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Review article

Temporal regulation of embryonic M-phases

Jacek Z. Kubiak[1], Franck Bazile[1], Aude Pascal[1], Laurent Richard-Parpaillon[1], Zbigniew Polanski[2], Maria A. Ciemerych[3], Franck Chesnel[1]

[1]CNRS/University of Rennes 1, Institute of Genetics & Development, Rennes cedex, France
[2]Department of Genetics and Evolution, Institute of Zoology, Jagiellonian University, Krakow, Poland
[3]Department of Cytology, Institute of Zoology, University of Warsaw, Poland

Abstract: Temporal regulation of M-phases of the cell cycle requires precise molecular mechanisms that differ among different cells. This variable regulation is particularly clear during embryonic divisions. The first embryonic mitosis in the mouse lasts twice as long as the second one. In other species studied so far (*C. elegans*, *Sphaerechinus granularis*, *Xenopus laevis*), the first mitosis is also longer than the second, yet the prolongation is less pronounced than in the mouse. We have found recently that the mechanisms prolonging the first embryonic M-phase differ in the mouse and in *Xenopus* embryos. In the mouse, the metaphase of the first mitosis is specifically prolonged by the unknown mechanism acting similarly to the CSF present in oocytes arrested in the second meiotic division. In *Xenopus*, higher levels of cyclins B participate in the M-phase prolongation, however, without any cell cycle arrest. In *Xenopus* embryo cell-free extracts, the inactivation of the major M-phase factor, MPF, depends directly on dissociation of cyclin B from CDK1 subunit and not on cyclin B degradation as was thought before. In search for other mitotic proteins behaving in a similar way as cyclins B we made two complementary proteomic screens dedicated to identifying proteins ubiquitinated and degraded by the proteasome upon the first embryonic mitosis in *Xenopus laevis*. The first screen yielded 144 proteins. To validate our strategy we are verifying now which of them are really ubiquitinated. In the second one, we identified 9 novel proteins potentially degraded via the proteasome. Among them, TCTP (Translationally Controlled Tumor Protein), a 23-kDa protein, was shown to be partially degraded during mitosis (as well as during meiotic exit). We characterized the expression and the role of this protein in *Xenopus*, mouse and human somatic cells, *Xenopus* and mouse oocytes and embryos. TCTP is a mitotic spindle protein positively regulating cellular proliferation. Analysis of other candidates is in progress.

Key words: CDK1 - Cell cycle - Cyclins - Embryo - MPF - Polyubiquitination - Proteasome - TCTP

Early embryonic mitoses are regulated differentially

The duration of the first two embryonic mitoses differs significantly in various species we studied so far. Curiously, the first mitosis lasts always longer than the second one. In nematode *Caenorhabditis elegans* the first one lasts about 4.5 min, while the second one 3.5-4 min. In sea urchin *Sphaerechinus granularis* the differences become more pronounced and the first mitosis takes about 20 min while the second one 15 min. In frog *Xenopus laevis* we estimated the mitotic duration for the first embryonic mitosis on 25 min, vs. 15 min for the second one [1,2]. The most dramatic difference was observed in the mouse Mus musculus embryos. The first mitosis takes about 120 min, while the second one approximately 70 min [3-5].

These differences in mitotic timings are not trivial since they are also observed in parthenogenetic embryos and in embryos with reduced volume of cytoplasm [3]. Even cytoplasmic fragments of mouse zygotes persisted in a mitotic stage for longer time than similar cytoplasmic fragments obtained from 2-cell stage dividing blastomeres, as judged by the behaviour of microtubules and the measurement of the activity of the major M-phase factor (MPF or M-phase Promoting Factor) [3,6]. This means that at least in the mouse embryo an internal, cytoplasmic, clock regulates differentially the duration of the first two mitoses.

The first two mitoses are most strikingly different in their duration. However, also the next embryonic

Correspondence: J.Z. Kubiak, CNRS/University of Rennes 1, Institute of Genetics and Development, UMR 6061, Mitosis & Meiosis Group, Faculty of Medicine, 2 Ave. Prof. Léon Bernard, CS 34317, 35043 Rennes cedex, France; tel. (+3302) 23234698, fax.: (+3302) 23 23 44 78, e-mail: jacek.kubiak@univ-rennes1.fr

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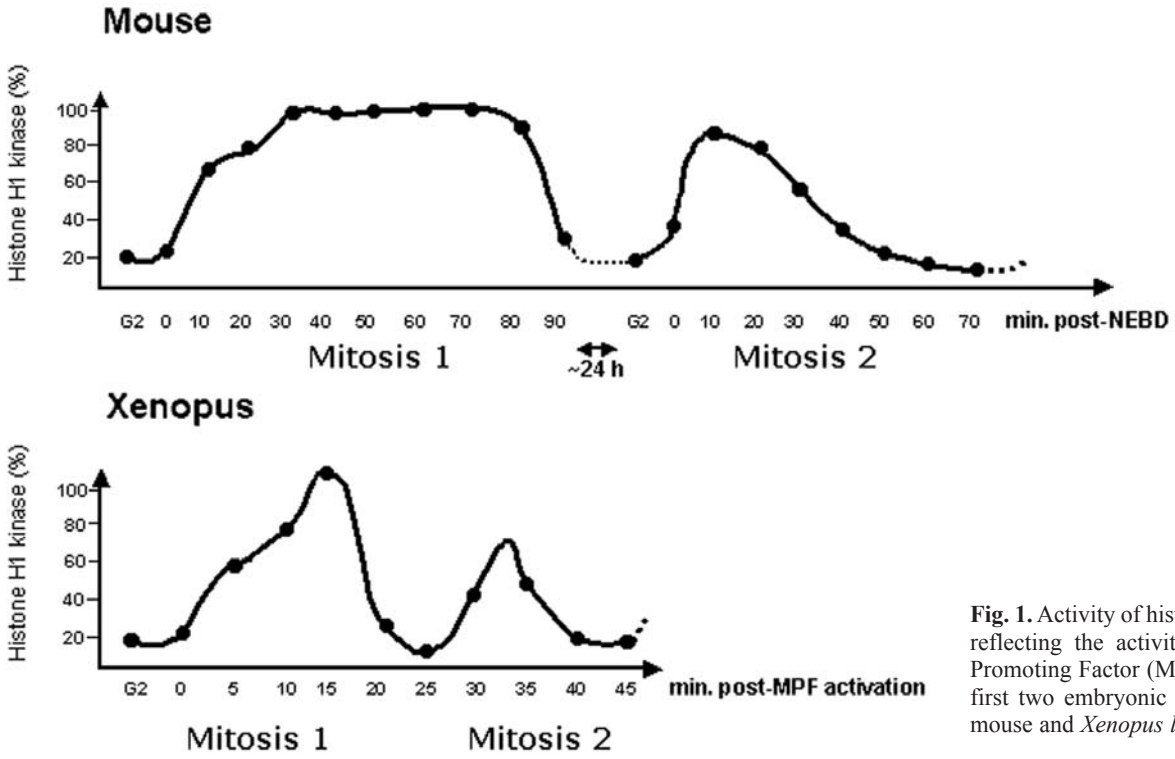

Fig. 1. Activity of histone H1 kinase reflecting the activity of M-phase Promoting Factor (MPF) during the first two embryonic mitoses in the mouse and *Xenopus laevis*.

mitoses become shorter in frog and in the mouse embryos [1,3]. It seems therefore, that early mitotic divisions become shorter gradually at least for the first few of them.

We are intrigued by understanding the molecular mechanisms underlying the prolongation of the first mitotic division. To better understand this issue we studied the first embryonic mitosis in two developmental models, the mouse and *Xenopus*, and took advantage of their distinct features. The relatively small and transparent mouse embryo is a perfect model to approach this study by cytological methods, like immunofluorescence. On the other hand, *Xenopus* embryo is an excellent tool for biochemical studies due to its large size, high quantity of proteins and the possibility to study, and to interfere with, cell cycle events in cell-free extracts.

First two embryonic mitoses in the mouse

The pattern of MPF activity during the first two embryonic mitoses in the mouse differs significantly. A plateau of this activity taking about 30-40 min is observed during the first M-phase (Fig. 1) [3]. The MPF activity profile during the second mitosis is similar to that of the somatic mitosis, *i.e.* it rises steadily and falls down immediately after attaining the maximum. Thus, MPF is stabilized only during the first but not the second M-phase. We did not know however, whether the mechanism stabilizing MPF impinges on prometaphase or metaphase during the first division. Thus, we analyzed chromosome movements and localization of spindle assembly checkpoint marker *i.e.* kinetochore protein Mad2 (present on kinetochores only during prometaphase) during the first two mitoses [7]). We found that the prometaphase length was very similar for the two mitoses, *i.e.* it lasted about 20 min in each case [5]. This observation suggested strongly that the extended duration of the first mitotic division is achieved through prolongation of the metaphase and not the prometaphase. The mechanism is yet unknown, however we hypothesize that it acts similarly to the mechanism operating in the oocytes physiologically arrested at metaphase by the Cytostatic Factor (CSF) during the second meiotic division. The determination of the timing of prometaphase delineated by the localization of Mad2 on kinetochores gave additional support to this hypothesis. During the first embryonic mitosis Mad2 was present on the kinetochores only at the beginning of the M-phase and disappeared later on, thus showing similar behaviour as in oocytes arrested at the second meiotic metaphase [5]. However, the checkpoint-dependent mechanism seems not to play an important role in the establishment of CSF arrest since its inactivation does not perturb the MII-arrest [8]). Nevertheless, this showed clearly that the first mitotic metaphase in the embryos resembles the meiotic MII arrest of oocytes *i.e.* in both cases the second part of the M-phase is prolonged by an activity stabilizing MPF rather then by the fact that spindle assembly checkpoint preventing the completion of M-phase

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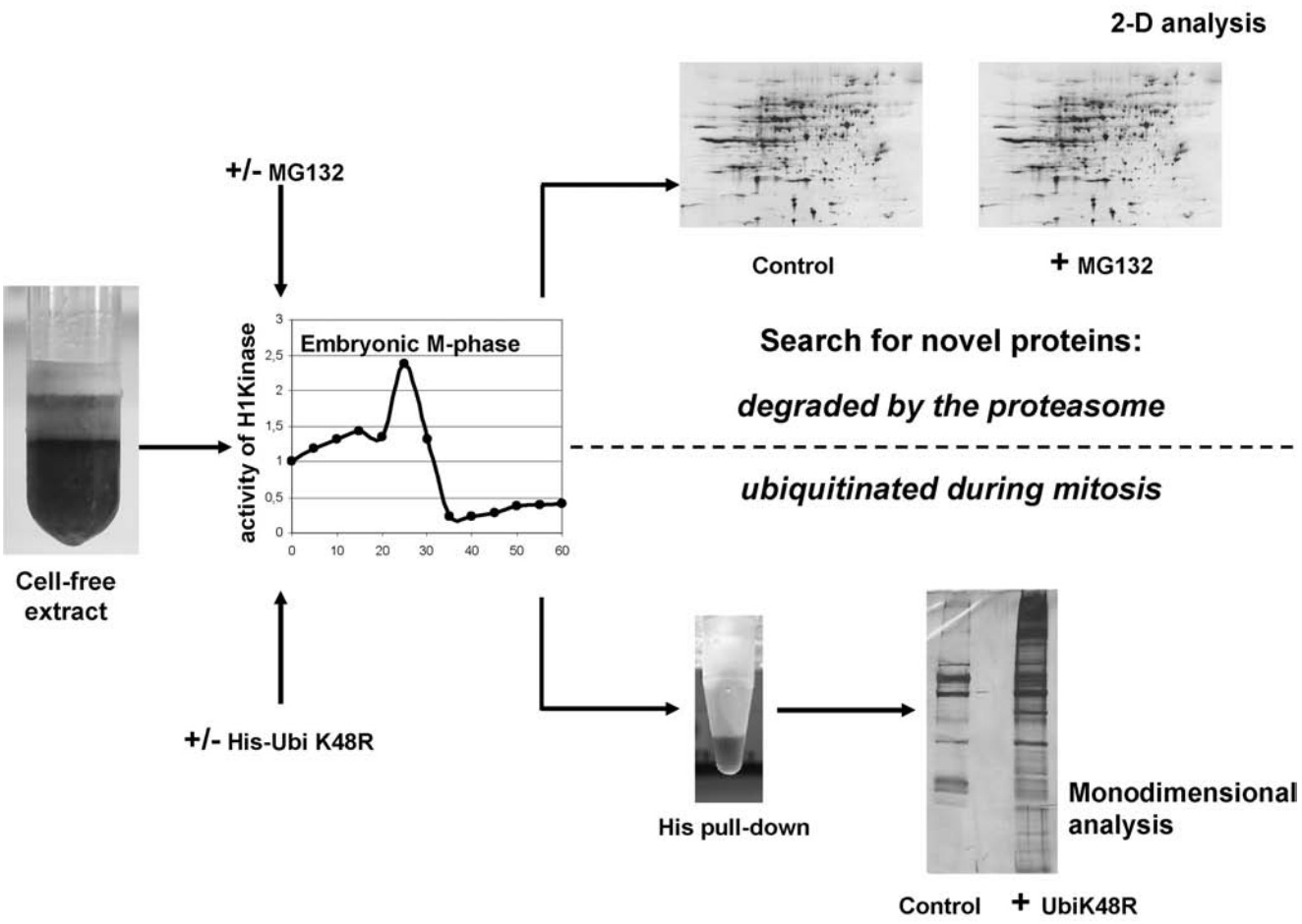

Fig. 2. Schematic representation of two screens devoted to identification of proteins ubiquitinated and degraded by proteasome during the first embryonic M-phase of *Xenopus laevis*.

is active. In MII-arrested oocytes this factor was identified as CSF [9], reviewed in [10], while in mouse zygotes it remains unidentified [5]. However, we cannot exclude that it resembles CSF. Among the factors playing an important role in the CSF activity are ERK1 and ERK2 MAP kinases [11,12]. However, during the first mitosis of the mouse embryo these kinases remain in unphosphorylated, thus inactive, state [13]. We cannot, however, exclude that the canonical MAP kinase pathway may be modified in zygotes. One of MAP kinases ERK1 and ERK2 direct substrates, p90rsk which also is a subsequent kinase in this pathway, is partially phosphorylated during the first mitosis of the mouse embryo [14]). Although p90rsk does not participate in CSF activity in the mouse oocytes [15], it was reported as indispensable element of CSF, acting downstream of ERK2 MAP kinase in *Xenopus laevis* MII oocytes [16,17]. Thus, we cannot exclude that this, or a similar kinase, could participate in prolongation of this particular M-phase, through some unidentified MAP kinase-independent pathway. However, this hypothesis awaits to be explored.

First two embryonic mitoses in *Xenopus*

The first two mitoses in *Xenopus laevis* embryo have distinct features than in the mouse. MPF activity during the two mitoses rises and falls down without plateau (Fig. 1) [1,2]. Using the cell-free extracts reflecting the first and the second M-phase respectively we found that the regulatory subunit of MPF, cyclin B accumulates to much higher degree during the first mitosis than during the second one [2]. Also the catalytic subunit of MPF, CDK1, was phosphorylated on Tyr15 (inhibitory phosphorylation) to clearly higher degree during the first M-phase. This combination of higher levels of cyclins B and different mode of CDK1 dephosphorylation on Tyr15 could result in higher MPF activity during the first mitosis. Higher level of MPF could in turn prolong MPF activity which results in longer duration of the whole M-phase.

To verify this hypothesis, we modified cyclin B levels in mitotic extracts. We either added exogenous cyclin B2 or inhibited protein synthesis (thus also cyclins B synthesis) shortly before the entry into the

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M-phase and observed that exogenous cyclin B2 indeed prolonged M-phase duration, whereas protein synthesis inhibition shortened its duration [2]. Therefore, we showed that cyclin B levels are involved in regulating the duration of embryonic M-phases in *Xenopus laevis*.

Inactivation of MPF depends on the efficiency of cyclins B proteolytic degradation. This process depends on the polyubiquitin/proteasome pathway. Using an inhibitor of the proteolytic activity of proteasome, MG132, we slowed down efficiently cyclin B degradation in the extract. However, no change in the M-phase duration was observed [18]. This result shows that MPF inactivation upon mitotic exit primarily depends on the dissociation of cyclin B from CDK1 molecule, and the process is mediated by the proteasome itself, and the cyclin B degradation follows this dissociation and the MPF inactivation, as shown before for meiotic M-phase [20]. The activity dissociating cyclin B from CDK1 is independent from the proteolytic activity of the proteasome and therefore is not inhibited by MG132 [18,20,21].

Another way to interfere with the polyubiquitin/proteasome pathway we used, namely addition of mutated recombinant ubiquitin (K48R) which blocks the elongation of the polyubiquitination chain (process mediated by ubiquitin ligase APC/C or Anaphase Promoting Complex/Cyclosome; reviewed in [22] appeared successful in stabilizing MPF activity. It acts on MPF activity by slowing down the targeting of cyclin B/CDK1 complex to the proteasome and the subsequent degradation of cyclin B by the proteasome [23]. This experiment demonstrated that interfering with the polyubiquitin/ proteasome pathway upstream from the proteasome enables to stabilize the MPF activity during mitosis.

Stability of cyclin B and the efficiency of the polyubiquitination/proteasome pathway may not be the only mechanisms regulating M-phase duration in *Xenopus*. Further studies should answer this question.

Screening for novel mitotic regulatory proteins

The experimental results for which we used either MG132 or mutated ubiquitin were focused on cyclin B metabolism and MPF activity. However, other proteins are known to be sequentially degraded during mitosis (*e.g.* Fig. 7 in [19], reviewed in [22]). We reasoned that if MG132 and K48R ubiquitin interfere with cyclin B dissociation/degradation, other still unknown proteins could behave in a similar way and may affect mitotic progression. Therefore we designed two proteomic screens dedicated to identification of novel proteins ubiquitinated and degraded upon mitosis.

We compared the proteomes of mitotic cell-free extracts in the presence and absence of K48R ubiquitin or with and without MG132 (Fig. 2). Using monodimensional (for ubiquitinated proteins - preceded by a enrichment of ubiquitinated proteins through a affinity chromatography step) or 2-D (for proteins degraded by the proteasome) electrophoretic separation followed by mass spectrometry, we identified 144 proteins potentially ubiquitinated during mitosis in the presence of K48R ubiquitin and 5 (among 9 surnumerary spots) present in MG132-supplemented extract in comparison to the MG132-free control sample. The first protein we chose to analyse in details was the Transcriptionally Controlled Tumour Protein (TCTP or Tpt1) which was found in the second screen. We confirmed that a part of the cytoplasmic pool of TCTP in *Xenopus laevis* cell-free extract as well as in oocytes is indeed degraded upon mitotic and meiotic exit, respectively. TCTP is localized to the mitotic spindle with higher concentration on the spindle poles. In mouse oocytes, it decorates pericentriolar foci forming the meiotic spindle poles. It seems that some post-translational modification targets a part of the pool of cytoplasmic TCTP for degradation. Local degradation of TCTP within the spindle could be important for the mitotic progression. We are currently studying a pattern of TCTP phosphorylation sites and kinases involved to identify the potential pathway acting in TCTP degradation.

Conclusions

The results briefly presented in this review suggest that at least two different mechanisms could be involved in regulation of the duration of embryonic M-phases in the mouse (transitional metaphase arrest) and in *Xenopus* (differential cyclin B accumulation). It seems that during evolution different strategies might have developed to prolong the first embryonic M-phase. However, further analysis should answer whether these two apparently different strategies indeed depend on different molecular mechanisms. It seems that in the two cases cyclin B metabolism is affected and in the mouse the mechanism may be simply more efficient than in *Xenopus*. Since the prolongation of the first embryonic mitosis is conserved among distant species and becomes more pronounced in mammals than in other vertebrates and invertebrates we think that it plays an important role during early embryonic development. These studies allowed us also to screen for novel proteins involved in temporal regulation of mitotic progression. We hope that analysis of some of them will deliver important information about mitotic control.

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