Greatwall and Polo-like Kinase 1 Coordinate to Promote Checkpoint Recovery

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Checkpoint recovery upon completion of DNA repair allows the cell to return to normal cell cycle progression and is thus a crucial process that determines cell fate after DNA damage. We previously studied this process in *Xenopus* egg extracts and established Greatwall (Gwl) as an important regulator. Here we show that preactivated Gwl kinase can promote checkpoint recovery independently of cyclin-dependent kinase 1 (Cdk1) or Plx1 (*Xenopus* polo-like kinase 1), whereas depletion of Gwl from extracts exhibits no synergy with that of Plx1 in delaying checkpoint recovery, suggesting a distinct but related relationship between Gwl and Plx1. In further revealing their functional relationship, we found mutual dependence for activation of Gwl and Plx1 during checkpoint recovery, as well as their direct association. We characterized the protein association in detail and recapitulated it *in vitro* with purified proteins, which suggests direct interaction. Interestingly, Gwl interaction with Plx1 and its phosphorylation by Plx1 both increase at the stage of checkpoint recovery. More importantly, Plx1-mediated phosphorylation renders Gwl more efficient in promoting checkpoint recovery, suggesting a functional involvement of such regulation in the recovery process. Finally, we report an indirect regulatory mechanism involving Aurora A that may account for Gwl-dependent regulation of Plx1 during checkpoint recovery. Our results thus reveal novel mechanisms underlying the involvement of Gwl in checkpoint recovery, in particular, its functional relationship with Plx1, a well characterized regulator of checkpoint recovery. Coordinated interplays between Plx1 and Gwl are required for reactivation of these kinases from the G$_2$/M DNA damage checkpoint and efficient checkpoint recovery.

Various types of DNA damage are frequently induced by both endogenous and exogenous agents, posing enormous threats to the cell and its genomic integrity. The cell responds to the occurrence of DNA damage by engaging DNA repair machineries to restore normal DNA structure and by activating the checkpoint mechanism through complex networks of signaling transduction to halt cell cycle progression (1). Eventually, if the cell successfully repairs its damaged DNA, checkpoints are to be turned off to allow resumption of cell cycle progression. This process, termed “checkpoint recovery,” is contrasted by permanent checkpoint arrest or programmed cell death (senescence or apoptosis, respectively), both of which are believed to result from un repaired DNA damage and sustained DNA damage signaling (2, 3).

The turn-off mechanism of the DNA damage checkpoint during recovery is poorly understood. Existing studies suggest that protein dephosphorylation and proteolysis are effective ways to deactivate checkpoint signaling. The involvement of numerous serine/threonine phosphatases in checkpoint recovery is not surprising given the crucial role of protein phosphorylation and kinase cascades in checkpoint activation (4). The wild-type p53-induced phosphatase Wip1 (PP2Cδ or PPM1D), as the best studied example of a DNA damage response phosphatase, has been shown to bind and dephosphorylate a number of key DNA damage response factors, including ATM, γ-H2AX, Chk1, Chk2, and p53. Wip1 expression is up-regulated after DNA damage in a p53-dependent manner and is required for efficient checkpoint inactivation and recovery (5). In addition to protein dephosphorylation, ubiquitin and proteasome-mediated degradation of key checkpoint activators has also been linked to checkpoint recovery. In particular, several studies have found that both Claspin, a mediator protein required for Chk1 activation, and Wee1, a tyrosine kinase responsible for inhibitory phosphorylation of Cdk1, are targeted for proteolysis during checkpoint recovery. Proteolysis of these proteins, referred to as a crucial step of G$_2$/M checkpoint recovery, is triggered by their phosphorylation by Polo-like kinase 1 (Plk1), a well characterized mitotic kinase that plays essential roles in multiple aspects of mitotic progression. Plk1-dependent phosphorylation creates docking sites for β-TrCP-SCF Skp1-Cul1-F-box ligase containing the F-box protein beta-transducin repeat-containing protein ubiquitin ligase complex in Claspin and Wee1 to mediate their proteolysis (6–10). Interestingly, Plk1-dependent phosphorylation of 53BP1, another mediator protein required for Chk2 and Chk1 activation, may disrupt its function by displacing it from chromatin (11). Collectively, these studies established Plk1 as a key regulator of cell recovery from the G$_2$/M DNA damage checkpoint.

In addition to Plk1, other mitotic kinases may also participate in checkpoint recovery. For example, it has been recently discovered that Aurora A kinase is required for cell recovery from the G$_2$/M checkpoint arrest. Interestingly, the study also indicated that Aurora A functions in checkpoint recovery through Plk1: when complexed to its co-factor, Bora, Aurora A phos-

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2 The abbreviations used are: Plk1, Polo-like kinase 1; CDK/Cdk, cyclin-dependent kinase; Gwl, Greatwall; KD, kinase-dead; MPF, maturation promoting factor; Plx1, *Xenopus* Polo-like kinase 1.
phosphorylates Plk1 at its T-loop activation site, leading to Plk1 activation (12, 13). Moreover, we recently reported another mitotic kinase, named Greatwall (Gwl), involved in checkpoint recovery (14). First identified in *Drosophila* and then extensively studied in *Xenopus* egg extracts and human cells, Gwl has been shown to be an essential mitotic kinase activated by phosphorylation (15–19). Joint efforts from multiple laboratories during the past a few years have provided valuable insights into Gwl function. It has become clear that Gwl contributes to mitotic entry and maintenance by inhibiting a key protein phosphatase complex, PP2A/B55δ, which otherwise would dephosphorylate CDK substrates regardless of CDK status (20–24). Gwl-dependent inhibition of PP2A/B55δ is exerted via α-endosulfine (Ensa) or cAMP-regulated phosphoprotein, 19-kDa (Arpp-19), two related proteins that have been recently identified as key substrates of Gwl kinase in *Xenopus* egg extracts. Ensa and/or Arpp-19, once phosphorylated by Gwl, specifically bind and inhibit PP2A/B55δ, yet spare PP2A complexed to other targeting subunits (25–27). Although these elegant studies have gone a long way in revealing Gwl functions, it is still largely unclear how Gwl activation is regulated or whether Gwl may also function through other, as yet unknown substrates. Interestingly, we have shown previously that Gwl is a negative regulator of DNA damage checkpoint signaling (14). Depletion of Gwl from interphase *Xenopus* egg extracts resulted in elevated phosphorylation of checkpoint proteins; conversely, supplementation with recombinant, wild-type, but not kinase-dead, Gwl rendered the extract insensitive to DNA damage. Importantly, depletion of Gwl impeded checkpoint recovery, as shown by sustained phosphorylation of checkpoint proteins and failure in reactivation of Cdk1, in a manner that can be fully reversed by adding back WT Gwl (14).

Our previous results thus established Gwl as a key regulator of checkpoint recovery, in somewhat comparable fashion with the well-characterized role of Plk1 (Plx1 in *Xenopus*) in this pathway. In the current study, we find that the involvement of Gwl in checkpoint recovery is both distinct from, and related to that of Plx1. Gwl and Plx1 rely on each other for reactivation from DNA damage checkpoint arrest. Moreover, we discover and characterize direct interaction between Gwl and Plx1 and confirm direct phosphorylation of Gwl by Plx1. Gwl interaction with Plx1 and its phosphorylation by Plx1 appear elevated during checkpoint recovery, which process is promoted by Plx1-mediated phosphorylation of Gwl. Finally, we identify an indirect route of Plx1 regulation by Gwl during checkpoint recovery: Gwl does not directly phosphorylate Plx1, but rather controls activation of Aurora A, which has been recently shown to serve as the upstream, T-loop kinase of Plx1 (12, 13).

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Antibodies against human Chk1 Ser(P)-345 (*Xenopus* Chk1 Ser-342) and human Aurora A Thr(P)-288 (*Xenopus* Aurora A Thr-295) were obtained from Cell Signaling Technology (Beverly, MA). Human Plk1 Thr(P)-210 (*Xenopus* Plx1 Thr(P)-201) antibody was obtained from Abcam (Cambridge, MA). Antibodies to Smc1 and Smc1 Ser(P)-957 were purchased from Bethyl Labs (Montgomery, TX). GST antibody was purchased from Sigma. Cdc27 antibody was purchased from BD Transduction Laboratories (San Jose, CA). Antibodies to *Xenopus* Gwl, Plx1, and Aurora A were characterized in Refs. 14, 17, and 28–30.

**GST Fusion Protein Expression and Pulldown**—Eight segments (N, amino acids 1–340; M, amino acids 335–660; C, amino acids 656–887; N1, amino acids 34–340; N2, amino acids 186–340; N3, amino acids 34–190; N4, amino acids 1–190; N5, amino acids 1–39) of Gwl were subcloned into pGex 4T-1 vector (GE Healthcare). The resulting constructs were transformed into bacteria strain BL21 (New England Biolabs) for expression. GST fusion proteins were purified on glutathione-Sepharose beads (New England Biolabs) from BL21 lysates. For GST pulldown assays, glutathione-Sepharose beads with specific recombinant proteins were incubated with *Xenopus* egg extracts for 30 min, isolated by centrifugation, washed, and eluted by boiling in Laemmli sample buffer (Bio-Rad).

**Immunoblotting**—Protein samples were denatured by boiling in 2× Laemmli sample buffer, resolved by SDS-PAGE, and then electrotransferred to PVDF membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat dry milk in 1× TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h and incubated with specific primary antibodies for 2 h. Membranes were then washed three times in 1× TBST before horseradish peroxidase (HRP)-conjugated secondary antibodies (Sigma) were added. After 1-h incubation with the secondary antibody, membranes were washed three times in TBST and immunoreactive signals detected using Enhanced Chemiluminescence (ECL) substrate kit (Pierce).

**Immunodepletion**—For Gwl immunodepletion, anti-mouse or anti-rabbit magnetic beads (New England Biolabs) were pre-washed three times in washing buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, and 0.5% Tween 20) and then incubated for 1 h with antibodies. Beads conjugated to the antibody were washed three times in washing buffer and then mixed with *Xenopus* egg extracts. After a 30-min incubation, the beads were removed with a magnet and the remaining extract collected.

**Immunoprecipitation**—For immunoprecipitation, anti-mouse or anti-rabbit magnetic beads (New England Biolabs) were conjugated to antibodies as described above and then mixed with egg extracts. After a 30-min incubation, the beads were removed with a magnet and washed three times in washing buffer before elution with 2× Laemmli sample buffer and analyzed by immunoblotting.

**Kinase Assay**—Plx1 and Gwl kinase assays were performed as previously described (14, 29). Briefly, the indicated kinases and substrates were resuspended in a final volume of 30 μl of kinase buffer (20 mM HEPES, pH 7.5, 2 mM DTT, 10 mM MgCl2, 0.1 mM EGTA, 100 μM cold ATP, 2 μCi of [γ-32P]ATP), and incubated for 20 min at 30 °C. The kinase reaction was stopped by boiling in 2× Laemmli buffer.

**Xenopus Egg Extracts**—Cytostatic factor extracts were freshly prepared as previously described (31, 32). The extract was released into interphase by supplementation with 0.4 mM CaCl2, and incubated for 30 min at room temperature. For checkpoint activation and recovery, biotinylated d(A-T) oligonucleotides were prebound to M-280 streptavidin Dynabeads (Invitrogen) following the standard protocol provided by the
manufacturer, and the beads were then added to the extracts to produce a final concentration of 20 μg/ml d(A-T). After 30 min, the beads were removed with a magnet to initiate checkpoint recovery, and the removal point is referred to as the 0 min time point of recovery.

RESULTS

Gwl Deactivates Checkpoint Signaling Independent of Cdk1 and Plx1

—Xenopus egg extract has become a widely used experimental system to study activation of DNA damage checkpoint signaling (33, 34). We have recently developed this system further to recapitulate the process of G2/M checkpoint recovery (14). We first add biotin-labeled double-stranded oligonucleotides into egg extracts on streptavidin beads to activate the DNA damage response and then remove the beads from extracts to mimic the completion of DNA repair. As expected, the remaining extract, over time, deactivates checkpoint signaling and reinitiates cell cycle progression into mitosis (supplemental Fig. S1). This system allows us to study biochemical progression of checkpoint recovery in a homogeneous and synchronized manner.

We have previously taken advantage of the above described system and discovered a novel involvement of Gwl kinase in checkpoint recovery (14). During normal G2/M transition, Gwl depletion prevents Cdk1 activation via accumulation of inhibitory phosphorylation on Cdk1 (17). Similarly, we have shown that reactivation of Cdk1 during checkpoint recovery is dependent on Gwl (14). Therefore, it is possible that Gwl promotes checkpoint recovery solely through activation of Cdk1. To test this, we supplemented recovery extracts with roscovitine, a Cdk inhibitor, at a concentration that has been previously shown to abolish Cdk1 activation in egg extracts (15) and asked whether the subsequent addition of preactivated recombinant Gwl kinase still promotes checkpoint recovery. As shown in Fig. 1A, despite the lack of Cdk1 activity, Gwl still efficiently accelerates dephosphorylation of Smc1 and Chk1.

We then extended this study to examine whether Gwl promotes checkpoint recovery through Plx1, known as a central regulator of this process (35, 36). As shown in Fig. 1B, in extracts in which Plx1 was completely removed by immunodepletion, preactivated recombinant Gwl is still capable of promoting dephosphorylation of Smc1 and Chk1. Taken together, these results show that Gwl, once activated, can facilitate checkpoint recovery in the absence of Cdk1 or Plx1.

Depletion of Plx1 and Gwl Does Not Synergistically Affect Checkpoint Recovery

—Current knowledge about checkpoint recovery underscores the critical involvement of Plx1, which phosphorylates a number of essential checkpoint activators, leading to their inactivation (35, 36). The notion that Gwl, another mitotic kinase, promotes deactivation of checkpoint signaling independent of Plx1 is very exciting because it suggests the existence of parallel mechanisms. To delineate further the involvement of Plx1 and Gwl in this process, we compared checkpoint deactivation in extracts depleted of Plx1, Gwl, or both. As expected, depletion of either Plx1 or Gwl clearly led to sustained phosphorylation of Smc1 and Chk1 and cell cycle arrest in interphase (Fig. 2), consistent with previously reported functions of these kinases in various experimental systems (6–10, 14). Surprisingly, simultaneous depletion of both Plx1 and Gwl did not cause a more severe deficiency in checkpoint deactivation compared with a single depletion of either Plx1 or Gwl (Fig. 2), suggesting that Plx1 and Gwl operate in an overlapping or interdependent manner.

FIGURE 1. Active Gwl promotes checkpoint recovery independently of Cdk1 and Plx1. A, preactivated Gwl purified from okadaic acid-treated Sf9 cells, as previously described (17), was added into recovery extracts with roscovitine (250 μm), as shown in the left panel. Extracts were harvested at indicated time points and analyzed by Western blotting using the indicated antibodies. B, Gwl was added into recovery extracts that were mock-depleted (−) or Plx1-depleted (+), as shown in the left panel. Extracts harvested at the indicated time points were analyzed by Western blotting using the indicated antibodies.
Reactivation of Plx1 and Gwl during Checkpoint Recovery—

In light of the above results (Figs. 1 and 2), we speculated that Gwl and Plx1, though each, once activated, can promote checkpoint recovery alone, rely on each other for activation. It has been shown that both Gwl and Plx1 are inhibited by DNA damage checkpoint signaling, whereas their reactivation during recovery, apparently initiated before the turn-off of checkpoint signaling, is poorly understood (14, 37). Interestingly, reactivation of Gwl is dependent on Plx1, whose depletion prevented Gwl activation, as judged by its phosphorylation. Add-back of purified WT Plx1 at least partially restored Gwl activation, confirming that loss of Gwl activation is indeed due to Plx1 depletion (Fig. 3A).

Although the above observation could suggest Plx1 as an upstream kinase of Gwl during checkpoint recovery, it should be noted that we also found that activation of Plx1 is dependent on Gwl. As shown in Fig. 3B, activation of Plx1 during checkpoint recovery, indicated by phosphorylation of its T-loop activation site, is not evident without Gwl, but can be partially rescued by adding back purified WT Gwl.

Gwl and Plx1 Association—The above discovered mutual dependence of Gwl and Plx1 activation during checkpoint recovery may reflect indirect modulation through other kinases or activators. However, as shown in Fig. 4A, we immunoprecipitated Gwl from interphase egg extracts and observed co-immunoprecipitation of a portion of Plx1. Conversely, we immunoprecipitated Plx1 from extracts and confirmed the presence of Gwl in its immunocomplex (Fig. 4B). Therefore, we report here direct association of these two kinases, which argues for the existence of a direct functional relationship between them.

To characterize further the association between Gwl and Plx1, we sought to identify the Plx1-binding motif in Gwl. Gwl contains a split kinase domain separated by a long and less conserved internal region. We expressed Gwl in three segments: N, a segment containing the N terminus of Gwl; M, a segment of the internal region; and C, a segment of the C-terminal portion of Gwl (Fig. 4C). All segments of Gwl were fused to GST to facilitate purification. As shown in Fig. 4D, the N segment efficiently pulls down Plx1, which is not evident with the M segment, and only seen at a much lower level with the C segment (Fig. 4D). The N segment is thus the main Plx1-binding motif in Gwl, so we then further mapped the association by introducing a series of truncations from either or both ends of the N segment (Fig. 4C). As shown in Fig. 4E, N3, the kinase domain, or N1 and N4, which contain the kinase domain, associate with Plx1; whereas neither N2 nor N5, regions flanking the kinase domain, shows Plx1 binding. Therefore, the N-terminal portion of the kinase domain in Gwl is both required and sufficient for Plx1 binding.

To determine whether Gwl and Plx1 association reflects direct interaction, we added together purified Plx1 and Gwl proteins, as shown in Fig. 4F, both WT and kinase-dead (KD)
Gwl interact with Plx1, whereas both WT and KD (N172A) Plx1 co-purified with Gwl (Fig. 4G). A reciprocal IP using Plx1 antibody also efficiently co-immunoprecipitated purified Gwl (supplemental Fig. S2A). To our surprise, KD Plx1 exhibits a stronger interaction with Gwl compared with the WT (Fig. 4G). Taken together, these results demonstrated direct interaction between Gwl and Plx1 and that the kinase activity of neither Gwl nor Plx1 is required for their interaction. This notion is also confirmed by pull down of FLAG-tagged Gwl from extracts, in which both WT and KD Gwl recovered a portion of Plx1 (supplemental Fig. S2B).

**Increased Gwl and Plx1 Interaction during Checkpoint Recovery**—Interestingly, the interaction between Gwl and Plx1, although readily detectable in interphase egg extracts, appears elevated during checkpoint recovery. As shown in Fig. 5A, Plx1 was immunoprecipitated from either interphase or recovery extract (made from interphase extracts in which damaged DNA was added and then removed, as described under “Experimental Procedures” and supplemental Fig. S1). Although a comparable amount of Plx1 was recovered from these extracts, Gwl protein co-immunoprecipitated from the recovery extract was clearly more abundant (Fig. 5A). Similarly, Gwl immunocom-
plex isolated from the recovery extract contains more Plx1 compared with that from interphase extracts (Fig. 5B). Moreover, GST pulldown using N-terminal Gwl consistently brings down more Plx1 from the recovery extract than from the interphase extracts (Fig. 5C). The last line of evidence is of particular interest, as it indicates that the N-terminal Gwl contains the regulatory element in Gwl that is responsible for the increased interaction with Plx1.

Gwl Phosphorylation by Plx1—An immediate possibility hinted at by the interaction between Gwl and Plx1 is that these kinases phosphorylate each other or each other’s co-factors. It has been shown previously that both MPF and Plx1 can phosphorylate Gwl in vitro (17). Consistently, we show in Fig. 6A that both WT and KD Gwl are efficiently phosphorylated by WT Plx1 in vitro. Importantly, as shown in Fig. 6B, although Gwl immunoprecipitated from the interphase extract can be phosphorylated by Plx1, Gwl immunoprecipitated from the recovery extract was more efficiently targeted. Notably, in Figs. 5 and 6B, early stage recovery extracts (30 min after the removal of damaged DNA) were used, whereas reactivation of Plx1 generally becomes detectable after 60 min. Therefore, certain molecular events take place at the early stage of checkpoint recovery to enhance/stabilize Gwl/Plx1 interaction and render Gwl more favorable for Plx1-dependent phosphorylation. Moreover, Gwl isolated from recovery extracts of this stage should not be prephosphorylated by Plx1 due to the lack of endogenous Plx1 activity.

Plx1-mediated Phosphorylation of Gwl Stimulates Checkpoint Recovery—Our results showing direct interaction between Gwl and Plx1 are in support of a functional relationship between these two kinases. More importantly, the interaction and Gwl phosphorylation by Plx1 are enhanced during checkpoint recovery, implying their involvement in this process. We thus sought to test whether phosphorylation of Gwl by Plx1 promotes checkpoint recovery. As shown in Fig. 7, although recombinant Gwl can promote dephosphorylation of Smc1 upon addition into recovery extracts, it does so more efficiently if prephosphorylated by Plx1; the KD mutant of Gwl, albeit also phosphorylated by Plx1, does not elicit a similar response as the WT, suggesting a requirement of Gwl kinase activity.

DISCUSSION

For the cell to recover from the G2/M DNA damage checkpoint and resume cell cycle progression into mitosis, it needs to turn off checkpoint signaling and reactivate mitotic kinases that are required for mitotic progression. An important lesson learned from previous studies on Plk1 is that this mitotic kinase, although known to be inhibited by checkpoint signaling, becomes reactivated during checkpoint recovery. Activated
Plk1 targets and inactivates multiple elements of the G2/M DNA damage checkpoint, leading to recovery (6–10). Notably, cells in which Plk1 is dispensable for mitotic entry during normal cell cycle fail to recover from the G2/M DNA damage checkpoint and to reenter mitosis without Plk1, suggesting a more stringent requirement of Plk1 for checkpoint recovery than for unperturbed cell cycle progression (7). Interestingly, we have recently shown that another mitotic kinase, Gwl, is involved in checkpoint recovery as a negative regulator of DNA damage signaling, as judged by its ability to accelerate dephosphorylation of checkpoint proteins and its essential role in reactivation of Cdk1 (14). With this discovery arose two important questions regarding the involvement of Gwl in checkpoint recovery: 1), how is Gwl activated and regulated during checkpoint recovery; and 2), what are the downstream pathways of Gwl in this process? Although this study is mainly focused on the first aspect, we also showed here that preactivated Gwl can promote checkpoint inactivation in the absence of Plx1 (as shown here), suggesting that Gwl, upon preactivation, can bypass the requirement of Plx1. We therefore speculated that Gwl and Plx1 may rely on each other for activation and confirmed the hypothesis by showing that depletion of Gwl abolished activation of Plx1, and vice versa. The mutual dependence of Gwl and Plx1 activation is consistent with the lack of synergistic effect between Gwl and Plx1 depletion because deletion of either one also eliminates the activation of the other, whose deletion thus does not further affect checkpoint recovery. This relationship between Gwl and Plx1 reflects coordinative and positive feedback actions and is in line with existing studies of their functions in promoting mitotic entry (6–10, 15–19, 35, 36), but not with a genetic study in Drosophila that revealed a mutually antagonistic relationship between these two kinases (16). We are uncertain about the cause of this apparent difference between the studies; however, it should be noted that the later study examined different biological processes, namely, embryonic development and mitotic/meiotic abnormalities (16).

Although it is possible that Gwl and Plx1 indirectly regulate each other, for instance, through Cdk1, or other intermediate substrates or factors, we present here novel evidence that these two kinases are physically associated, possibly through direct interaction, because purified Gwl and Plx1 are capable of binding each other. Further analysis showed that the N-terminal portion of the Gwl kinase domain is the major Plx1-binding motif. Kinase-inactivating mutations in Gwl or Plx1 do not disrupt the interaction, suggesting that the activity of neither kinase is required. Unfortunately, the region in Gwl that binds Plx1 is also required for its own kinase activity, we thus are unable to assess directly the functional significance of the inter-
action. A possibility, however, is that the interaction enables these kinases to phosphorylate each other or additional factors that are present in the complex. We then confirmed that Plx1 can efficiently phosphorylate Gwl in vitro. The in vitro phosphorylation of Gwl by Plx1 was also shown in a previous report compared with that by MPF (17). These authors reported that MPF, but not Plx1-mediated phosphorylation of Gwl significantly augmented its kinase activity in vitro. Importantly, MPF-phosphorylated Gwl, upon addition into interphase egg extracts, was sufficient to induce mitosis, an effect not seen with Gwl phosphorylated by Plx1. Therefore, compared with Plx1, MPF seems to be a better candidate for the upstream kinase responsible for Gwl activation during normal mitotic entry (17). Interestingly, during the preparation and publication of this work, an independent study re-investigated this phosphorylation event and reached a different conclusion: the authors found that Gwl, when phosphorylated by Plx1 in vitro, exhibits significantly enhanced kinase activity, which can be further stimulated in the presence of the phosphorylated hydrophobic motif of Rsk2. The latter study also identified a site in Gwl that is essential for Gwl activation and can be phosphorylated by both Plx1 and MPF in vitro (38). It thus remains to be further clarified whether and how Plx1-dependent phosphorylation contributes to Gwl activation, and more importantly, if Plx1-mediated phosphorylation does not render Gwl active in promoting mitosis (17), what would be its physiological relevance?

Interestingly, we show here that both Gwl and Plx1 interaction and Gwl phosphorylation by Plx1 are enhanced during the early stage of checkpoint recovery, suggesting a potential involvement of this regulation in checkpoint recovery. This notion is further strengthened by our finding that Gwl phosphorylated by Plx1 exhibits stronger activity in promoting checkpoint recovery compared with the unphosphorylated control. Collectively, our results indicate a direct relationship between Gwl and Plx1 and provide novel insights into the functional aspect of Plx1-mediated phosphorylation of Gwl in the process of checkpoint recovery. Although similar regulation may also take place during normal cell cycle progression, this is, nevertheless, a possibility that this regulation is of more significance to checkpoint recovery; after all, previous studies have reported a differential requirement of Plx1 between checkpoint recovery and normal cell cycle progression (7). Additionally, MPF, although capable of activating Gwl during normal mitotic entry, is likely to be kept inactive during the initial stage of checkpoint recovery. It has been shown that, during checkpoint recovery, Aurora A and Plx1 are activated before the onset of mitosis and promote checkpoint deactivation and cell cycle progression into mitosis (12). The similar role of Gwl in promoting G2/M checkpoint recovery may also suggest its activation prior to mitosis, or Cdk1 activation, in which scenario, its activation may be initially promoted by Plx1-mediated phosphorylation.

With respect to the Gwl dependence of Plx1 activation during recovery, we show that Gwl is unable to phosphorylate Plx1, thus the regulation is likely to be achieved indirectly. Inspired by previous reports showing that Plx1 is phosphorylated and activated by Aurora A kinase (12, 13), we found that Gwl is required for activation of Aurora A during checkpoint recovery and that reconstitution of WT Aurora A activity in Gwl-depleted extracts efficiently restored Plx1 reactivation. We therefore conclude that Gwl regulates Plx1 through Aurora A. It has been a matter of debate whether Aurora A activation is dependent on Cdk1 during cell cycle progression (39–41). The apparent discrepancy among these studies could result from the use of different experiment systems or cell lines. Interestingly, a recent report distinguished the initial activation of Aurora A in G2, which does not require Cdk1, from its mitotic, and Cdk1-dependent activation (42). Moreover, Aurora A is involved in feedback regulation with Plx1 through its co-factors, such as Tpx2, and Bora (29, 43, 44). In light of these studies, the requirement of Gwl for Aurora A activation during checkpoint recovery, as discovered here, needs to be investigated further.

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