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Canonical and alternative pathways in Cyclin-Dependent Kinase 1/cyclin B inactivation upon M-phase exit in *Xenopus laevis* cell-free extracts

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Abstract

Cyclin-Dependent Kinase 1 CDK1 is the major M-phase kinase known also as the M-phase Promoting Factor or MPF. Studies performed during last decade have shown many details of how CDK1 is regulated and also how it regulates the cell cycle progression. *Xenopus laevis* cell-free extracts were widely used to elucidate the details and to obtain a global view of the role of CDK1 in M-phase control. CDK1 inactivation upon M-phase exit is a primordial process leading to the M-phase/interphase transition during the cell cycle. Here we discuss two closely related aspects of CDK1 regulation in *Xenopus laevis* cell-free extracts: firstly, how CDK1 becomes inactivated and secondly, how other actors, like kinases and phophatases network and/or specific inhibitors cooperate with CDK1 inactivation to assure timely exit from the M-phase.

1. Introduction

The cell cycle regulation comprises a network of numerous kinases and phosphatases. CDK1 is a major kinase necessary both for the S-phase and M-phase progression. Identification at the end of XX century of the CDK1 as a major regulator of the cell cycle made the understanding of its own regulation a fascinating topic. The use of *Xenopus laevis* cell-free extracts has had a large impact on these studies.

CDK1 belongs to the family of Cyclin-Dependent Kinases (CDKs). It has been the first CDK described in yeast (as a product of *cdc2* or *cdc28* gene depending on species) and human (called $p34^{cdc2}$). The name of CDKs comes from the association of these kinases with the regulatory subunits called cyclins. Similarly to all other kinases CDK1 has in its amino-terminal domain a xGxPxxxxREx sequence (where x represents any amino-acid). This conserved region corresponds to a cyclin binding domain [1]. There are twenty one CDK-coding genes in the human genome [2]. However, only a few of them are involved in cell cycle regulation. In *Xenopus laevis* oocytes and early embryos a handful of CDKs are expressed (CDK1, CDK2, CDK5, CDK9; our unpublished data). Two major CDKs taking part in cell cycle regulation are CDK1 and CDK2. Only CDK1 seems to be involved in M-phase regulation, while CDK2 is a major player in S-phase progression. The similarity in three-dimensional structure of these two kinases helps to understand some aspects of their regulation.

CDK1 activity picks only for a very short period of time upon G2/M transition and falls down rapidly at the M-phase exit. The structure of CDK1 is bilobate, similar to the cyclic AMP-dependent protein kinase, but contains a unique helix-loop segment that interferes with ATP and protein substrate binding. In its monomeric inactive form, CDK1 binds to the ATP in a conformation, which prevents a nucleophilic attack by hydroxyl substrate on the β-γ phosphate bridge of ATP [3].

Cyclins, the regulatory subunits of CDKs, are encoded by at least 15 different genes in human genome [2]. Only some of them are expressed in oocytes and early embryos of *Xenopus laevis*. B-type cyclins are main regulators of CDK1. In *Xenopus laevis* early developmental stages five of B-type cyclins (B1-B5) were identified [4]. B1 and B2 cyclins play a major role in M-phase regulation in *Xenopus laevis* since they are associated with the majority of CDK1. Upon M-phase exit cyclins are degraded sequentially beginning from cyclin B1 and ending with cyclin B5. This sequential degradation of cyclins reflects inactivation of successive parts of CDK1. Thus just a brief look at the composition and metabolism of CDK1/cyclin B complexes illustrates the complexity of the system, and this is just the tip of the iceberg.

2. M-phase control in *Xenopus laevis* oocytes via MPF, CSF and calcium signalling

Amphibian oocytes have been excellent model system allowing the discovery of the basic principles of M-phase regulation. Most of the molecules regulating M-phase entry, maintaining and exit were identified with the help of the amphibian experimental model. The enzymatic complex of CDK1 and cyclin B is the universal regulator of the M-phase. It was first discovered as an activity called Maturation Promoting Factor (MPF) by Masui and Markert in 1971 [5]. MPF drives both meiotic and mitotic cell cycle via M-phase entry induction. Masui and Markert [1] discovered the MPF activity in experiments involving a cytoplasmic transfer between mature and immature oocytes of *Rana pipiens* and *Xenopus laevis*. Such a transfer invariably induced resumption of meiotic maturation, i.e. M-phase entry in G2-arrested immature oocytes. Initially, the MPF activity was called a Maturation Promoting Factor because of its ability to induce maturation upon injection into immature oocytes. However further studies have shown that the very same factor induces also the mitotic M-phase. Thus the name of Maturation Promoting Factor was changed to M-phase Promoting Factor. Soon

after, the MPF was shown to have an M-phase-inducing activity regardless of the species. This suggested the key role of this molecule in the induction of the M-phase of cell cycle in all eukaryotic cells. After numerous efforts, MPF was identified as a complex of Cyclin-Dependent Kinase 1 (CDK1) and its regulatory subunit cyclin B [6-9].

Masui and Markert [5] demonstrated the presence of another factor that stabilized MPF in MII-arrested oocytes. They called it the CSF for CystoStatic Factor. Identification of the molecular identity of CSF took more time and energy than identification of MPF. The key player in the CSF pathway was discovered recently as a protein called Early mitotic inhibitor 2 (Emi2) [10-13]. Emi2 arrests the Ubiquitin/Proteasome System (UPS) by an inhibitory association with APC/C^{Cdc20} ubiquitin ligase necessary for cyclin B ubiquitination and targeting the CDK1/cyclin B to proteasomes where cyclin B becomes degraded. Thus, CSF holds APC/C in an inactive state assuring MII-arrest in oocytes, and Emi2 is the most downstream effector.

Amphibian oocytes, similarly to the majority of other vertebrate oocytes, are ovulated in MII-arrested state. Mos/…/ERK2 MAP kinase pathway stabilizes Emi2 during this period. The stabilization of Emi2 is achieved via phosphorylation on Thr 336, Ser 342 and Ser 344 by the most downstream enzyme of ERK2 MAP kinase pathway, the Rsk90 kinase. These phosphorylations promote Emi2-PP2A interaction and thus antagonize Emi2 phosphorylation by CDK1/cyclin B [14]. Other residues, namely T545 and T551, are phosphorylated by CDK1/ cyclin B [15]. These phosphorylations are removed by protein phosphatase 2A (PP2A) assuring the turnover of Emi2 phosphorylation during the oocyte MII-arrest (Fig. 1).

Upon fertilization, the spermatozoon entering the oocyte induces a burst of free calcium in the ooplasm. The calcium signaling plays a major role in triggering the developmental program of the embryo. The rapid increase in calcium concentration inactivates CSF. The first step is activation of calcium-dependent kinase 2 (CaMK2), which phosphorylates Emi2. This

turns Emi2 into a substrate for Plk1, the kinase which is already active in MII oocytes before fertilization, but unable to phosphorylate Emi2 before it becomes modified by CaMK2 (Fig. 1). The double CaMK2- and Plk1-mediated modification of Emi2 following fertilization makes this protein recognizable by $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase. $SCF^{\beta\text{-}TRCP}$ triggers Emi2 polyubiquitination followed by its proteasome-dependent degradation and disappearance from the ooplasm. This complex process is necessary to remove the CSF activity and to release the MII-arrest of oocytes upon fertilization via APC/C activation and triggering cyclin B polyubiquitination [10-12].

Despite the major role of the Mos…/ERK2 MAP kinase pathway resulting in Emi2 stability other alternative CSF pathways seem also involved in MII arrest induction and maintenance. For example CDK2/cyclin E pathway was shown to induce a CSF-like arrest in *Xenopus laevis* cell-free extracts [16]. A checkpoint kinase Mps1 (Monopolar spindle 1) is necessary for this action of CDK2/cyclin E [16]. Further details of the role of this alternative CSF pathway and especially its elimination upon oocyte activation remain unknown.

The CSF inactivation is not the only effect of the rise in calcium concentration upon fertilization. The calcium burst also triggers a transient activation of a phosphatase called calcineurin [17, 18], which is absolutely necessary for oocyte activation. However, it is unknown whether calcineurin acts on CDK1 or on some of its substrates. Nevertheless, these results pinpoint the network of kinases and phosphatases involved in phosphorylation and dephosphorylation events of numerous proteins as major mean to regulate the cell cycle transition necessary to initiate the embryo development.

3. CDK1 inactivation without cyclin B degradation

Proteolytic degradation of cyclin B via UPS plays a major role in cell cycle regulation. Its perturbation disorganizes the cell cycle progression [19-21]. Inhibition of the proteolytic

activity of proteasome with inhibitors like MG115, MG132 or ALLN arrests cells in M-phase with high cyclin B content and equally high CDK1 activity. The same inhibitors block also cyclin B degradation in *Xenopus laevis* cell-free extracts, however, they neither arrest CDK1 inactivation nor provoke the M-phase-arrest [22-24]. This important difference between the reaction of intact cells and cell-free extracts strongly suggests that CDK1 inactivation proceeds without cyclin B degradation at least in *Xenopus laevis* oocytes and embryo extracts. The reason for such a different reaction of intact cells and cell-free extract to proteasome inhibition remains unclear. It seems reasonable to speculate that in the case of somatic cells some up-stream substrate of proteasome pathway must be degraded before cyclin B could be targeted for degradation. In this case the proteosome inhibition would inhibit cyclin B degradation indirectly, via action of remaining up-stream substrate of UPS. However, the identity of a potential UPS substrate conditioning cyclin B degradation in intact cells remains unknown.

The first step in cyclin B degradation is its polyubiquitination by APC/C ubiquitin ligase (Fig. 2). This process takes place when cyclin B is still associated with CDK1. Thus, APC/C-mediated polyubiquitination targets to the proteasome not only cyclin B but the whole complex, which is still active when cyclin B is in the polyubiquitinated state. The proteasome induces or catalyses the dissociation of cyclin B from its CDK1 partner. Nishiyama and colleagues [22] have shown that the activity of the 26S proteasome involved in cyclin B dissociation from CDK1 is associated with its 19S regulatory subunit. It seems that the lid of the proteasome could be involved in this process. The 19S subunits of proteasome may also deubiquitinate, and concomitantly denature cyclin B before it becomes loaded into the 20S proteasome catalytic chamber. This hypothetical modification of cyclin B may, as a side effect, trigger its dissociation from CDK1. Takeo Kishimoto's group named the dissociating

activity of the proteasome a “non-proteolytic activity” to distinguish it from the classical proteolytic activity [22].

The APC/C activity is itself positively regulated in large part by CDK1/cyclin B-dependent phosphorylations of its numerous subunits [25, 26]. These phosphorylations trigger cyclin B polyubiquitination, dissociation from CDK1 and degradation resulting in CDK1 inactivation. In addition, the substrate specificity of APC/C changes during mitosis, due to the switch in its regulatory subunit, from Cdc20 (Fizzy) to Cdh1 (Fizzy-related). This switch is possible after Cdc20 poluybiquitination by APC/C and its subsequent degradation. Thus, CDK1/cyclin B indirectly regulates itself via controlling APC/C activity at least at two different levels, which in turn determines the stability of cyclin B (reviewed in [27, 28]).

4. CDK1 inactivation is inhibited by interference with cyclin B polyubiquitination

Ubiquitin is a highly evolutionary conserved small (76 amino acids) polypeptide. Its COOH terminus is covalently linked to lysine residues of a substrate (e.g. cyclin B) *via* an isopeptide bond (for a review see [30]). Polyubiquitination proceeds *via* multiple rounds of ubiquitination during which the COOH terminus of a new ubiquitin molecule forms an isopeptide bond with the lysine residue of ubiquitin previously attached to the substrate. This process is mediated by three sequentially acting enzymes (E1, E2 and E3, the last being an ubiquitin ligase). Lysine 48 of ubiquitin molecule is one of the major residues involved in polyubiquitination, mediating subsequent targeting of substrates to the proteasome. However, all seven lysine residues present within the molecule of ubiquitin are able to form isopeptide bonds [29-31]. A mutation of lysine 48 (K48) to arginine (R) severely affects the process of polyubiquitination [32,33]. Such ubiquitin mutant (UbiK48R) was used to perturb ubiquitination of proteins during the M-phase in the cell-free mitotic extracts upstream from the inhibition of the proteolytic activity of the proteasome. The interference with the

polyubiquitination pathway via UbiK48R arrests cyclin B dissociation from CDK1 and its degradation [34]. Therefore, the polyubiquitination pathway appears to be necessary for the targeting of cyclin B complexed with CDK1 to the proteasome. This in turn results in the maintenance of high activity of CDK1 and keeping the extract in the M-phase. Thus UPS inhibition at the level of plyubiquitination and not at the level of the proteasome proteolytic activity inhibits effectively CDK1 inactivation. This points to the importance of cyclin B dissociation from CDK1 and not the degradation of cyclin B.

5. Alternative pathways to inactivate CDK1

5A. Dephosphorylation of CDK1 threonine 161

The phosphorylation of threonine 161 residue of CDK1 is necessary for activation of CDK1. Thus, the dephosphorylation of this site may inactivate CDK1 independently of cyclin B dissociation and proteolysis [35,36]. The dephosphorylation is catalyzed by the okadaic acid (OA)-sensitive type 2C protein phosphatases (PP2Cs) [36]. The comparison of the dynamics of CDK1 inactivation with the dynamics of CDK1 Thr161 dephosphorylation upon M-phase exit has shown that the latter follows CDK1 inactivation [24]. The detailed analysis of the interaction between CDK1 and cyclin B2 (one of five cyclins B potentially present in *Xenopus laevis* early embryos) upon M-phase exit revealed that the dissociation of cyclin B2 from CDK1 perfectly correlates with the dynamics of CDK1 inactivation and not with CDK1 threonine 161 dephosphorylation [24]. Thus, CDK1 threonine 161 residue dephosphorylation is a relatively late step in CDK1 inactivation and perhaps plays a role in the ultimate switching off of the CDK1, thus protecting the cell against unscheduled and premature reactivation of the kinase after the M-phase exit.

5B. Transient inhibitory phosphorylation of CDK1 on threonine 14 and tyrosine 15

A study in *Xenopus laevis* cell-free extracts has shown that a transient phosphorylation of CDK1 on tyrosine 15 (and most probably on threonine 14) could also participate in CDK1 inactivation. D'Angiolella and colleagues have shown that in cycling cell-free extracts the cyclin B1-associated histone H1 kinase activity diminishes more rapidly than the level of cyclin B1 protein [37]. This was not the case for cyclin A-associated activity and the diminution of the level of cyclin A protein. Detailed analysis of M-phase exiting extracts both during mitosis and meiosis (CSF extract treated with calcium) has revealed a transient re-phosphorylation of cyclin B-associated CDK1 on tyrosine 15 following the initial drop in CDK1 inactivation. This short lasting increase in tyrosine 15 phosphorylation could be mediated by a transient increase in association of CDK1 with Wee1 kinase catalysing the reaction of phosphorylation at this site. However, another study has shown that CDK1 dephosphorylation at tyrosine 15 precedes the kinase inactivation [38] (Fig. 3). Thus, it is not entirely clear to what extend this kind of regulation influences the dynamics of CDK1 inactivation.

5C. Specific inhibitors may participate in CDK1 inactivation

Data from yeast and HeLa cells suggest that the specific inhibitors could also be involved in CDK1 inactivation upon mitotic exit. Cdc6 protein, well know as a key S-phase regulator, could play this role [39, 40] (Fig. 3). There is no data supporting such a role of Cdc6 in *Xenopus laevis* cells. However, since Cdc6 was shown to inhibit CDK1 both in yeast and in human cells it seems possible that it also plays the same role in amphibians. CDK1 inhibition specifically during the M-phase exit may lead, together with the transient CDK1 phosphorylation on tyrosine 15, to more rapid and more effective inhibition of CDK1.

5D. Cooperation of CDK2 and PKA in CDK1 inactivation

The next alternative pathway of CDK1/cyclin B inactivation implies the participation of CDK2. It was shown in 2001 that CDK2/cyclin E enables to maintain the high CDK1/cyclin B activity during mitosis in *Xenopus laevis* cell-free extract [41]. Premature inactivation of CDK2 during the mitotic M-phase induces an increase in protein kinase A (PKA) activity and speeds up CDK1/cyclin B inactivation and cyclin B degradation. Thus, CDK2/cyclin E seems to be coupled with PKA activity in assuring a correct timing of CDK1/cyclin B inactivation and the M-phase exit. However, it remains unknown how CDK2 and PKA act in concert on CDK1 and cyclin B degradation pathway.

Crystallographic studies of another CDK2 enzymatic complex, namely CDK2/cyclin A that has fundamental role in S-phase regulation, have shown details of the mechanism underlying inactivation of this kinase [2, 42]. CDK1/cyclin B complex has never been studied with such accuracy. As the two complexes are closely related the mechanism proposed for CDK2 may also apply to CDK1. The active site of CDK1 could undergo conformational changes in its PSTAIRE helix and T-loop upon the dissociation of cyclin B, as it happens with CDK2 upon dissociation of cyclin A. Such a conformational change prevents proper interaction of the enzyme with ATP and inactivates the kinase [2, 42]. The cyclin subunit determines substrate specificity of CDKs (reviewed in [43]). Thus, the loss of cyclin subunit should be immediately followed by efficient inactivation of CDK1 to protect the cell against possible phosphorylation of undesired substrates. A part of this job may be performed by the transient inhibitory phosphorylation of tyrosine 15. Another possibility to eliminate unspecific activity of cyclin-free CDK1 would be a direct inhibition via its association with a specific inhibitor, for instance Cdc6.

6. Kinase/phosphatase network upon M-phase exit

In HeLa cells CDK1 inactivation is not sufficient to assure the successful transition to interphase. The efficient transition to interphase requires a phosphatase activity (or activities) dephosphorylating CDK1 mitotic substrates [44]. The activation of these phosphatases is clearly proteasome-dependent, but independent of cyclin B degradation. PP2A was shown to be the major phosphatase dephosphorylating CDK1 substrates in interphase *Xenopus laevis* eggs extract [45]. PP2A is a heterotrimer composed of the catalytic C-, scaffolding A- and regulatory B-type subunits represented by different isoforms. The B-type subunits are responsible for the substrate-specificity of the whole complex [46]. Mochida and colleagues [45] have shown that PP2A containing the B55δ subunit is the major phosphatase controlling the exit from the M-phase via dephosphorylation of CDK1 phospho-substrates. It was proposed that the newly discovered Greatwall kinase could play a role of a phosphatase suppressor [47]. Greatwall kinase was discovered in a screen for Drosophila mutants defective in chromosome condensation [48]. Greatwall is a ubiquitous evolutionarily conserved protein kinase, known in humans as MAST-L kinase, belonging to the AGC family of Ser/Thr kinases [49]. Depletion of this kinase from M-phase extracts induced activation of an okadaic-sensitive phosphatase that acts on CDK1 substrates and on the mitotic exit. Addition of this kinase to interphase extracts inhibited dephosphorylation of CDK1 substrates [50, 51]. Moreover, the inability of Greatwall-depleted cell-free extracts to enter M-phase was reverted by removal of PP2A-B55 delta [50]. It was shown recently by two independent laboratories that a small protein called cAMP-regulated phosphoprotein-19 (ARPP-19), a close relative of another small protein alfa-Endosulfine (ENSA), was an ideal substrate for Greatwall kinase in *Xenopus laevis* cell-free extracts [52, 53]. Thus, the network of kinases and phosphatases governing CDK1 substrates dephosphorylation, and thus, the transition to interphase following the M-phase was discovered.

8. Conclusions

Several pathways control CDK1 inhibition upon M-phase exit. The major pathway called here canonical, involves dissociation of cyclin B from CDK1 and is followed by cyclin B degradation and disappearance from the cell. Other, probably minor pathways, including phosphorylation and dephosphorylation of CDK1 at different sites, and active inhibition of CDK1 kinase activity, may play supplementary role in shortening the process of CDK1 inactivation. This hypothetical role of CDK1 accelerator could be of particular importance for very fast cleaving embryos such as amphibian embryos. CDK2/cyclin E and PKA seem also to exercise an important control over the timing of CDK1/cyclin B inactivation. The alternative pathways in CDK1/cyclin B inactivation may be important in certain unique conditions when the canonical pathway becomes ineffective. For example, in rat one-cell embryos treated with MG132 the M-phase exit probably occurs without cyclin B degradation [54]. Also in mouse oocytes undergoing maturation and fertilized in vitro by numerous spermatozoids CDK1/cyclin B is inactivated rather via threonine 161 dephosphorylation than full cyclin B degradation [55]. Surprisingly, recent studies of the minimal control of CDK network in fission yeast suggest that modulation of CDK1 activity and not its proteolytic or phosphorylation-dependent regulation could play an ancestral role during evolution [56]. This discovery will certainly stimulate further studies on non-canonical pathways regulating M-phase exit and it is possible that the canonical pathway will become non-canonical, and *vice versa*.

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Figures

Figure 1.

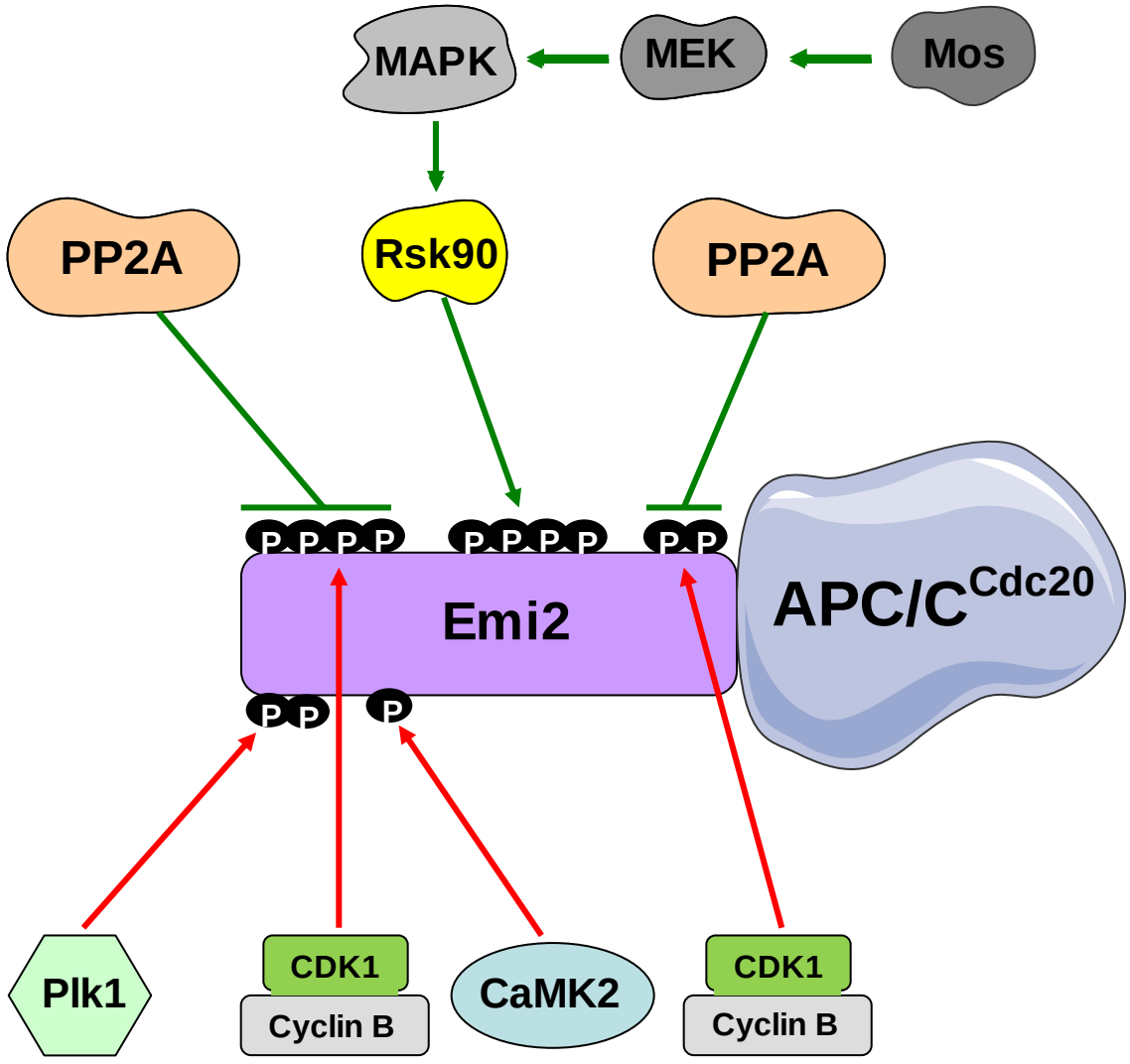


Figure 2.

Fig. 2

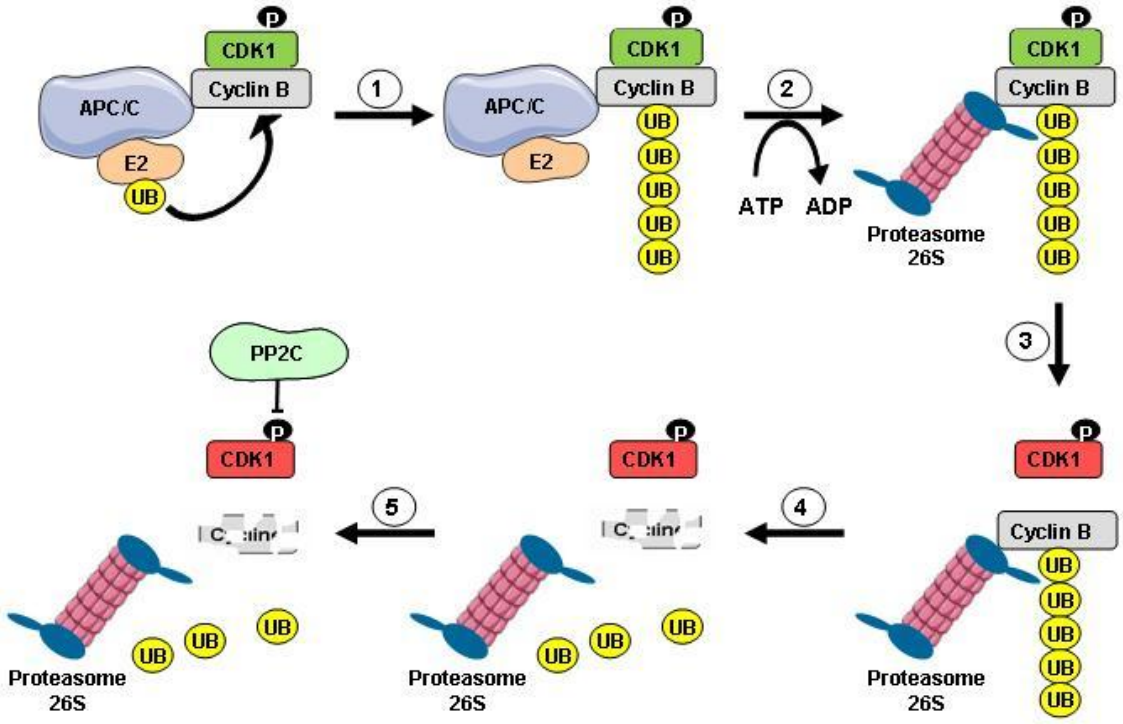
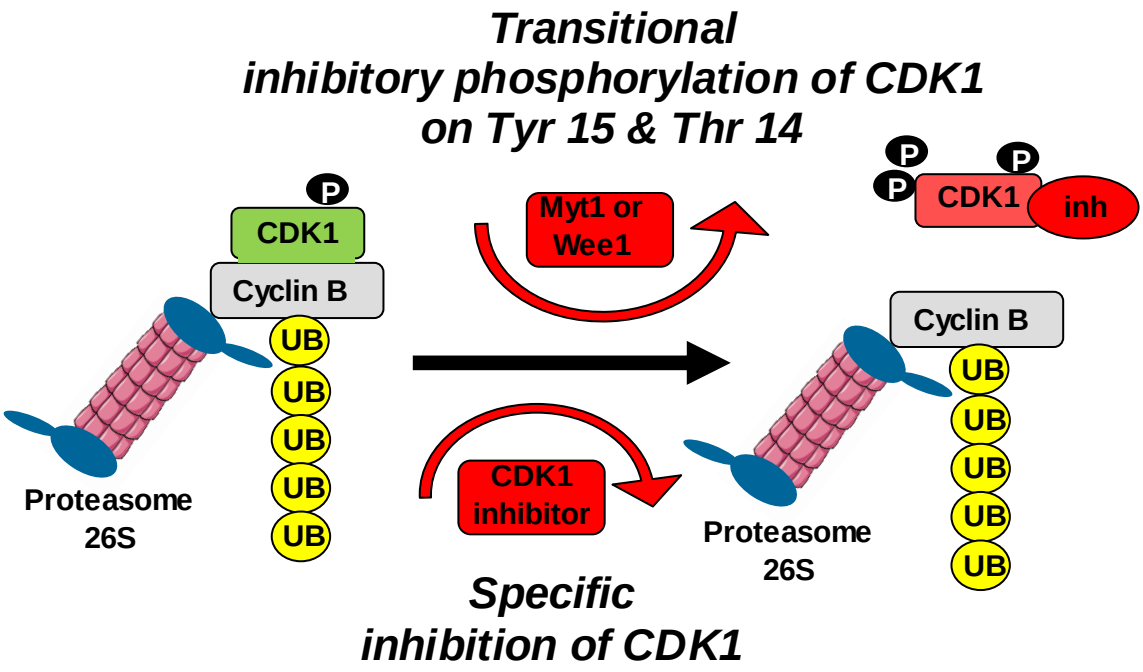


Figure 3



Legend to the figures:

Fig.1. Regulation of Emi2 association with APC/C^{Cdc20}. Phosphorylation sites in the upper part of Emi2 are inhibitory for the association and are protected during MII –arrest (green arrows and symbols of inhibition), while the sites in the bottom part of Emi2 are activatory for the association and the CSF-arrest exit (red arrows).

Fug. 2. Canonical pathway of CDK1/cyclin B inhibition.

Fig. 3. Hypothesis of alternative pathways involvement during CDK1/cyclin B dissociation.