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Mitotic timing is differentially controlled by A- and B-type cyclins and by CDC6 associated with a *bona fide* CDK inhibitor Xic1 in *Xenopus laevis* cell-free extract

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ABSTRACT The timing of the M-phase is precisely controlled by a CDC6-dependent mechanism inhibiting the mitotic histone H1 kinase. Here, we describe the differential regulation of the dynamics of this mitotic kinase activity by exogenous cyclin A or cyclin B in the *Xenopus laevis* cycling extracts. We show that the experimental increase in cyclin A modifies only the level of histone H1 kinase activity, while the cyclin B increase modifies two parameters: histone H1 kinase activity and the timing of its full activation, which is accelerated. On the other hand, the cyclin A depletion significantly delays full activation of histone H1 kinase. However, when CDC6 is added to such an extract, it inhibits cyclin B-associated histone H1 kinase, but does not modify the mitotic timing in the absence of cyclin A. Further, we show via p9 co-precipitation with Cyclin-Dependent Kinases (CDKs), that both CDC6 and the *bona fide* CDK1 inhibitor Xic1 associate with the mitotic CDKs. Finally, we show that the Xic1 temporarily separates from the mitotic CDKs complexes during the peak of histone H1 kinase activity. These data show the differential coordination of the M-phase progression by cyclin A- and cyclin B-dependent CDKs, confirm the critical role of the CDC6-dependent histone H1 kinase inhibition in this process, and show that CDC6 acts differentially through the cyclin B- and cyclin A-associated CDKs. This CDC6- and cyclins-dependent mechanism likely depends on the precisely regulated association of Xic1 with the mitotic CDKs complexes. We postulate that: i. the dissociation of Xic1 from the CDKs complexes allows the maximal activation of CDK1 during the M-phase, ii. the switch between cyclin A- and cyclin B-CDK inhibition upon M-phase initiation may be responsible for the diauxic growth of mitotic histone H1 kinase activity.

KEY WORDS: *Mitosis, cyclin, CDK1, CDC6, Xic1*

Introduction

The mitotic cell cycle is composed of four phases: G1, S, G2, and M, during which the eukaryotic cell produces two diploid, genetically identical daughter cells. The main two families of proteins involved in cell cycle control are cyclin-dependent kinases (CDKs) and cyclins. Several CDKs have been identified as being active during the cell cycle (Walker and Maller, 1991). The main CDKs that act during interphase are CDK1, CDK2, CDK4, and CDK6,

Abbreviations used in this paper: CDKs, Cyclin Dependent Kinases; CKIs, Cyclin-dependent Kinase Knhibitors; XB, Xenopus Buffer.

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while the CDK1 and CDK2 are essential for the M-phase regulation.

Cyclins are regulatory subunits of CDKs involved both in G1/S cyclins and G2/M transitions (D'Angiolella *et al.*, 2001; Girard *et al.*, 1991; Müller-Tidow *et al.*, 2004). The G1/S cyclins bind to CDK2 and CDK4-6 during the G-phase. This binding is necessary for the entry into and progression of the S-phase. The mitotic cyclins, which associate with CDK1 and CDK2 during the G2 phase, are required for entry into the M-phase (Walker and Maller, 1991). The levels of mitotic cyclins A and B fluctuate during the cell cycle, in contrast to CDK1 and CDK2 levels, which remain stable during the whole cell cycle. The increase or decrease in cyclins' level allows the cycling activation of CDKs with which they are associated (Evans *et al.*, 1983; Pines, 1995; Pines and Hunter, 1991). The activity of CDKs is also regulated by the CDK inhibitors known as CKIs. The CKIs bind to CDKs alone or the CDK/cyclin complex and down-regulate the kinase activity. Two distinct classes of CDKs inhibitors have been identified: INK4 and Cip/Kip (Blain *et al.*, 1997; Sherr and Roberts, 1999). In *Xenopus laevis*, the Cip/Kip-type of CKI named Xic1 inhibits DNA replication by binding to CDK2-cyclins complexes (You *et al.*, 2002). So far, there are no data available on the involvement of the CKI in the mitotic CDKs regulation in *Xenopus laevis* embryos or oocytes; however, such regulation was described in yeasts (Örd *et al.*, 2019).

Cyclins are coded by at least 30 different genes in the human genome (Gopinathan *et al.*, 2011). Only some of these are expressed in *Xenopus laevis* oocytes and embryos. B-type cyclins are major regulators of CDK1 during the M-phase (Arellano and Moreno, 1997; King *et al.*, 1994). A-type cyclins interact with CDK1 and CDK2, promoting entry into the M-phase (Lorca *et al.*, 1992; Pagano *et al.*, 1992). Both cyclins A and B contain a destruction box required for their proteolysis via ubiquitination during the pertinent period of the cell cycle (Glotzer *et al.*, 1991; Rechsteiner and Rogers, 1996). At prometaphase-metaphase transition, the ubiquitin ligase APC/C^{Cdc20} complex becomes activated, inducing degradation of cyclin B and securin, necessary for the transition from the metaphase to the anaphase.

The cyclins are not the only factors driving the embryonic cell cycle, and determining the timing of the M-phase. CDK1 activation is regulated indirectly at different levels by the network of kinases and phosphatases; among the latter, the major players are PP2A, Ensa, ARPP19, Greatwall, CDC25, WEE1, PP1, PLK1, and Bora. However, there is another important factor involved in this process: the Cell Division Cycle 6, CDC6, which appears to inhibit histone H1 kinase activity of mitotic CDKs in a more direct manner (Örd *et al.*, 2019). We showed previously that the inhibitory activity linked to CDC6 determines the timing of M-phase entry and mitotic progression in *Xenopus laevis* cell-free extract, and in the mouse embryo (Borsuk *et al.*, 2017; El Dika *et al.*, 2014b).

The CDC6 protein is an evolutionarily conserved member of the AAA + ATPase family that plays a key role in many cell functions, such as folding, unfolding, degradation of proteins, vesicular transport, and the assembly of macromolecules for DNA replication (Duderstadt and Berger, 2008). The CDC6 contains the 200-250 amino acid long ATPase domain. The N-terminal region of CDC6 has one phosphorylation site for PLK1 and three consensus sites for phosphorylation by CDKs. These sites are phosphorylated in the S-phase. It also has a leucine zipper domain, which interacts with other proteins, and a so-called cyclin binding domain, which allows association with cyclins (Örd *et al.*, 2019). The cyclin-binding motif is present in different substrates and inhibitors of CDK2 (Chen *et al.*, 1996; Zhu *et al.*, 1995) and binds specifically to the hydrophobic region of cyclin A (Russo *et al.*, 1996). This improves the interaction between CDK2 and its targets. The D-boxes and Ken-boxes, present in CDC6, are necessary for the ubiquitination by APC/C^{Cdh1}, which induces CDC6 degradation by the proteasome during the G1/G0 phase (Petersen *et al.*, 2000). During the M-phase, in HeLa cells, the CDC6 is phosphorylated by Polo-like kinase 1 (Plk1) (Yim and Erikson, 2010). CDC6 associates with Plk1 and localizes to the mitotic spindle throughout the metaphase and anaphase. The high CDC6 phosphorylation level correlates with the high Plk1 activity, and conversely, CDC6 is dephosphorylated in cells depleted of Plk1. Phosphorylation of CDC6 by Plk1 is required for the efficient interaction with CDK1 and its inhibition. In yeast, CDC6 homolog has been shown to regulate CDK1 inactivation during the M-phase

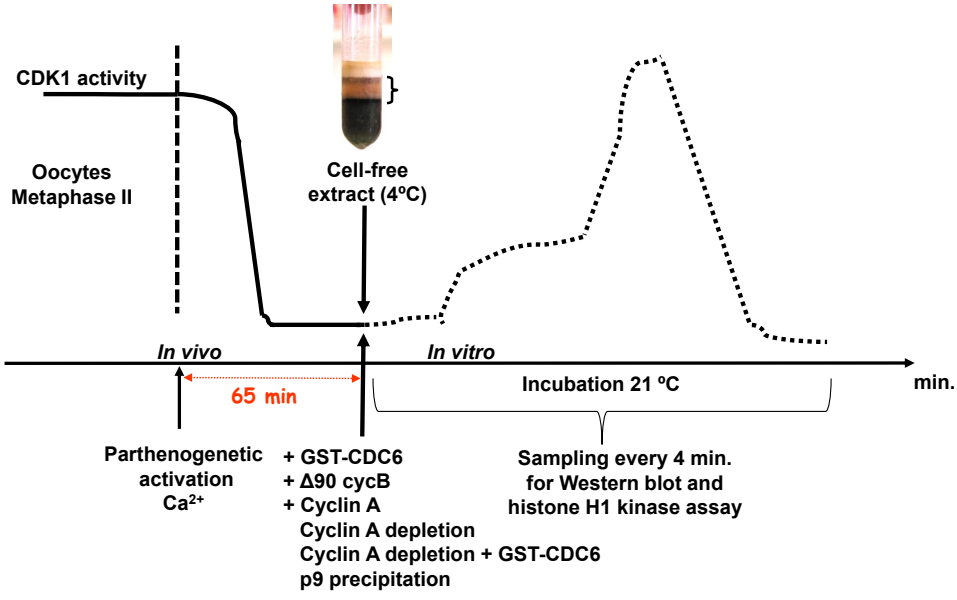

Fig. 1. Outline of the experiments. *CDK1 activity drops after oocytes activation (solid line) and reactivates during the M-phase entry of the first embryonic mitosis (dotted line). We prepared the cell-free extracts from parthenogenetic embryos 65 min. post oocytes parthenogenetic activation, i.e. before the time of CDK1 activation and incubated them at 21°C and sampled for histone H1 kinase assay or CDC27, MCM4 Western blotting during the mitotic period every 4 minutes. Before incubation, the cell-free extracts were depleted of cyclin A, or different proteins were added in the combinations listed above.*

exit. The N-terminal region of CDC6 interacts with CDK1/cyclin B during M-phase exit and inhibits histone H1 kinase activity *in vitro* (Elsasser *et al*., 1996). The deletion of the CDK1 interaction domain in CDC6 slows down the M-phase exit (Calzada *et al*., 2001). As mentioned above, CDC6 in somatic cells undergoes cyclic degradation and reaccumulation. However, in *Xenopus laevis* oocytes and early embryos, no massive degradation is observed during the cell cycle progression (our unpublished data).

It has been shown recently in *Xenopus laevis* embryos that cyclin A participates in the initiation of the M-phase by triggering the activation of CDK1/cyclin B complex (Vigneron *et al*., 2018). The question is whether CDC6 regulates Cyclin A-dependent CDKs during M-phase in *Xenopus laevis* embryos. Here we demonstrate the presence of the regulatory network between cyclin A, cyclin B, mitotic CDKs (CDK1 and CDK2), and CDC6 and a potential role of CDK1/2 *bona fide* inhibitor Xic1 in the progression of the M-phase. This regulatory mechanism is especially important for the coordinated activation of the mitotic CDKs, the control of timing and amplitude of histone H1 kinase during early embryo cleavage divisions, when the precise timing of cell cycle events is required for the coordination with the genetic developmental program of the embryo.

Results

The exogenous Cyclin A and cyclin B differentially regulate M-phase progression in X. laevis *embryo cycling extract*

To investigate the effects of cyclin A and cyclin B on the M-phase timing, we first verified when the cytoplasmic extracts, prepared from the parthenogenetically activated eggs, enter the M-phase. There were 3 groups of extracts: 1. control, without the addition of any purified protein; 2. with the addition of three concentrations of purified human cyclin A (a gift from Daniel Fisher, IGMM, Montpellier, France); 3. with the addition of non-degradable Δ90 cyclin B from sea urchin (a gift from Marie-Anne Felix, IJM, Paris, France). The prepared extracts were then incubated at 21°C, and samples were collected every 4 min for Western blot analysis with primary antibodies against CDC27, and histone H1 kinase assay. The CDC27, a subunit of the APC/C, is a direct substrate for CDK1 phosphorylation. On Western blot, the phosphorylation of CDC27 is visible as the up-shifted band resulted from the phosphorylation by active CDK1 (Huang *et al*., 2007). In Fig. 2A, the top panel shows the timing of the CDC27 up-shift in extracts with cyclin A addition. In Fig. 2A, the bottom panel shows the timing of the CDC27 up-shift in the extract with cyclin B addition.

In Fig. 2A, the top panel, in the control extract, the up-shift of CDC27 (caused by phosphorylation by the active CDK1) appeared at 24 min of the incubation, and the down-shift, corresponding to the dephosphorylation and thus inactivation of CDK1, at 40 min of incubation. 7.5 nM purified cyclin A does not change the timing of CDC27 phosphorylation during the whole M-phase duration, which remains exactly as in the control. In the presence of 15 nM cyclin A, CDC27 becomes phosphorylated, which marks the M-phase entry, at the same time as in the control, i.e. at 24 min of incubation. However, at 40 min of incubation, there was no down-shift of the CDC27 band, which suggests the arrest of the M-phase progression. Interestingly, the addition of 22 nM cyclin A to the extract accelerates CDC27 phosphorylation by 4 min then arrests the M-phase progression in similar fashion to the 15 nM cyclin A.

As shown in figure 2A (bottom panel), the timing of the initial up-shift (phosphorylation) of CDC27 accelerated in a dose-dependent manner with the increasing concentration of Δ90 cyclin B in the extracts. The up-shifted band of phosphorylated CDC27 (labeled with red asterisks on Fig. 2A) appears after 44, 40, and 36 min when Δ90 cyclin B is added to the extracts in 7.5, 15 and 22.5 nM concentration, respectively. This shows that supplementation with exogenous cyclin A and B acts differently on the timing of the initial CDC27 phosphorylation, which, indirectly, shows the effect on the timing of histone H1 kinase activation.

To investigate directly how the exogenous cyclin A or cyclin B modifies biochemical mitotic processes in the extract, and how they impact the mitotic progression, we measured the histone H1 kinase activity under the conditions of increasing concentrations of cyclin A or Δ90 cyclin B added to the extracts before the M-phase (Fig. 2B). We found that the concentration of the added cyclin A or B positively correlated with the increase in the activity of H1 kinase. However, the dynamics of histone H1 kinase activity were different for each added cyclin, as already suggested by the timing of CDC27 phosphorylation (shown above in figure 2A). The increasing cyclin A-concentration in the extract paralleled the increase in the mitotic H1 kinase activity (Fig. 2B, top panel). Interestingly, in all concentrations of cyclin A, the peaks of H1 kinase activation, which mark the peaks of CDK1 activity during the M-phase, were always observed at 24 min of incubation (Fig. 2B, top panel). This indicates that the timing of biochemical events was not modified, despite significant differences in the levels of histone H1 kinase activities at different concentrations of the cyclin A. On the other hand, in the Δ90 cyclin B addition experiment, the increase of H1 kinase activity was gradual, paralleling the increasing concentration of cyclin B. The fastest increase in histone H1 kinase activation was observed for the highest concentration of added cyclin B (i.e. 22.5 nM). This indicates the acceleration of biochemical events by the increased cyclin B level. Importantly, the time points of the peaks of H1 kinase activity are positively correlated with cyclin B concentrations in the extracts and accordingly shifted in time. The CDK1 activity peaked the soonest in the extract with the maximal dose of cyclin B. Thus, the timing of mitotic events in the extracts is regulated by cyclin B in a dose-dependent manner (Fig. 2B, bottom panel). These differences show that only the increased levels of cyclin B, but not those of cyclin A, accelerate M-phase progression. Increased cyclin A levels augment H1 kinase activity substantially, but the timing of biochemical mitotic processes remains unchanged. This suggests different roles of A- and B-type of cyclins in the regulation of the timing of mitotic events in the cell-free extract.

The results presented in figures 2A and 2B were obtained from two different extracts (note different time points of incubation for each experiment), therefore some differences between two independent experiments are possible. For this reason, Fig. 2C compares the effect of adding 15 nM cyclin A or 15 nM Δ90 cyclin B to the same control extract. This experiment clearly shows the gradual increase of H1 kinase activity upon cyclin A increase (as in the previous experiment shown in fig. 2B, top panel), and the slower initial dynamics of H1 kinase activation followed by a rapid acceleration (as in the previous experiment shown in figure 2B, bottom panel) upon cyclin B increase. Moreover, the amplitudes of histone H1 kinase activity are higher in the extracts supplemented with 15 nM cyclin A (Fig. 2C, top panel) than in the control extract,

and become even higher in the extract with 15 nM Δ90 cyclin B (Fig. 2C, top panel). The peaks of this activity occur at the same time (24 min) in the control and cyclin A-supplemented extracts, while in the extracts with the cyclin B the peak is accelerated by 4 minutes (20 min time point, Fig. 2C, top panel). The H1 kinase activity measurements performed on the same gel and in a single assay, shown in the bottom panel of Fig. 2C, confirm the results shown in the upper panel of Fig. 2C. These results confirm that the exogenous cyclin A and cyclin B have distinct effects on the mitotic extract, and thus differentially regulate M-phase entry and

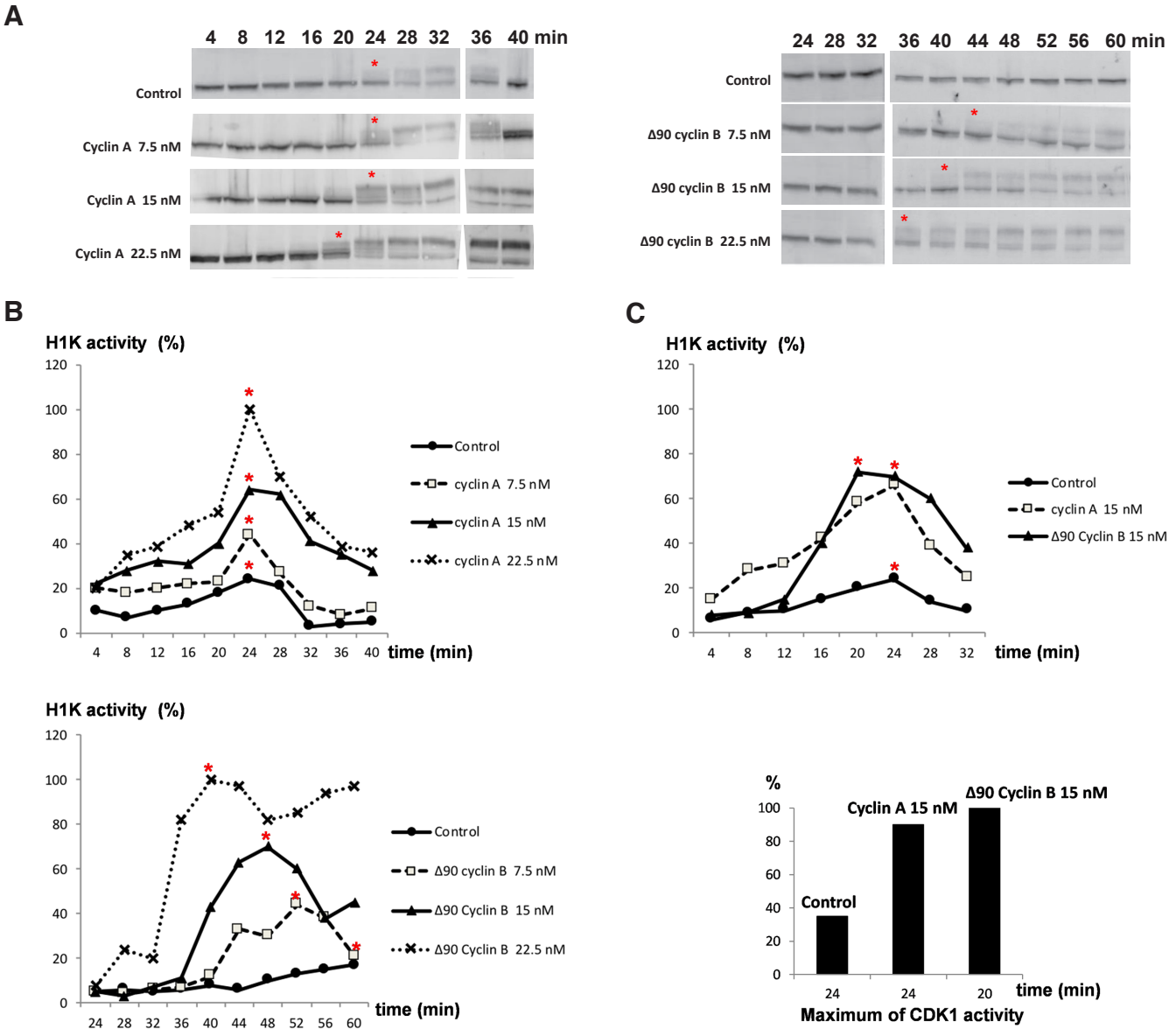

Fig. 2. Dose-dependent effects of cyclin A and Δ90 cyclin B addition on the M-phase entry and progression. (A) *The cytoplasmic extract was incubated at 21°C in the presence of three concentrations of purified cyclin A or Δ90 cyclin B (7.5, 15, and 22.5 nM) then sampled every 4 min. Samples were analyzed by 8% SDS-PAGE followed by CDC27 Western blot. The dynamics of CDC27 phosphorylation were compared to control. Red asterisks indicate the time points when a substantial shift in CDC27 migration was observed, and which were assumed to be the M-phase entry. Note that the Western blots shown here are the compilation of two separate membranes in each lane because the samples of 4-32 min time points were run and blotted separately from the 36–40 min samples in the series with cyclin A, and the samples of 24-32 min were run and blotted separately from the 36–60 min samples in the series with Δ90 cyclin B. Note different time points of incubation for each experiment (cyclin A* vs. *Δ90 cyclin B) because the timing of M-phase differed from one lot of embryos to the other.* **(B)** *The histone H1 kinase assays of the control extract, and the extracts containing increased concentrations of purified cyclin A or Δ90 cyclin B (7.5, 15, and 22.5 nM) corresponding to the CDC27 Western blot shown in Fig. 2A for. Asterisks mark the peaks of histone H1 kinase activity. Note that the growing histone H1 activity in the control extract does not reach its maximum during the period of 60 min. when all Δ90 cyclin B-supplemented extracts achieve their maxima.* **(C)** *The histone H1 kinase assays were performed with samples from the same cell-free extract supplemented with purified cyclin A or Δ90 cyclin B (15 nM each) to avoid differences in M-phase timing in different extracts as in Fig. 2A and B. The top panel shows the progression of histone H1 kinase activity in each experimental variant. Asterisks mark the peaks of histone H1 kinase activity. The bottom panel shows histograms depicting the maximum of histone H1 kinase activity obtained in a single reaction series.*

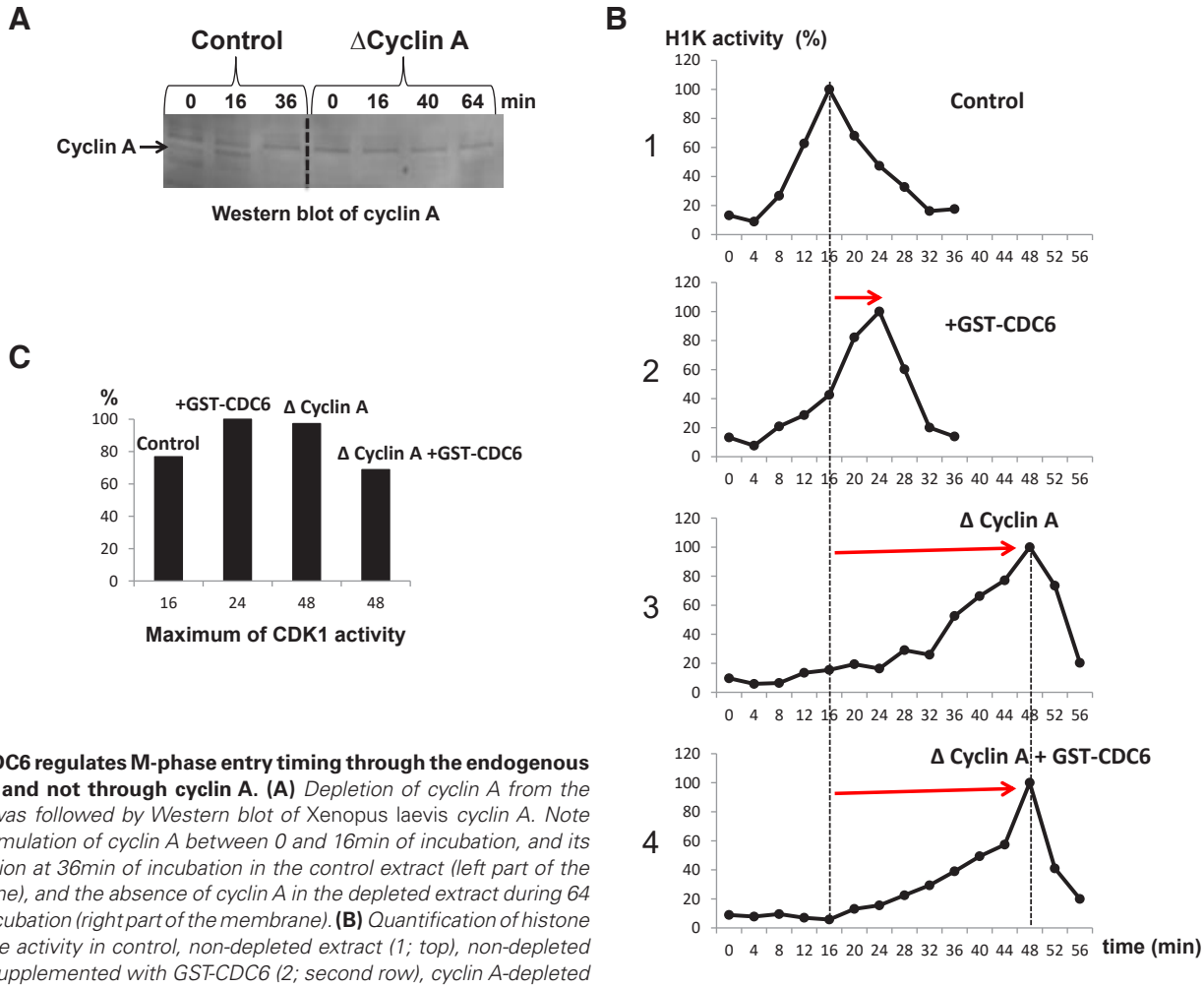

Fig. 3. CDC6 regulates M-phase entry timing through the endogenous cyclin B and not through cyclin A. (A) *Depletion of cyclin A from the extract was followed by Western blot of* Xenopus laevis *cyclin A. Note the accumulation of cyclin A between 0 and 16min of incubation, and its degradation at 36min of incubation in the control extract (left part of the membrane), and the absence of cyclin A in the depleted extract during 64 min of incubation (right part of the membrane).* **(B)** *Quantification of histone H1 kinase activity in control, non-depleted extract (1; top), non-depleted extract supplemented with GST-CDC6 (2; second row), cyclin A-depleted extract (3; Δ cyclin A; third row), and cyclin A-depleted extract supplemented with GST-CDC6 (4; bottom. Red horizontal arrows point to the delay in the timing of the peak of histone H1 kinase activity in the experimental extracts (supplemented with CDC6 and/or depleted of cyclin A) in comparison to the timing in the control extract (the vertical dotted line).* **(C)** *The comparison of the values of the peaks of histone H1 kinase activity obtained in a single series of histone H1 kinase assay reactions. We checked by Western blot analysis that the endogenous cyclin B was present and that its dynamics were unchanged in all samples (data not shown).*

progression. While the exogenous cyclin B accelerates the peak of histone H1 kinase activity and increases its amplitude, the exogenous cyclin A only increases the amplitude of H1 kinase activity, without modifying the timing of its activation.

CDC6 regulates the timing of M-phase progression through inhibition of the endogenous cyclin A-dependent kinase

Previously, we have shown that CDC6, acting as histone H1 kinase inhibitor, regulates the timing and amplitude of CDK1 activity during the M-phase (El Dika *et al.*, 2014b). However, we did not distinguish whether CDC6 inhibits cyclin A- or cyclin B-dependent kinase, or both. To differentiate between these possibilities, we compared the timing of M-phase progression in the control extract containing A- and B-type cyclins, and in the extract depleted of cyclin A, therefore containing only B-type cyclins. By adding recombinant CDC6 to these extracts and following the evolution of histone H1 kinase, we were able to ascertain the impact of cyclin A- and cyclin B-dependent kinases. We thus wanted to check whether CDC6 acts differently in the presence of the endogenous cyclin B only (cyclin A-depleted extract) and the variant when both cyclins are present. We knew from previously reported data that cyclin A depletion delays the M-phase entry in the extract indicating its role in the initiation of the M-phase entry (Vigneron *et al.*, 2018). To this end, we supplemented the cell-free extract with the pre-immune or immune cyclin A serum at 1:100 dilutions. The pre-immune serum-treated extract served as a control and the immune cyclin A serum highly reduced the level of cyclin A in the extract (Fig. 3A).

Fig. 3B shows that the addition of 5 nM GST-CDC6 to the control extract delayed M-phase entry (from 16 min time point in the control to 24 min in the presence of exogenous CDC6; Fig. 3B), as shown before (El Dika *et al.*, 2014b). Next, we compared this effect in cyclin A-depleted extract and upon addition of 5 nM GST-CDC6 addition to such an extract. The cyclin A depletion showed, as expected from previously published data (Vigneron *et al.*, 2018), that the absence of cyclin A in the extract significantly (32 min.) delayed the M-phase entry. However, the addition of 5 nM GST-CDC6 to cyclin A-depleted extract did not alter the time course of the M-phase (Fig. 3B, compare the third and the fourth

row). These results suggest that the time regulation of the M-phase progression by CDC6 is exercised only through the cyclin A-dependent kinases, but not through the cyclin B-dependent ones. However, the effects of the addition of exogenous cyclins A and B presented above suggested the contrary, i.e. that the timing of mitotic events is regulated by cyclin B-dependent mechanism, and not by cyclin A. Both experiments show that this regulation is more complex and is differentially exercised in extracts containing both A- and B-type cyclins, or only the B-type cyclin. Nevertheless, the cyclin A depletion experiment shows that cyclin A is absolutely essential for the regular mitotic timing control via CDC6 inhibition.

To assess more accurately the relationship between the levels of histone H1 kinase activity, we measured the respective maximums of the activity in a single histone H1 kinase reaction. The highest histone H1 kinase activity (designated as 100%) in this experiment was observed in the presence of GST-CDC6 at the 24 min time point of incubation (Fig. 3C). The maximum peak of H1 kinase activity in the control extract took place at 16 min time point and was lower than in the presence of added GST-CDC6. At first glance, this seems paradoxical, because CDC6 inhibits histone H1 kinase activity and one could expect a lower activity in the GST-CDC6-supplemented extract. However, as shown before (El Dika *et al.*, 2014b), CDC6 has a concomitant effect on H1 kinase activity and the timing of the biochemical events. Accordingly, as the peak of histone H1 kinase activity appeared 8 minutes later in the GST-CDC6-supplemented extract than in the control extract, more endogenous cyclin B was accumulated in this extract, resulting in the observed difference in the amplitude of H1 kinase activity. The threshold necessary to attain the maximal H1 kinase activity is different in each experimental case and results from the interplay between the inhibitory action of exogenous CDC6 and the stimulating action of newly synthesized and accumulated endogenous cyclin B. The maximum activity of histone H1 kinase in the cyclin A-depleted (Δ cyclin A) extract was higher than in the extract supplemented with 5 nM GST-CDC6 (Δ cyclin A+GST-CDC6; Fig. 3C). In these two extracts, the peak of H1 kinase activity appeared at the same time -at 48 min of incubation - which suggests that they had the same level of accumulated endogenous cyclin

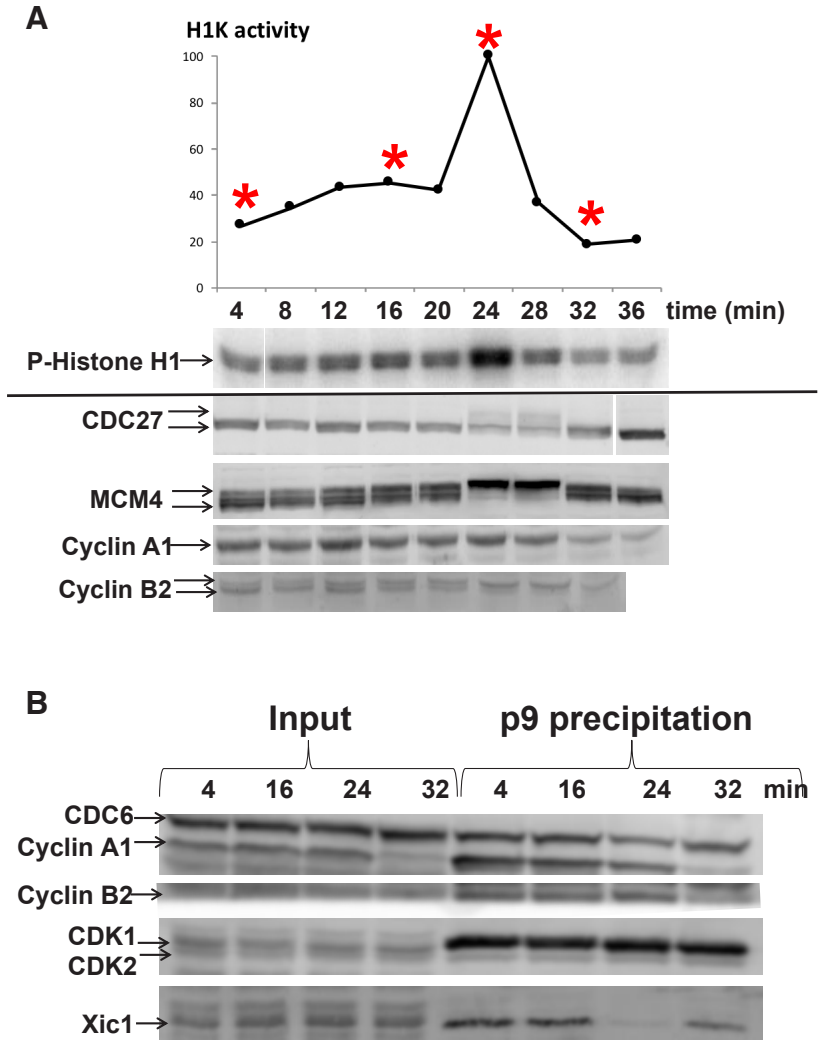

Fig. 4. CDC6 and Xic1 associate with p9-precipitated CDK complexes containing also cyclin A and cyclin B. (A) *The cytoplasmic extract was incubated at 21° and sampled every 4 min for histone H1 kinase assay and Western blot analysis. The same samples were split for the histone H1 kinase activity assay, and for 8% SDS-PAGE, followed by CDC27, MCM4, cyclin A1, and cyclin B2 Western blotting. Red asterisks indicate the time points selected based on the basis of histone H1 kinase activity for further Sepharose p9 beads precipitation of CDK complexes during the M-phase. P-histone H1 kinase panel shows the results of the autoradiography assay. Technical remark: note that the first 4 min slot is separated from the following ones because it was revealed on another membrane and two membranes were combined for better visualization. CDC27 and MCM4 Western blots show the M-phase-typical shift of the bands of these proteins due to CDK1 phosphorylation. Technical remark: note that the last 36 min slot of CDC27 is separated from the preceding ones because they were blotted on two different membranes and combined for visualization. Cyclin A1 and cyclin B2 Western blots made from the same samples show accumulation and degradation during the M-phase progression in the extract. The cyclin B2 36 min. time point was lost during experimentation.* **(B)** *Samples of 10 µl of extracts were added to 10 µl p9 beads pre-equilibrated with a buffer supplemented with 1% BSA and protease inhibitors, and p9-associated proteins were precipitated. Samples were analyzed by Western blot with anti-XlCDC6, anti-cyclin A1, anti-cyclin B2, anti-PSTAIR antibody (which detects both CDK1 and CDK2), and anti-Xic1 antibody. Input is shown on the left 4-32 min slots, p9-precipitated complexes are shown on the right 4-32 min slots. In the p9-precipitated sample, note the stable levels of CDK1, CDK2, and CDC6, the degradation of cyclin A1 and B2 during the M-phase. Importantly, the drastic reduction of Xic1 is visible specifically at the 24 min time point, while the quantity of p27Xic1 is stable in the input. This indicates that the disappearance of the Xic1 at 24 min time point in p9-precipitated material, and its reappearance at 32 min time point is not due to degradation, but to the dissociation from the CDK complexes. Note that the membrane was hand-cut after transfer, according to the molecular weight standard positions, before probing with different antibodies to avoid cross-reactivity between them. This made the cyclin B2 Western blot unclear, but the principal information concerning cyclin degradation in the 32 min. time point in p9 precipitation (rightmost lane) is clear.*

B. Thus, the addition of exogenous CDC6 to the Δ cyclin A extract diminished the level of histone H1 kinase activity because it acted on the equal pool of endogenous cyclin B in each case, as occurs upon the addition of CDC6 to the control extracts containing both cyclin A and B (El Dika *et al*., 2014b). This result shows that CDC6 effectively inhibits cyclin B-dependent kinases, but such inhibition cannot modify the timing of biochemical events of M-phase progression in the absence of cyclin A. Thus, CDC6 acts on both cyclin A- and cyclin B-dependent kinases, but the effects of each type of kinases inhibition provides different outcomes in terms of M-phase progression and the control of the timing of biochemical mitotic events. Moreover, cyclin A appears necessary for correct timing via a CDC6-dependent mechanism.

CDC6 stably associates with CDK1/2 during the whole M-phase, while Xic1 dissociates from CDK1/2 complexes, specifically during the peak of CDK1 activity

Xic1 is a *bona fide* CDK inhibitor. We wanted to check whether Xic1 associates with the CDK mitotic complexes by precipitating all CDKs by Sepharose p9 beads, and analyze whether Xic1 is associated with these complexes, as we did before for proteomic analysis of CDK complexes in *Xenopus laevis* embryos (Marteil *et al*., 2012). To do so, we first determined the timing of the M-phase in the cell-free extract by histone H1 kinase assay, to find the critical time points during the M-phase from which the samples for p9 precipitation would be tested. Additionally, Western blotting analysis of the marker proteins CDC27, MCM4, cyclin A1, and cyclin B2 was performed. Subsequently, the selected samples with known mitotic status were used for p9 precipitation, followed by Western blotting analysis of cyclin A1, cyclin B2, CDC6, CDK1, CDK2, and Xic1 on the same Western blot.

The top part of Fig 4A shows the progression of histone H1 kinase activity during the M-phase in the extract. It starts with a typical slow increase in histone H1 kinase activity until the 20 min time point. Then, it reached the maximum activity peak at 24 min of incubation, followed by an immediate fall in this activity, reaching the minimum at 32 min of incubation. This dynamic was corroborated by the CDC27 and MCM4 phosphorylation state, reflected by the electrophoretic mobility shifts, visualized by Western blotting of the same extract. The maximum up-shift of CDK1-phosphorylated CDC27 and MCM4 was observed between 20 and 24 min of incubation. After 32 min of incubation, the CDC27 and MCM were dephosphorylated (down-shifted bands).

We also assessed the dynamics of cyclin A1 and cyclin B2 in the extract. Fig 4A (the bottom part) shows that cyclin A1 accumulates gradually and decreases noticeably due to degradation at 32 min of incubation. Cyclin B2 also accumulates gradually and gradually changes its phosphorylation status, visible as a gradual up-shift up to 28 min of incubation, followed by degradation at the 32 min time point. These results allowed us to choose extract samples for further analysis by Sepharose p9 precipitation and Western blotting.

Fig. 4B shows the comparison of Western blotting of the input extract (left) and the p9 precipitation (right). It shows that CDC6 is stably associated with the two mitotic CDKs. Cyclin A1 degradation occurs at the same time in the input and in the p9-precipitate (32 min). Cyclin B2 behaves in the same way. The levels of CDK1 and CDK2 in the input and p9 precipitated material remain stable during the whole M-phase period between 4 and 32 minutes of incubation. However, Xic1, which is stably present during the whole M-phase in the input, is absent from the p9 precipitate at 24 min, and rapidly re-associates with CDKs complexes at the 32 min time point. This dynamic shows that while CDC6 remains stably associated with CDKs complexes, the Xic1 dissociates specifically at the time when CDK1 attains maximum activity.

Discussion

We showed here that the cyclin A and cyclin B differentially regulate the timing and progression of the M-phase. The increase in the cyclin A levels positively correlates with the increase in the mitotic histone H1 kinase activity, but does not change the timing of this kinase activation or inactivation; thus, the time when peaks of this activity appear remains unchanged despite variations in cyclin A levels. This is in contrast to the increase in the cyclin B levels, which also positively correlates with the increase of histone H1 kinase activity, but contrary to the cyclin A increase, accelerates the peak of the maximum of H1 kinase. We also showed, by comparing A- and B-type cyclins containing extract (control extract) with the extract containing only B-type cyclins (cyclin A-depleted), that the CDC6-dependent mechanism delaying M-phase entry acts through the cyclin A, and not through the cyclin B. Moreover, the lower level of the histone H1 kinase induced by CDC6 addition to the extract containing B-type cyclins only demonstrated that CDC6 indeed inhibits cyclin B-dependent kinases in cyclin A-depleted extract, but that this inhibition is unable to change the mitotic timing. These results show that the CDC6 exerts its inhibitory action on both cyclin A and cyclin B-dependent kinases, but only cyclin A-dependent kinases regulate the timing of mitotic events. At the first glance, different outcomes in terms of the mitotic timing regulation in A- and B-type cyclins containing extracts *vs*. only B-type cyclins containing extracts appear paradoxical. However, this rather suggests that the real control involves interactions between the two types of kinases associated with A- and B-types cyclins, and that in the extract with increased cyclin B, the acceleration of the peak of histone H1 kinase correlated with cyclin B level, but was dependent on the endogenous cyclin A. Indeed, Vigneron and colleagues (Örd *et al*., 2019) have shown that cyclin A-dependent kinase acts as a trigger for activation of cyclin B/CDK1 upon mitotic entry. Thus, our results confirm that, physiologically, both types of kinases collaborate in the normal regulation of the mitotic timing, but each plays a different role in this control.

A simple mechanism regulating the timing of mitotic divisions based on the accumulation and degradation of cyclin had already been proposed in 1989 (Murray *et al*., 1989; Murray and Kirschner, 1989). However, this mechanism did not address the precision of the temporal control of the cell division in the early embryo. Cyclin B accumulation to a threshold level is necessary to trigger the M-phase entry. Nevertheless, as shown previously, the CDC6-dependent mechanism controls in parallel the timing of the mitotic entry through the regulation of the dynamics of the mitotic histone H1 kinase activation (El Dika *et al*., 2014b). CDC6 makes it possible to set the correct timing of M-phase entry and controls the level of CDK1 activity in cooperation with the continuously increasing level of cyclin B. It is also responsible for the correct shape of the curve of histone H1 kinase activity during entry into the M-phase, which takes the form of a diauxic curve with typical inflection points (Dębowski *et al*., 2019). Until now, it was not known whether these functions of CDC6 are fulfilled via cyclin A- and/or

cyclin B-dependent complexes. Our current results demonstrate that CDC6 controls biochemical mitotic events through cyclin A- and cyclin B-dependent kinases, although with different effects on the timing of mitotic progression. We postulate that the switch between cyclin A-CDK (most probably CDK2) and cyclin B-CDK1 upon the entry into the M-phase may be responsible for the change in the dynamics of the histone H1 kinase curve, resulting in the diauxic character of this growth. This resembles the diauxic growth of bacteria initially described by Jacques Monod (Monod, 1949), which results from the switch in the types of sugars metabolized by the growing population of microorganisms. Cyclin A-CDK may be inhibited by a CDC6-dependent mechanism with different efficiency from cyclin-B-CDK1, or the affinity of a CDC6-inhibitory complex may be different for A- and B-type associated kinases, and this/these difference(s) is/are likely to induce the diauxic growth of the histone H1 kinase curve.

The CDC6 protein is not a *bona fide* CDK inhibitor. Moreover, it acts as an activator of CDK2 during the S-phase (Kan, Jinno, Yamamoto, *et al*., 2008; Kan, Jinno, Kobayashi, *et al*., 2008; Uranbileg *et al*., 2012). This occurs via the separation of CDK2 from p27, allowing CDK2 activation. The only *bona fide* inhibitor of CDK identified to date in *Xenopus laevis* embryos is the Xic1, a *Xenopus* p21cip1/p27kip1 family member (Finkielstein *et al*., 2002; Su *et al*., 1995; You *et al*., 2002). For this reason, during this study we checked whether it is associated with the mitotic CDKs/cyclin/CDC6 complexes during the M-phase. We demonstrated here that in the *Xenopus* embryo cycling extracts, the CDC6 and Xic1 are indeed in the same complexes. This reinforces the hypothesis that CDC6 may serve as a platform for the assembly of CDK1 or CDK2 and Xic1 to promote their inhibition. As we showed here, CDC6-dependent inhibition acts on both cyclin A- and cyclin B-dependent kinases, so it is plausible that it can inhibit both CDK1/cyclin B and CDK2/cyclin A. During the S-phase, CDK2 kinase activity is regulated by CDC6 indirectly (Kan, Jinno, Yamamoto, *et al*., 2008; Kan, Jinno, Kobayashi, *et al*., 2008; Uranbileg *et al*., 2012). CDC6 activates CDK2 in somatic cells by the removal of the CDK inhibitor p27 from the CDK2, triggering the kinase activation necessary for the initiation of the S-phase. Our previous studies demonstrated that, during mitosis, the CDC6 has an opposite effect on CDK1 (El Dika *et al*., 2014b), and in the present paper we show that this can be also the case for CDK2. We postulate that in the M-phase, CDC6 regulates CDK1 and CDK2 by targeting the CKI inhibitors to these CDKs. A recent study detailing the association of CDK1 with cyclin B, CDC6, and p27 (Cks1) homologs in yeasts (Örd *et al*., 2019) has shown that, indeed, the CDC6 behaves like a platform gathering all the components of the CDK1 complex. Moreover, these studies showed that the association of these components is coordinated by phosphorylation and dephosphorylation of the LxF motif of CDC6, which confirms the multifunctional regulatory role of CDC6 during the M-phase. Our data suggest that in *Xenopus laevis* cell-free extract, this regulation may be very similar to that present in yeast, and that Xic1 may play the role of yeast Cks1. Our observation that Xic1 dissociates from CDKs complexes specifically when the CDK1 activity level achieves the maximum, suggests that CDC6 and Xic1 actively participate in the rapid activation of CDK1 immediately before it peaks. We postulate that, in *Xenopus laevis* cell-free extracts, the rapid and transitional release of Xic1 from the mitotic CDKs complex allows the achievement of the extremely steep curve of the CDK1 activity in the M-phase. Our model allows us to unify the role of CKI in the M-phase regulation in lower eukaryote, the yeast, and the vertebrate *Xenopus laevis*. We postulate therefore that in both cases CDK1 is actively inhibited during the M-phase by the CDC6-dependent mechanism including CKI, the Xic1 in the case of *Xenopus laevis*. In addition, in *Xenopus laevis* embryos, CDK2/cyclin A is also subject to CDC6-dependent inhibition, and this interaction is particularly important for the regulation of the mitotic timing and the dynamics of mitotic progression.

Material and methods

Xenopus *egg collection and activation*

Xenopus laevis eggs were collected from the overnight spawning, dejellied with 2% L-cysteine pH 7.81 in XB buffer (100 mM KCl, 1 mM MgCl2, 50 mM CaCl2, 10 mM HEPES, and 50 mM sucrose pH 7.6), washed in XB, activated with calcium ionophore A23187 at 0.5 mg/ ml, and then washed in XB as described above (El Dika *et al*., 2014a).

Xenopus *cell free extracts*

Cycling cell-free extracts were obtained from calcium ionophore-activated one-cell embryos as described previously (El Dika *et al*., 2014b) using the method allowing biochemical analysis prior to the M-phase entry (Fig. 1).

Anti-XlCDC6 production

Complementary DNA encoding wild-type *X. laevis* CDC6, recombinant protein, and the corresponding polyclonal antibodies were produced as previously described (El Dika *et al*., 2014b).

Cyclin A immunodepletion

Immunodepletion of Cyclin A from *Xenopus* embryo extracts was carried out using AffiPrep Protein A beads (Sigma) conjugated with the *Xenopus laevis* anti-Cyclin A1 (a gift from Daniel Fisher, IGMM, Montpellier France) or with the pre-immune serum, overnight at 4˚C. 200ml of beads were washed four times with XB buffer (pH 7.6) and incubated with 400 ml of the pre-mitotic extracts. Following 30 min incubation at 4˚C, extracts were spun down, beads were removed, and the supernatant was recovered. Two runs of the immunodepletion were necessary to remove 90% of Cyclin A from the extract.

Immunoblotting of X. laevis *proteins*

Proteins in the extracts were separated by SDS-PAGE (8 to 12.5% gels), transferred to the nitrocellulose membranes (Hybond C, Amersham Biosciences), and probed with primary antibodies against cyclin A1 (gift from Daniel Fisher, IGMM, Montpellier France), cyclin B2 and CDC27 (gift from Thierry Lorca, CRBM, Montpellier, France), MCM4 (gift from Marcel Méchali, IGH, Montpellier, France), Xic1 (gift from Anna Philpott, University of Cambridge, UK), CDC6 (produced by us), and PSTAIR (Sigma). The secondary antibodies were alkaline phosphatase- conjugated anti-rabbit or anti-mouse IgG. Bands were visualized with Enhanced Chemifluorescence reagent (ECF; Amersham Biosciences) and quantified with ImageQuant 5.2 software (Amersham Biosciences).

Recombinant proteins added to extracts

Purified human cyclin A (a gift from Daniel Fisher, IGMM, Montpellier, France) or non-degradable Δ90 cyclin B from sea urchin (a gift from Marie-Anne Felix, IJM, Paris, France) were added to the extracts.

Sepharose p9 beads precipitation

The p9-Sepharose beads (a gift from L. Meijer and O. Lozach from the Marine Station, Roscoff, France) were used to precipitate CDK complexes (CDK1 and CDK2 in *X. laevis* embryo). The 10 μl of extracts were mixed with 10 μl p9 beads pre-equilibrated with the homogenization buffer (MOPS pH 7.2, 60 mM β-glycerophosphate, 15 mM EGTA, 15 mM MgCl2, 2 mM dithiothreitol, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM

disodium phenyl phosphate) containing 1% BSA, 1mM AEBSF, aprotinin, leupeptin, pepstatin, and chymostatin (10 μg/ml each). Subsequently, the samples were gently agitated for 2.5 h at 4˚C. Following the first brief spin down (5,000 g, 1 min, 4˚C), the supernatant was collected for the Western blot analysis, while the p9 beads pellet was washed four times with 1 ml of washing buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5mM sodium fluoride and 0.1% Nonidet-P 40) supplemented with 0.5 mM AEBSF, aprotinin, leupeptin, pepstatin, and chymostatin (10 μg/ml each). Finally, the beads were resuspended in 12 μl of 2× Laemmli sample buffer and heated at 85˚C for 5 min.

Histone H1 kinase activity assay

CDK1 activity was measured as previously described (El Dika *et al.*, 2014a; El Dika *et al.*, 2014b). In short, samples of extracts were diluted in the MPF buffer supplemented with 0.5 mM sodium orthovanadate and protease inhibitors, containing 0.4 mg/ml H1 histone (type III-S), 1 μCi [γ32P] ATP (specific activity: 3000 Ci/mmol; Amersham Biosciences) and 0.8 mM ATP. They were incubated at 30˚C for 30 minutes, mixed with Laemmli sample buffer, and heated at 85˚C. Following the SDS-PAGE, the histone H1-incorporated radioactivity was measured with STORM phosphorimager (Amersham Biosciences). Data were analyzed with ImageQuant 5.2 software. The outline of the experiments and the histone H1 kinase activity curve representing CDK1 activity is shown in Fig. 1.

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