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Temporal regulation of the first mitosis in *Xenopus* and mouse embryos

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Abstract

Cell cycle regulation in Eukaryotes is based on common molecular actors and mechanisms. However, the canonical cell cycle is modified in certain cells. Such modifications play a key role in oocyte maturation and embryonic development. They can be achieved either by introduction of new components, pathways, substrates, changed interactions between them, or by elimination of some factors inherited by the cells from previous developmental stages. Here we discuss a particular temporal regulation of the first embryonic M-phase of *Xenopus* and mouse embryo. These two examples help to understand better the general regulation of M-phase of the cell cycle.

Keywords: Cell cycle, Cyclin, Embryo, Mitosis, mouse, MPF, *Xenopus*.

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References

1. Introduction

Cell cycle regulation of meiotically maturing oocyte and mitotically dividing embryo at the very beginning of development differs in many important aspects. The obvious difference concerns

suppression of DNA replication during the oocyte meiotic maturation; more precisely during meiosis I/meiosis II transition. Besides this, mechanisms governing M-phases during this period are also significantly modified. In vertebrates, such as mammals, the first meiotic M-phase takes many hours (8-10 in the mouse), while the second can be even longer since at this stage, the oocyte becomes arrested waiting for fertilization to occur (the last one is true both for mammals and amphibians). Less studied, but probably equally important, are modifications of the first embryonic mitotic M-phases. In the current review we discuss recent evidences documenting that the temporal progression of the first mitotic divisions both in *Xenopus* and mouse embryos is precisely controlled by molecular mechanisms that in some aspects differ from the canonical mechanisms described both in somatic and germ cells. Specific control of the first embryonic mitosis may be important for the proper development of the embryo.

2. Specific temporal control of the first embryonic mitosis

The first and the second embryonic mitoses differ by their duration in four animal species we studied so far i.e. nematode (*Caenorhabditis elegans*), sea urchin (*Sphaerechinus granularis*), anuran amphibian (*Xenopus laevis*) and mammal i.e. mouse (*Mus musculus*) (Fig.1). In each of these embryos the first mitosis lasts longer than the second one. This difference is particularly pronounced in the mouse embryo, in which the first mitosis is almost twice as long as the second one (120 vs 70 min) (Ciemerych et al., 1999; Kubiak and Ciemerych, 2001; Sikora-Polaczek et al., 2006). After fertilization of mouse oocytes and at the beginning of the first cell cycle, the two sets of chromosomes from paternal and maternal origin remain separated. Since in mammals pronuclear fusion does not happen, the two parental genomes meet for the first time during the first mitosis. The two sets of chromosomes do not mix up upon the first cleavage, but they form two clearly distinguishable entities within the metaphase plate and later in the nucleus (Mayer et al., 2000). Moreover, paternal and maternal genomes of mammals differ functionally and carry

specific imprint (John and Surani, 2000). Thus, it is possible that the prolongation of the first M-phase could be a prerequisite for the proper reorganisation of future embryonic chromatin composed of these two different genomes. The prolonged period spent in the M-phase could, therefore, favour a specific mode of the mitotic apparatus formation as well as proper reorganisation of chromatin ensuring correct organisation of embryonic nuclei.

However, the first embryonic mitosis takes also longer time in non-mammalian species in which pronuclear fusion precedes the first embryonic division and imprinting is not observed. By measuring the period of cleavage division i.e. starting from nuclear envelope breakdown (NEBD) until the onset of cytokinesis, we found that in sea urchin embryos (*Sphaerechinus granularis*) the first mitosis takes 20 min, while the second one 15 min (J.Z. Kubiak and P. Cormier, unpublished observation). Moreover, the first mitosis in nematode embryos (*Caenorhabditis elegans*) takes 4.5 min vs. 3.5-4 for the second mitotic division (J.Z. Kubiak, F. Chesnel and P. Gönczy, unpublished observations). Since due to pigmentation and opacity of amphibian embryos (*Xenopus laevis*) the determination of NEBD is not trivial we decided to focus on the changes in the activity of the M-phase Promoting Factor (MPF), which controls entry into M-phase. To this end we measured histone H1 kinase activity reflecting the MPF activity. Differences in the profile of histone H1 kinase during each mitosis of the *Xenopus* embryo suggested that the first M-phase is longer than the second one (25 min vs. 15-20 min; Chesnel et al., 2005a). More precise analysis of M-phase durations in *Xenopus* embryos were possible using cell-free extracts specific for each embryonic M-phase. Again, levels of histone H1 kinase activity estimated in these extracts showed that the first one lasted 25 min and the second one 15 min (Chesnel et al., 2005b). Moreover, the period of mitotic phosphorylation of certain proteins (like MCM4, a protein involved in S-phase regulation and most probably inactivated during each M-phase via specific phosphorylation mediated by CDK1, or Eg3 kinase) was twice as long during the first M-phase than during the second one (10 min vs. 5 min at 21°C). All these observations suggest existing of mechanism(s) that induces a

specific increase in the duration of the first embryonic M-phase. It is possible that this mechanisms operates not only in *Xenopus* but also in three other evolutionarily very distant species.

Curiously, we found that the proportions in the degree of the shortening of the second embryonic mitosis change gradually accordingly to the evolutionary position of the studied species. The duration of the second mitosis in *C. elegans* embryos represents 77% of the duration of the first mitosis, 75% in sea urchin, 60% in *Xenopus* and finally less than 60% in the mouse. At least in the mouse and *Xenopus*, not only the second mitosis is shorter than the first one, but also the third one seems shorter than the second one (Ciemerych, 1995; Chesnel 2005a). It is intriguing that early mitotic divisions tend to shorten gradually to a certain extent as development proceeds.

We asked therefore which are the molecular mechanisms involved in the prolongation of the first mitotic division and whether different and evolutionary distant species share similar regulatory features. To approach this issue we focused on the first embryonic mitosis in *Xenopus* and in the mouse.

3. Duration of M-phase: the affair of MPF stability

Each eukaryotic cell enters M-phase upon activation of MPF, i.e. upon activation of a complex of a kinase CDK1 (known also as p34^{cdc2}) and its regulatory subunit cyclin B. Activation of MPF is due to the formation of a complex between newly synthesized cyclin B and CDK1. Additionally, CDK1 must be phosphorylated on Thr-161 and dephosphorylated on Thr-14 and Tyr-15 to be fully active. Inactivation of MPF is due to the dissociation of CDK1 from its subunit, cyclin B (Chesnel et al., 2006; Chesnel et al., 2007; Nishiyama et al., 2000) which is subsequently degraded via the ubiquitin/proteasome pathway (for review see Irniger, 2002). Studies using different experimental systems such as yeast, in vitro cultured mammalian somatic cells, or

meiotically dividing mammalian germ cells showed that M-phase duration clearly depends on the stability of MPF activity. Two major mechanisms stabilize MPF via inhibition of the polyubiquitination/proteasome pathway: the spindle assembly checkpoint (SAC) and the Cytostatic Factor (CSF) (Gillett and Sorger, 2001; Lew and Burke, 2003; Schmidt et al., 2006).

The SAC is a mechanism regulating cell division in many organisms such as yeast (Chen et al., 1999), insects (Logarinho et al., 2004), nematodes (Encalada et al., 2005), amphibians (Gorbsky, 1997), or mammals (Skoufias et al., 2001). It also acts in meiotically dividing germ cells (Homer, 2006; Homer et al., 2005; Marston and Amon, 2004; Tsurumi et al., 2004). Certain differences might exist between cells and species, but a factor common to all of them is Mad2. Mad2 binds to Cdc20, a regulator of the anaphase promoting complex/cyclosome (APC/C) and thus prevents the initiation of MPF inactivation and anaphase. APC/C is an E3 ubiquitin ligase which polyubiquitinates cyclin B targeting it for its degradation by the 26S proteasome (Chen et al., 1998; Fang et al., 1998; Li and Benzra, 1996). The SAC is activated during prometaphase in response to the absence of proper attachment of spindle microtubules to the kinetochores. This in turn leads to the kinetochore localization of checkpoint proteins including Mad2, inhibiting APC/C activity and preventing precocious inactivation of MPF (Shannon et al., 2002; Skoufias et al., 2001).

In contrast, CSF activity acts exclusively in meiotically dividing vertebrate oocytes and is responsible for arresting the progression of meiosis in metaphase of the second meiotic division (Masui, 2000). CSF activity results from the activation of various pathways in which the APC/C inhibitor Emi2 plays a key role (Shoji et al., 2006; Tung et al., 2005). The Mos/MEK1/ERK1 and ERK2 MAP kinases cascade is also involved, but its exact mode of participation in the CSF activity remains not fully understood (Araki et al., 1996; Colledge et al., 1994; Hashimoto et al., 1994; Phillips et al., 2002; Sagata et al., 1989; Verlhac et al., 1996). Recent papers by T. Kishimoto's and N. Sagata's groups have shown that phosphorylation of Emi2 by p90^{Rsk}, the

downstream substrate of ERK2, in *Xenopus* oocytes is a key regulatory event in stimulating Emi2 activity (Inoue et al., 2007; Nishiyama et al. 2007). Moreover, it was postulated that CDK2/cyclin E kinase also participates in the CSF activity inhibiting APC/C (for review see Liu et al., 2007). These data suggest that CSF may require more than one APC/C interfering pathway.

CSF inactivation, differently from the SAC, requires an external stimulus provided by fertilization or parthenogenetic activation (Liu and Maller, 2005; Marangos and Carroll, 2004). There is no data suggesting that CSF is active in other cells than oocytes even if there is a number of indications that Mos participates in ERK2 mild activation during the final stages of the first embryonic M-phase in *Xenopus* embryo (Yue and Ferrell, 2006), or might eventually participate in ERK1 and ERK2 activation on kinetochores in dividing somatic cells (Shapiro et al., 1998; Wang et al., 1997; Zecevic et al., 1998). Thus, the first embryonic M-phase could be prolonged by one of the pathways composing the CSF activity.

4. Lesson from *Xenopus*: more cyclin B, longer M-phase

The molecular basis of the prolongation of the first embryonic mitosis of *Xenopus laevis* (Chesnel et al., 2005a,b) may involve cyclin B availability and therefore MPF activity and stability. Thus we compared in details the regulation of the CDK1/cyclin B complex during these two M-phases. Cyclin B accumulation is much more pronounced during the first mitosis than during the second one. Also the proportion of CDK1 phosphorylated on Tyr15 (inhibitory phosphorylation) is clearly higher during the first M-phase. This combination results in higher MPF activity in the first mitosis (Fig. 2).

This observation led to the question whether the duration of M-phase could be lengthened or shortened by experimentally modifying cyclin B levels. The up-regulation could be theoretically achieved in cell-free extract either by addition of exogenous cyclin B or by inhibiting

the ubiquitin-proteasome pathway responsible for cyclin B degradation upon mitotic exit, while the down-regulation via reducing cyclin B synthesis with protein synthesis inhibitors. Indeed, the addition of exogenous cyclin B2 into the mitotic extract induces an increase of M-phase duration whereas cycloheximide applied right before the M-phase entry shortens its duration (Chesnel et al., 2005b).

Intriguingly, the inhibition of the ubiquitin/proteasome pathway using an inhibitor of the proteolytic activity of proteasome, MG132, indeed slows down cyclin B degradation but no change in the M-phase duration is observed (fig. 3; Chesnel et al., 2006). Detailed analysis has shown that the primary cause of MPF inactivation upon mitotic as well as meiotic exit is not cyclin B degradation, but the dissociation of cyclin B from CDK1 molecules in which a part of the proteasome itself plays a pivotal role (Nishiyama et al., 2000). This dissociating activity is independent from the proteolytic activity of the proteasome and therefore is not inhibited by MG132 (Chesnel et al., 2006; Chesnel et al., 2007; Nishiyama et al., 2000). To efficiently prevent CDK1 inactivation in mitotic embryo extracts we used mutated recombinant mutated ubiquitin (K48R) which blocks the elongation of the polyubiquitination chain. This protein added to the extract inhibits efficiently the polyubiquitination of cyclin B and also its targeting to the proteasome complex. This slows down cyclin B degradation and prolongs the MPF activity of CDK1 as expected (fig. 3) (Bazile et al., 2007). Therefore, we confirmed that interfering with the polyubiquitin/proteasome pathway upstream from the proteasome indeed enables to stabilize the MPF activity for a prolonged period.

These results could also suggest that high levels of cyclin B could participate in the prolongation of MPF activity. Accumulation of this protein increases MPF activity (Chesnel et al., 2005b), and by saturating the ubiquitylation machinery, could also indirectly reduce the efficiency of polyubiquitination of other proteins whose degradation is required for mitotic progression (cyclin B included).

Cyclin B accumulation as well as high MPF activity could also induce inhibition of APC/C. To test this hypothesis we depleted the APC/C inhibitor protein Mad2 from the mitotic extract. Indeed, such depletion shortens the M-phase (Chesnel et al., 2005a). This strongly suggests that Mad2 present in the cytoplasm could slow down APC/C activation and cyclin B targeting to the proteasome. However, we cannot exclude that Mad2 depletion also diminishes levels of other proteins via non-specific depletion or co-depletion of Mad2-associated factors. Since SAC is not effective in *Xenopus* embryo and chromosomes (kinetochores) are absent in the extract, we attribute the potential inhibitory role of APC/C to the cytoplasmic pool of Mad2. It was shown that such a cytoplasmic pool of Mad2 regulate anaphase onset in tissue culture cells (Meraldi et al., 2004). We believe therefore that a similar mechanism might operate in *Xenopus* embryos explaining, at least partially, the presence and role of Mad2 in developing embryo (Chesnel et al., 2005a) despite the lack of SAC activation in blastomeres upon mitotic spindle destruction.

Apart from the precise control of both the amount and stability of cyclin B, other mechanisms might be involved in the regulation of M-phase duration. One of them is most probably linked to the calcium metabolism since the decrease in the level of free calcium in the cytoplasm prolongs MPF activity (Chesnel et al., 2005a). Calcium ions play crucial role as second messengers in the regulation of cell cycle progression. Indeed, they act upstream of the mechanisms controlling cyclin B accumulation/stability by more general modulation of the plethora of enzymatic activities (Beckhelling et al 1999).

5. Lesson from the mouse: neither SAC nor CSF?

In the mouse, despite an even more pronounced difference in the duration of the first two embryonic mitoses compared to the one observed in *Xenopus* embryos, the levels of MPF activity (our unpublished data) and cyclin B (Ciemerych et al., 1998) are equal in one-cell and two-cell

embryos contrary to *Xenopus*. Also, the pattern of MPF activity during the two mitoses differs significantly between embryos of the two species. In the mouse a clear plateau of MPF activity is observed during the first M-phase (Ciemerych et al., 1999). In *Xenopus* there is no plateau of this activity, and MPF activity simply rises slower and reaches an higher level than during the second M-phase, while its inactivation is equally rapid during the two M-phases (Chesnel et al., 2005a,b). These observations suggest that different mode of control could be operating in these two species in order to modulate M-phase duration.

In our studies focused on mouse embryos, we therefore paid particular attention to the role of SAC in the prolongation of the first mitosis. We monitored active checkpoint via the analysis of chromosome movements and kinetochore localization of Mad2 (Fig. 4). Mad2 presence on kinetochores delineated the timing of prometaphase and showed that it was very similar, i.e. lasted 20 min, during both the first and the second embryonic mitosis (Sikora-Polaczek et al., 2006). Such rapid disappearance of these proteins from kinetochores, marking the entry into metaphase, does not result in the swift anaphase onset as it would happen in somatic cells (Meraldi et al., 2004). Importantly, the anaphase onset occurs after additional 100 minutes during the first mitosis, the period that is substantially longer than during the second one (40-50 min) (Sikora-Polaczek et al., 2006). To summarize, the prometaphases during the first two mitoses are equally long but the mechanisms prolonging the first mitosis likely operate during metaphase and do not seem to be related to SAC activity.

In search for the mechanism(s) prolonging the first mitosis, we turned to the mouse MII-arrested oocytes. We showed that in these cells Mad2 disappears gradually from the kinetochores. Thus, Mad2 disappearance from kinetochores and SAC inactivation does not precede directly the anaphase onset since metaphase II oocytes can remain arrested as long as they are not activated by sperm entry (Sikora-Polaczek et al., 2006). This observation led us to the conclusion that the first mitotic metaphase in the embryos resembles to some extent the meiotic MII arrest of oocytes i.e.

in both cases the initial SAC dependence becomes replaced by another activity (Sikora-Polaczek et al., 2006; Tsurumi et al., 2004). Thus, we hypothesize that during the first embryonic mitosis mechanism(s) different from SAC preserve the M-phase state.

A few lines of evidences suggested that mechanism prolonging the first mitotic division could relay on maternal factors remaining within the cytoplasm of the one cell embryo. ERK1 and ERK2 MAP kinases, which activity can be assayed indirectly by the analysis of their phosphorylation status (only phosphorylated forms are catalytically active) or by their ability to phosphorylate exogenous substrate i.e. myelin basic protein (MBP), could serve as good candidates. However, an important argument against it comes from the fact that during the first mitosis of the mouse embryo ERK1 and ERK2 MAP kinases remain unphosphorylated. Since the MBP kinase activity is, however, well detectable at this stage, it is possible that other pathways different from MAP kinases ERK1 and ERK2 could be involved (Verlhac et al., 1994). It is also possible that the canonical MAP kinase pathway is modified at this stage of development. Moreover, one of MAP kinases ERK1 and ERK2 substrate - p90^{rsk}, is partially phosphorylated during the first mitosis of the mouse embryo suggesting a potential role of this kinase in the control of this particular M-phase (Kalab et al., 1996). Thus, the question whether ERK1 and ERK2 MAP kinases or other related kinases play a role in the regulation of the first mitosis in the mouse embryo remains open.

6. Concluding remarks: common lesson from mouse and *Xenopus*

The comparison between MPF regulation in *Xenopus* and in mouse embryos suggests that different molecular strategies might develop in different species during evolution to reach the same objective: extension of the first embryonic M-phase. If different mechanisms prolonging the first embryonic mitosis by either increasing or stabilizing MPF activity appeared, it means that this

prolongation might play an important role in the process of early embryo development. Identification and detailed analysis of potential molecular mechanism(s) at play in *Xenopus* and mouse embryos should enable to check this hypothesis. Further comparison of of cyclin B regulation, the role of ERK1 and ERK2 MAP kinases pathways, the potential role of p90^{rsk} and non-canonical MAP kinases cascades are among the most obvious candidates which should allow us to understand this phenomenon.

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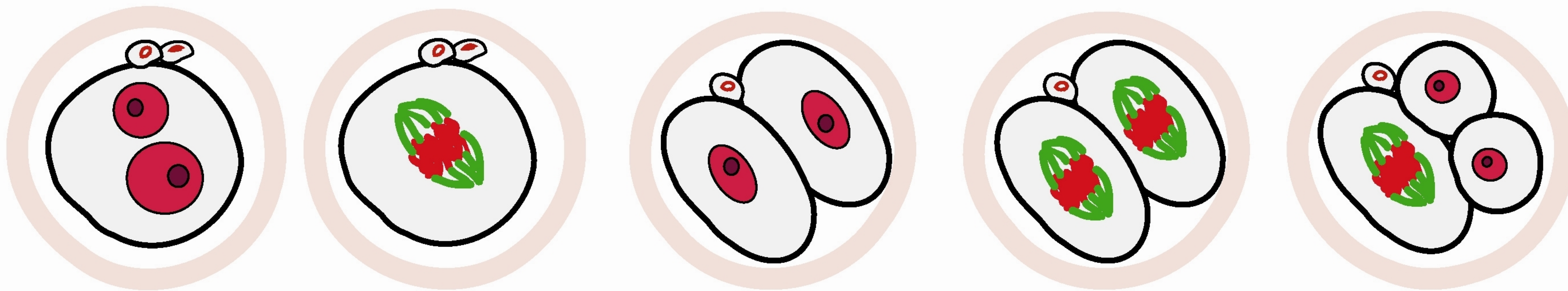
Figure legends

Fig. 1. Duration of the first two mitoses in four species studied so far.

Fig. 2. MPF activity, cyclin B levels and CDK1 inhibitory phosphorylation on Tyr-15 differs during the first two mitoses in *Xenopus laevis* embryo.

Fig. 3. Opposite effects of the inhibition of cyclin B degradation in *Xenopus laevis* embryo cell-free extract. Cyclin B degradation inhibited using proteasome proteolytic activity inhibitor MG132 does not modify MPF activity. The interference with polyubiquitination using mutated ubiquitin K48R prolongs the MPF activity. In both cases cyclin B degradation is efficiently blocked.

Fig. 4. Mad2 during the first embryonic mitosis in mouse zygote. A. In prometaphase Mad2 localizes on kinetochores. B. In metaphase Mad2 disappears from kinetochores. Mad2 in green, DNA in red. bar = 20 μm .



← first mitosis →

← second mitosis →

M. musculus

120 min

60 min

X. laevis

25 min

15 min

S. granularis

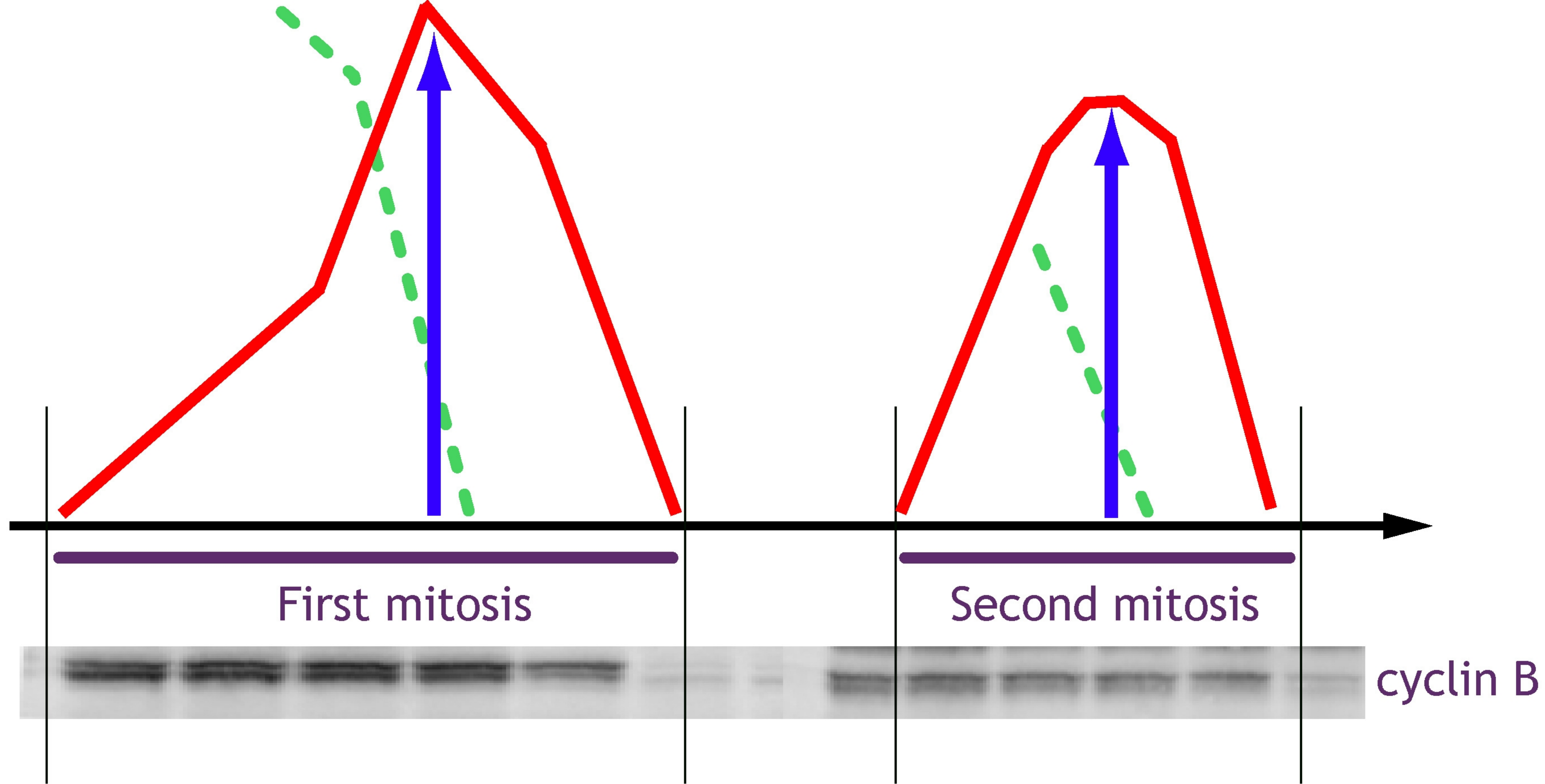
20 min

15 min

C. elegans

5 min

4 min

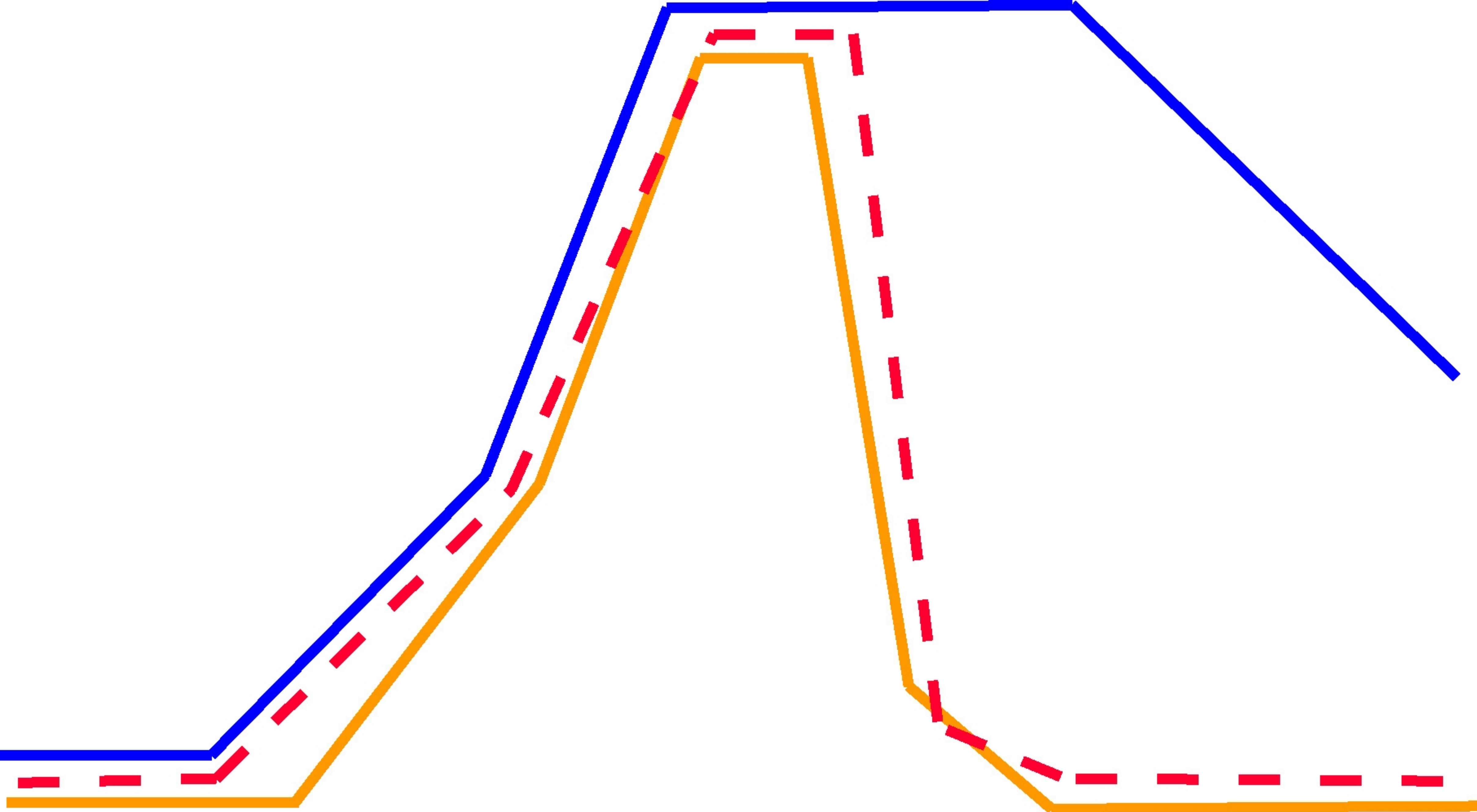


↑ cyclin B level



--- Tyr15 phosphorylation of CDK1

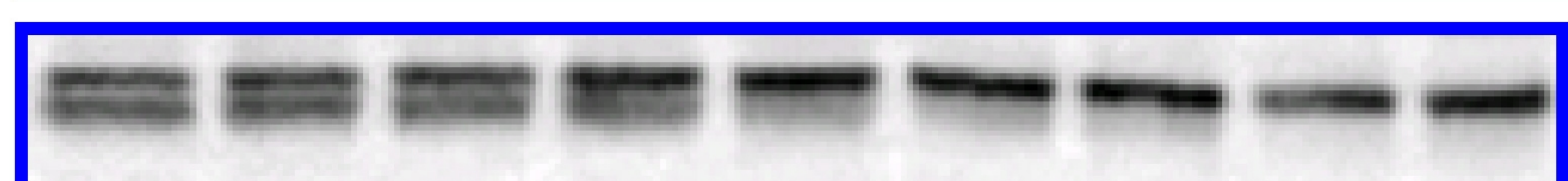
— histone H1 kinase activity (MPF)



WB cycB2 control



WB cycB2 MG132



WB cycB2 K48R

