

Protein phosphatase 1 is essential for Greatwall inactivation at mitotic exit

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Abstract

Entry into mitosis is mediated by the phosphorylation of key cell cycle regulators by cyclin-dependent kinase 1 (Cdk1). In *Xenopus* embryos, the M-phase-promoting activity of Cdk1 is antagonized by protein phosphatase PP2A-B55. Hence, to ensure robust cell cycle transitions, Cdk1 and PP2A-B55 must be regulated so that their activities are mutually exclusive. The mechanism underlying PP2A-B55 inactivation at mitotic entry is well understood: Cdk1-activated Greatwall (Gwl) kinase phosphorylates Ensa/Arpp19, thereby enabling them to bind to and inhibit PP2A-B55. However, the re-activation of PP2A-B55 during mitotic exit, which is essential for cell cycle progression, is less well understood. Here, we identify protein phosphatase PP1 as an essential component of the PP2A-B55 re-activation pathway in *Xenopus* embryo extracts. PP1 initiates the re-activation of PP2A-B55 by dephosphorylating Gwl. We provide evidence that PP1 targets the auto-phosphorylation site of Gwl, resulting in efficient Gwl inactivation. This step is necessary to facilitate subsequent complete dephosphorylation of Gwl by PP2A-B55. Thus, by identifying PP1 as the phosphatase initiating Gwl inactivation, our study provides the molecular explanation for how Cdk1 inactivation is coupled to PP2A-B55 re-activation at mitotic exit.

Keywords Greatwall; mitosis; PP1; PP2A B55; *Xenopus* embryos

Subject Categories Cell Cycle; Post translational Modifications, Proteolysis & Proteomics

Introduction

Cell cycle transitions are driven by changes in the phosphorylation state of cell cycle proteins. Cdk1 in complex with cyclin B triggers entry into mitosis by phosphorylating numerous structural as well as regulatory proteins. To facilitate full mitotic commitment and avoid futile cycles of phosphorylations and dephosphorylations,

activation of Cdk1/cyclin B at mitotic entry must be accompanied by the simultaneous inhibition of antagonizing phosphatases. In *Xenopus laevis* oocytes and embryos, a key antagonist of Cdk1 mediated substrate phosphorylation is protein phosphatase 2A with its regulatory B55 subunit (PP2A B55) [1,5]. At mitotic entry, Cdk1/cyclin B mediates the inactivation of PP2A B55 via the atypical AGC type kinase Greatwall (Gwl) [6,7]. According to the current model [8,9], Cdk1/cyclin B phosphorylates *Xenopus* Gwl at T193 and T206 and these phosphorylations enable Gwl to autophosphorylate itself at a C terminal serine residue (S883) resulting in Gwl activation. Active Gwl phosphorylates Arpp19 and the closely related endosulfine α /Ensa (collectively referred to as Arpp19) and thereby converts them into potent inhibitors of PP2A B55 [10,11].

Cdk1/cyclin B inactivation at mitotic exit is mediated by the selective destruction of cyclin B via the anaphase promoting complex/cyclosome (APC/C) [12,13]. The concomitant re-activation of PP2A B55 depends on the dephosphorylation of both Arpp19, to relieve its inhibitory effect on PP2A B55, and Gwl, to stop the replenishment of phosphorylated Arpp19. The identity of the phosphatase dephosphorylating Arpp19 remains controversial with Fcp1 and PP2A B55 being potential candidates [1,14,15]. According to Williams *et al* (2014), Arpp19 is not only an inhibitor of PP2A B55, but also a substrate that binds extremely tightly to PP2A B55 and is dephosphorylated at a very low rate. Based on these findings, an “inhibition by unfair competition” model has been postulated according to which phosphorylated Arpp19 inhibits PP2A B55 by blocking access of other substrates while being slowly dephosphorylated. PP2A B55 has also been suggested to dephosphorylate Gwl at a Cdk1 site (T193 in *Xenopus*) in the activation loop [14]. However, PP2A B55 is unlikely to be the phosphatase that initiates Gwl inactivation at mitotic exit because it is inhibited in this situation by the product of Gwl, that is, phosphorylated Arpp19 (pArpp19) which is present in molar excess over PP2A B55 [15]. Such a system would be futile with PP2A B55 not being able to inactivate Gwl as long as pArpp19 levels are high and pArpp19 levels remaining high as long as Gwl is active. And indeed, recent mathematical modeling studies postulated that an additional phosphatase termed PPx exists that triggers the inactivation of Gwl at mitotic exit [16]. Here, we identify PP1 as the sought after phosphatase essential for Gwl inactivation at mitotic exit in extract of *Xenopus laevis* early embryos. Inhibition of PP1 blocks dephosphorylation and consequentially the inactivation

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of Gwl. We provide evidence that the autophosphorylation site of Gwl is the critical target of PP1 and dephosphorylation of this site largely inactivates Gwl. Thus, this study proposes a model for how PP1 in concert with PP2A B55 dephosphorylates and inactivates the Gwl Arpp19 module to allow faithful exit from mitosis.

Results and Discussion

PP1 activity is required for the dephosphorylation of Gwl during mitotic exit

To gain insights into the mechanism of Gwl inactivation at mitotic exit, we used a previously established *Xenopus* embryo extract system [17]. Interphasic extract was prepared from embryos that were in the process of exiting the second mitotic division as evidenced by cleavage furrow ingression (Fig 1A). The interphasic stage of the extract was confirmed by immunoblotting (IB) for the APC/C inhibitor XErp1 and Cdc27 which both display a faster mobility in SDS PAGE during interphase than during mitosis. (Fig 1B, lane 1). Mitotic entry was induced by the addition of non-degradable cyclin B1 ($\Delta 90$), which resulted in retarded mobility of XErp1 and Cdc27 in SDS PAGE as well as APC/C activation indicated by the destruction of endogenous cyclin B2 (lane 2). To mimic mitotic exit, Cdk1 was inactivated by the addition of roscovitine. As expected, Cdk1 inhibition resulted in fast and efficient dephosphorylation of Cdc27 and XErp1 (lane 3-8). Collectively, these experiments validate *Xenopus* embryonic extract as an appropriate system to analyze mitotic exit.

A prerequisite for the reactivation of PP2A B55 at mitotic exit is the inactivation of Gwl by dephosphorylation. Previously, it was reported that PP2A B55 is required for the dephosphorylation of a critical site in the activation loop of Gwl during mitotic exit [14]. To investigate whether PP2A B55 is the only relevant Gwl phosphatase for *Xenopus* embryonic divisions, mitotic exit was induced in extract supplemented with increasing concentrations of Arpp19 that was *in vitro* pre-phosphorylated by the hyperactive Gwl mutant K71M (Fig 1C). To ensure efficient PP2A B55 inhibition, the slowly hydrolysable ATP analogue ATP γ S was used for Arpp19 phosphorylation (ThioArpp) [15]. Notably, even at the highest concentration of ThioArpp which was about fivefold the concentration of endogenous Arpp19 [15] Gwl dephosphorylation upon Cdk1 inactivation

was not completely but only partially inhibited (Fig 1D). This effect was specific for PP2A B55 inhibition because no effect on Gwl dephosphorylation was observed when the extract was supplemented with high concentrations of mutant Arpp19 (S67A) that could not be phosphorylated by Gwl *in vitro* (Fig 1C and D). In line with the previous reports [15,16], we conclude that a phosphatase in addition to PP2A B55 mediates Gwl dephosphorylation at mitotic exit.

PP1 is under the negative control of Cdk1/cyclin B [18], making it an attractive candidate phosphatase that could contribute to Gwl dephosphorylation once Cdk1 activity declines due to cyclin B destruction. To test this idea, Gwl dephosphorylation was monitored in extract treated with both ThioArpp and the PP1 specific inhibitory protein Inhibitor 2 (I 2) [19]. Notably, under these conditions, dephosphorylation of Gwl was completely prevented (Fig 1E). Gwl dephosphorylation was also completely prevented when ThioArpp was added together with a His tagged wild type (wt) fragment of NIPP1 (nuclear inhibitor of PP1, Fig EV1A), which as shown previously efficiently inhibits PP1 [20]. Addition of His NIPP1 with a mutated PP1 binding motif (RVxF \rightarrow RAxA) had no effect (Fig EV1A). In summary, these data suggest that PP1 together with PP2A B55 acts on the Gwl Arpp19 B55 module during mitotic exit.

PP1 mediates the dephosphorylation of Gwl rather than of Arpp19

Next, we investigated how PP1 contributes to Gwl dephosphorylation during mitotic exit. Within the Gwl Arpp19 B55 module, two potential mechanisms are conceivable: First, PP1 assists PP2A B55 in dephosphorylating Arpp19 resulting in activation of PP2A B55, which according to a previous report dephosphorylates Gwl [14]. Second, PP1 acts on Gwl itself. To test whether PP1 acts on Arpp19, we analyzed the dephosphorylation of Arpp19 in the presence (buffer) or absence (I 2) of PP1 activity. To monitor Arpp19 dephosphorylation, we prevented its Gwl mediated re-phosphorylation by depleting Gwl from $\Delta 90$ treated extract (Fig 2A). Samples were taken during (samples A and B) and after (samples C and D) the immunodepletion, and Phos tagTM SDS PAGE was performed to increase the shift in electrophoretic mobility of phosphorylated Arpp19 (Fig 2B, asterisk indicates cross reacting band). Cdc27 IB confirmed that depletion of Gwl or Arpp19 (see below) induced

Figure 1. PP1 contributes to Gwl dephosphorylation at mitotic exit.

- Scheme for the preparation of interphasic embryo extract.
- Mitotic exit in embryo extract. Interphasic embryo extract prepared according to (A) was supplemented with non-degradable cyclin B $\Delta 90$ ($\Delta 90$) to induce mitotic entry. Thirty minutes later, mitotic exit was induced by addition of the Cdk inhibitor roscovitine (Rosco). Extract samples were taken at the indicated time points and immunoblotted for cyclin B2, XErp1 and Cdc27. α tubulin serves as a loading control. Where indicated, samples were treated with lambda phosphatase.
- In vitro* thio phosphorylation of Arpp19. Constitutively active, full length Flag Gwl (K71M) was expressed from mRNA in mitotic embryo extract, purified via Flag IP and incubated with recombinant full length His Arpp19 wt or mutated to alanine at the Gwl site S67 in the presence of ATP γ S. Supernatant containing thio phosphorylated His Arpp19 (ThioArpp) was separated from Flag Gwl beads and dialyzed to remove residual ATP γ S. To enhance electrophoretic retardation of ThioArpp, samples were analyzed by Phos tagTM SDS PAGE followed by Coomassie Brilliant Blue (CBB) staining.
- Mitotic exit in the presence of ThioArpp. Mitotic embryo extract was prepared as in (B). Buffer (control), wt or S67A ThioArpp from (C) was added simultaneously with roscovitine at the indicated concentrations. Samples were taken at indicated time points and immunoblotted for Gwl. α tubulin serves as a loading control.
- Mitotic exit in the presence of ThioArpp and/or I 2. Mitotic embryo extract was prepared as in (B). Buffer (control), ThioArpp from (C) and/or PP1 Inhibitor 2 (I 2) was added simultaneously with roscovitine at the indicated concentrations. Samples were taken at the indicated time points and immunoblotted for Gwl and exogenous ThioArpp. To confirm mitotic exit, Cdk1 assays were performed by supplementing samples with histone H1 and γ ³²P ATP. Samples were analyzed by SDS PAGE followed by CBB staining and autoradiography. α tubulin serves as a loading control.

Source data are available online for this figure.

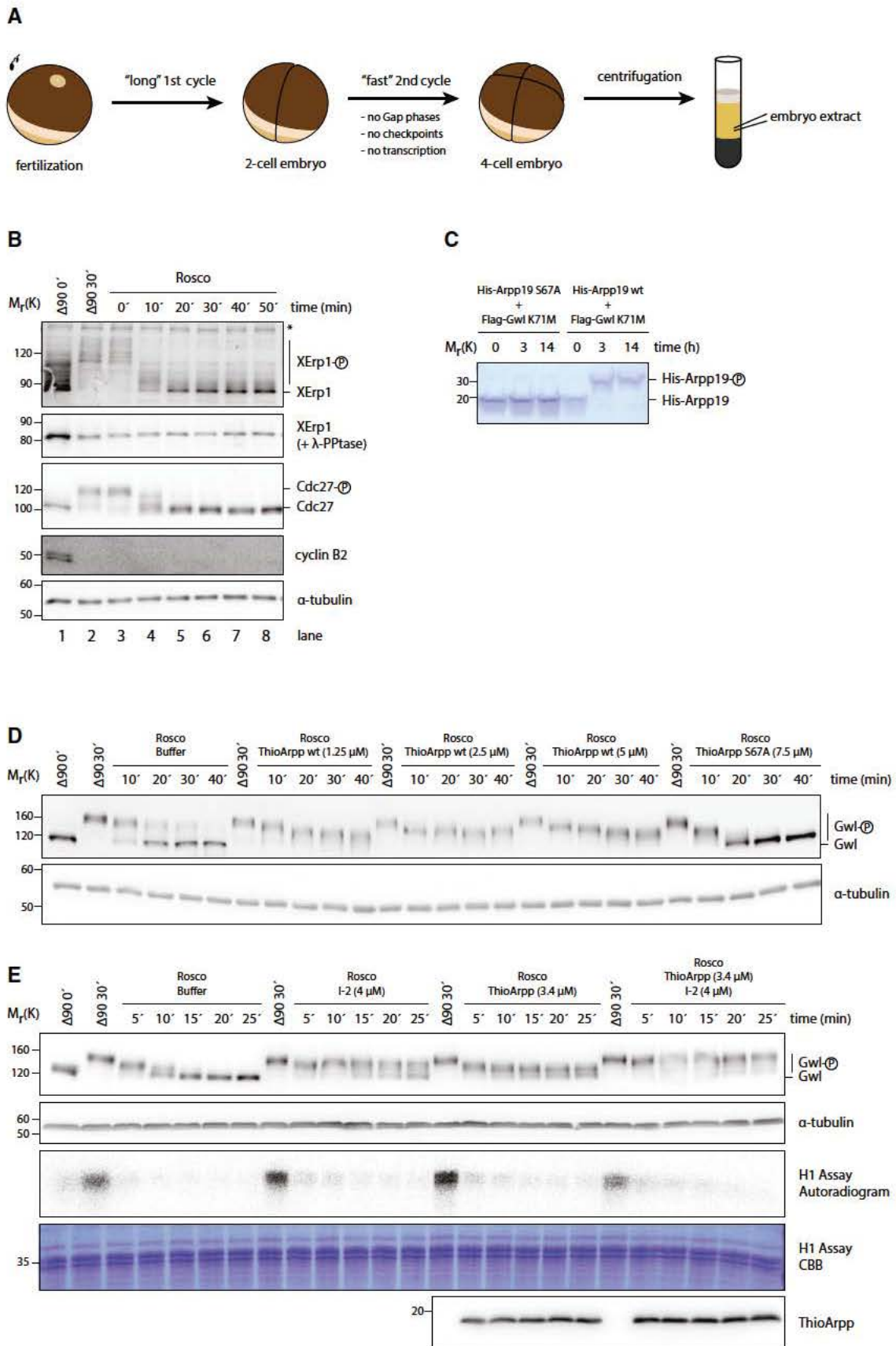


Figure 1.

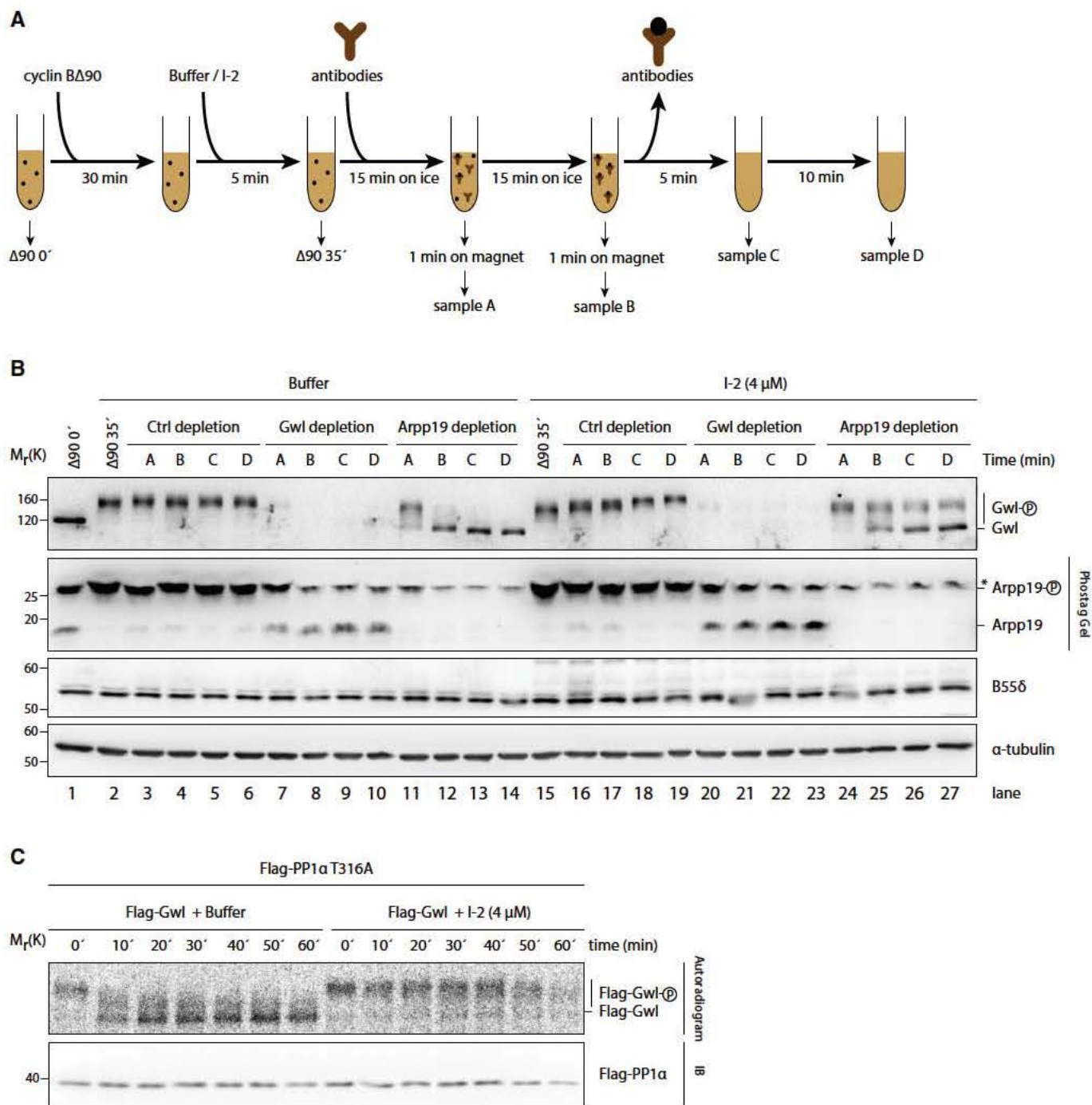


Figure 2. PP1 is important for Gwl but not Arpp19 dephosphorylation.

A Experimental outline of the experiment shown in (B).

B Interphasic embryo extract was prepared ($\Delta 90$ 0') and supplemented with $\Delta 90$ to induce mitotic entry. Thirty minutes later, mitotic extract was split into half and treated with buffer (control) or I 2 for 5 min ($\Delta 90$ 35'). Both reactions were split into three aliquots and treated with control, anti Gwl or anti Arpp19 antibodies coupled to magnetic beads. Extracts were incubated on ice, and samples from the supernatant were taken after 15 min (sample A) and 30 min (sample B). Subsequently, supernatants were isolated from beads and incubated at 20°C. Samples from the depleted supernatants were taken after 5 min (sample C) and 15 min (sample D). All samples were immunoblotted for Gwl, B55 δ and Arpp19 (Arpp19 immunoblot after Phos tag™ SDS PAGE). α tubulin serves as a loading control.

C PP1 dephosphorylates Gwl *in vitro*. Mitotic embryo extract was supplemented with mRNA coding Flag PP1 α T316A. Flag PP1 α T316A was purified by Flag IP. To phosphorylate *in vitro* translated ³⁵S labeled Flag Gwl, it was incubated in mitotic embryo extract and purified by Flag IP. Immunopurified ³⁵S Flag Gwl and Flag PP1 α were incubated in the presence or absence of I 2. At the indicated time points, samples were taken and immunoblotted for Flag. ³⁵S Flag Gwl was analyzed by SDS PAGE followed by CBB staining and autoradiography.

Source data are available online for this figure.

M phase exit (Fig EV2A). As expected, in extract depleted of Gwl, dephosphorylated, that is, faster migrating, Arpp19 was detectable (Fig 2B, samples C and D, lanes 9/10 and 22/23). Notably, the addition of anti Gwl antibodies was sufficient to detect Arpp19 dephosphorylation (samples A and B, lanes 7/8 and 20/21), indicating that they might act in an inhibitory manner. Importantly, I 2 had no detectable effect on Arpp19 dephosphorylation, suggesting that PP1 is unlikely to act on Arpp19 (compare lanes 7 10 with 20 23). This finding is supported by our observation that ThioArpp which would be largely inert to PP1 dephosphorylation only partially prevented Gwl dephosphorylation, while the addition of both ThioArpp and I 2 completely prevented Gwl dephosphorylation (Fig 1E). Next, we analyzed the dephosphorylation of Gwl. Notably, addition of I 2 to control treated extract induced a hypershift of Gwl (lanes 16 19), suggesting that PP1 might be at least partially active in mitotic extract. To induce the activation of PP2A B55, we depleted Arpp19 from $\Delta 90$ treated extract. Addition of anti Arpp19 antibodies was sufficient to trigger Gwl dephosphorylation (Fig 2B, samples A and B, lanes 11/12 and 24/25), probably by titrating Arpp19 away from PP2A B55. Consistently, depletion of Arpp19 did not result in the co depletion of PP2A B55 (samples C and D, lanes 13/14 and 26/27). Intriguingly, addition of I 2 significantly impaired the dephosphorylation of Gwl (compare lanes 11 14 with 24 27). We conclude that PP1 in addition to PP2A B55 is involved directly or indirectly in the dephosphorylation of Gwl during mitotic exit.

Next, we investigated whether Gwl is a direct substrate of PP1. Unfortunately, immunoprecipitation (IP) experiments failed to detect an interaction between PP1 and Gwl in embryo extract. We therefore tested whether purified PP1 is capable of dephosphorylating Gwl. Active PP1 was obtained by supplementing mitotic embryo extract with mRNA coding Flag tagged PP1 α that carried a non phosphorylatable mutation at the Cdk1 inhibitory phosphorylation site T316 (T316A) [18]. Subsequently, Flag PP1 α was isolated from the embryo extract by Flag IP. Mitotically phosphorylated Gwl was obtained by incubating *in vitro* translated (IVT) ^{35}S labeled wt Flag Gwl in mitotic embryo extract, followed by Flag IP of ^{35}S Flag Gwl. Indeed, ^{35}S Flag Gwl was quickly dephosphorylated upon incubation with immunopurified Flag PP1 α as evidenced by the change in its electrophoretic mobility (Fig 2C). Notably, addition of I 2 significantly suppressed the dephosphorylation of ^{35}S Flag Gwl (Fig 2C).

We conclude that purified PP1 α can directly dephosphorylate mitotic Gwl.

PP1 mediates rapid inactivation of Gwl at mitotic exit

According to the current model [8,9], Gwl activation at mitotic entry is initiated by Cdk1/cyclin B mediated phosphorylations of T193 and T206 (*Xenopus* Gwl) and these priming phosphorylations enable Gwl to activate itself by autophosphorylating its C terminal tail at S883. Based on these data, we speculated that Gwl dephosphorylation at mitotic exit occurs as well in a sequential manner. We therefore investigated first the order of events mediating Gwl dephosphorylation at mitotic exit. To this end, PP1 or PP2A B55 was inhibited at the same time mitotic exit was induced by roscovitine addition or with a five minute delay and the dephosphorylation kinetics of Gwl were analyzed by IB analysis. Notably, the inhibition of PP1 had only a significant effect on the dephosphorylation of Gwl when I 2 was added simultaneously with roscovitine, but not when I 2 was added with a five minute delay (Fig 3A, lanes 7 16). In contrast, addition of ThioArpp significantly affected the dephosphorylation of Gwl even when added five minutes after roscovitine (Fig 3A, lanes 17 26). We conclude that PP1 mediates an early event in the dephosphorylation cascade of Gwl during mitotic exit. Next, we established a Gwl kinase assay to analyze the kinetics of Gwl inactivation during mitotic exit. In brief, extract samples were supplemented with phosphatase inhibitors together with [γ ^{32}P] ATP and His tagged full length (FL) Arpp19 which was wt for the Gwl phosphorylation site (S67), but carried non phosphorylatable serine (S) to alanine (A) mutations (2A) at known Cdk1 (S28) and PKA (S109) sites [11]. To control for unspecific background (BG) phosphorylation, the identical assay was performed using FL His Arpp19 with an additional S \rightarrow A mutation at the Gwl site (S67A, 3A). Autoradiography analyses confirmed that His Arpp19 2A but not His Arpp19 3A was efficiently phosphorylated in control (Ctrl) depleted mitotic extract and phosphorylation of His Arpp19 2A was significantly reduced upon Gwl depletion (Fig 3B, note that mitotic extract was treated with phosphatase inhibitors prior to Gwl/Ctrl immunodepletion to maintain the mitotic state, see Cdc27 IB). After validating the Gwl assay, we determined Gwl activity during mitotic exit. Unfortunately, addition of roscovitine to extracts incubated at

Figure 3. PP1 activity is necessary for early Gwl inactivation at mitotic exit.

- Timing of Gwl dephosphorylation. Interphase extract ($\Delta 90$ 0') was treated for 30 min with $\Delta 90$ to induce mitotic entry ($\Delta 90$ 30'). Mitotic exit was induced by adding roscovitine (Rosco). Buffer (control), I 2 or thio phosphorylated His Arpp19 (ThioArpp) was added either simultaneously with roscovitine or 5 min later. Samples were taken at the indicated time points and immunoblotted for Gwl. α tubulin serves as a loading control.
- Gwl kinase activity assay. Mitotic extracts were prepared as in (A) and treated with okadaic acid and I 2 to maintain the mitotic state. Control (Ctrl) or Gwl depleted extracts were immunoblotted for Gwl. Cdc27 IB confirms that both extracts maintained the mitotic state. Ctrl or Gwl depleted extracts were incubated with γ ^{32}P ATP and recombinant FL His Arpp19 that was wt for the Gwl phosphorylation site (S67), but non phosphorylatable mutant for S28 and S109 (2A). To control for unspecific phosphorylation, samples were incubated with His Arpp19 carrying an additional non phosphorylatable mutation at S67 (3A). Analysis of Gwl kinase activity by SDS PAGE followed by CBB staining and autoradiography. α tubulin serves as a loading control.
- Kinetics of Gwl inactivation at mitotic exit. Mitotic embryo extract was prepared as in (A). To increase temporal resolution, extracts were incubated on ice prior to roscovitine addition. Samples were taken at the indicated time points and immunoblotted for Gwl. In parallel, samples were supplemented with His Arpp19 2A and γ ^{32}P ATP to monitor Gwl activity. As in (B), His Arpp19 3A was used for background correction. Values given as mean \pm standard deviation of three independent experiments. α tubulin serves as a loading control.
- Gwl kinase activity at mitotic exit. Mitotic embryo extract was prepared as in (A) and treated for 15 min with roscovitine and where indicated with ThioArpp or I 2. Samples were immunoblotted for Gwl and assayed for Gwl kinase activity as in (C). α tubulin serves as a loading control.
- Quantification of the Gwl kinase activity in (D). Background phosphorylation signal of His Arpp19 3A was subtracted from the one of His Arpp19 2A . Values given as mean \pm standard deviation of six independent experiments. Statistical significance was probed using unpaired two sided t test with Welch's correction.

Source data are available online for this figure.

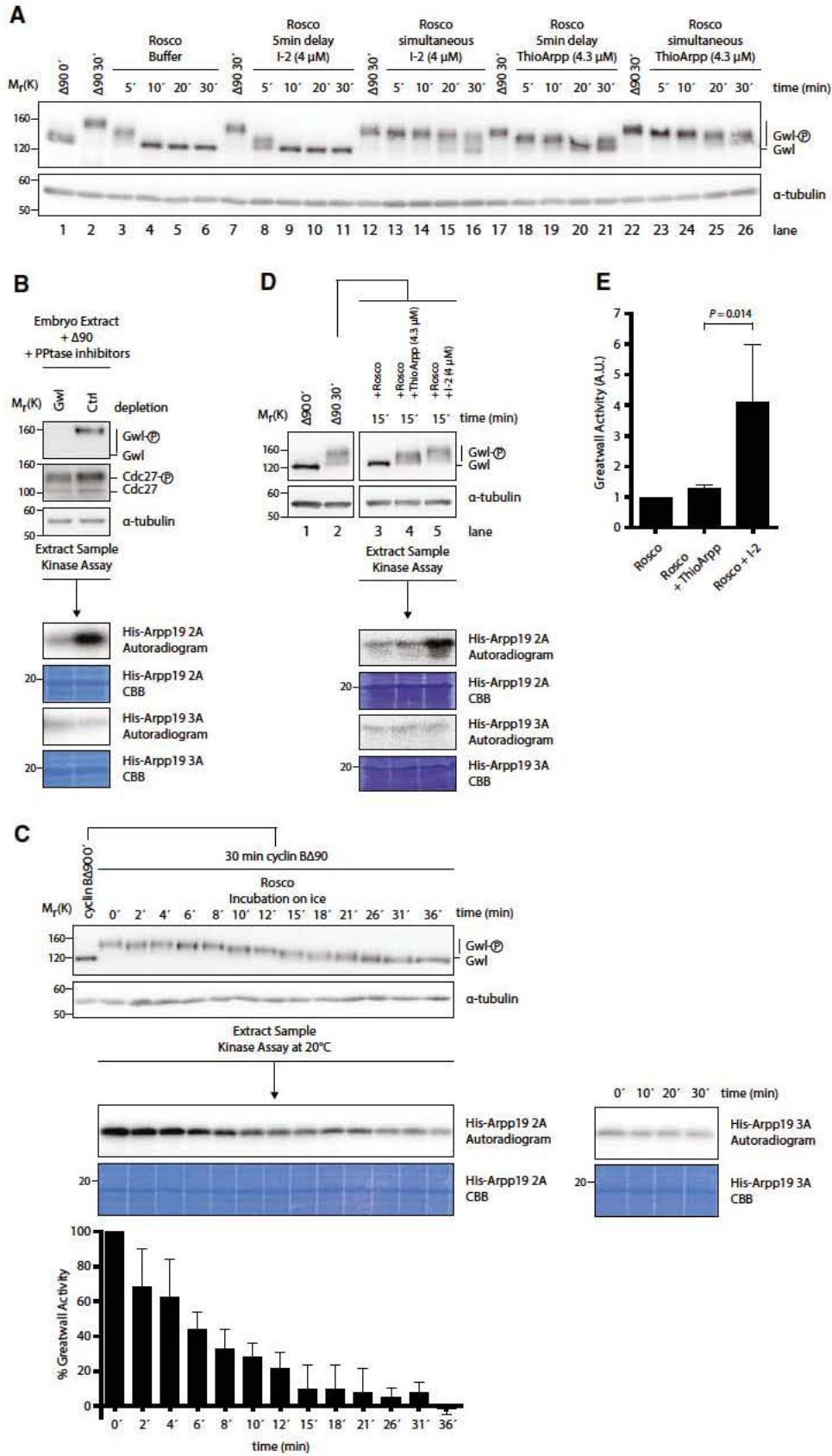


Figure 3.

room temperature induced Gwl inactivation with such fast kinetics that we could not temporally resolve it. Thus, to increase the temporal resolution, roscovitine was added to extracts incubated on ice, and at the indicated time points, samples were taken, supplemented with [γ 32 P] ATP and His Arpp19 2A and incubated at 20°C. To quantify Gwl activity, phosphorylation of His Arpp19 3A was used for BG correction. Autoradiography analyses of three independent experiments revealed that Gwl activity quickly declined within 15 min after roscovitine addition (Fig 3C). Notably, the decline in Gwl activity strongly preceded full dephosphorylation of Gwl which occurred about 31 min after induction of mitotic exit. Thus, these data suggest that Gwl inactivation occurs primarily during its initial dephosphorylation phase quickly after induction of mitotic exit, whereas the subsequent complete dephosphorylation of Gwl was not associated with a major decline in Gwl kinase activity. Based on this series of data, we further speculated that PP1 mediates the fast inactivation of Gwl during the initial phase of Gwl dephosphorylation, whereas the activity of PP2A B55 accounts for the subsequent complete dephosphorylation of Gwl. To test this hypothesis, we investigated whether inhibition of PP1 or PP2A B55 during mitotic exit affected the inactivation of Gwl. As expected, Δ 90 extracts supplemented with [γ 32 P] ATP and His Arpp19 2A fifteen minutes after roscovitine addition displayed low Gwl activity (Fig 3D, lane 3) and this was confirmed by the quantification of six experiments using His Arpp19 3A for BG correction (Fig 3E). Inducing mitotic exit in the presence of high concentrations of ThioArpp did not significantly prevent Gwl inactivation (lane 4). However, Gwl maintained substantial activity toward His Arpp19 2A when mitotic exit was induced in the presence of I 2 (lane 5). Thus, this series of experiments is consistent with the idea that Gwl inactivation is primarily mediated by PP1.

PP1 acts on the autophosphorylation site of Gwl

Next, we analyzed which phosphorylation site of Gwl is dephosphorylated by PP1. Previously, it was shown that a critical Cdk1 dependent site in the Gwl activation loop (T194 in human; T193 in *Xenopus*) is dephosphorylated by human PP2A B55 [14], a finding which is in line with the concept that PP2A B55 is the phosphatase that mediates the dephosphorylation of many Cdk1 substrates during mitotic exit [2,4,6,21,22]. Based on these findings, we

speculated that PP1 might catalyze the dephosphorylation of the autophosphorylation site. To test this idea, we first aimed to analyze the impact of PP1 on the dephosphorylation of S883. Unfortunately, none of the antibodies generated against phosphorylated S883 were phosphorylation specific. To test by an alternative approach whether PP1 mediates the dephosphorylation of autophosphorylation sites, we made use of a well characterized kinase dead mutant of Gwl (G41S in *Xenopus laevis*) [8]. Since this mutant lacks autophosphorylation activity, the electrophoretic mobility of Gwl G41S is exclusively affected by the phosphorylation state of sites phosphorylated by kinases other than Gwl. Like the endogenous protein, the mitotic form of wt 35 S Flag Gwl displayed a strong retardation in SDS PAGE compared to its interphasic form (Fig 4A, lanes 1, 2 and 8). As expected, induction of mitotic exit caused rapid dephosphorylation of 35 S Flag Gwl wt and this was significantly impaired when roscovitine was added together with I 2 (lanes 2 7 and 8 13). The electrophoretic mobility of kinase dead 35 S Flag Gwl G41S was markedly less affected by the addition of Δ 90 (lanes 14, 15 and 21) consistent with the finding that Gwl phosphorylates itself in an autocatalytic manner. Addition of roscovitine resulted in rapid dephosphorylation of 35 S Flag Gwl G41S (lanes 15 20). Notably, however, the presence of I 2 during mitotic exit had no detectable effect on the dephosphorylation of 35 S Flag Gwl G41S (lanes 21 26). Thus, our data support the model that PP1 initiates the inactivation of Gwl at mitotic exit by dephosphorylating it at the autophosphorylation site and thereby facilitates complete dephosphorylation of Gwl by PP2A B55 which acts on sites phosphorylated by kinases other than Gwl. A key corollary of this model is that sustained PP1 activity during mitotic exit is required to prevent the re activation of Gwl by autophosphorylation as long as Gwl has not been fully inactivated by the action of PP2A B55. To test this idea, we induced mitotic exit in the presence of PP1 activity and analyzed whether the subsequent inhibition of PP1 results in the reactivation of Gwl. As expected, addition of I 2 could not induce Gwl re phosphorylation when it was added to extract in which Gwl was already completely inactivated as evidenced by its electrophoretic mobility (Fig 4B, lanes 9 13). Gwl activity assays using His Arpp19 2A as substrate confirmed that PP1 inhibition under these conditions did not result in the re activation of Gwl (Fig 4C, lanes 3 6). Notably, however, when mitotic exit was induced in the presence of ThioArpp, that is, conditions where Gwl inactivation could be initiated by PP1 but not

Figure 4. PP1 directly affects dephosphorylation of Gwl autophosphorylation sites.

- PP1 acts on the autophosphorylation site of Gwl. Interphasic embryo extract (Δ 90 0') was supplemented with 35 S labeled *in vitro* translated Flag Gwl wt or G41S (kinase dead), and mitotic state was induced by the addition of cyclin B Δ 90 (Δ 90 60'). Roscovitine (Rosco) was added simultaneously with buffer (control) or I 2. At the indicated time points, 35 S Flag Gwl was analyzed by SDS PAGE followed by CBB staining and autoradiography. Addition of neither wt nor G41S 35 S Flag Gwl affected roscovitine induced mitotic exit as evidenced by Cdc27 IB. α tubulin serves as a loading control.
- PP1 is required to prevent Gwl rephosphorylation during mitotic exit. Mitotic embryo extract was prepared as in (A). Extract was split into half and treated for 25 min with roscovitine plus control buffer or thio phosphorylated His Arpp19 (ThioArpp). Both reactions were split into half and treated with control buffer or I 2. Samples were taken at the indicated time points and immunoblotted for Gwl. α tubulin serves as a loading control.
- PP1 is required to prevent Gwl reactivation during mitotic exit. Mitotic embryo extract was prepared as in (A). Extract was split into half and treated for 10 min with roscovitine plus control buffer or ThioArpp. Reactions were split and treated with control buffer or I 2 as indicated. Samples were taken at the indicated time points and supplemented with His Arpp19 2A and γ 32 P ATP to measure Gwl activity. Analysis of Gwl kinase activity by SDS PAGE followed by CBB staining and autoradiography.
- Working model for mitotic regulation of Greatwall activity during the early embryonic cycles of *Xenopus laevis*. Greatwall (Gwl, red; inactive, green: active), cyclin dependent kinase 1 (Cdk1), cyclin B (cycB), protein phosphatase 1 (PP1), protein phosphatase 2A regulatory subunit B55 (B55), protein phosphatase 2A scaffold/catalytic subunit (PP2A), cAMP regulated phosphoprotein 19 (Arpp19).

Source data are available online for this figure.

completed due to PP2A B55 inhibition (Fig 4B, lane 15), subsequent inhibition of PP1 resulted in enhanced retardation of Gwl indicative of Gwl re phosphorylation (Fig 4B, lanes 21–25). As expected, re phosphorylation of Gwl upon PP1 inhibition was accompanied by its re activation (Fig 4C, lanes 9–12). We conclude that PP1 is essential to initiate the inactivation of Gwl and to keep the autophosphorylation site in its dephosphorylated state during mitotic exit to prevent the autocatalytic re activation of Gwl.

Our study identifies PP1 as an essential component of the Gwl inactivation pathway at mitotic exit in *Xenopus* embryo extract. Based on our data, we propose the following model (Fig 4D): The first event resulting ultimately in tipping the balance in favor of PP2A B55 re activation at mitotic exit is the inactivation of Cdk1 by APC/C mediated destruction of cyclin B. Cdk1 inactivation enables PP1 to dephosphorylate itself at an inhibitory Cdk1 site [18,23–25] resulting in PP1 activation under conditions where PP2A B55 would still be inhibited by the molar excess of phosphorylated Arpp19. Active PP1 would then initiate Gwl inactivation by dephosphorylating it at the autophosphorylation site S883. Dephosphorylation of S883 results in a major decline in Gwl activity facilitating PP2A B55 to activate itself by dephosphorylating Arpp19. Active PP2A B55 completes the dephosphorylation and inactivation of Gwl, and this step is critical to prevent the reactivation of Gwl by autophosphorylation during mitotic exit and to finally reset the system to its interphasic state. While we favor the idea that the autophosphorylation site of Gwl is dephosphorylated by PP1, we do not formally exclude the possibility that this site is also targeted by PP2A B55, once the pool of phosphorylated Arpp19 has reached a critical minimal threshold. Irrespective of the contribution of PP2A B55 or yet an additional unidentified phosphatase, the identification of PP1 as the phosphatase initiating Gwl inactivation provides key insights into how Cdk1 inactivation at mitotic exit is coupled to the re activation of PP2A B55 which as shown previously contributes to resetting the cell divisions machinery to its interphasic state.

Materials and Methods

Detailed descriptions of the constructs, reagents and methods can be found online in the Appendix.

Xenopus laevis embryo extract

Embryo extract was prepared as described before [17]. When indicated, 6xHis cyclin BΔ90 (aa91–397) was added to a concentration of 75 nM, roscovitine to 1 mM and PP1 Inhibitor 2 to 4 μM. Unless otherwise stated, experiments were carried out at 20°C.

Antibodies

Anti XErp1 and anti Cdc27 antibodies were described before [17]. Anti cyclin B2 antibody was purchased from Biozol (MBL K01893). Anti His tag antibody was purchased from Dianova (DIA 900). Anti Greatwall antibody for IB was a gift from T. Lorca [7]. Anti Greatwall antibody for IP was purified against GST XI Greatwall (wt, FL) from serum obtained by the European *Xenopus* Research Centre. Anti Arpp19 antibody was a gift from O. Gruss. Anti Arpp19 antibody for IP was generated by immunizing rabbits with

6xHis XI Arpp19 (wt, FL) and purification against GST XI Arpp19 (wt, FL). Anti B55δ antibody was a gift from S. Mochida [4]. Anti Flag antibody for IB was purchased from Sigma (F1804). Anti Flag antibody for IP was generated by immunizing rabbits with Flag peptide (NH₂ CMDYKDHDGDYKDHDIDYKDDDDK COOH) and purification against Flag peptide lacking the N terminal cysteine.

Greatwall/Arpp19 immunodepletion from mitotic embryo extract

A total of 4 μg α Gwl or α Arpp19 antibodies were coupled to Dyna beads Protein G and incubated with 20 μl embryo extract. Immuno precipitations were carried out on ice for 30 min.

Greatwall kinase assay in extract samples

A total of 1 μl embryo extract was diluted in 16.5 μl reaction buffer (80 mM β glycerophosphate; 20 mM EGTA; 15 mM MgCl₂; 1 mM DTT; 10 μM okadaic acid; 1× protease inhibitors Complete (Roche); 100 μM NaATP; 100 nCi/μl γ P³² ATP; 0.3 μg/μl His XI Arpp19 S28A S109A or His XI Arpp19 S28A S67A S109A). Reactions were carried out at 20°C for 30 min. Samples were processed by SDS PAGE, and radioactive signals were detected with a Typhoon FLA 7000 IP (GE Healthcare Life Sciences). Band intensities were quantified using ImageJ.

In vitro phosphatase assay

A total of 2.5 μl ³⁵S Flag Gwl (wt, FL) was incubated at 20°C for 30 min in 35 μl embryo extract supplemented with 0.2 U/μl creatine phosphokinase (Sigma), 1 μM Ac DEVD (Enzo Life Sciences) and 75 nM 6xHis cyclin BΔ90. Extract was transferred to 4 μg α Flag antibodies coupled to Dynabeads Protein G and incubated at room temperature for 1 h. Beads were washed once with PBS + 0.1% Triton X 100, once with PBS + 300 mM NaCl and four times with phosphatase buffer (20 mM TrisCl pH 7.4; 150 mM NaCl; 1 mM DTT; 1 mM MnCl₂; 0.1% NP 40). Beads were resuspended in 35 μl phosphatase buffer. A total of 4.5 μg mRNA coding 3xFlag XI PP1α (T316A, FL) was incubated at 20°C for 1 h in 100 μl embryo extract supplemented with 0.2 U/μl creatine phosphokinase, 1 μM Ac DEVD and 75 nM 6xHis cyclin BΔ90. Extract was transferred to 10 μg α Flag antibodies and incubated at room temperature for 1 h. Beads were washed six times with phosphatase buffer. The Flag PP1α beads were resuspended with the Flag Gwl bead suspension and incubated at 30°C to start the phosphatase reaction. Samples were processed by SDS PAGE and radioactive signals were detected with a Typhoon FLA 7000 IP (GE Healthcare Life Sciences).

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Author contributions

AH and AK performed all experiments. AH, AK and TUM were involved in designing the study, analyzing experimental data and writing the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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