected and subjected to co-immunoprecipitation using a GFP antibody. (H) GFP-tagged wild-type, S16A or S16E mutant Pin1 were re-expressed in stable Pin1-knockdown U2OS cells. The endogenous expression of Bora was examined. (I) Nocodazole-synchronized FLAG-Bora-expressing HEK293T cell extracts were subjected to a GST pull-down assay with either GST beads as a negative control or various WW-domain-containing GST-fusion proteins including WWOX, YAP, FE65 and Pin1.

We next investigated whether the interaction of Pin1 with Bora is phosphorylation dependent. The up-shifted FLAG-Bora band no longer existed upon treatment with calf intestinal phosphatase (CIP), indicating that it is a phosphorylated form of Bora (Fig. 4E, Input lane). Most importantly, treatment with CIP was found to completely abolish the interaction between Pin1 and Bora in a GST pull-down assay (Fig. 4E), further indicating that the interaction between Pin1 and Bora is phosphorylation dependent. Moreover, we demonstrated in our current experiments that only Pin1 or its WW domain can interact with Bora, because *in vitro* GST pull-down analyses reveal that this association is absent or markedly reduced for the Ser16-phosphorylation-mimicking mutant of Pin1 (S16E) and other WW domain mutants (W34A and Y23A) (Fig. 4F).

Pin1 Ser16 phosphorylation abolishes the interaction of Pin1 with Bora and thereby increases Bora expression

We demonstrate that Aurora A can phosphorylate Pin1 at Ser16 and this phosphorylation event can suppress the binding ability of Pin1 (Fig. 2). In addition, we reveal from our current analyses that upon Pin1 and Bora interaction, Pin1 promotes the premature degradation of Bora (Fig. 3). Collectively, these results suggest that Pin1 Ser16 phosphorylation is essential for the interaction and degradation of Bora. To further validate these findings, we re-expressed wild-type Pin1, and its S16A and S16E mutants in stable Pin1-knockdown U2OS cells to further investigate the interaction between Pin1 with Bora and also the endogenous expression of Bora. The co-immunoprecipitation results showed that compared with wild-type Pin1, the Pin1 S16A mutant markedly enhanced the interaction of Pin1 with Bora, whereas this interaction was totally abolished in the case of the Pin1 S16E mutant (Fig. 4G). Together with our finding that the GST-Pin1-S16E protein also failed to interact with Bora (Fig. 4F), the evidence suggests that Pin1 cannot interact with Bora upon Ser16 phosphorylation, either *in vivo* or *in vitro*.

The expression of Bora was increased in Pin1-knockdown cells compared with control cells, indicating that a loss of Pin1 function causes an accumulation of Bora (supplementary material Fig. S1C). We next restored Pin1 expression, GFP-Pin1 and GFP-Pin1-S16A (with substrate-binding ability) and GFP-Pin1-S16E (without substrate-binding ability) in Pin1-knockdown cells and found thereafter that the expression of endogenous Bora was lower in WT and Pin1-S16A-expressing cells than in Pin1-S16E-expressing cells (Fig. 4H). Together with our observation that Pin1 S16E loses its ability to bind Bora (Fig. 4G), our current data suggest that Pin1 reduces Bora expression depending on the interaction between Pin1 with Bora. Furthermore, in a GST pull-down assay, Bora also exhibited interaction specificity for the Pin1 WW domain but not for other WW-containing proteins, such as FE65, WWOX1 and YAP (Fig. 4I). We conclude from these results that Pin1 interacts with Bora in a phosphorylation- and cell-cycle-dependent manner, and that Pin1 Ser16 phosphorylation can regulate the interaction of Pin1 with Bora.

Both pSer274 and pSer278 are important Pin1-binding sites on Bora

Bora is highly phosphorylated in the G2/M phase. We therefore used LC-MS/MS-based phosphopeptide mapping analysis to identify novel *in vivo* Bora phosphorylation sites. A total of 13 Bora phosphorylation sites were identified from G2/M-arrested HEK293T cells. To identify the Pin1-binding sites of Bora, site-directed mutagenesis and GST pull-down assays were used. The results of these GST pull-down assays (Fig. 5A; supplementary material Fig. S5D) showed that S41A, S112A, S252A, S270A and S299A Bora mutants still interacted with Pin1, whereas S268A, S274A and S278A Bora variants had a reduced interaction with Pin1 compared with WT Bora (43%, 11% and 23% reductions, respectively). We further determined whether the triple Bora mutants S270A/S274A/S278A (S3A in Fig. 5A) and S268A/S274A/S278A (S3A in supplementary material Fig. S5D) or the double mutant S274A/S278A (S2A in Fig. 5A) could interact with Pin1. The S274A/S278A double mutant exhibited a marked reduction in Pin1 binding (73%), whereas the S270A/S274A/S278A and S268A/S274A/S278A triple mutants did not exhibit any additional reduction in Pin1 binding (a 75% and 46% reduction, respectively). This suggests that pSer274 and pSer278 are the important Pin1-binding sites on Bora *in vivo*.

S274A/S278A mutations reduce the Pin1-mediated effects on Bora

We found that a gain of Pin1 function promotes the premature degradation of Bora by β-TrCP and alters the subcellular location of Bora, and that pSer274 and pSer278 are important Pin1-binding sites on Bora, we speculated that mutations in Pin1-binding sites might reduce the subcellular translocation and polyubiquitylation of Bora and its interaction with β-TrCP. The results in Fig. 5B show that the GFP-S274A/S278A-Bora mutant is expressed in the nucleus and the cytoplasm of cells in a similar pattern to that found for GFP-Bora. However, whereas in DsRed-Pin1-co-expressing cells, GFP-Bora cells exhibited a significant degree of cytoplasmic translocation, this was not the case for the GFP-S274A/S278A-Bora mutant ($P<0.05$). These results suggested that S274/S278 phosphorylation is not functionally relevant to the cytoplasmic translocation of Bora. The cytoplasmic translocation of Bora was found to be dependent upon Pin1 binding. Moreover, together with the results shown in Fig. 5C,D, the Pin1-mediated polyubiquitylation of Bora and the subsequent interaction with β-TrCP were also markedly reduced in cells expressing the S274A/S278A Bora mutant. These results indicate that pSer274 and pSer278 are the key Pin1-binding sites on Bora and that Pin1 can promote the β-TrCP-mediated degradation of Bora through specific interactions.

Discussion

The role of Pin1 as an inhibitor of premature mitotic entry has been demonstrated in both *Xenopus* and humans – two completely different genetic systems (Lu et al., 1996; Shen et al., 1998). Although many other studies including our present report have confirmed that Pin1 plays a role in preventing premature entry into mitosis, the mechanism by which Pin1 inhibits the G2/M transition remains unclear. Our current findings identify a novel regulatory mechanism involving the Aurora-A–Bora complex and Pin1 to regulate the transition from G2 to M phase (Fig. 6). Aurora A interacts with and phosphorylates Pin1 at Ser16 and this modification can suppress Pin1 G2/M function by disrupting Pin1-binding ability (Fig. 2). In view of this finding, and the observed prolonged G2 phase in Pin1-overexpressed cells (Fig. 1), it was logical to speculate that a gain of Pin1 function will override Aurora-A-mediated Pin1 suppression. Indeed, we found that Pin1 overexpression alters the cytoplasmic translocation of Bora and

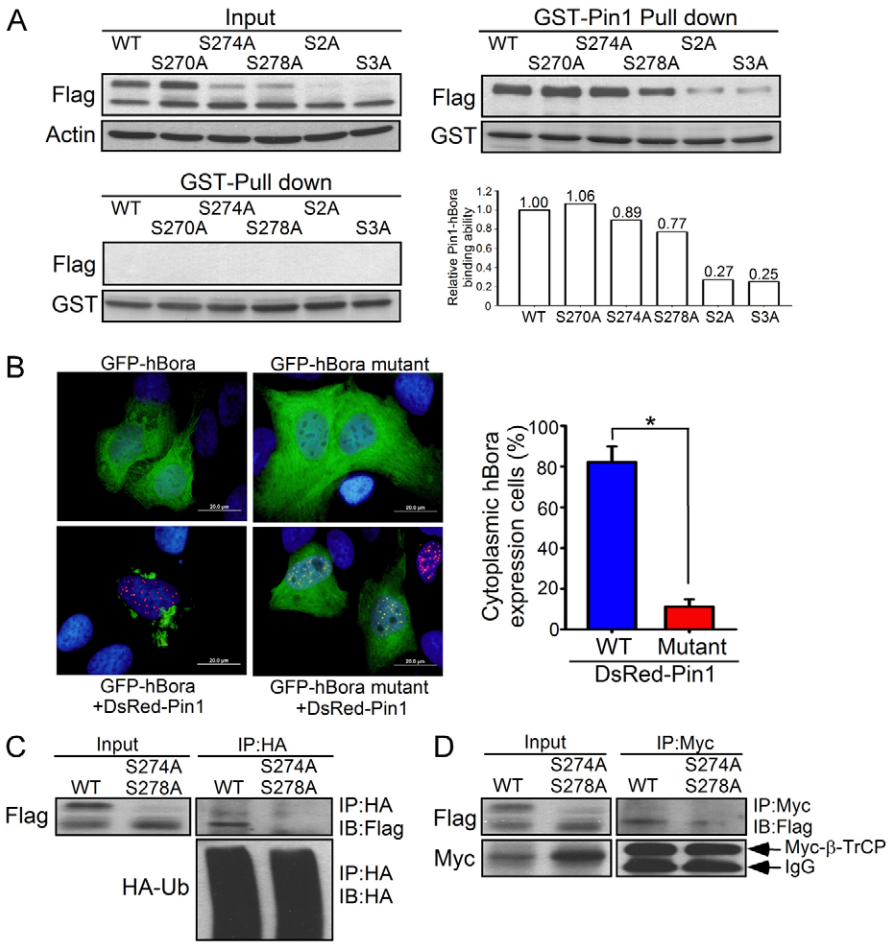

Fig. 5. The effects of Pin1 on Bora are mediated through Pin1 binding to the pSer274 and pSer278 sites of Bora. (A) Extracts of nocodazole-synchronized HEK293T cells expressing FLAG-Bora mutants were subjected to a GST pull-down assay. S2A, S274A/S278A Bora; S3A, S270A/S274A/S278A Bora. (B) GFP-Bora or the GFP-S274A/S278A Bora mutant were co-transfected or not with DsRed-Pin1 in U2OS cells. The subcellular distribution of Bora was examined using immunofluorescence microscopy. Scale bars: 20 μm. The percentages of cytoplasmic only Bora-expressing cells were quantified in GFP-Bora and GFP-S274A/S278A Bora cells. *$P<0.05$. (C,D) HEK293T cells were transiently co-transfected with FLAG-WT or the S274A/S278A Bora mutant with HA-ubiquitin and GFP-Pin1 or with Myc-β-TrCP and GFP-Pin1. Immunoprecipitation assay performed with either anti-HA or anti-Myc antibodies.

promotes its premature degradation by β-TrCP (Fig. 3). This occurs through the binding of Pin1 to phosphorylated Ser274/Ser278 Bora sites (Figs 4, 5). The downregulation of Bora can downregulate Aurora A activity by reducing Aurora-A–Bora complex formation, which then suppresses the activation of Plk1 to delay mitotic entry (Fig. 1).

An interesting question in relation to cell cycle regulation is the nature of the factor(s) that control the cell cycle transition. In the case of the G2/M transition, Aurora-A–Bora-dependent phosphorylation is essential for Plk1 to activate the Cyclin-B1–Cdk1 complex and promote mitotic entry. Here, we show that Aurora A can suppress Pin1, a negative regulator of G2/M function. Aurora A was selected for the following reasons. First, Aurora A is a Ser/Thr kinase that recognizes the consensus R/K/N-R-x-S/T-B site, where B denotes any hydrophobic residue with the exception of Pro (Ferrari et al., 2005). Ser16 locates in the sequence of Pin1, ^{13}KRMSR^{17}, is a potential Aurora A consensus phosphorylation site. Second, Aurora A levels and activity are abundant during the G2/M phase, and that is consistent with our hypothesis that Pin1 is phosphorylated at Ser16 and is functionally repressed during the G2/M transition. Third, depletion of Aurora A can markedly impair the ability of cells to enter mitosis, which is similarly observed in cells displaying a gain of Pin1 function. Furthermore, the elevated Ser16 phosphorylation profile of Pin1 coincides with the pSer/Thr-Pro immunoreactivity of Bora during the G2/M phase (Fig. 2A; Fig. 4A). Collectively, these findings strongly suggest that Aurora A activity and Pin1 function are inversely correlated in the regulation of mitotic entry and support our proposal that Aurora A phosphorylates Pin1 at Ser16 during the G2/M phase to suppress Pin1 binding to phosphorylated Bora and hence block the premature degradation of Bora.

Bora was first identified in the *Drosophila* peripheral nervous system as a binding partner of Aurora A during the regulation of mitosis onset (Hutterer et al., 2006); two independent studies also demonstrated that, during G2, Aurora-A–Bora-mediated Plk1 activation can promote mitotic entry both during unperturbed growth (Seki et al., 2008b) and after recovery from a DNA-damage checkpoint (Macůrek et al., 2008). These findings reveal that Bora expression is required for mitotic entry. In other words, the downregulation of Bora will suppress Aurora A activity by reducing the Aurora-A–Bora complex formation needed to activate Plk1. Our present study shows that Pin1 overexpression downregulates Bora by promoting its premature degradation by β-TrCP, resulting in delayed mitotic entry. The timing of normal mitotic entry could therefore be influenced by Pin1, which must be inhibited for G2/M transition. Here, we show that the interaction between Pin1 and Bora is disrupted through phosphorylation of Pin1 at Ser16 by Aurora A. Therefore, a rise in Aurora A activity in G2, leads to an increase in Bora protein that further stimulates Aurora A, enabling mitotic entry. In summary, therefore, the inhibition of Pin1 function by Ser16 phosphorylation and accumulation of Bora expression at the G2/M transition are two important events in the regulation of mitotic entry.

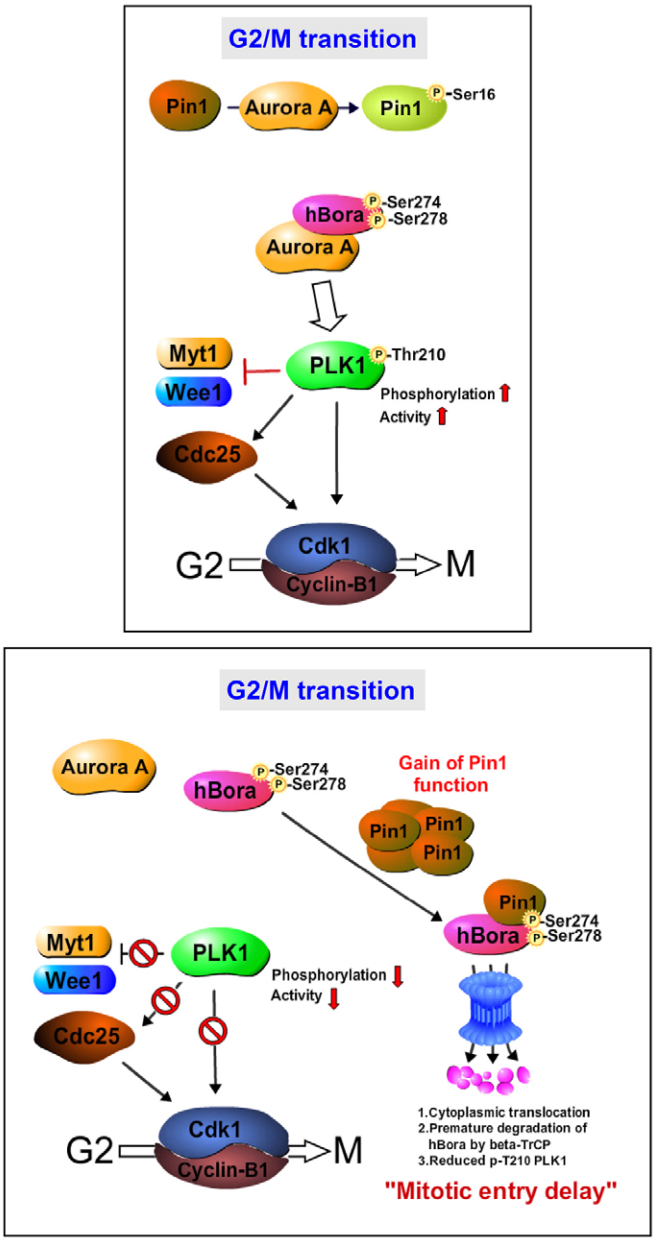

Fig. 6. Molecular mechanisms regulating the interplay of Aurora-A–Bora with Pin1 during mitotic entry. At the G2/M transition, the Aurora-A–Bora complex activates Plk1 through Thr210 phosphorylation and controls mitotic entry. Aurora A suppresses Pin1 G2/M function by phosphorylating Pin1 at Ser16 to disrupt its binding ability (top panel). Gain of Pin1 function through overexpression overrides the inhibition of suppression of Pin1 by Aurora A (bottom panel). Pin1 binds to phosphorylated Bora at Ser274/Ser278. Upon this interaction, Pin1 alters the Bora cytoplasmic translocation, and promotes the premature degradation of Bora by β-TrCP. The downregulation of Bora can downregulate Aurora A activity by reducing the formation of Aurora-A–Bora complexes and as a consequence, suppresses the activation of Plk1 at the G2/M transition. Overexpression of Pin1 can delay mitotic entry and this explains how Pin1 acts as a negative regulator of the G2/M transition.

The physiological degradation of Bora by β-TrCP has also been found to be important for promotion of normal mitotic progression (Chan et al., 2008; Seki et al., 2008a). Our present results raise the issue that if Pin1 is inhibited by Aurora A phosphorylation, then Bora should not be degraded in mitosis. However, we did not clarify the status of Pin1 Ser16 phosphorylation during the mitotic phase in our present experiments and we speculate that Pin1 Ser16 phosphorylation is reduced in mitosis. First, Lu and colleagues reported that Ser16 is a major phosphorylation site of Pin1. The total phosphorylation level of Pin1 is elevated in growing HeLa cells but is then clearly dephosphorylated upon mitotic arrest (Lu et al., 2002), suggesting that Ser16 phosphorylation is reduced in the mitotic phase, even at the point of maximal Aurora A activity. Second, Pin1 is a regulator of proper mitotic progression through its regulation of the levels of pSer/Thr-Pro antigens (Shen et al., 1998). Ser16 phosphorylation can regulate the binding of Pin1 to its pSer/Thr-Pro antigens. Collectively, our results show that Ser16 phosphorylation does not coincide with maximal Aurora A activity in mitosis and that the de-phosphorylation of Pin1 Ser16 is instead essential for the mitotic function of Pin1. In mitosis, the loss of Pin1 function causes a striking accumulation of Bora and defects in both mitotic progression and exit (supplementary material Fig. S4). Together with the results that the interplay of Aurora A and Pin1 at the G2/M transition can inhibit Pin1 function, we suggest that after mitotic entry, Pin1 might have a physiological role in the feedback regulation of Bora destruction in mitosis. In conclusion, we believe that the phosphorylation of Pin1 at Ser16 is dynamically regulated in cells and that this is a primary mechanism of Pin1 regulation.

Pin1 specifically interacts with many important regulators of Cdk1 including Cdc25 and Plk1, whose activation is believed to trigger entry into mitosis. Although the interaction has been demonstrated (Crenshaw et al., 1998; Shen et al., 1998), the functional regulation of mitotic entry through Pin1–Plk1 binding has not been analyzed in detail. Pin1 overexpression can block mitotic entry (Fig. 1), whereas the depletion of Pin1 can promote mitotic entry (supplementary material Fig. S1). That leads to at least two possible explanations for the Pin1–Plk1 binding effects at the G2/M transition. First, Pin1 could suppress Plk1 function through its direct interaction. Second, Pin1 might negatively affect Plk1 regulatory molecules and indirectly suppress Plk1 function. Our results support the contention that Pin1 negatively and indirectly suppresses Plk1 function through β-TrCP-mediated Bora degradation (Fig. 3). Furthermore, Plk1 can phosphorylate Pin1 at Ser65 in its PPIase domain, and increase Pin1 stability by reducing its ubiquitylation and degradation in mitosis (Eckerdt et al., 2005). Our current findings indicate that Pin1 exerts a functional interplay with the Aurora-A–Bora complex at the G2/M transition and that Aurora A suppresses Pin1 function and forms a complex with Bora to activate Plk1. The collective evidence thus suggests the existence of a feedback system for Plk1 that affects the stability and function of Pin1, although this remains to be investigated further.

Through the isomerization of the pSer/Thr-Pro motif of Cdc25C during mitosis, Pin1 can control whether Cdc25C exists in an active conformation and can thus turn Cdc25C activity on and off (Shen et al., 1998; Stukenberg and Kirschner, 2001). The phosphorylation of two distinct Pin1-binding sites at Thr48 and Thr67 of Cdc25C can inhibit phosphatase activity and that supports our present observation that the gain of Pin1 function can inhibit mitotic entry (Shen et al., 1998). By contrast, Crenshaw and co-workers have found that Pin1 binding does not suppress the *in vitro* phosphatase activity of Cdc25 and

concluded that Pin1 inhibition of entry into mitosis does not occur through a direct inhibition of Cdk1-directed Cdc25 phosphatase activity (Crenshaw et al., 1998). Whether Cdc25 phosphorylation and activity are associated with the negative role of Pin1 at the G2/M transition remains to be clarified in the future. Although the sequential molecular interplay of Plk1 and Cdc25 is involved in mitotic entry and these factors have been identified as Pin1-binding proteins, the nature of these interactions with Pin1 is unclear given that Pin1 acts as a negative regulator of G2/M transition. The cause and effect of the Pin1-mediated premature degradation of Bora is suggested through the direct interaction these proteins (Fig. 4), which Pin1 prevents Bora from efficiently cooperating with Aurora A to activate Plk1. Accordingly, in addition to Cdc25 and its upstream regulator Plk1, we believe that Pin1 has a role in regulating upstream molecules of the Plk1 and Cdc25 pathways, such as Bora, to negatively regulate mitotic entry.

The principle of cell cycle regulation is the appropriately timed structural modification of proteins through phosphorylation/dephosphorylation and ubiquitin-mediated protein degradation (Fisher et al., 2012; Vermeulen et al., 2003). Abnormalities in the turnover or post-translational modifications of cell-cycle-related proteins can cause dysregulated cell cycle progression. Our present results demonstrate a novel mechanism of post-translational regulation of Pin1 by Aurora A. Phosphorylation of Pin1 at Ser16 has a physiological role in the progression of cells from G2 into mitosis and this pathway could explain how Pin1 negatively regulates the G2/M transition.

Materials and Methods

Cells culture, transfection, synchronization

Pin1$^{+/+}$ and *Pin1*$^{-/-}$ mouse embryonic fibroblasts, U2OS and HEK293T cells were cultured as previously described (Macůrek et al., 2008). Transfections were performed by electroporation (Neon® Transfection System) and with Lipofectamine 2000 (Invitrogen). Plasmids used for transfection were: pCMV-FLAG-Aurora-A and its K162M mutant, pEGFP-Aurora-A; pEGFP-Pin1; pDsRed-Pin1; Myc-β-TrCP and pcDNA3.1-HA-Ub. The pCMV-Bora-S41A, Bora-S112A, Bora-S252A, Bora-S268A, Bora-S270A, Bora-S274A/S278A, Bora-S270A/S274A/S278A, Bora-S299A and peGFP-S274A/S278A vectors were constructed using site-directed mutagenesis. G418 was used to generate the GFP and GFP-Pin1 stable cell lines.

For synchronization, the cells were treated with 2.5 mM thymidine for 24 hours to block cells at the G1/S boundary. Nocodazole 200 ng/ml was used to synchronize cells in the G2/M phase and the nocodazole-arrested mitotic cells were collected by mechanical shake-off method. The methods for cell collection and lysate preparation were similar to those previously described (Shen et al., 1998). The cell samples were subsequently subjected for immunoprecipitation or western blot analyses.

Production of recombinant proteins, *in vitro* kinase assay and antibodies

GST-fusion proteins (Pin1, Pin1-WW domain, S16E, Y23A and W34A) and His-tagged proteins (tau and Aurora A) were produced in BL21 bacteria and purified by glutathione agarose beads or Ni-beads, respectively (Lu et al., 2002). *In vitro* Aurora A kinase assays were performed at room temperature in buffer supplemented with 10 μM ATP and 2 μCi [γ-^{32}P]ATP (Seki et al., 2008b). The reaction products were visualized using SDS-PAGE followed by autoradiography or specific Pin1 antibodies for phospho-Ser16.

Antibodies were obtained from commercial sources: anti-pSer10 histone H3 (Cell Signaling Technology); anti-actin, anti-FLAG (Sigma-Aldrich); anti-Cyclin-B1, anti-Plk1, anti-HA, anti-Myc, anti-Pin1, anti-GFP (Santa Cruz Biotechnology); anti-Aurora-A, anti-pSer16-Pin1, anti-Aurora-B (Abcam), anti-Bora (ProSci); anti-pThr288 Aurora A (Cell Signaling); anti-pThr210 Plk1 (BD) and anti-MPM2 (Millipore). D6068, cycloheximide (239763), Juglone (H47003), nocodazole (M1404) and thymidine (T1895) were purchased from Sigma-Aldrich; MLN8054 was purchased from selleckBio.com. Lactacystin (CAS1258004) was purchased from Merck Millipore.

Cell-fractionation and protein-stability assays

U2OS cells were transiently transfected with GFP or GFP-Pin1. Co-transfection with GFP-Bora and DsRed-Pin1 (DsRed vector as control) was used to study the Pin1-mediated cytoplasmic translocation of Bora (Crenshaw et al., 1998). For the protein-stability assay, control and stable GFP-Pin1-expressing cells were treated with cycloheximide (100 ng/ml) and the cells were harvested at 30, 60, 90, 120, 150 minutes for immunoblot analyses.

LC-MS/MS-based phosphopeptide mapping analysis

HEK293T cells were transfected with FLAG-Bora and synchronized in the G2/M phase by nocodazole. FLAG-Bora was immunoprecipitated using FLAG antibody following the in-gel tryptic digestion (Shevchenko et al., 1996), and the *in vivo* phosphorylation sites of Bora were identified by MALDI-TOF mass spectrometry (Hoffmann et al., 1999).

Protein-binding assays

GST pull-down and immunoprecipitation analyses were performed as previously described (Lu et al., 2002). Briefly, the relevant proteins were expressed in HEK293T or U2OS cells by transient transfection. GST recombinant proteins were bound on GSH-agarose beads and incubated with cell lysates at 4℃ for 2–3 hours before SDS-PAGE and immunoblot analyses (Lu et al., 2002). For immunoprecipitation reactions, 0.5–1 mg of nocodazole-arrested mitotic cell lysates was used per sample and binding was performed using the indicated antibodies (2–4 μg). Wild-type Pin1 (or its mutants) was co-expressed with FLAG-Bora in stable Pin1-knockdown U2OS cells for examination of the binding of Pin1 to Bora.

Immunofluorescence microscopy

U2OS cells were grown on coverslips and fixed with ice-cold 100% methanol for 10 minutes before permeabilization. The primary antibodies used were anti-Bora, anti-Aurora-A and anti-Pin1. The secondary fluorescence antibodies used were anti-mouse Alexa Fluor 488 (green) and anti-rabbit Alexa Fluor 633 (red). To examine the colocalization and cytoplasmic translocation of Bora and the S274A/S278A Bora mutant with Pin1 in cells, GFP-Bora or GFP-S274A/S278A Bora mutant were co-transfected with DsRed-Pin1 into U2OS cells. Confocal laser-scanning microscope was used to examine the protein–protein colocalization.

Statistical analysis

Statistical analyses were performed using the Student's *t*-test. Data are presented as the mean ± s.d.; *P* values less than 0.05 were considered statistically significant.

Author contributions

Y.-C.L. designed the study, drafted and wrote the manuscript. J.Q., L.-C.L. and L.-Y.H. provided materials. P.-C.L. provided LC/MS/MS-based phosphopeptide mapping analysis. Y.-C.L. provided materials and reviewed the manuscript. Y.-C.C. and J.-T.L. reviewed the manuscript. Y.-P.L. and K.-H.L. helped to organize the data and reviewed the manuscript. M.H., C.-Y.H. and P.-J.L. gave conceptual advice, provided materials and reviewed the manuscript.

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