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Protein phosphatase 2A controls the order and dynamics of cell cycle transitions

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Running title: PP2A controls cell cycle transitions

Summary

Bistability of the CDK1-Wee1-Cdc25 mitotic control network underlies the switch-like transitions between interphase and mitosis. Here, we show by mathematical modelling and experiments in *Xenopus* egg extracts that protein phosphatase 2A (PP2A), which can dephosphorylate CDK1 substrates, is essential for this bistability. PP2A inhibition in early interphase abolishes the switch-like response of the system to CDK1 activity, promoting mitotic onset even with very low levels of cyclin, CDK1 and Cdc25, while simultaneously inhibiting DNA replication. Furthermore, even if replication has already initiated, it cannot continue in mitosis. Exclusivity of S- and M-phases does not depend on bistability only, since moderate PP2A activity prevents replication without inducing mitotic onset. In these conditions, interphase-level mitotic kinases inhibit cyclin E-Cdk2 chromatin loading, blocking initiation complex formation. Therefore, by counteracting both CDK1 activation and activity of mitotic kinases, PP2A ensures robust separation of S-phase and mitosis, and dynamic transitions between the two states.

Introduction

How the S-phase and M-phase of the eukaryotic cell cycle are ordered, and what makes transitions between them irreversible, are fundamental questions. Much data suggests that cyclin-dependent kinase (CDK) activity is the key element of this control. In fission yeast, a single mitotic CDK-cyclin complex (CDK1-cyclin B) can promote an ordered cell cycle, implying that different CDK1 activity thresholds ensure that S-phase and M-phase do not overlap (Coudreuse and Nurse, 2010; Fisher and Nurse, 1996). Mathematical modelling showed that changes in CDK1 activity may be dictated by hysteresis, characterized by different cyclin B thresholds for activation and inactivation (Novak and Tyson, 1993; Pomerening et al., 2003; Sha et al., 2003; Solomon et al., 1990). In an ultrasensitive system, hysteresis should generate bistability – with irreversible transitions between two mutually exclusive stable states. These predictions have been confirmed experimentally (Novak and Tyson, 1993; Pomerening et al., 2003; Sha et al., 2003; Solomon et al., 1990). Feedback loops, in which CDK1 inhibits its negative regulator, Wee1 (Mueller et al., 1995) and stimulates its activator, Cdc25 (Kumagai and Dunphy, 1992), contribute to ultrasensitivity but are insufficient to explain it entirely (Kim and Ferrell, 2007; Trunnell et al., 2010).

Albeit overshadowed by CDKs, phosphatases have long been known to regulate the cell cycle (Kinoshita et al., 1990). We propose that mitosis-regulating protein phosphatase activities (MPPs), that dephosphorylate Cdc25 and Wee1, should contribute to bistability. Inhibiting the MPP should abolish hysteresis, and therefore bistability (Novak and Tyson, 1993). This is consistent with experiments in egg extracts using phosphatase inhibitors such as okadaic acid (OA), which renders histone H1 kinase activity proportional to added cyclin concentrations (Solomon et al., 1990). Moreover, phosphatase inhibition can also inappropriately trigger mitosis (Izumi and Maller, 1995). The identity of the MPP is not known, although PP2A is a likely candidate as it restrains CDK1 activation and is required for

dephosphorylation of CDK1 substrates including Cdc25 and Wee1, the balance of which controls mitosis onset in interphase egg extracts (Clarke et al., 1993; Felix et al., 1990; Margolis et al., 2006; Solomon et al., 1990; Lorca et al., 2010). PP2A exists as a heterotrimer of catalytic (“C”), structural (“A”), and regulatory (“B”) subunits. In fission yeast, the product of the PP2A C gene, *ppa2*, is responsible for OA sensitivity, and acts in concert with Wee1 and in opposition to Cdc25 to inhibit mitotic CDK1 (Kinoshita et al., 1993). In budding yeast, PP2A-Cdc55 is required for dephosphorylation of the Cdc25 and Wee1 homologues (Wicky et al., 2011). In *Xenopus* egg extracts, PP2A is active in interphase and inhibited in mitosis, in a Greatwall kinase-dependent manner, which is required to maintain CDK1 substrates in a phosphorylated state (Castilho et al., 2009; Mochida et al., 2009; Vigneron et al., 2009; Yu et al., 2006). As Greatwall activity, in turn, depends on CDK1, this raises the possibility of a mutual antagonism between CDK1 and PP2A. However, it is possible that other phosphatases could contribute to MPP activity. PP1, for example, is regulated by CDKs, can dephosphorylate CDK1 substrates, and a fission yeast PP1 mutant enters mitosis prematurely (Kinoshita et al., 1990).

A simple way of ensuring that S-phase and M-phase do not overlap would be to employ the CDK1/MPP balance not only to promote mitosis, but also to prevent DNA replication. High CDK1 activity inhibits replication licensing, preventing inappropriate DNA replication (Mahbubani et al., 1997). Whether or not the CDK1/MPP activity ratio might affect other steps of DNA replication is unknown, although PP2A, like S-phase CDKs, is required for formation of replication pre-initiation complexes (Chou et al., 2002; Lin et al., 1998; Murphy et al., 1995; Zou and Stillman, 1998).

Based on these considerations, we reasoned that it should not be the level of CDK1 activity *per se*, but rather the bistable CDK1/MPP activity ratio that orders the cell cycle. Here, we show that elimination of the MPP abolishes bistability in the CDK1 activation network and allows entry into mitosis at very low CDK1 activity levels, bypassing the mitotic requirements for Cdc25. Secondly, the CDK1/MPP ratio regulates not only mitotic onset, but also DNA replication, and the mitotic state, with high CDK1 and low PP2A activity, also prevents post-licensing steps of DNA replication, including the chromatin-loading of S-phase CDKs. Thirdly, the major MPP is PP2A-B55. We demonstrate these findings theoretically and experimentally, using the *Xenopus* egg extract system that facilitates study of core cell-cycle system components. We propose a simple mathematical model in which PP2A is required for bistability of CDK1 activity and contributes to a robust separation of S-phase and mitosis.

Results

Bistability in the CDK1 control network requires PP2A activity

The mitotic CDK1 control network contains several regulators connected by feedback loops whereby, for example, CDK1 stimulates Cdc25 and inhibits Wee1 (Figure 1A). The network behaviour generates two stable steady states with low and high CDK1 activities, respectively, separated by unstable steady states (Novak and Tyson, 1993; Pomerening et al., 2003). Transitions between these states are switch-like and occur at different cyclin thresholds for CDK1 activation and inactivation, creating S-shaped balance curves (Figure 1). Bistability is partly caused by the CDK1-Cdc25 / Wee1 feedback loops, but earlier modelling predicted that it also requires a protein phosphatase (which we name MPP) to counteract effects of CDK1 on Wee1 and Cdc25 (Novak and Tyson, 1993). Recent experiments suggest that PP2A might be such an MPP. Thus, Wee1 and Cdc25 are phosphorylated by CDK1 and would be dephosphorylated via the CDK1-regulated MPP, PP2A, creating an extra double-negative feedback loop, and coherent feed-forward loops in the network (Figure 1A; see also Novak et al., 2010). This should increase the distance between the two cyclin thresholds and make the bistable switch more robust. To analyse the network behaviour in response to MPP inhibition, we calculated steady-state levels of cell cycle regulators (Figure 1B-F). The low CDK1 and high MPP state constitutes S-phase while the high CDK1 and low MPP activity state corresponds to M-phase (Figure 1B, C). Entry into mitosis depends on mitotic cyclin

accumulation. The S-phase steady state with low cyclin levels and CDK1 activity is replaced by the mitotic steady state at cyclin levels of 0.6 (Figure 1D & E). Between cyclin levels of 0.3 and 0.6 the control system is bistable (Figure 1D & E). The CDK1/MPP activity ratio changes more than two orders of magnitude between the two steady states, which translates into a switch-like change in the phosphorylation state of generic CDK substrates (Figure 1F, thick line). We then analysed the behaviour of the network in response to MPP inhibition (Figure 1B-F, +OA). In this situation, bistability is lost (Figure 1C) and the mitotic state will be induced at low cyclin levels with S-phase timing (Figure 1F). In this case, the S-phase steady state allowing DNA replication (Figure 1G) has been lost (Figure 1H). Interestingly even lower CDK1 activity than normally present in early interphase (for example, after CDK1 partial depletion or inhibition) should allow mitotic entry, although with a delay (Figure 1I).

To experimentally test these predictions, we used early interphase *Xenopus* egg extracts, that have low CDK activity (Krasinska et al., 2008a), supplemented with sperm chromatin, and cycloheximide to prevent cyclin synthesis. If MPP activity is mainly due to PP2A, OA inhibition should lead to inhibition of S-phase and induction of mitosis in the absence of cyclin translation. Addition of 1 μ M OA prevented DNA replication (Figure 2A), as expected (Chou et al., 2002; Lin et al., 1998; Murphy et al., 1995), and also provoked condensation of chromatin, breakdown of the nuclear membrane and disassembly of lamina (Figure 2B). All nuclei showed this phenotype. OA addition also caused dephosphorylation of CDK1Y-15 and the electrophoretic mobility shifts of Cdc25, Wee1 and Gwl, a hallmark of their mitotic hyperphosphorylation (Figure 2C) that always correlated with nuclear breakdown and was subsequently used as a mitotic marker. Addition of recombinant cyclin B lacking its N-terminal 90 residues (cyclin B Δ 90) reproduced the effects of OA (Figure S1A and B), in accordance with mathematical modelling (Figure 1F and Figure S1C). Fostriecin, a potent PP2A-family inhibitor with 10,000-fold selectivity over PP1 (Walsh et al., 1997), also abolished DNA replication while inducing mitotic onset (Figure 2D). OA generated three-fold higher CDK activity than is normally present in early interphase egg extracts, that was sufficient to trigger mitosis despite being much lower than that obtained by cyclin B Δ 90 addition (Figure 2E). Partial PP2A inhibition reduced the concentration of cyclin B needed to promote complete phosphorylation of CDK1 substrates and therefore mitotic onset (Figures 1F, 2F, and S1D). From these data we conclude, firstly, that PP2A is a major determinant of the threshold CDK activity required for mitotic entry; secondly, that inhibiting PP2A unmasks the mitotic potential of the CDK activity already present in early interphase. This activity is probably due to both cyclins A and B, as CDK1-cyclin A is active in interphase *Xenopus* egg extracts (Krasinska et al., 2008a), and we observed low levels of cyclin B2 (Figure S1E).

Our mathematical model predicts that if PP2A is inactivated by OA, even a major reduction in CDK activity should only delay, but not abolish, mitotic onset (Figure 1I). To test this, we investigated CDK requirements for mitotic onset when PP2A is inhibited in early interphase. In *Xenopus* egg extracts CDK2 activity promotes CDK1 activation by phosphorylating Cdc25 (Margolis et al., 2003). However, CDK2 depletion had no effect on mitotic entry induced by 1 μ M OA (Figure 3A), indicating that its role is bypassed, as expected if MPP is PP2A. Depletion of the majority of CDK1, however, delayed but did not prevent mitosis (Figure 3A). Additionally removing CDK2 using the small CDK-binding protein p13^{Suc1} did not accentuate the delay, indicating that roles of CDK1 and CDK2 are different (Figure 3B). We obtained similar results using specific inhibitors NU6102 and RO-3306, which in extracts inhibit CDK2 and CDK1, respectively (Krasinska et al., 2008a,b). Increasing concentrations of the two inhibitors progressively reduced CDK activity back to interphase levels while increasingly delaying, and, at the highest concentrations, preventing OA-induced mitotic onset (Figure 3C). The same result was obtained when we added recombinant p21^{Cip1/waf1} protein, which inhibits both CDK1 and CDK2 (Figure 3D). These results confirm that the balance of PP2A and CDK1 activities determines the dynamics of mitotic entry. We imagined that loss of switch-like activation of CDK1 might lead to asynchrony of mitotic

onset. Indeed, whereas with OA alone mitotic onset was synchronous, upon decreasing CDK activity both mitotic and interphase nuclei were present at some time points (Figure S2).

Entry into mitosis normally requires positive feedback in which CDK1 activates Cdc25 and inhibits Wee1. Mathematical modelling predicts that MPP inhibition should reduce requirements for Cdc25 in mitotic onset, because Wee1 should still be inhibited (Figure 4A). We therefore compared effects of depleting Cdc25, and/or inhibiting ERK1/2, as it was reported that ERK1/2 kinases can phosphorylate and stimulate Cdc25 activity (Wang et al., 2007). UO126, which inhibits ERK1/2 activation, only marginally reduced CDK1 activity and did not prevent OA-induced mitotic onset (Figure 4B). In contrast, immunodepletion of around 90% of Cdc25 strongly reduced CDK1 activity. Nevertheless, as predicted, Cdc25 depletion had no effect on mitotic onset induced by OA, even when combined with ERK1/2 inhibition (Figure 4B). These results reinforce our conclusion that CDK1 activity requirements for mitosis are very low when MPP is inhibited, and show that ERK1/2 cannot substitute for CDK1 activity to trigger mitotic onset in the presence of OA.

Because the Cdc25/ Wee1 balance is a target of the checkpoint that restrains mitotic entry during DNA replication (Dasso and Newport, 1990), we then asked whether the checkpoint would affect MPP requirements. Mathematical modelling predicted that unreplicated DNA should increase the cyclin threshold for CDK1 activation by increasing MPP activity (Novak and Tyson, 1993; Pomerening et al., 2003). If so, decreasing the amount of unreplicated DNA should allow entry into mitosis with lower OA levels. This was confirmed experimentally: at nuclear concentrations that mimic early embryonic development (1400 nuclei/ μ l), 1 μ M OA is required to promote mitosis, whereas 0.5 μ M OA is sufficient at 140 nuclei/ μ l (Figure 4C). Note that the biochemical state characteristic of mitosis does not require the presence of DNA. Therefore, unreplicated DNA alters the balance between CDK1 and PP2A activity as expected, providing further evidence that PP2A functions as the MPP.

The S-phase stable steady state: replication licensing and initiation stages are differentially sensitive to PP2A inhibition

In early interphase, PP2A is active, CDK1 activity is low, and DNA synthesis can take place. The bistability model predicts that this state should be robust, and small increases in cyclin or PP2A levels should not affect DNA replication. We verified these predictions experimentally: replication rates are neither reduced by addition of recombinant cyclin B Δ 90 at levels that do not provoke mitosis entry (Figure S1D) nor enhanced by addition of purified PP2A (Figure S3A). We next investigated the mechanisms of the block to DNA replication upon PP2A inhibition. Chromatin loading of pre-replication complex (pre-RC) proteins, including Cdc6, Cdt1 and MCMs, occurred very early, but Cdc6 later disappeared from chromatin, coinciding with electrophoretic retardation of cytosolic Cdc6 and other pre-RC proteins, indicating the mitotic state (Figure 5A). Thus, mitotic hyperphosphorylation "unlicenses" origins, preventing their conversion to pre-initiation complexes (pre-ICs). We next asked whether the mitotic state is required for the inhibition of DNA replication. In agreement with simulations (Figure S3B), low concentrations of PP2A inhibitor decreased replication rates but did not prevent replication (Figure S3C). Interestingly, when we reduced PP2A to intermediate levels (50-100 μ M fostriecin or 0.7 μ M OA), onset of mitosis did not occur, but DNA did not replicate (Figure 5B and S3D). Replication also did not occur when we prevented OA from inducing mitosis by CDK inhibition (Figure S3E). These results suggest that either there is an intermediate steady state in which the CDK1/MPP activity ratio is too low for mitotic onset but too high for S-phase, which is not expected from the bistability model, or PP2A promotes replication by counteracting other kinases along with CDK1. Either way, this could provide a buffer ensuring robust separation of S-phase and mitosis. To investigate the nature of this state, we analysed pre-RC and pre-IC formation when PP2A is partially inhibited. Figure 5C shows that licensing can occur, but pre-IC components are absent from chromatin. Pre-IC loading requires S-phase CDKs (Mimura and Takisawa, 1998; Zou and Stillman, 1998),

which in *Xenopus* is fulfilled by CDK2-cyclin E, and cyclin E and CDK2 were absent from chromatin at intermediate PP2A activity levels (Figure 5C, E, F). We could detect PP2A subunits on chromatin early during DNA replication where they directly interacted with cyclin E (Figure S3F, G). These results suggest that PP2A acts on chromatin to control S-phase CDK loading. Indeed, OA no longer had any effect when added at the point where CDKs become dispensable for DNA replication (Figure S3H). One possibility is that an intermediate CDK1/PP2A ratio prevents loading of S-phase CDK onto chromatin and thus also prevents DNA replication, an unexpected variation on the simple bistability model. If so, then modelling predicts that sufficiently reducing CDK1 activity should restore DNA replication (Figure 5D). When we titrated OA to minimal levels that inhibit replication, we could partly restore replication by inhibiting CDK1 with RO-3306 (figure S3I).

PP2A counteracts additional mitotic kinases to allow cyclin E-CDK2 loading onto chromatin in S-phase

One explanation for the incomplete replication rescue by CDK1 inhibition is that additional kinases opposed by PP2A ensure that DNA replication and mitosis do not overlap. Given that chromatin binding of cyclin E is inhibited by phosphorylation (Furstenthal et al., 2001), and that cyclin E can be phosphorylated by multiple kinases including GSK3, ERK1/2, CDK1, and CDK2 itself (Furstenthal et al., 2001; Welcker et al., 2003), we suspected that PP2A counteracts not only CDK1 but also another kinase to allow cyclin E loading and replication origin firing. To test this idea, we used staurosporine, a broad-spectrum kinase inhibitor. In the absence of OA, 2 μ M staurosporine reduced replication efficiency (Figure S3J), presumably because it also partly inhibits S-phase CDKs. Nevertheless, whereas 0.7 μ M OA alone abolished DNA replication, addition of 2 μ M staurosporine restored cyclin E-CDK2 loading onto chromatin and allowed DNA replication in some nuclei (Figure 5E). Inhibition of GSK3 with AR-A014418 had no effect (data not shown). Preventing ERK1/2 activation with UO126 restored most cyclin E loading (Figure 5F), although had little effect on replication (figure S3K). Combining UO126 and RO-3306, to counteract both ERK1/2 and CDK1, fully restored cyclin E levels on chromatin, and around 40% of DNA was replicated (Figure 5F). Further adding staurosporine to the inhibitor cocktail, however, was not effective (Figure S3K), perhaps because CDK activity is overly reduced. We then added a recombinant CDK2-cyclin E complex in which we mutated all ERK/CDK consensus phosphorylation sites (supplementary information). Although active, this did not rescue the block of DNA replication due to partial OA-inhibition (Figure S3L). It has also been reported that caffeine, which inhibits ATM/ATR checkpoint kinases, could restore DNA replication in the absence of PP2A (Petersen et al., 2006). In our hands, caffeine alone did not affect the block to DNA replication by OA (Figure S3M), but, once the OA block is overcome using UO126 and RO-3306, caffeine slightly boosts replication rates (Figure S3N). Finally, we noticed that partial PP2A inhibition provoked strong chromosome condensation and histone H3 phosphorylation on serine 10, even in the absence of mitosis (Figure 5B and S3O), providing a potential hindrance to replication fork progression. Although inhibition of Aurora kinases with ZM4474439 could abrogate the increase in H3S10 phosphorylation, it could not restore replication. We conclude that there are multiple additional kinases, superimposed onto the bistable network, which restrain replication when PP2A is partially inhibited, and that replication and mitosis are incompatible at many levels.

PP2A-B55 complexes are the major PP2A complexes involved in control of DNA replication and mitosis

Inhibition of the PP2A-B55 δ complex is required for mitotic entry, but its depletion does not provoke mitotic entry in interphase extracts without cyclin synthesis (Castilho et al., 2009; Mochida et al., 2009), unlike complete inhibition by OA or fostriecin (Figure 2). Therefore, other PP2A complexes suffice to maintain the interphase state, or the low levels of PP2A-

B55 δ remaining after depletion are sufficient. To distinguish between these possibilities we attempted to deplete the four B subfamilies using antibodies raised against B55 δ , B56 ϵ , B56 γ and B γ /PR48, and assayed DNA replication. The B55 δ antibody only slightly reduced abundance of the two bands recognised. Figure 6A shows that none of the depletions significantly affected DNA replication. Multiplex depletions were not efficient, so we used microcystin covalently immobilised onto agarose beads, reported to specifically remove PP2A from *Xenopus* extracts (Maton et al., 2005). To determine the composition of any phosphatase complexes removed, we analysed the material isolated by the control and microcystin agarose depletions by semi-quantitative tandem mass spectrometry (Figure S4). The major phosphatase identified was indeed PP2A, although PP1, PP4, PP5 and PP6 subunits were also present, to a lesser extent. Interestingly, the predominant B subunits of PP2A identified were B55 α and B55 δ , although several B56 subunits were also present, at lower levels (Figure S4B). Western blotting analysis showed that microcystin depletion led to nearly complete depletion of PP2A catalytic and structural subunits (Figure 6B). The PP2A family members PP4 and PP6 were also significantly depleted, but PP1 and PP5 less so. Both bands recognised by the PP2A-B55 δ antibody, which we infer from proteomics data to be the highly related B55 α and B55 δ , were depleted, but no change was detected for the other B subunits. The result of the depletion was either mitotic onset from interphase, or, in other experiments, inhibition of replication without mitotic onset (Figure 6C, left and right panels respectively), as expected. Unlike inhibition of PP2A by OA or microcystin depletion, inhibition of PP1 by tautomycin, which has a similar IC₅₀ for PP1 as OA for PP2A, or inhibitor-2 protein, a specific PP1 inhibitor (Cohen, 1989), did not affect DNA replication nor induce mitosis (Figure 6D); on the contrary, PPI2 delayed cyclin B Δ 90-induced mitotic onset (Figure 6E). At low concentrations, due to its high affinity essentially all OA will be complexed to PP2A. We therefore titrated OA to the minimal replication-inhibitory concentration for individual extracts, and then added purified PP2A complexes. This partially rescued replication (Figure 6F). Finally, to definitively demonstrate the role for PP2A-B55, we exploited the recent discovery that Greatwall-phosphorylated Arpp19/endosulfine is a specific inhibitor of PP2A complexed with B55 subunits (Mochida et al., 2010; Gharbi-Ayachi et al., 2010). To reduce specific phosphatase activity to a level allowing inhibition by recombinant proteins, we primed the extract with 0.1 μ M OA, which had no phenotype alone. We immunoprecipitated Greatwall kinase from a mitotic extract and used it to thiophosphorylate recombinant Arpp19, which we then titrated into the extract. High or intermediate Arpp19 levels inhibited DNA replication and caused mitotic entry, whereas low levels blocked replication in interphase (Figure 6G). Therefore, while we cannot formally rule out redundancy with PP2A in complex with other B subunits, and/or PP1, PP4, PP5 or PP6, we show that PP2A-B55 complexes are essential in promoting replication and preventing mitosis.

All stages of DNA replication are incompatible with mitosis

Our results indicate that the cell cycle has several mechanisms preventing overlap of S- and M-phase. Nevertheless, one report found that mitotic chromatin can replicate if a concentrated S-phase nucleoplasmic extract is added (Prokhorova et al., 2003). We therefore asked whether, once initiated, replication can continue in mitosis. First, we allowed chromatin to form with or without OA for the time normally taken to form active pre-ICs, then transferred it into control extracts, or extracts rendered mitotic either by OA treatment or addition of cyclin B Δ 90. Replication in the donor and recipient extracts was monitored by two-colour immunofluorescence microscopy and quantified by incorporation of ³³P-dCTP (Figure 7A). Pre-ICs formed in control extracts replicated only briefly in mitotic extracts, which probably reflects the lag before the nucleus breaks down and chromatin becomes mitotic, before arresting (Figure 7A, B, S5A, B). To formally exclude the possibility that replication forks can progress in the mitotic state, we first allowed origins to fire by incubating chromatin

in an egg extract containing aphidicolin to prevent any elongation. Chromatin was then transferred to a mitotic extract with aphidicolin until it became mitotic. This mitotic post-initiation chromatin was reisolated and transferred to a third extract, also mitotic but without aphidicolin, thereby potentially allowing elongation, which was quantified (Figure 7C). Elongation did not occur (Figure 7C). Taken together, these results show that all steps of DNA replication, pre-RC assembly, pre-IC formation, and elongation, are incompatible with mitosis, ensuring a robust ordering of the cell cycle.

Discussion

PP2A appears to act as a central modulator of phosphorylation to generate two stable states, interphase and mitosis. To illustrate this, an analogy is presented in Figure 7D, which depicts a traditional Japanese fountain known as a *shishi odoshi*. A water flow generates oscillations between two states, due to the cylinder filling up until it overbalances, whereupon it empties and swings back again. In our analogy, the flow represents phosphorylation due to CDK1. PP2A is represented by the cylinder, which continuously dephosphorylates mitotic CDK1 substrates. In interphase, PP2A prevails, preventing mitotic onset and allowing S-phase (Figure 7D, left). But as a result of increasing CDK1 activity, which activates Greatwall kinase to generate a PP2A inhibitor, PP2A becomes overwhelmed and phosphorylation of mitotic CDK1 substrates can occur, bringing about mitosis while simultaneously preventing DNA replication (Figure 7D, right). Several points of this analogy, supported by our experimental observations, are worth highlighting. Firstly, S-phase and mitosis are inversely regulated by mitotic CDK1-phosphorylation and never overlap, whether PP2A is active or not. Secondly, artificial inhibition of PP2A (the cylinder forced right) should allow even low-level CDK1 activity to bring about mitosis, whose timing should depend on CDK1 activity. Thirdly, since CDK1 substrates are involved in the feedback loops, PP2A should be a critical component of these loops. As such, it is likely to contribute to the ultrasensitivity of the regulatory network to increasing CDK1 activity. Several mechanisms for ultrasensitivity have been proposed, involving multisite phosphorylation of the regulatory enzymes, including allostery and competition among phosphorylation sites, but ultrasensitivity found in extracts could not be recapitulated with a minimal network *in vitro* (Kim and Ferrell, 2007; Trunnell et al., 2010). Although inclusion of a CDK1-regulated phosphatase could theoretically generate ultrasensitivity (Novak et al., 2010), so far this has not been experimentally proven; addition of purified PP2A catalytic subunit alone did not restore the ultrasensitivity found in extracts (Trunnell et al., 2010). The most likely reason is that in this case, the phosphatase activity is not regulated by CDK1. We nevertheless find that inhibiting PP2A eliminates ultrasensitivity in the extract. We suggest that to reproduce physiological ultrasensitivity *in vitro*, it would be necessary to reconstitute a network containing PP2A-B55, the PP2A inhibitor Arpp19/ENSA, Greatwall kinase, and the currently unknown components allowing Greatwall to be regulated by CDK1.

We find that mitosis and DNA replication are fundamentally incompatible due to opposite regulation by phosphorylation. One aspect of this incompatibility might be the requirement for a nucleus to replicate. In yeast, unlike metazoans, the nuclear membrane remains intact during mitosis, suggesting that replication and mitosis might in some experimental circumstances become compatible. To date, although the present and many other studies performed in different model systems have found that the mitotic state can be experimentally induced before completion of DNA replication, only one report, to our knowledge, describes ongoing replication in mitosis (Prokhorova et al., 2003). Interestingly, in this study, replication was induced using a concentrated nucleoplasmic extract, which bypasses the need for a nuclear membrane (Walter et al., 1998). We find by serial transfer of post-initiation chromatin that replication elongation does not occur in mitotic extracts. Taken together, these observations suggest that addition of concentrated nucleoplasmic extracts must also somehow modify the mitotic state – perhaps by restoring some PP2A activity, although this would be expected to cause exit from mitosis.

In our model, the CDK1/PP2A activity ratio determines whether S-phase or M-phase will occur. There is an interesting parallel in budding yeast. A distinct phosphatase, Cdc14, reverses CDK1 phosphorylation at the exit from mitosis, and has recently been found to be required for DNA replication, where it acts to permit a sufficient accumulation of replication factors in the nucleus (Dulev et al., 2009). *Xenopus* Cdc14 homologues also have roles in control of mitosis and can dephosphorylate Cdc25 (Krasinska et al., 2007), although whether they are required for S-phase is not clear. If so, they could potentially provide another barrier to DNA replication within mitosis. Our *shishi odoshi* analogy also suggests a mechanism for "phase-locking" of the Cdc14 autonomous oscillator to the CDK oscillator, as proposed recently (Lu and Cross, 2010). If Cdc14 rather than PP2A oscillations are represented by the cylinder, it can be seen that they indeed do not require oscillation of the water flow; nevertheless, the water flow itself is required, just as Cdc14 oscillations disappear if all CDK1 activity is abolished. Furthermore, the frequency of oscillations will depend on the water flow-rate. The phase-locking comes about because of the double-negative feedback between the oscillator and the water flow. Although not represented in the analogy, in reality both Cdc14 and PP2A activities lead to CDK1 inactivation – in other words, the water flow is not constant, but increases and decreases with the same frequency as the autonomous oscillator, locking the two oscillation frequencies together. Certainly, closer study of the relationships between Cdc14 and PP2A in yeast and vertebrates will be important in order to build a universal model of how phosphatases control the cell cycle.

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Experimental procedures

Xenopus egg extracts and replication reactions

Interphase egg extracts, chromatin isolation and replication assays were prepared as described (Krasinska et al., 2008a). Where indicated, 1:100 dilution of okadaic acid, fostriecin, RO-3306, AR-A014418, tautomycin, NU6102, protein phosphatase inhibitor-2 (I-2 protein, 4 μ M final), UO126, recombinant GST-cyclin B Δ 90 (1 μ g/ μ l; Krasinska et al., 2007), or DMSO solvent only was added. Aphidicolin was used at 50 μ g/ml. Caffeine was freshly prepared in 10 mM Pipes pH 8.0, and used at 5mM. p21^{Cip1/waf1} (recombinant GST-fusion) was used at 1 or 2 μ M; PP2A at 0.012 U/ μ l. Thiophosphorylated Arpp19 was produced by incubating Greatwall kinase immunoprecipitated from 20 μ l extract rendered mitotic by GST-cyclin B Δ 90 (40ng/ μ l), with 5 μ g Arpp19 and 2 μ l kinase buffer containing 1mM ATP γ S.

Depletion and immunoprecipitation

20 μ l packed protein G-Sepharose beads were incubated with 40 μ l of pre-immune (Mock), anti-XCDK1, or XCDK2 serum at 4°C, for 2 hours. For XCDC25 depletion, 40 μ l Protein G Dynabeads and 2.5 μ l anti-XCdc25 antibodies were used and incubated for 30 min at room temperature. Elsewhere, 20 μ l of packed p13^{Suc1} agarose conjugate was used where stated. Beads were washed in PBS and XB (Krasinska et al., 2008a), resuspended in 50 μ l egg extract, incubated at 4°C for 40 min, pelleted and the supernatant used for further experiments. Two rounds of depletions were performed unless otherwise stated. Microcystin-agarose was produced as described (Moorhead et al., 2007). For depletion from 50 μ l of extract, 20 μ l of packed Protein A-agarose or microcystin-agarose beads were used. For mass spectrometry analysis, the same procedure was used with 125 μ l extract.

Proteomics

Detailed protocols are given in supplementary information. Briefly, proteins eluted from microcystin or mock beads were precipitated and separated by SDS-PAGE. Coomassie stained bands were excised, destained, dehydrated and dried. After digestion, peptides were extracted and samples were analysed by RPLC coupled via electrospray to a maXis qTOF mass spectrometer. Relative abundance between samples was estimated by spectral counting.

Kinase assays

2µl of packed p13^{Suc1} beads washed in PBS were incubated with 5µl of extract and 45µl IP buffer for 30 min on ice, with agitation. Beads were washed in IP buffer and wash buffer and resuspended in 20µl kinase buffer. Reactions were carried out in duplicate for 20 min at 25°C, spotted onto P81 filters, placed into 1% orthophosphoric acid, washed twice, dried and counted by scintillography.

λ phosphatase treatment.

2µl of control interphase extract or extract incubated for 90 min with 1 µM OA, was treated 30 min at 30°C with or without 100U λ PPase.

Cdc6 phosphorylation.

Sperm chromatin was incubated in 10µl extract for 35 min, re-isolated and the pellet resuspended in 18µl kinase buffer, with 0.5µl of recombinant CDK1/cyclin B, and incubated for 15 min. The pellet was re-isolated and proteins analysed by western blotting.

Immunofluorescence

Immunofluorescence was performed as previously described (Krasinska et al., 2008a). Where indicated, 20µM biotin-dUTP or FITC-dATP, and inhibitors or DMSO, were used. DNA was stained with 1 µg/ml Hoechst 33258. Secondary antibodies were AlexaFluor conjugates.

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Figure legends.

Figure 1.

A model for mitotic CDK regulation. (A) A diagram of mitotic CDK1 regulation. See text for details. (B) Calculated changes of activity of cell cycle regulators Wee1, MPP, Greatwall

(Gwl) and Cdc25 as a function of CDK1 activity. (C) The antagonism of CDK1 and MPP on the phase plane. Steady state levels of CDK1 and MPP (*i.e.* $\partial\text{CDK1}/\partial t = \text{CDK1}' = 0$, in black, or $\partial\text{MPP}/\partial t = \text{MPP}' = 0$, in blue). The CDK1 balance curve depends on total cyclin level and the MPP balance curve is OA-sensitive (see +OA). There are three intersections: steady states for both CDK1 and PP, two of which are stable (filled circles, S-phase or M-phase). (D, E, F) Steady state levels of CDK1 (D), MPP (E) and the phosphorylated fraction of a generic CDK1 substrate (S_p), which is proportional to the CDK1/MPP ratio (F), as functions of total cyclin level (Cyc_T) at different concentrations of OA (multiples of the IC_{50}). (G, H, I) Numerical simulation of DNA replication (DNA) and changes in activity of cell-cycle regulators in extracts, without (G) and with (H) OA (99IC_{50}), or with OA and CDK1 inhibitor RO-3306 (RO) (I) (RO-3306 concentration = IC_{50}). DNA replicates in the control extract but not with OA, which induces mitosis even with low concentrations of CDK inhibitor. Time zero corresponds to addition of chromatin. The interphase level of Cyc_T is assumed to be 0.2.

Figure 2.

Inhibition of PP2A induces *bona fide* mitosis at low CDK activity. (A) DNA replication time course in control interphase extract or with $1\mu\text{M}$ OA. (B) Sperm nuclei after 60 min in extract with or without $1\mu\text{M}$ OA. States of chromatin, nuclear membrane and lamina were assessed by staining with Hoechst 33258 (DNA), nucleoporin (mAb414) and Lamin B3 antibodies. Bar, $10\mu\text{m}$. Right: all nuclei show this phenotype. Bar, $20\mu\text{m}$. (C) Immunoblots at different time-points of extract incubated with sperm-nuclei, control (Ctl) or with $1\mu\text{M}$ OA, against the indicated proteins. Arrows, shifts due to phosphorylation; * non-specific bands. Right: lambda phosphatase treatment eliminates shifts. (D) Replication time-course and chromatin state at 60 min (below) after sperm DNA incubation in extracts with or without $150\mu\text{M}$ fostriecin (Fostr). (E) p13^{Suc1}-associated Histone H1 kinase activity assessed at 60 min in control extract or with $1\mu\text{M}$ OA or GST-Cyclin B Δ 90 (CycB Δ 90). (F) Adding sub-threshold cyclin B reduces the OA concentration causing mitotic phosphorylation: compare lanes 2 with 7 and 3 with 8; see also Figure 1F and Figure S1.

Figure 3.

Inhibition of PP2A eliminates switch-like behaviour of the mitotic oscillator. (A) Interphase egg extract immunodepleted (Δ) with control antibodies or antibodies against CDK2 (left) or CDK1 (right) and containing sperm nuclei, incubated with or without $1\mu\text{M}$ OA. Aliquots were removed at indicated times and analysed by immunoblotting. Control of depletion efficiency with anti-PSTAIR antibody, recognising CDK1, upper band, and CDK2, lower band. (B) After depletion with control (Mock) or p13^{Suc1} beads, extracts were incubated with sperm DNA, with or without $1\mu\text{M}$ OA. Aliquots at different time-points were immunoblotted against the proteins indicated. Below, supernatants blotted with anti-PSTAIR antibody. (C) Interphase extract with sperm nuclei incubated with or without $1\mu\text{M}$ OA, and increasing concentrations of NU6102 (NU) and RO-3306 (RO). Aliquots were blotted with Greatwall and Wee1 antibodies (upper panel). P13^{Suc1} associated Histone H1 kinase activity assessed at 90 min (lower panel). (D) Interphase extract with sperm nuclei incubated with or without $1\mu\text{M}$ OA, and increasing concentrations of GST-p21^{Cip1/Waf1}. Aliquots at different time-points were blotted for Greatwall. See also Figure S2.

Figure 4.

Inhibiting PP2A largely bypasses requirements for Cdc25. (A) Mathematical modelling, as in Figure 1, of the effect of depleting Cdc25 to different degrees (depletion of 90%, top; 95%, middle; 99%, bottom) on activity of cell cycle regulators as a function of CDK1 activity (left), on CDK1 activation (centre) and the phosphorylated fraction of a generic CDK1 substrate (S_p) (right), as functions of total cyclin level (Cyc_T). (B) Left, p13^{Suc1}-associated Histone H1 kinase activity at 90 min in control or Cdc25-depleted (Δ) extract, with or without $1\mu\text{M}$ OA or

UO126 (UO). Right, aliquots were immunoblotted for indicated proteins. Below: Cdc25 depletion control; *, non-specific band. (C) Interphase extract incubated with zero, 140 or 1400 nuclei/ μ l (n/μ l) and 0.5 μ M OA. Mitotic entry was assessed by blotting for Greatwall and Wee1.

Figure 5.

DNA replication requires PP2A to counteract multiple mitotic kinases. (A) Chromatin binding (left) and electrophoretic mobility in total extract (right) of replication factors, analysed by immunoblotting during DNA replication time-course in control or 1 μ M OA-treated extract. Bottom right: Cdc6 can be phosphorylated by CDK1-cyclin B on chromatin. (B) Left: DNA replication time course in control interphase extract, with 0.75 μ M OA or 100 μ M fostriecin. Right, chromatin state (Hoechst) at 60 min. (C) Chromatin-bound proteins at different time-points, in control replicating extract, and in extract containing 0.7 μ M OA. (D) Simulation of partial MPP inhibition (OA = 5 x IC₅₀) which allows low level DNA replication. (E) Left: immunoblot of chromatin-bound proteins at different time-points, in control and 0.7 μ M OA containing extract, with or without 2 μ M Staurosporine (S). Right: immunofluorescent analysis of nuclei (DNA (Hoechst), top; replication monitored by biotin-dUTP incorporation, bottom) after 60 min incubation in control extract (Ctl), with 100 μ M fostriecin (F) or 0.7 μ M OA, with or without 2 μ M Staurosporine. Insets: enlargements of chromatin. Bar, 10 μ m. (F) Left, immunoblots of chromatin-bound proteins during DNA replication in control extract, or in extracts treated with OA (0.7 μ M), 40 μ M RO-3306 (RO), and 100 μ M UO126 (UO), as indicated. Right, % of input DNA replicated at different time-points. See also Figure S3.

Figure 6.

PP2A-B55 complexes are the major PP2A complexes involved in control of DNA replication and mitosis. (A) Replication time-courses in Mock or B-subunit depleted (Δ) extracts; mean of 4 independent experiments. Below, depletion controls by immunoblot. * Cross-reacting band. (B) Microcystin agarose (μ C) depleted extract immunoblotted against indicated proteins. Non-depleted subunits serve as loading control. (C) Microcystin agarose depletion prevents DNA replication with (microscopic analysis of nuclei, left; bar, 10 μ m) or without (absence of shift of mitotic markers on Western blot, right) entry into mitosis. (D) Comparison of effects of 0.6 μ M tautomycin, OA and PPI2 on DNA replication. (E) PPI2 delays onset of mitosis induced by recombinant GST-cyclin B Δ 90 (cycB), as shown by Western blotting of Greatwall. (F) Purified PP2A subunits partly restore replication upon minimal OA (0.25 μ M in this extract) inhibition. (G) Arpp19 thiophosphorylated by Greatwall kinase inhibits DNA replication, and at higher concentrations provokes mitotic onset, when added to extracts primed with 0.1 μ M OA, which has no phenotype alone. See also Figure S4.

Figure 7.

All steps of DNA replication are incompatible with mitosis. (A) Sperm chromatin was added to control interphase extract or extract containing 1 μ M OA and incubated for 40 min with biotin-dUTP. Chromatin was then transferred into a control interphase extract, or extract pre-treated for 45 min with 1 μ M OA, containing FITC-dATP. Left, diagram of the experimental procedure. Right, nuclei after 60 min incubation in the recipient extract, monitored for chromatin state and replication in initial (biotin-dUTP) and recipient (FITC-dATP) extract. (B) Nuclei formed in control interphase extract containing FITC-dATP for 40 min, transferred to control extract or extract rendered mitotic with cyclin B Δ 90 for 45 min, containing biotin-dUTP. Chromatin state and replication in first (FITC-dATP) and recipient (biotin-dUTP) extracts visualised in nuclei isolated 60 min after transfer. (C) Sperm DNA incubated for 90 min in interphase extract containing aphidicolin, transferred to control extract, or extract rendered mitotic by 45 min incubation with 1 μ M OA or cyclin B Δ 90, with aphidicolin. After mitotic onset, nuclei were transferred to a third extract, control or pre-incubated for 45 min with 1 μ M OA or cyclin B Δ 90,

containing FITC-dATP. Left, diagram of the procedure. Right, replication efficiency, chromatin state, and replication (FITC-dATP) at 60 min in the final extract. Bars, 10 μ m. (D) Model presenting an analogy between a *shishi odoshi* and the oscillations between S- and M-phases due to regulation by PP2A. See text for explanation and Figure S5.

Figure 1.

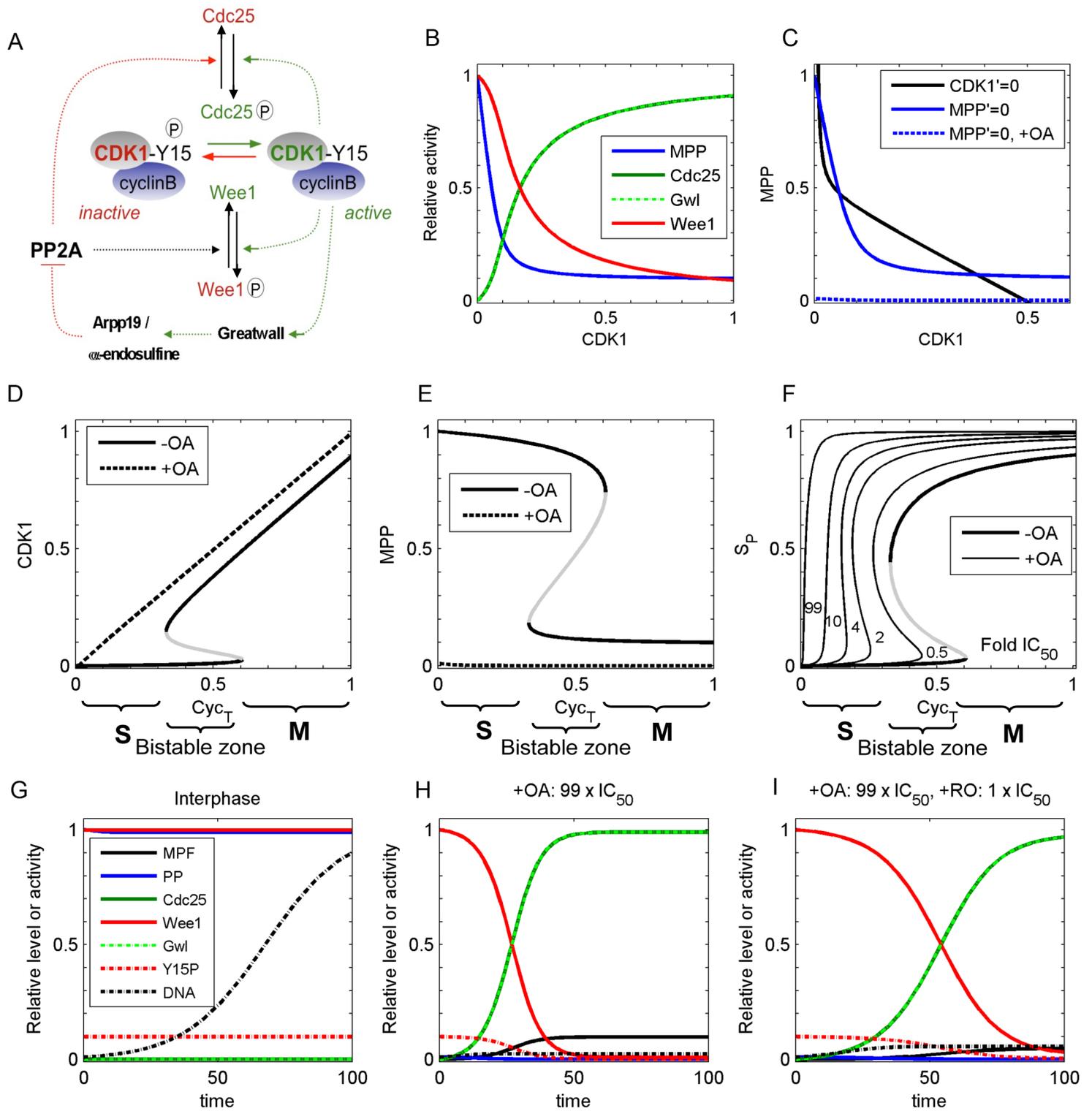


Figure 2.

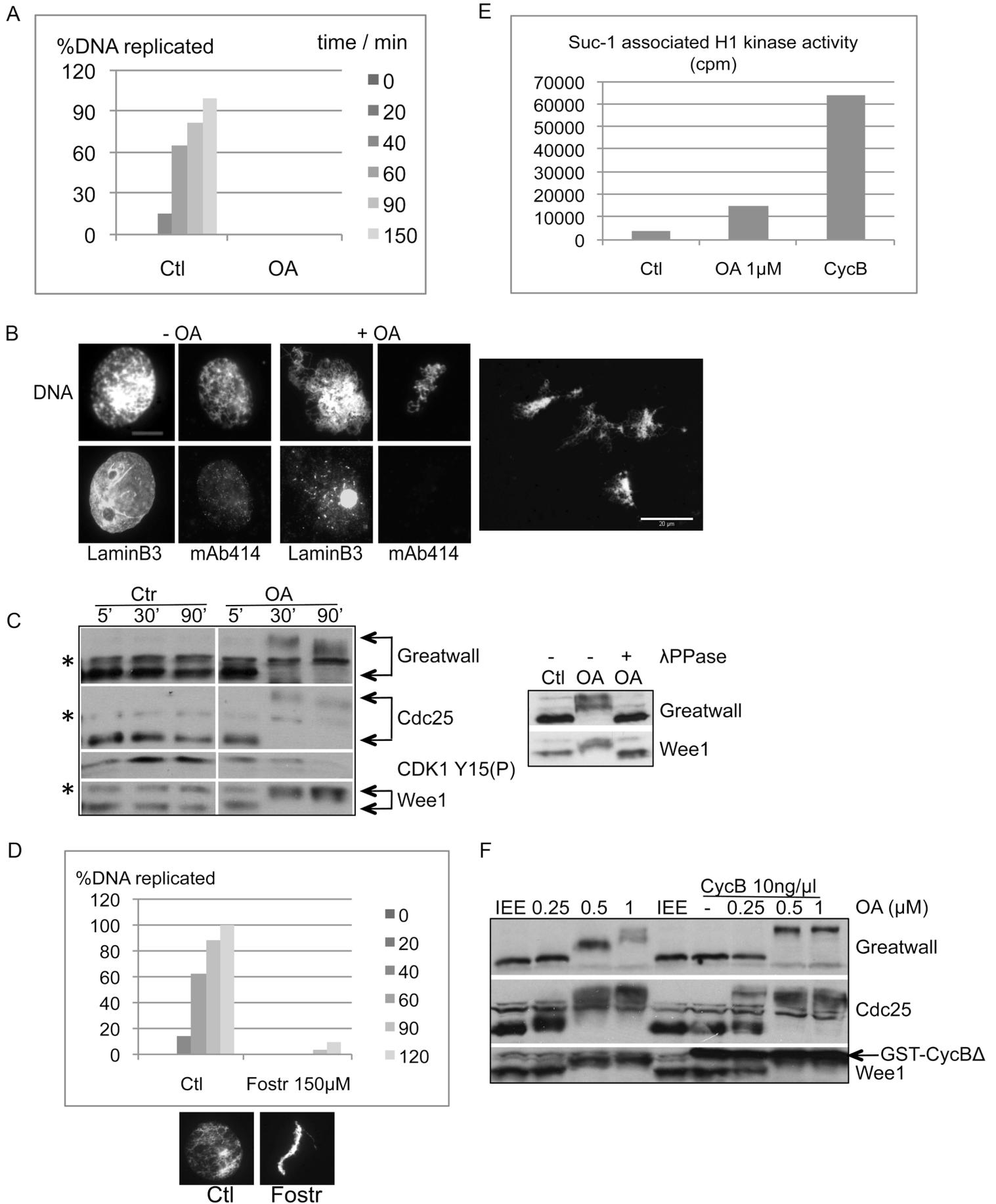
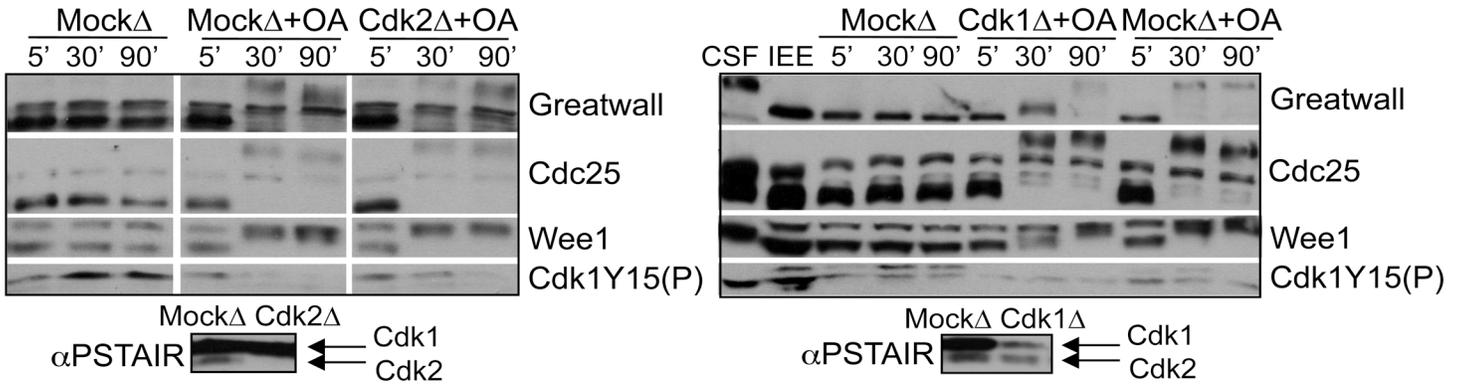
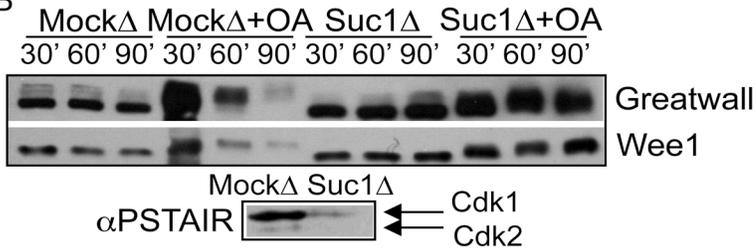


Figure 3.

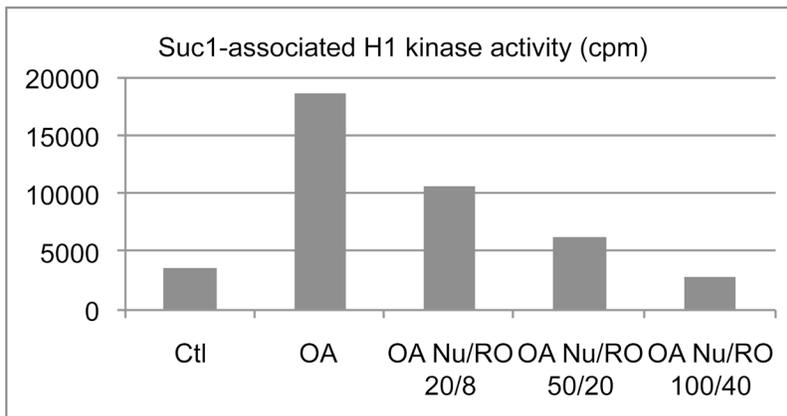
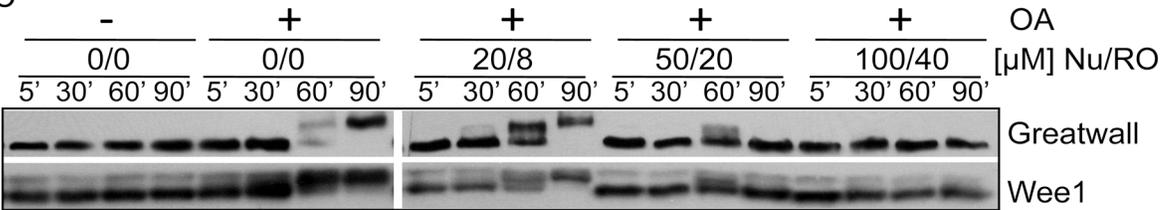
A



B



C



D

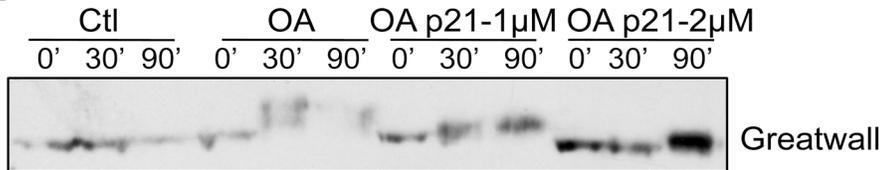


Figure 4.

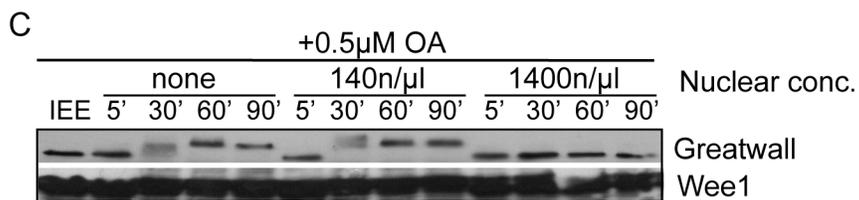
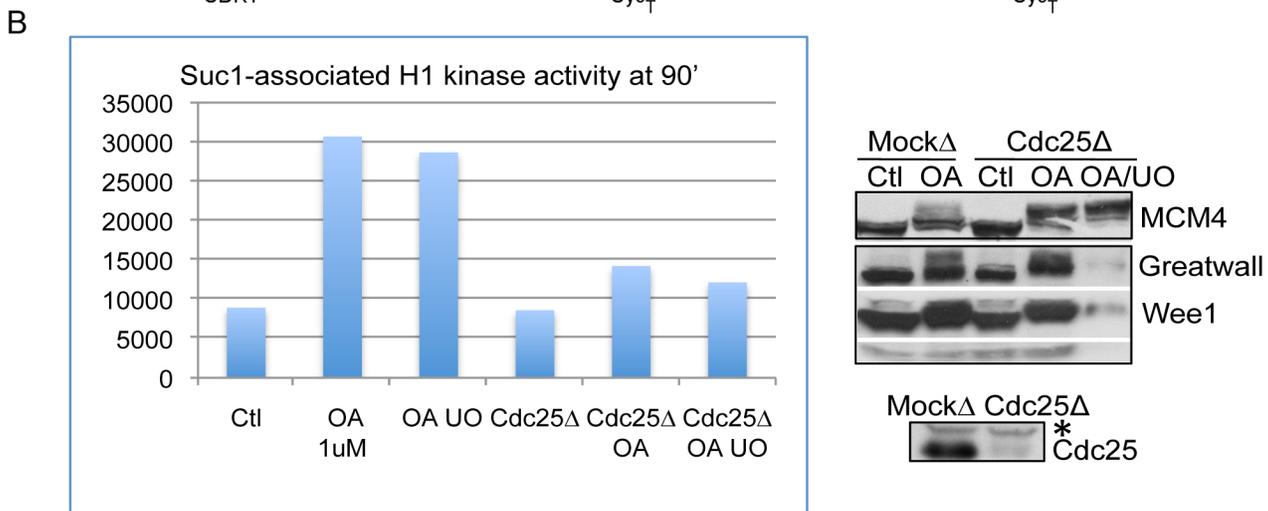
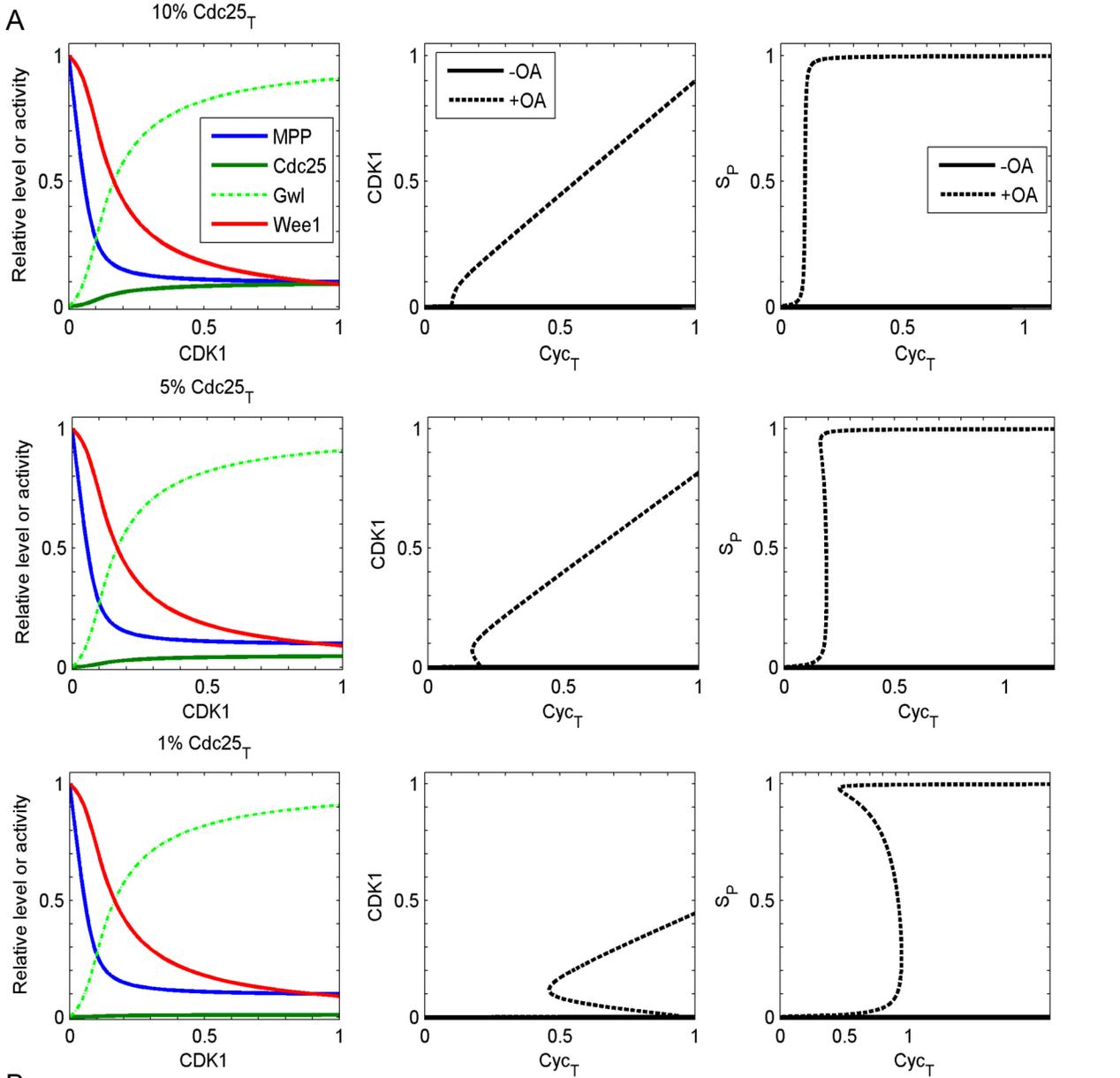


Figure 5.

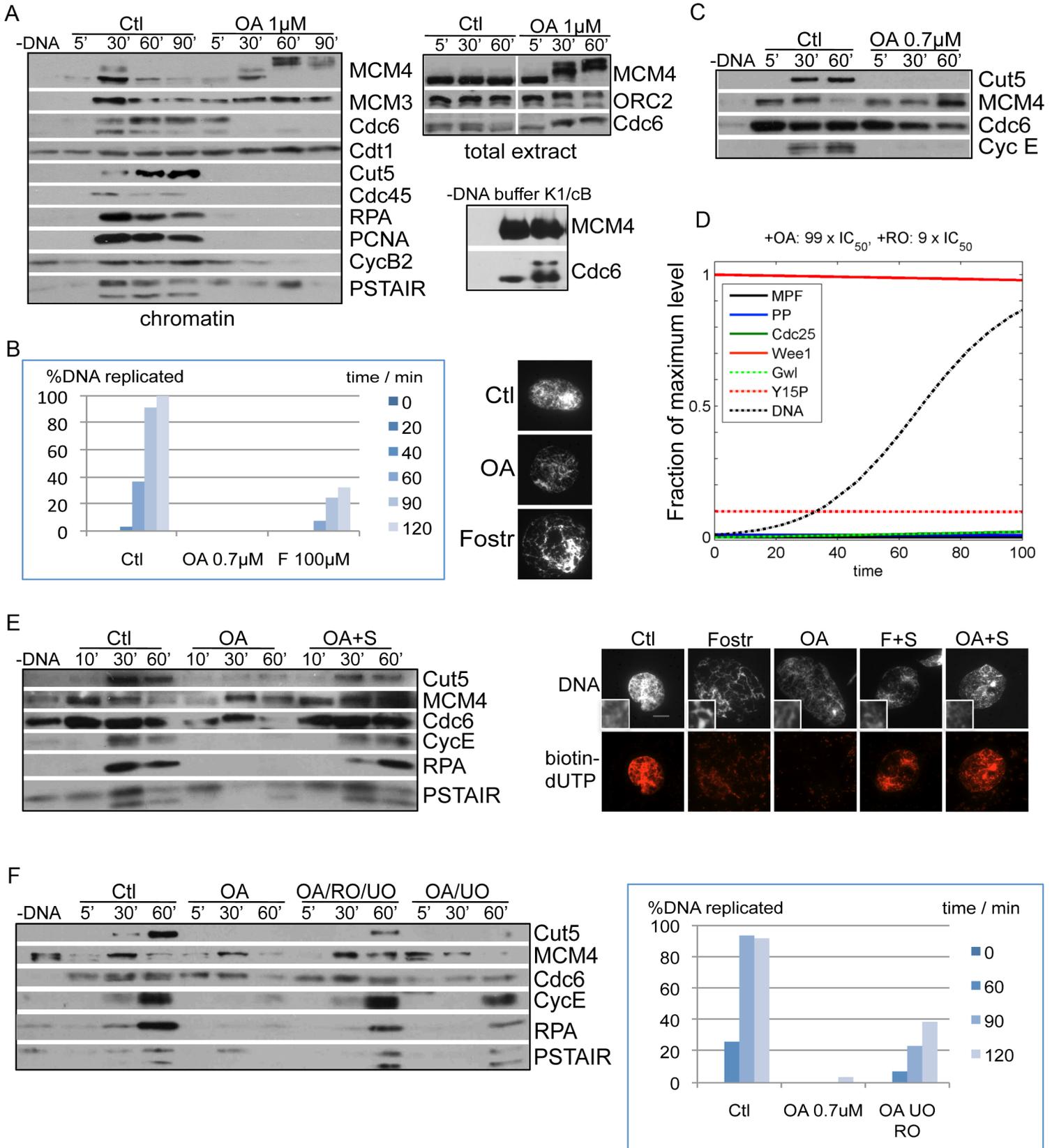


Figure 6.

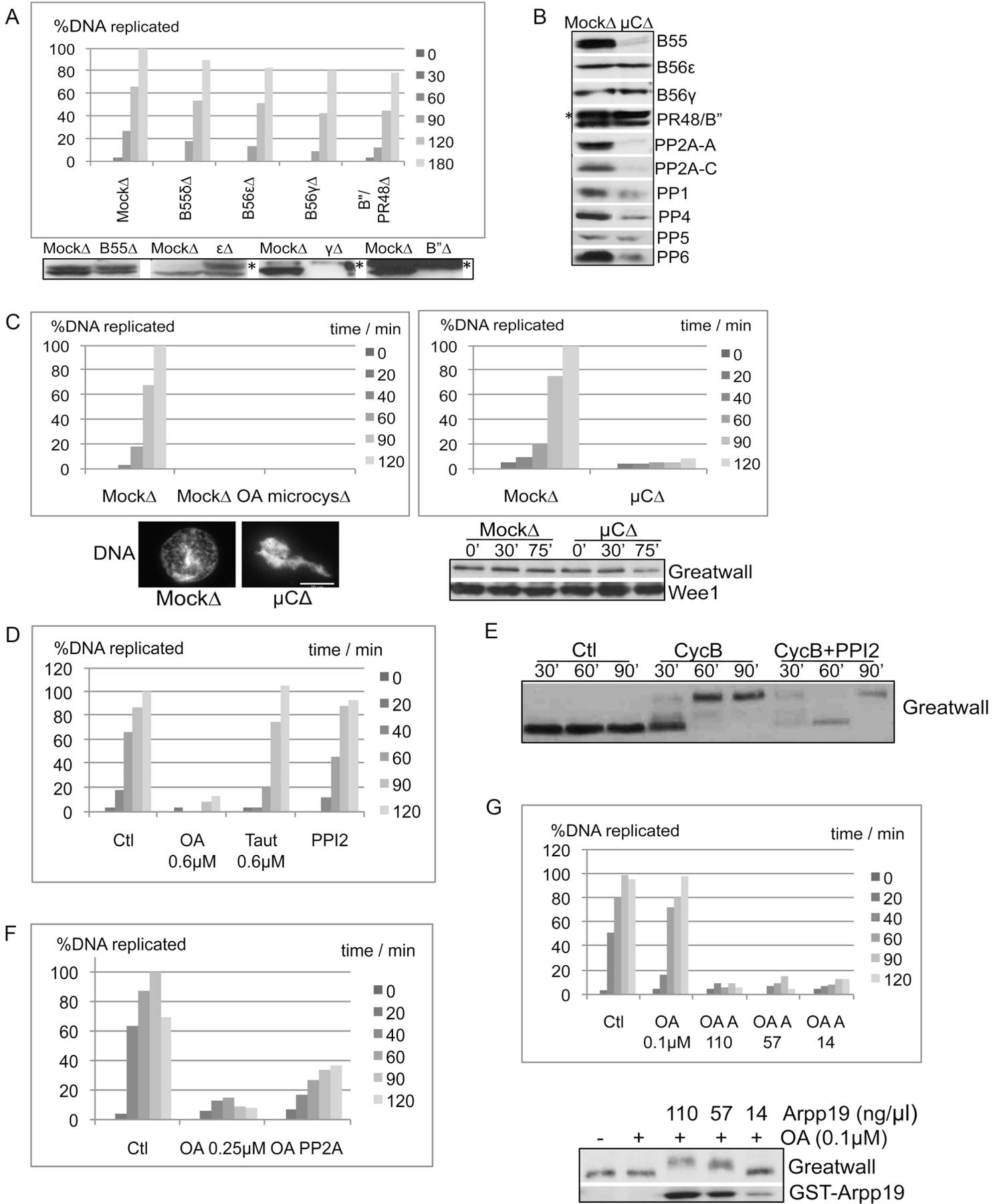


Figure 7.

