Role for regulated phosphatase activity in generating mitotic oscillations in *Xenopus* cell-free extracts

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Although current textbook explanations of cell-cycle control in eukaryotes emphasize the periodic activation of cyclin-dependent protein kinases (CDKs), recent experimental observations suggest a significant role for the periodic activation and inactivation of a CDK-counteracting protein phosphatase 2A with a B55δ subunit (PP2A:B55δ), during mitotic cycles in frog-egg extracts and early embryos. In this paper, we extend an earlier mathematical model of embryonic cell cycles to include experimentally motivated roles for PP2A:B55δ and its regulation by Greatwall kinase. Our model is consistent with what is already known about the regulation of CDK and PP2A:B55δ in frog eggs, and it suggests a previously undescribed role for the Greatwall-PP2A:B55δ interaction in creating a toggle switch for activation of the anaphase-promoting complex as embryonic cells exit mitosis and return to interphase.

mitotic control | bistability | hysteresis

In all eukaryotic cells, progression through the cell cycle (G1–S–G2–M) is orchestrated by periodic activation of cyclin-dependent kinases (CDKs), which trigger DNA synthesis (S phase) and mitosis (M phase) in succession, and of the anaphase-promoting complex/cyclosome (APC/C), which promotes exit from mitosis and return to G1 phase (1). In most present-day eukaryotic cells, CDKs and the APC/C are involved in complex regulatory networks involving transcriptional controls, posttranslational modifications, stoichiometric activators and inhibitors, and regulated proteolysis (2–4). However, the cell-division cycles of early embryos (and the mitotic cycles observed in frog-egg extracts) are considerably simpler because they lack transcriptional regulation and stoichiometric CDK inhibitors (3).

From careful considerations of frog embryonic cell cycles—both experimental (5–7) and theoretical (8–10)—some general principles of cell-cycle control have emerged. First of all, cyclin synthesis during interphase drives increasing CDK activities, which initiate first DNA synthesis and then mitosis. Secondly, high CDK activity in mitosis initiates a time-delayed activation of the APC/C, which degrades mitotic cyclins, allowing the cell to return to G1 phase (when cyclin concentrations are low). The time delay between CDK activation and APC/C activation seems to be crucial to the proper sequence of events during mitosis and cell division. The molecular basis of this time delay is the subject of this paper: in particular, the role of regulated protein phosphatases in delaying activation of the APC/C.

CDK refers to a family of protein kinases (Cdk1, Cdk2, …) that form heterodimers with members of the cyclin family (cyclin A, cyclin B, …). The cyclin partner activates the kinase subunit and directs its activity toward specific substrates. Although considerable effort has gone into characterizing the roles of specific Cdk*n*:Cyc*X* complexes in cell-cycle events, it is crucial to recognize that a single CDK heterodimer, Cdk1:CycB, can drive all events of the cell cycle in fission yeast cells (11, 12).

The APC/C is a multisubunit ubiquitin-ligase that targets specific proteins for proteosomal degradation (13). The APC/C requires an auxiliary protein, Cdc20, to direct its activity to specific mitotic substrates (14). The APC/C:Cdc20 complex is activated in M phase, and it promotes degradation of securin, a protein inhibitor of separase. Separase is a specific protease that, when activated, cleaves the cohesin rings that have been holding together sister chromatids since S phase of the cell cycle (15). After cohesin cleavage, the sister chromatids are free to be pulled to opposite poles of the mitotic spindle by microtubules. APC/C:Cdc20 also promotes the degradation of cyclin B as cells exit from mitosis: thus its other name, the "cyclosome" (13, 16–19). Near-complete degradation of cyclin B is necessary to reset the cell cycle to G1 phase and to relicense origins for another round of DNA replication.

APC/C:Cdc20-dependent ubiquitination of securin and cyclin B depends on high activity of Cdk1:CycB (6, 20). Therefore, the activation of cyclin B-dependent kinase as cells enter mitosis sets the stage for its own destruction by APC/C:Cdc20 as cells exit mitosis. Such a negative feedback loop is necessary for periodicity of the cell cycle, but only if the loop experiences a sufficient time delay (21). The activation of APC/C:Cdc20 must occur sometime after the rise in Cdk1:CycB activity to allow sufficient time for replicated chromosomes to align on the mitotic spindle (especially in early embryonic cell cycles that lack a spindle-assembly checkpoint). Furthermore, the inactivation of cyclin B-directed APC/C activity must continue for some time after Cdk1:CycB activity falls to allow for complete degradation of cyclin B and return to G1 phase.

The required time delay might be provided by additional components in the feedback loop, like multistep activation of APC/C:Cdc20 by Cdk1:CycB (22), or by amplification mechanisms based on positive feedback on APC/C or CDK (21). Somatic cells contain a Cdc20-homolog, Cdh1, which also promotes APC/C-dependent degradation of cyclin B. A double-negative feedback loop between Cdk1:CycB and APC/C:Cdh1 maintains a high rate of cyclin B degradation during mitotic exit and throughout G1 phase, despite the degradation of nearly all cyclin B. However, this mechanism is not functional in early embryos, like *Xenopus*, which lack G1 phase.

Significance

A long-standing puzzle in the cell-cycle field is how it can be that the anaphase-promoting complex/cyclosome (APC/C) gets activated only when cyclin-dependent kinase 1 (Cdk1) activity reaches a very high level in metaphase but stays active even after Cdk1 activity drops to much lower levels as cells exit mitosis. In this manuscript, we propose that the regulation of a protein phosphatase 2A (PP2A:B55δ) by Greatwall kinase might provide a solution to this puzzle. Our proposal is consistent with what is already known about the regulation of Cdk1 and PP2A:B55δ in frog eggs, and it leads to testable predictions.

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Another possibility for positive feedback on Cdk1:CycB activity is inhibitory phosphorylation of Cdk1 by Wee1 kinase and subsequent activation by Cdc25 phosphatase. These interactions are also governed by double-negative and positive feedback loops, respectively, and they play a well-known role in generating CDK oscillations in *Xenopus* cell-free extracts (9). However, inhibitory Cdk1 phosphorylation is not observable during cycles 2–11 in intact *Xenopus* embryos (23) so there must be an alternative mechanism responsible for time-delayed activation and inactivation of APC/C:Cdc20.

Ciliberto et al. (24) attributed the time delay to a double-negative feedback loop between APC/C:Cdc20 and Cdk1:CycB. Their model was based on early reports suggesting that Cdc20 phosphorylation by Cdk1:CycB inhibits its binding to the APC/C (25, 26). If so, then Cdc20 would be regulated in a similar fashion to Cdh1, with the important difference that Cdc20 binding requires the APC/C to be phosphorylated whereas Cdh1 binding does not. In Ciliberto's model, the complex of unphosphorylated Cdc20 with phosphorylated APC/C promotes cyclin B degradation. Therefore, Ciliberto's model is characterized by intertwined feedback loops of negative sign (Cdk1:CycB → APC/C-P → Cdc20:APC/C-P ⊣ Cdk1:CycB) and of net-positive sign (Cdk1:CycB ⊣ Cdc20 → Cdc20:APC/C-P ⊣ Cdk1:CycB; note that two inhibitory interactions in series is equivalent to a net-positive feedback). Because of the positive feedback loop, the rate of cyclin B degradation is low when Cdk1:CycB activity is high and Cdc20 is phosphorylated. This delayed degradation of cyclin B in Ciliberto's model can provide the required time delay for the negative feedback loop to oscillate. To generate limit cycle oscillations, the positive-feedback loop must operate on a faster timescale than the negative feedback loop; i.e., phosphorylation and dephosphorylation of APC/C must be slower than phosphorylation and dephosphorylation of Cdc20. To ensure this requirement, Ciliberto et al. (24) assumed a slow and constant rate of dephosphorylation of APC/C.

In the current manuscript, we drop the assumption of a constant rate of APC/C dephosphorylation, based on recent experimental evidence suggesting that the APC/C might be dephosphorylated by a regulated phosphatase (27). The regulated phosphatase is likely to be PP2A:B55δ, one of the Cdk1-counteracting phosphatases in *Xenopus* (28, 29). PP2A:B55δ activity is indirectly repressed by Cdk1:CycB, which could account for the slow rate of APC/C dephosphorylation in M phase required by Ciliberto's model. Other data suggest roles for PP2A:B55δ in Wee1 activation and Cdc25 inactivation (27, 29, 30). Altogether, these data suggest an active role for PP2A:B55δ in the control of entry into and exit from mitosis.

To provide a comprehensive picture of mitotic control by both kinases and phosphatases, we incorporate regulation of PP2A:B55δ into Ciliberto's model of CDK-APC/C feedback loops. With a suitable choice of parameter values, the extended model reproduces in quantitative detail all of the experimental data cited above. Moreover, the extended model suggests that the activation and inactivation of APC/C:Cdc20, as a function of Cdk1:CycB activity, functions as a bistable switch. As elaborated later, a bistable switch has several appealing properties. For example, in a bistable switch, the transition from the inactive state to the active state is abrupt, which provides a natural explanation for the abrupt activation of APC/C:Cdc20 as Cdk1:CycB activity increases during M phase, as recently quantified by Yang and Ferrell (20). In addition, bistability ensures irreversibility of exit from mitosis (31). Once a bistable APC/C:Cdc20 switch flips to the active state, it will not readily return to the inactive state as Cdk1:CycB activity is destroyed (by cyclin B degradation). Therefore, APC/C:Cdc20 remains active until cyclin B is reduced to a level low enough to reset the cell in G1 (i.e., relicense origins of DNA replication).

In this way, the regulation of PP2A:B55δ might provide a potential solution for a long-standing puzzle in the cell-cycle field. Namely, how can it be that the APC/C gets activated only when Cdk1 activity reaches a very high level in metaphase but stays active even after Cdk1 activity drops to much smaller levels as cells exit mitosis? The regulation of PP2A:B55δ is especially important in frog eggs, where the spindle-assembly checkpoint is absent. The two properties of our model (bistability and irreversibility in the regulation of PP2A:B55δ) that resolve this puzzle are readily testable predictions.

Results

Model for Cdk1:CycB and APC/C:Cdc20 Regulation. Here, we propose a model (its "influence diagram" in Fig. 1 and its "wiring diagram" in Fig. S1) for the interrelationships among proteins regulating mitotic cycles in *Xenopus* embryos and cell-free extracts. At the top-center of the diagram is Cdk1:CycB, which is referred to in the model as MPF (M-phase–promoting factor). MPF is phosphorylated (inactivated) by Wee1 kinase and dephosphorylated (activated) by Cdc25 phosphatase. The phosphorylated, inactive form of MPF is also known as preMPF. The activities of both Wee1 and Cdc25 are regulated by MPF-catalyzed phosphorylation, which inactivates Wee1 and activates Cdc25. These interactions create parallel positive circuits in the activation of MPF.

The degradation of cyclin B is catalyzed by active APC/C:Cdc20 complex (17). Following Ciliberto's model (24), we assume that the active ubiquitin-ligase is a complex of MPF-phosphorylated APC/C and unphosphorylated Cdc20. Because Cdk1:CycB also phosphorylates Cdc20, it both activates (via APC/C) and inhibits (via Cdc20) the formation of APC/C:Cdc20. To approximate multisite phosphorylation of APC/C subunits (22), we assume four phosphorylations of APC/C by Cdk1, which is a departure from Ciliberto's model (where APC/C is singly phosphorylated). Unphosphorylated Cdc20 binds only to fully phosphorylated APC/C (APC/C-P_4), and their complex (APC/C-P_4:Cdc20) is the active ubiquitin-ligase promoting cyclin B degradation.

In Ciliberto's model, all Cdk1:CycB phosphorylations are counteracted by phosphatases with constant activities. For the case of Cdc20 dephosphorylation, this assumption has recently received experimental support. Using *Xenopus* cell-free extracts, Yamano and co-workers (32) showed that phosphorylation of three out of five Cdk1-sites on Cdc20 significantly reduces its binding to APC/C. They also showed that okadaic acid, an inhibitor of type 2A protein phosphatases, blocks APC/C:Cdc20 activity, suggesting that a PP2A dephosphorylates Cdc20. Unlike PP2A:B55δ, the PP2A that dephosphorylates Cdc20 does not show reduced activity in mitosis. Also, the PP2A:B55δ-targeted site (S50) of Cdc20 does not influence APC/C binding (32). These findings provide strong support for Ciliberto's assumption of constant, rapid turnover of Cdc20 phosphorylation; therefore, we keep this feature of the model.

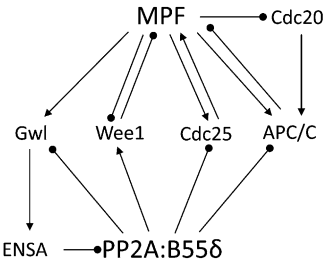

Fig. 1. Influence diagram of the model. MPF activates APC/C (indicated by an arrow head) but inhibits Cdc20 (indicated by a blunt connector). APC/C then inhibits MPF after its activation by Cdc20. Wee1 and MPF inhibit each other whereas Cdc25 and MPF activate each other. In addition, MPF activates Greatwall kinase (Gwl); Gwl activates ENSA; ENSA inhibits PP2A:B55δ, which then inhibits Gwl. The phosphatase PP2A:B55δ is assumed to oppose the effects of MPF on Gwl, Wee1, Cdc25, and APC/C, but not on Cdc20.

The dephosphorylation of other CDK substrates in our model is known to be repressed when cells enter mitosis and to be activated by depletion of endosulfin-alpha (Ensa), a PP2A:B55δ inhibitor. For example, Ensa depletion keeps APC3 (an APC/C subunit) unphosphorylated and cyclin B stabilized (27), suggesting that PP2A:B55δ is the phosphatase that dephosphorylates and inactivates the APC/C. Consistent with this idea, recent data suggest that PP2A:Cdc55 dephosphorylates APC/C in budding yeast (33, 34). High PP2A:B55δ activity (upon addition of PP2A:B55δ to or depletion of Ensa from frog egg extracts) also results in delayed activation (dephosphorylation) of preMPF (27), suggesting that PP2A:B55δ dephosphorylates Cdc25 (35, 36) and Wee1. Therefore, we assume in Fig. 1 that these three Cdk1 targets (Cdc25, Wee1, and APC/C)—but not Cdc20—are dephosphorylated by PP2A:B55δ.

Model for Regulation of PP2A:B55. During M phase, MPF activates another mitotic kinase called Greatwall (Gwl) (Fig. 1) (28, 36, 37), and one function of Gwl is to phosphorylate and activate two phosphatase inhibitors, Ensa and Arpp19 (27, 30). (In our model, we lump together Ensa and Arpp19 in a single variable, called ENSA.) The phosphorylated forms of ENSA bind and inhibit PP2A:B55δ. Because PP2A:B55δ is a major Cdk1-counteracting phosphatase, this regulation comprises a coherent feed-forward loop, by which MPF directly phosphorylates its target proteins and indirectly inhibits their dephosphorylation by PP2A:B55δ. In addition, we propose, based on the following arguments, that PP2A:B55δ is involved in a positive feedback loop with Gwl.

Gharbi-Ayachi et al. (30) studied Gwl phosphorylation in *Xenopus* extracts that were arrested in interphase by cycloheximide (a drug that inhibits protein synthesis) and further depleted of Cdc27 (a component of APC/C) and Cdc25 by antibody precipitation. In this extract, Cdk1:CycB is inactive, as indicated by low H1 kinase activity and unphosphorylated Gwl. Addition of phosphorylated phosphatase inhibitors (Ensa or Arpp19) to this extract causes rapid and permanent Gwl phosphorylation (figure 2B in ref. 30), suggesting that the phosphatase responsible for Gwl dephosphorylation is PP2A:B55δ. If this is indeed the case, then Gwl and PP2A:B55δ are involved in a double-negative feedback loop (Gwl inactivates PP2A:B55δ via ENSA, but PP2A:B55δ dephosphorylates and inhibits Gwl). Auto-activation of the phosphatase could also work if PP2A:B55δ inhibits ENSA by dephosphorylation. However, recent evidence suggests that PP2A:B55δ is indeed required to revert phosphorylation of Gwl by Cdk1 (38).

Oscillatory Behavior of *Xenopus* Extracts. To assess the fidelity of our model of mitotic control in *Xenopus* cell-free extracts, we compare numerical simulations of the differential equations (*SI Text S1* and Table S1) to recently observed temporal dynamics of the regulatory components (27, 29). We use experimental observations from a single laboratory to avoid confusion caused by different protocols in preparing frog extracts. To facilitate the direct comparison between experimental observations and model simulations, we present time-course simulations (Fig. 2) in exactly the same format as published experimental panels. The advantage of this presentation style will be evident if readers lay side-by-side the experimental and simulation figures.

Fig. 2 presents the model's simulation of an undisturbed oscillatory frog egg extract. Fig. 2 *A* and *B* is to be compared with figure 3C of Mochida et al. (27), and Fig. 2 *C* and *D* is to be compared with figure S2 of the same publication (27). The simulation starts from interphase when cyclin B level and MPF activity are low and PP2A:B55δ phosphatase activity is high. As cyclin B accumulates, Cdk1:CycB complexes first accumulate in the preMPF form, with the Cdk1 subunit phosphorylated by Wee1. We assume that preMPF is significantly less active than MPF against all substrates in the model. Cdk1 activity is measured experimentally by histone-H1 phosphorylation, which we identify as proportional to [MPF] + 0.3*[preMPF]. The residual kinase activity of preMPF explains the significant rise in H1-kinase activity before the abrupt dephosphorylation of preMPF, as seen in figure 3C of Mochida et al. (27). (Note: the kinase activity that we assign to preMPF may be due in part to the extract's supply of Cdk1:CycA, whose temporal profile is similar to that of preMPF.)

The partial activity of preMPF causes the slight decrease of PP2A:B55δ activity during interphase. At ~30 min, preMPF is abruptly converted to MPF, PP2A:B55δ is quickly inactivated, and the extract enters mitosis. Under these conditions, APC/C gets phosphorylated on multiple sites (because MPF is active and PP2A:B55δ is inactive). Multiphosphorylated APC/C combines with the pool of unphosphorylated Cdc20 to form active APC/C-P_4:Cdc20, which degrades cyclin B, inactivates MPF, and triggers mitotic exit. During the initial stage of mitotic exit, APC/C remains active because its dephosphorylation by PP2A:B55δ is slow. However, as the cell degrades cyclin B and inactivates MPF, Gwl and ENSA get progressively dephosphorylated and inactivated. Therefore, in the final stage of mitosis, PP2A:B55δ is fully active, and APC/C is quickly dephosphorylated and inactivated.

With our current parameter settings (Table S2), Cdc20 is only ~50% phosphorylated in metaphase, despite high MPF activity (Fig. S2). This aspect of the model is consistent with observations [figure 5E of Labit et al. (32)] that removal of CDK-phosphorylation

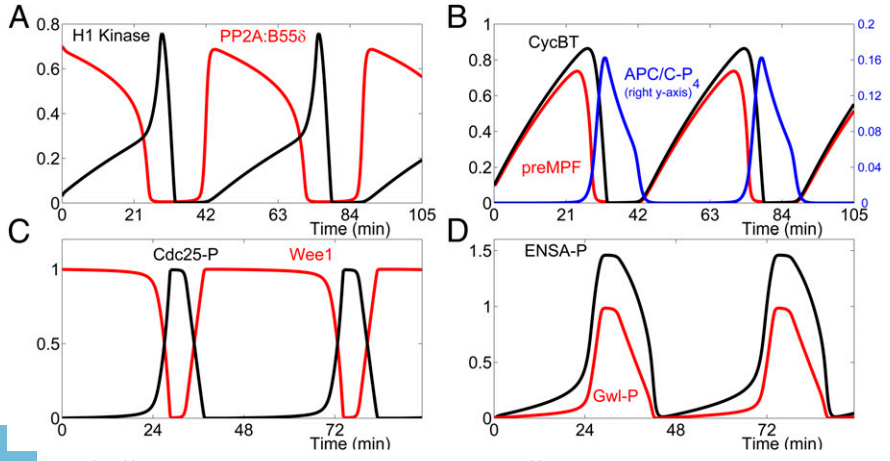

Fig. 2. Simulations of normal mitotic cycles in a frog-egg extract. (*A*) Time courses of PP2A:B55δ activity and H1 kinase activity ([MPF] + 0.3*[preMPF]). Compare with figure 3C of Mochida et al. (27). (*B*) Time courses of total cyclin B and preMPF (scale on the left), and of APC/C-P_4 (scale on the right). When the extract enters mitosis, preMPF is dephosphorylated and APC/C is phosphorylated. Notice that preMPF is dephosphorylated around 30 min. Later, cyclin B is degraded and the extract exits mitosis. Compare with figure 3C of Mochida et al. (27). (*C*) Time courses of Cdc25-P and Wee1. As the extract enters mitosis, both Cdc25 and Wee1 are phosphorylated. Compare with figure S2 of Mochida et al. (27). (*D*) Time courses of phosphorylated ENSA and phosphorylated Gwl. Compare with figure S2 of Mochida et al. (27). All concentrations are dimensionless, i.e., "arbitrary units." Notice that, to show the simulation curves of different components as clearly as possible, different *y* scales are used on the panels.

sites from Cdc20 causes little change to the dynamics of MPF oscillations. So the double-negative feedback between MPF and Cdc20, which plays a prominent role in Ciliberto's model (24), does not seem to be as important as we once thought. In its place, the present model emphasizes the regulation of phosphorylated forms of APC/C by Cdk1:CycB and PP2A:B55δ.

The Importance of PP2A:B55δ Regulation by Greatwall-ENSA. In Ensa-depleted *Xenopus* extracts, PP2A:B55δ activity stays constantly high, and Cdk1 activation brings about a sustained, mitotic state without cyclin B degradation, as seen in figure 3D of Mochida et al. (27). Cyclin B degradation is blocked in this extract, and APC3 stays dephosphorylated (after a short and transient phosphorylation). To mimic these experiments, we set $ENSAT = 0$ in the model equations, assuming that the Ensa-directed antibody used in the experiments cross-reacts significantly with Arpp19. In our simulations, setting $ENSAT = 0$ results in sustained and high PP2A:B55δ activity (Fig. 3*A*). H1 kinase (Cdk1:CycB) activity shows its characteristic abrupt rise (Fig. 3*A*), which partially activates APC/C-P_4:Cdc20 and causes partial degradation of cyclin B (Fig. 3*B*). The partial drop in Cdk1:CycB activity, together with the elevated activity of PP2A:B55δ in this extract, leaves APC/C in the dephosphorylated (inactive) state. The fact that APC/C locks in a hypo-phosphorylated state in Ensa-depleted extracts, despite high H1 kinase activity, strongly suggests that PP2A:B55δ is the phosphatase that counteracts MPF phosphorylation of APC/C core components.

The regulation of PP2A:B55δ activity by Greatwall and ENSA is essential for cell cycling in *Xenopus* extracts and for oscillations in our model (Fig. 3 *A* and *B*). In our model, PP2A:B55δ regulation is essential because the level of PP2A:B55δ is high ($B55T = 0.7$). If the total level of PP2A:B55δ is reduced below half of the original value ($B55T < 0.35$), Greatwall and ENSA become dispensable for cell-cycle oscillations (Fig. 3 *C* and *D*). This simulation explains why Greatwall and ENSA are dispensable during mitotic cycles of *Drosophila* embryos heterozygotic for PP2A:B55δ (39) and in *Caenorhabditis elegans* (40). This finding also explains why previous cell-cycle models [e.g., Ciliberto's (24)] with constitutive Cdk1 counteracting phosphatases were permissive for oscillations.

Effects of Up- and Down-Regulation PP2A:B55δ Levels. The effects of Ensa depletion in *Xenopus* extracts (27) are consistent with the idea that PP2A:B55δ regulates MPF activation (35). Recall that, in our model, in the presence of ENSA, dephosphorylation of the inhibitory residues of Cdk1 takes place at ~30 min (Fig. 2*B*). By contrast, in $ENSAT = 0$ simulations, preMPF dephosphorylation is delayed to ~45 min (Fig. 3*B*), similar to the observations in figure 3D of Mochida et al. (27). Delayed activation of Cdk1 in the presence of constitutively active PP2A:B55δ confirms our assumption that PP2A:B55δ targets the enzymes (Wee1, Myt1, and Cdc25) that regulate the inhibitory phosphorylations of Cdk1. This conclusion is further supported by experiments in which extra PP2A:B55δ has been added to oscillating extracts (29). Twice the normal level of PP2A:B55δ delays Cdk1 activation but does not block activation of APC/C, and the extract continues to cycle [compare Fig. 4*A* with figure 5 of Mochida et al. (29)]. By contrast, three times the normal level of PP2a:B55δ blocks the extract in G2 phase, with Cdk1 phosphorylated (inhibited) and APC/C unphosphorylated (inactive) [compare Fig. 4*B* with figure 5 of Mochida et al. (29)].

As a consequence of our assumption that PP2A:B55δ dephosphorylates the enzymes (Wee1, Myt1, and Cdc25) that regulate the inhibitory phosphorylation of Cdk1, depleting PP2A:B55δ results in premature activation of H1 kinase in the model (Fig. S3) and in frog egg extracts [see figure 4D of Mochida et al. (29)]. In our simulation, MPF activation triggers activation of the APC/C, and the negative feedback loop between MPF and APC/C causes the simulated H1 kinase activity to approach a low steady state by damped oscillations (Fig. S3).

The successful reproduction of the Mochida–Hunt experiments (27, 29) strongly supports our assumptions that the APC/C and the Tyr-modifying enzymes of Cdk1 are dephosphorylated by PP2A:B55δ. In the next section, we analyze the dynamic consequence of our assumption that PP2A:B55δ dephosphorylates and inactivates Gwl.

The Role of Mutual Antagonism Between Gwl-P and PP2A:B55δ. Our assumption that PP2A:B55δ dephosphorylates and inactivates Gwl creates an additional double-negative feedback loop in the control network, with the potential to create a toggle switch. To analyze the significance of this double-negative feedback, we have eliminated all other positive circuits (MPF auto-regulation by Wee1 and Cdc25 as well as the antagonism between MPF and Cdc20) from the network. This simplified cell-cycle control network corresponds to a *Xenopus* extract in which both Cdk1 and Cdc20 are replaced by nonphosphorylable mutant proteins (Cdk1-AF and Cdc20-5A). The cell-cycle oscillations of PP2A:B55δ and MPF (identical to H1-kinase in the absence of inhibitory phosphorylation) persist with shorter periods and smaller amplitudes (Fig. 5).

It is instructive to plot the temporal oscillations in Fig. 5*A* in a different way. At each point of time, we read the values of PP2A:B55δ activity and of H1 kinase activity from the curves in Fig. 5*A*, and we plot these values (in Fig. 5*B*) as a point in "state space," PP2A:B55δ activity as a function of H1 kinase activity.

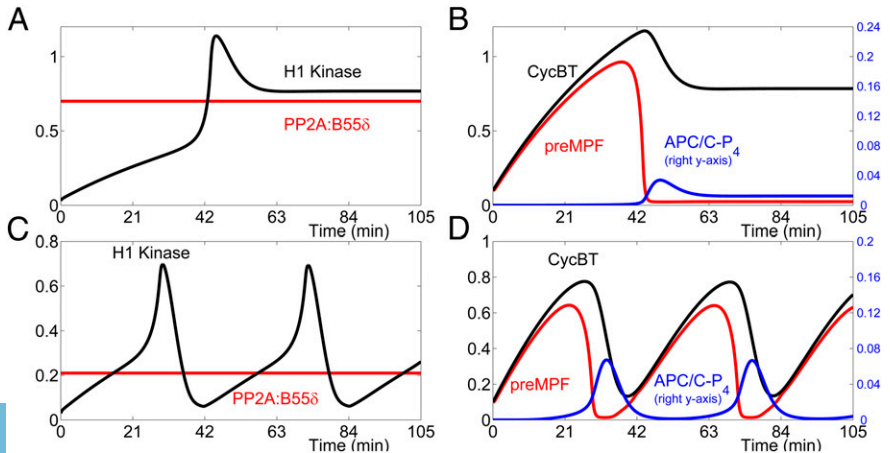

Fig. 3. Simulations of aberrant cycles in extracts that are biochemically manipulated. (*A* and *B*) ENSA depletion. When $ENSAT = 0$, both PP2A:B55δ and MPF are active, APC/C is unphosphorylated, cyclin B is stable, and Cdk1 dephosphorylation is delayed. Notice that preMPF is dephosphorylated around 45 min. Compare with figure 3D of Mochida et al. (27). (*C* and *D*) Gwl depletion plus PP2A:B55δ reduction (30%). When PP2A:B55δ level is low, the Gwl-ENSA pathway is no longer essential for cell-cycle oscillations. This model prediction remains to be tested.

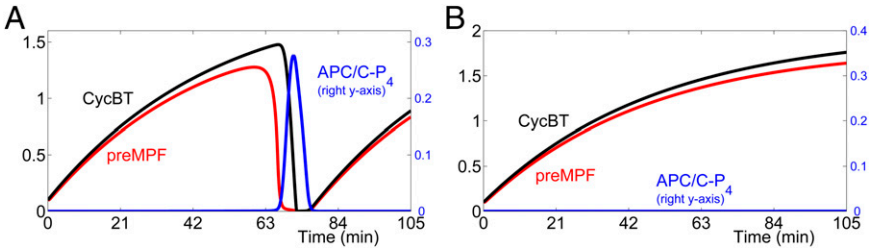

Fig. 4. Simulations of aberrant cycles in extracts with extra PP2A:B55δ. (*A*) Twofold excess of PP2A:B55δ delays mitotic entry (dephosphorylation of preMPF), as reported in figure 5 of Mochida et al. (29). Notice that preMPF is dephosphorylated around 65 min. (*B*) Threefold excess of PP2A:B55δ blocks mitotic entry, as reported in figure 5 of Mochida et al. (29). Notice that CycBT accumulates higher in the absence of APC/C:Cdc20 activation.

As time proceeds in Fig. 5*A*, the representative state point moves in Fig. 5*B*, tracing out a closed orbit (the black curve in Fig. 5*B*) as PP2A:B55δ and H1 kinase activities undergo a complete oscillation in Fig. 5*A*. The state point moves around the closed orbit in a clockwise direction; the landmark points 1, 2, . . ., 6 in Fig. 5*B* corresponding to the time points 1, 2, . . ., 6 in Fig. 5*A*. To understand the "forces" that drive the state point around the closed orbit, we plot the steady-state activity of PP2A:B55δ as function of MPF (the Z-shaped blue curve) in Fig. 5*B*.

At point 1, PP2A:B55δ is at its peak and MPF is increasing. As MPF accumulates, Gwl and ENSA are gradually activated and PP2A:B55δ drops only slightly, because the MPF-to-PP2A:B55δ ratio is low and so is the activity of Gwl. The low activity of Gwl is consistent with a stable steady state of high PP2A:B55δ activity (the solid blue curve between points 1 and 2). The dynamical trajectory (black curve) stays close to these attracting steady states.

At point 2 (where $MPF \sim 0.26$), the stable steady state with high PP2A:B55δ activity disappears. The only attracting state for $MPF > 0.26$ is the steady state of low PP2A:B55δ activity, and the dynamical trajectory moves toward this low steady state. As PP2A:B55δ inactivation proceeds, Gwl repression by PP2A:B55δ is relieved, and PP2A:B55δ inactivation is further accelerated (the black trajectory between points 2 and 3).

At point 3, MPF reaches its peak value. The high MPF-to-PP2A:B55δ ratio leads to activation of APC/C, followed by degradation of cyclin B, and MPF inactivation (the black trajectory between points 3 and 4). Because Gwl is active, PP2A:B55δ inactivation is fast, and the dynamical trajectory (the black curve) stays close to the stable steady states with low PP2A:B55δ activity (the solid blue curve with $B55 \sim 0$).

At point 4 (where $MPF \sim 0.11$), MPF activity is so low that the stable steady state with low PP2A:B55δ activity can no longer be maintained. However, because Gwl dephosphorylation depends on PP2A:B55δ activity, the reactivation of PP2A:B55δ takes a long time. During this time lag, APC/C:Cdc20 keeps degrading cyclin B, until MPF reaches its lowest point at 5. Then, the activation of PP2A:B55δ causes inactivation of APC/C:Cdc20 and reaccumulation of MPF. When PP2A:B55δ reaches its peak at point 6, the oscillation starts over.

We conclude that the double-negative feedback loop between Gwl kinase and PP2A:B55δ phosphatase has the potential to generate two coexisting steady states of low and high PP2A:B55δ activity (i.e., a bistable switch). Because PP2A:B55δ regulates APC/C activity, the PP2A:B55δ switch in turn results in two coexisting steady states of high and low APC/C activity (*SI Text S2* and Figs. S4 and S5). During the initial stage of MPF accumulation, Gwl activity is low and PP2A:B55δ activity is high. By keeping the APC/C dephosphorylated, PP2A:B55δ allows for a protracted state of MPF accumulation, providing enough time for the early embryonic cell to align its replicated chromosomes on the mitotic spindle. Eventually, MPF accumulates high enough to flip the Gwl-PP2A:B55δ switch to the inactive phosphatase state. APC/C can now be phosphorylated, leading to a rapid degradation of cyclin B and loss of MPF activity. However, because the Gwl-PP2A:B55δ interaction is a bistable switch, PP2A:B55δ is kept in the low-activity state for an extended period, allowing for the complete degradation of cyclin B before the interphase cell returns to the low kinase–high phosphatase steady state. In this way, a regulated phosphatase activity can ensure the proper sequence of events coming into and exiting from mitosis. The timing and irreversibility of these events is intimately connected to the bistability of the kinase-phosphatase double-negative feedback loop.

Discussion

Sustained oscillations of cell division in early embryos of *Xenopus* are driven by time-delayed negative feedback between Cdk1:CycB protein kinase (which drives entry into mitosis) and APC/C:Cdc20 ubiquitin ligase (which drives exit from mitosis and return to G1 phase of the cell cycle). The activities of these central drivers of the cell-division cycle in early embryos are controlled by a complex network of interacting proteins. All of these regulatory proteins are phosphorylated by protein kinases and dephosphorylated by protein phosphatases. The principal kinase is Cdk1:CycB itself (also called MPF), and one of its counteracting phosphatases is PP2A:B55δ. The mutual interactions (direct and indirect) of these components create a jumble of feedback and feed-forward loops. Earlier models of this control system neglected regulatory effects on the counteracting phosphatase, which are now known to play important roles in the control system. In this work, we present a mathematical model of the roles of PP2A:B55δ in regulating the activities of Cdk1:CycB and APC/C:Cdc20 in frog-egg extracts (see more details in *SI Text S3* and *SI Text S4*).

Recent experiments (27, 30) suggest that PP2A:B55δ activity is down-regulated by binding to a stoichiometric inhibitor, Arpp19, or the closely related protein, Endosulfine. To be effective, the inhibitor proteins must be phosphorylated by Greatwall kinase (Gwl), and Gwl is activated by phosphorylation by MPF. What

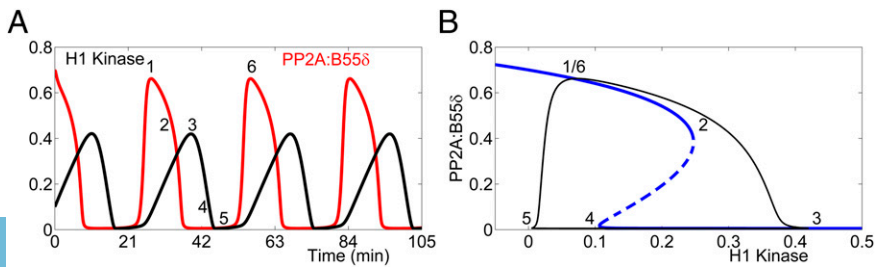

Fig. 5. Simulations of aberrant cycles in extracts where Cdk1 and Cdc20 cannot be phosphorylated. (*A*) Time courses of PP2A:B55δ activity and MPF (H1 kinase) activity. Compared with Fig. 2*A*, there are more oscillations in the same period. (*B*) MPF and PP2A:B55δ phase plot. The black trajectory is the time-dependent dynamics of MPF and PP2A:B55δ (taken from *A*). The blue curve is the steady state of PP2A:B55δ computed at different levels of MPF. Six reference points are labeled.

makes this signaling pathway interesting is evidence that PP2A:B55δ is the protein phosphatase that counteracts the phosphorylation of many Cdk1 substrates. In this case, Cdk1-dependent phosphorylation of substrate S is governed by a coherent feed-forward loop: MPF directly phosphorylates S, and MPF indirectly (through Gwl and Ensa) inactivates the phosphatase that dephosphorylates S-P.

In building our model of cell-cycle regulation in *Xenopus* eggs and egg extracts, we cite evidence that PP2A:B55δ counteracts MPF-dependent phosphorylation of Gwl kinase, of the Cdk1-modifying enzymes (Wee1 and Myt1 kinases and Cdc25 phosphatase), and of APC/C subunits—phosphorylations that are necessary for effective binding of Cdc20 to the APC/C.

We weave together these interactions in a regulatory network (Fig. 1) that has one negative feedback loop (MPF activates the APC/C, which promotes cyclin degradation) and four distinct positive feedback loops (PFLs): (*i*) MPF and Wee1 (also, possibly Myt1) are mutually antagonistic, (*ii*) MPF and Cdc25 are coactivating, (*iii*) MPF and Cdc20 are mutually antagonistic, and (*iv*) Gwl and PP2A:B55δ are mutually antagonistic. A common role of PFLs in protein interaction networks is to generate bistability (41). Indeed, PFLs *i* and *ii* are well-known to create bistability at the G2/M transition in frog-egg extracts (9, 42, 43). The coherent feed-forward loops from MPF through PP2A:B55δ to these substrates (Wee1 and Cdc25) contribute to robust bistability of the G2/M transition. On the other hand, PFL *iii* does not contribute to bistability for the parameter values we have chosen here (to fit the available data). We propose that, as a result of PFL *iv*, PP2A:B55δ responds to changing MPF activity as a bistable toggle switch.

If PP2A:B55δ does indeed exhibit bistable activation by MPF and if phosphorylated APC/C is indeed a substrate of PP2A:B55δ, then the activation of APC/C by MPF would also exhibit bistability and hysteresis. This prediction of our model is supported by experimental evidence that, in response to increasing MPF activity, APC/C:Cdc20 is activated in an exceedingly abrupt fashion (Hill exponent > 10) (20). We suggest that the abrupt activation of APC/C:Cdc20 with increasing MPF activity represents the activation threshold of a bistable switch. If so, then we predict that inactivation of APC/C:Cdc20 requires MPF activity to drop below an inactivation threshold that is smaller than the activation threshold (hysteresis), and two different APC/C:Cdc20 activities might coexist for the same MPF activity (bistability). If this proposal is correct, it would help to explain how embryonic cells achieve complete cyclin B degradation at the end of mitosis in the absence of other APC/C regulators (e.g., Cdh1). Because this proposal is of fundamental importance to mechanisms at the core of eukaryotic cell-cycle control, it should be tested experimentally.

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