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On the road to destruction: coupling Cyclin B degradation to proteolysis independent MPF inactivation

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Summary

At M-phase onset, rapid M-phase Promoting Factor (MPF; the cyclin B-cdk 1 complex) activation is granted by an activation loop in which the cdc25C phosphatase removes inhibitory phosphorylations of cdk1 at thr-14 and tyr-15, while MPF stimulates cdc25C activity and inhibits activity of wee1, the cdk1 tyr-15 kinase. At M-phase exit, ubiquitin-dependent cyclin proteolysis inactivates MPF. Here we show that timely activation of proteolysis requires interruption of the MPF activation loop. By sampling Xenopus egg extracts at very short intervals during both meiotic and mitotic exit, we found that cyclin B1-associated cdk1 underwent transient inhibitory phosphorylation at tyr-15 and that cyclin B1-cdk1 activity fell more rapidly the cyclin B1 content. Concomitantly, cdc25C underwent inhibitory phosphorylation at ser-287. In addition, we detected physical interaction between Wee1 and MPF in M-phase. MPF inactivation required Ca++/calmodulin-dependent kinase II (CaMKII) and cAMP-dependent protein kinase (PKA) activities at meiosis and mitosis exit respectively.

Supplying extracts with mutant cyclin B1-cdk1AF complex, refractory to inhibition by phosphorylation, altered M-phase exit. Loss of MPF activity was required for full binding of the ubiquitin ligase Anaphase Promoting Complex /Cyclosome (APC/C) to its co-activator Cdc20. Thus, M-phase exit requires a tight coupling of proteolysis-dependent and proteolysis-independent mechanisms of MPF inactivation.

Cdk-Cyclin complexes and their oscillatory properties

The primary task of the cell division cycle is to duplicate genetic information precisely through the process of DNA replication (S phase) and then to allocate this information equally to two daughter cells through mitosis. In eucariotic cells each phase of the cell cycle is controlled by several Cyclin Dependent Kinases with their obligate activating partners, the Cyclins. Once activated these kinases are known to modify various substrate whose activity is critical for cell cycle progression.

The activation of Cdks is mediated by the sequential accumulation of cyclins, that once bind to their specific partner subunits begin a wave of phosphorylations events that ultimately leads to the completion of a particular cell cycle phase. The end of one phase and the beginning of the other is mediated by the desctruction of the cyclin wave through proteolysis. Other mechanisms coupled tightly to degradation restrict CDK activity to a specific phase of the cell cycle, the controlled degradation of Cyclins is an irreversible mechanisms that blocks the way back, thus driving the cell cycle in only one direction. The tight control through proteolysis and localization of the Cyclin activity represents an important feature of the mammalian cell cycle, complex mechanisms called checkpoints restrict wave diffusion in a spatially and timely coordinated manner. The disruption of Cdk's activity control through defect in proteolysis or localization leads to abnormal completion of the cell cycle and the acquisition of a feature typical of the cancer cell: genomic instability.

Fig.2 depicts the cyclin waves during cell cycle progression, the model is simplified highlighting only the contribution of three maor cdk activity: S-cdk, M cdk and G1 cdk; the cell cycle is completed by the proteolysis of the M Cyclin wave mediated by the ubiquitin ligase APC/C(see below for further details).

In a simple organism like yeast is only the availability of nutrients that allows a cell in G1 to grow in size and enter the cell cycle. The decision to start the cell cycle is taken at a specific G1 checkpoint called START in yeast and renamed "restriction point" in mammalian cells. After this point the cell is committed to replicate its genome and divide. After G1, during the "S phase" (phase of DNA synthesis), the cell replicates its genome and then enters the short G2 phase during which key mechanisms control the completion and fidelity of DNA replication and prepare the cell for entering mitosis. Mitosis or "M-phase" is the shortest and the most dramatic phase since at this time the replicated genome is segregated to the opposite pole of the cell and the two daughter cells are generated.

The molecular mechanisms of cell cycle regulation were first reveled by the genetic dissection of the yeast cell cycle through the isolation and study of the cell division cycle (cdc) mutants. Of fundamental importance was the discovery that a gene called CDC28 in Saccharomyces Cerevisiae and cdc2 in Schizosaccharomyces Pombe is the master controller of both G1/S and G2/M transitions in these organisms (Nasmyth and Nurse 1981). It was then shown that cdc2/CDC28 encodes a serine/threonine kinase whose activity oscillates during the cell cycle. During different phases of the cell cycle cdc2/CDC28 associates with different activating subunits called cyclins because of their property of accumulating at specific phases of the cell cycle.

After those observations the purification of the MPF from the X. Laevis egg extract leaded to the isolation of the cdc2 and cyclin B

complex(Labbe, Capony et al. 1989) and the major acting route of oscillation of the Cyclin dependent kinases with their cyclin activating partners was uncovered. In '87 a family of mammalian kinases homologues to Cdc2/CDC28 was identified(Draetta, Brizuela et al. 1987)

In contrast to yeast, where the cell cycle and, therefore, proliferation is mainly controlled by the presence of nutrients, in mammalian cells a more complex system of growth control has evolved. The rules imposed by multicellular organization requires that proliferation, differentiation and programmed cell death (apoptosis) be restricted by extracellular events such as the presence of soluble growth. factors and cell-cell contact interaction. Nevertheless, control of the various phases of the cells cycle requires similar protein modules of kinases controlled by phosphorylation and de-phosphorylation events, unstable activating (cyclins) and inhibitory (ckis) subunits(Sherr and Roberts 2004).

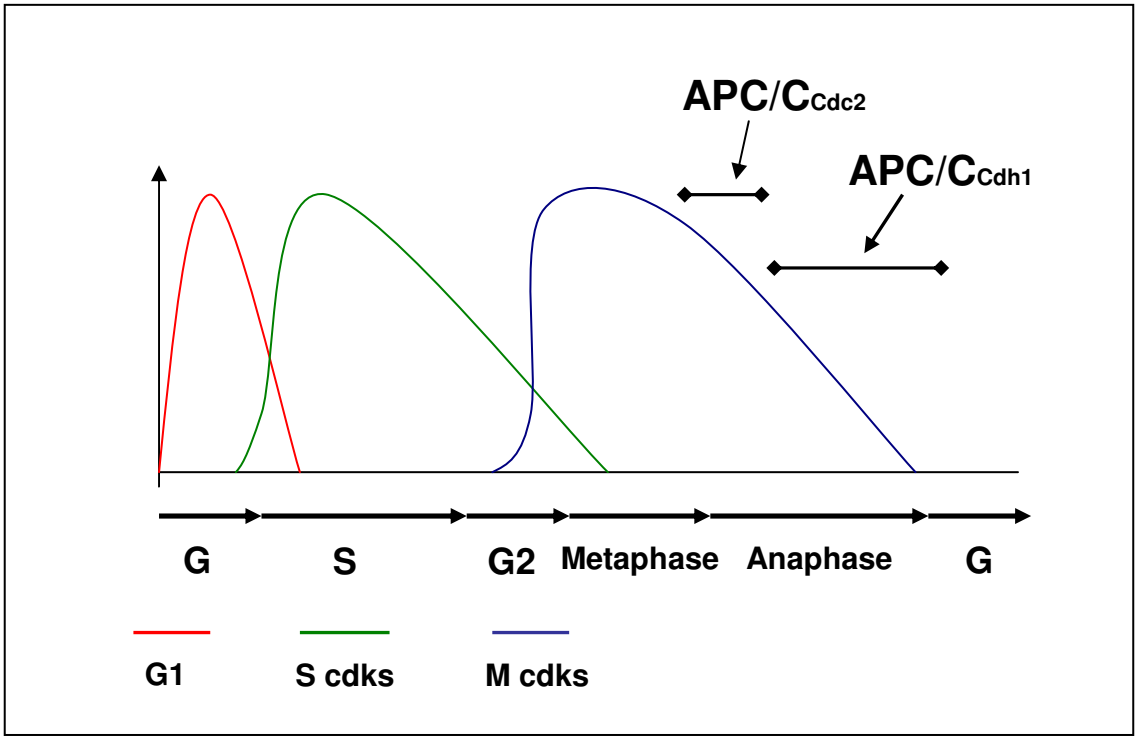

Fig.1 The picture depicts a simplified model of cell cycle transition that highlights the contribution of only three major cdk waves respectively called as G1 cdks, S cdks, Mcdks, The metaphase- anaphase transition is signed by the activation of the APC/C that degrades Cyclin B and interrupt the mitotic cyclin wave.

Use of Xenopus laevis system to study cell cycle transitions

During the last 10 years a great advancement in the comprehension of cell cycle mechanisms has been achieved, the presence of model systems like Xenopus laevis helped tremendously in the biochemical characterization of cyclin B degradation and mitosis exit. The system has been used in a multitude of approaches to address questions regarding not only cell cycle regulation but also DNA damage, apoptosis, chromatin remodeling and nuclear transport (for an extensive review see *methods mol bio 2006 vol.322*). The system is based on the embryonic cycle of the south African clawed frog Xenopus Laevis, and exploits the process of oocyte fertilization and the early embryonic division.

Fully grown, immature stage VI oocytes present in the ovaries of adult frogs are arrested at the G2/M transition of meiosis I. On progesterone (PG) secretion *in vivo* by the neighboring follicle cells of the ovary in response to pituitary hormones, or on PG addition in culture, the oocytes enter meiosis I and proceed with the process termed “oocyte maturation,” ultimately resulting in a fertilizable egg. The egg is arrested in metaphase II of meiosis by a factor called CSF (Cytostatic factor), whose component have been dissected during the past years(Tunquist and Maller 2003) (D'Angiolella, Costanzo et al. 2001). The CSF maintain an high activity of MPF and the addition of Calcium, mimicking the process of fertilization, allows the exit from metaphase II of meiosis. The extract is prepared from metaphase II arrested eggs, upon calcium addition *in vitro* a series of events stimulated by CAMKII trigger cyclin B degradation, partially phenocopying the process of mitosis exit in mammalian cells. The process of fertilization can also be triggered when the egg is intact by using calcium

ionophore. When the extract is prepared the eggs undergo different round of MPF oscillation, the process *in vitro* reproduces the early embryonic division after fertilization. Fig 3 explain the difference and the behaviour of a cycling extract and a CSF extract. It is shown the MPF activity oscillation upon calcium addition or after the preparation of cycling extract.

Another possibility is to study cell cycle checkpoint in Xenopus laevis egg extract, for example the spindle checkpoint can be induced by adding to the extract nuclei and nocodazole. Nuclei and nocodazole represent a source of unattached kinethochores that send a wait signal for cyclin B degradation and mitosis exit.

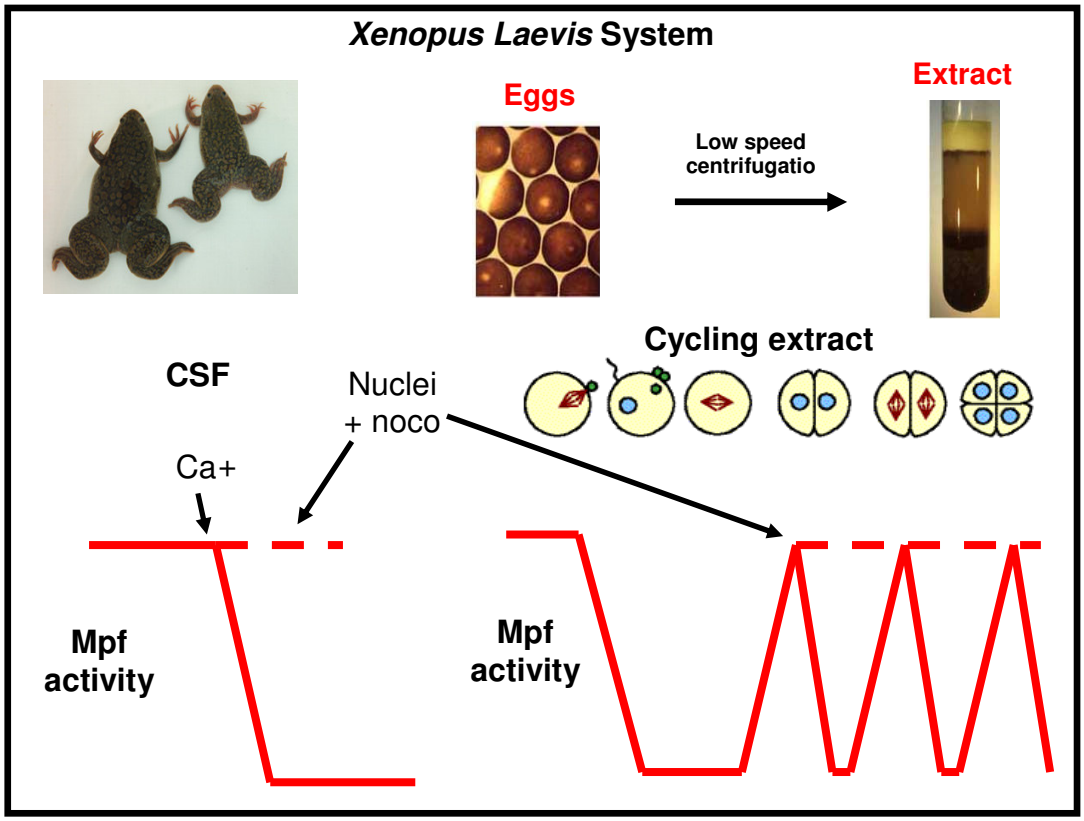

Fig.2 In the picture is shown a model of cell cycle transition during early embryogenesis of the African clawed frog Xenopus Laevis. The extract is prepared using eggs physiologically arrested in the metaphase II of meiosis or using the eggs that undergo different cycle of rapid S-M phase after parthenogenetic activation. The extract can be arrested in mitosis by adding nuclei and nocodazole. Nuclei and nocodazole simulate the signal present in vivo during an active "spindle checkpoint".

Unravelling frog and human control of G2/M transition

Over 30 years ago, Yoshio Masui and Clement Markert (1971) published an historic paper describing cytoplasmic control over the behavior of nuclei of both meiotic and mitotic cells. This paper described an activity in the cytoplasm of eggs from the leopard frog *Rana pipiens* that was able to initiate oocyte maturation when injected into immature G2-arrested oocytes, an activity they termed maturation-promoting factor (MPF).

Since MPF activity is also present in mitotic cells, MPF has subsequently come to stand for M-phase promoting factor. The essential component of MPF is the cyclin B-Cdk1 complex. Cyclin B levels are regulated transcriptionally, at the level of mRNA stability and by proteolysis. Cyclin B is not detectable until the end of S-phase. Once the cyclin B-Cdk1 complex is formed, it is stabilized by phosphorylation by the Cdk-activating kinase (CAK), the activity of which appears constitutive throughout the cell cycle(Thuret, Valay et al. 1996). The major control on Cdk1 Cyclin B complex in the G2/M phase of the cell cycle is exerted by phosphorylation in the ATP cleft of Cdk1 on the thr14 and tyr 15 residues by Wee1 and Myt1 while its dephosphorylation and hence activation is achieved by the Cdc25 phosphatases(Krek, Marks et al. 1992). Premature entry into mitosis is prevented by keeping the cyclin B-Cdk1 complex in its inactive, phosphorylated state. This is accomplished by enhancing Wee1 activity or by inhibiting the Cdc25 phosphatase activity. Studies on the Cdc25 proteins from *Xenopus,* humans, and fission yeast have shown that Cdc25 is hyperphosphorylated during mitosis relative to during interphase, and that this leads to a significant enhancement of its phosphatase activity (Dunphy 1994)(Fig. 3). Similar studies on *Xenopus* and human Weel have

also uncovered mitotic hyperphosphorylation, in this case leading to an inhibition of its kinase activity (Hoffmann, Clarke et al. 1993; McGowan and Russell 1995; Mueller, Coleman et al. 1995; Watanabe, Broome et al. 1995). Cdc2 contributes to these hyperphosphorylations, but other kinases also play a role in Cdc25 and Wee1 phosphorylation.

In human three different genes code for cdc25 phosphatases respectively named cdc25A, B and C; the three different forms exert specific function during the cell cycle and Cdc25c is mainly implicated in controlling mitosis entry. The control of Cdk1 cyclin B through Cdc25c ad Wee1 is extremely important to the timely progression of the cell cycle, the inhibition of cdk activity during S- phase prevents the beginning of mitosis if the DNA replication is not completed or if DNA is damaged. Any of this two checkpoints(Dna damage or Dna replication) activates a complex response that culminates in the activation of two different kinases called Chk1 ad Chk2(Sancar, Lindsey-Boltz et al. 2004). Both Chk1 and Chk2 can phosphorylate Cdc25c on Ser287 (human Ser216), which creates a binding site for 14-3-3. The mechanisms by which phosphorylation of this residue and 14-3-3 binding suppress Cdc25c's ability to dephosphorylate cdc2, including changes in its subcellular localization in some types of cell cycles are still being worked out.

Kumagai *et al*. (1998b) were the first to suggest that phosphorylation of Cdc25c on Ser287 might also play a role in normal cell cycles. This idea was based on observations that a significant amount of endogenous cdc25C coprecipitated with 14-3-3 from unperturbed *Xenopus* egg interphase extracts and that association of 14-3-3 with recombinant Cdc25c protein was dependent on Cdc25c phosphorylation on Ser287. In addition to the checkpoint kinases, several others can phosphorylate Cdc25c on Ser287. Protein kinase A (PKA), a well-known inhibitor of the G2/meiosis I transition, helps to maintain *Xenopus* oocytes in their natural G2 arrest

through phosphorylation of cdc25C on Ser287 (Duckworth, Weaver et al. 2002; Schmitt and Nebreda 2002). In fertilized *Xenopus* eggs, calmodulin-dependent protein kinase II (CaMKII) seems to be responsible for the majority of Ser287 phosphorylation during interphase of the first mitotic cell cycle (Hutchins, Dikovskaya et al. 2003)

During mitosis, Cdc25c becomes phosphorylated at multiple sites by Cdc2, leading to the formation of hyperphosphorylated, electrophoretically retarded forms (Izumi, Walker et al. 1992; Kumagai and Dunphy 1992). The Cdc2- dependent mitotic phosphorylation of Cdc25c on Ser285 (human Ser214) seems to prevent rephosphorylation of Ser287 during mitosis, presumably strengthening the positive feedback loop (Bulavin, Demidenko et al. 2003; Bulavin, Higashimoto et al. 2003). On Wee1 the regulation appears to be very similar to that of cdc25; the Ser549 residue of Wee1 is phosphorylated during checkpoint arrest and the phosphorylation increases Wee1 activity (Stanford, Lieberman et al. 2003; Stanford and Ruderman 2005). Fig.3 shows a model of cdk activation during the transition from G2 to mitosis, based on the different phosphorylation state of Cdc25c and Wee1, the mechanism is quite different “in vivo” as many other factors contribute to cdk regulation for example the relative localization of Cdc25c or cdk1, or the phosphorylation of different residues on Cdc25c and Wee1.

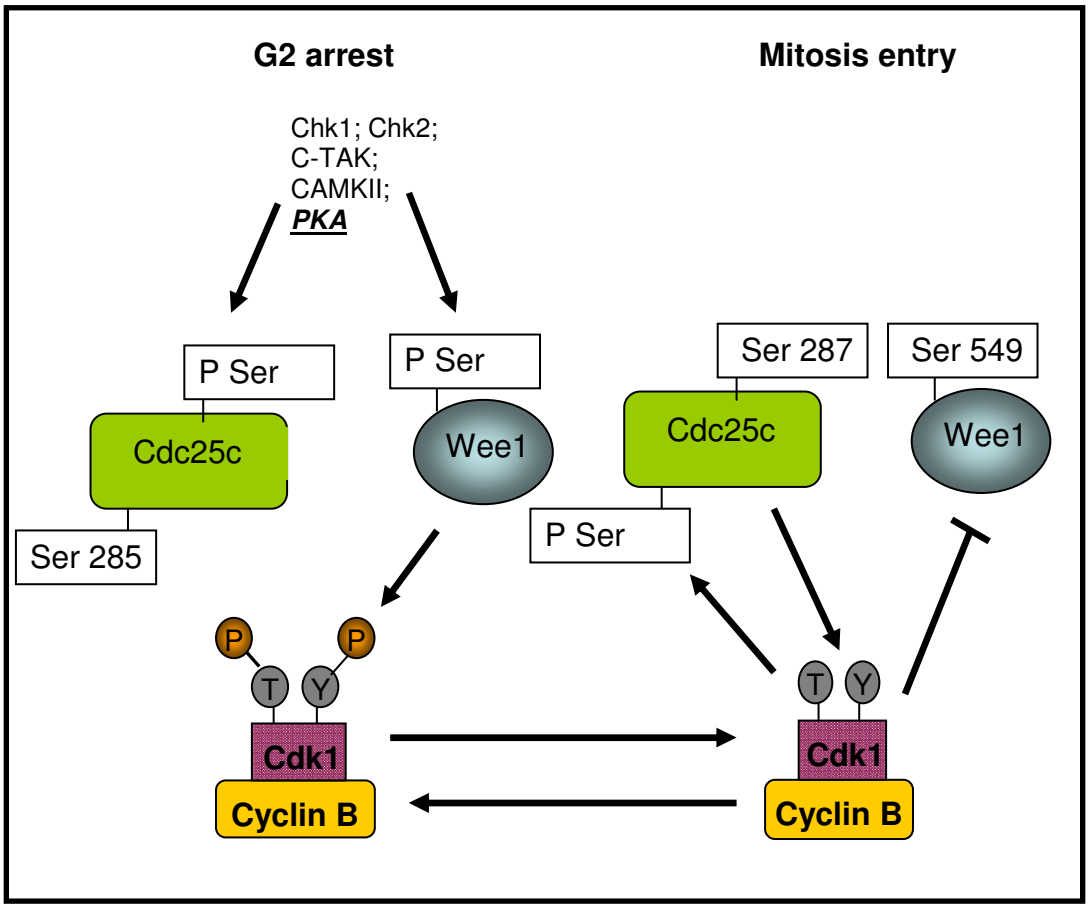

Fig.3 The picture shows the different phosphorilation state of cdc25c and Wee1 during g2 arrest and mitosis. During interphase cdc25c is inhibited and phosphorylated on the Ser285 residue (Ser216 in human) by different kinases, on the contrary wee1 is phosphorylated and activated, this keeps Cdk1/cyclin B in low activation state because maintain Tyr15 phosphorylation in the ATP cleft of Cdk1. The transition from G2 to mitosis is signed by an abrupt increase of cdk1 activity determined by the autoamplification loop exerted by the Cdk1/cyclin B complex, that phosphorylates cdc25c on Ser285 increasing its activity, hence providing an autoamplification loop required for cdk activation.

APC/C and SCF destruction in cell cycle control

Ubiquitin-dependent proteolysis ensures that specific protein functions are turned off at the right time, in the right place and in a unidirectional fashion. The high substrate specificity of the system is determined by a large family of ubiquitin ligases, which competes with the protein kinases to be the largest family of enzymes in mammals. Given the crucial function of the proteolytic machinery, altered degradation of cell cycle regulators contributes to the unchecked proliferation typical of cancer cells.

In order to keep a cell healthy, proteins need to be destroyed in a timely fashion. Regulated protein degradation offers the advantage of switching off a function in a specific subcellular compartment while leaving the others untouched, a task that the cell could not perform if protein abundance was regulated only by synthesis. In addition, proteolysis has the advantage of being fast, as a large number of different proteins can be eliminated in a matter of minutes. Finally, proteolysis is irreversible, an essential quality to permanently eliminate a protein in unidirectional processes such as cell cycle. Cells deploy the ubiquitin-proteasome system to degrade cellular regulatory proteins, thus monitoring their abundance. The targeting of a substrate to the 26S proteasome requires the covalent attachment of polyubiquitin chains to one or multiple lysine residues of the substrate. Upon association with the proteasome, the substrate is fed into the catalytic core where it is proteolyzed. Protein ubiquitinylation is a multistep process orchestrated by the concerted action of three enzymes: ubiquitin-activating enzyme (E1); ubiquitin-conjugating enzyme (Ubc or E2); and ubiquitin-protein ligase (E3). The latter recruits the target, guides the transfer of the ubiquitin from the Ubc to the substrate and allows for elongation of the ubiquitin chain (Hershko and Ciechanover 1998). Given the diversity of the target proteins, there is a correspondingly large number of E3s. Proteolysis of many core components of the cell cycle machinery is controlled by two major classes of ubiquitin ligases: the **SCF** (Skp1-Cul1-F-box protein + Roc1/Rbx1) complexes and the Anaphase Promoting Complex/Cyclosome (**APC/C**). Both complex share a similar core organization (the association of a cullin-like protein[Cdc53 in SCF and APC2 in APC] with a protein containing a particular zinc finger domain[Rbx1 in SCF and APC11 in APC]) and possibly a common origin (Vodermaier 2004). Despite these similarities they are regulated in different ways. The SCF complex is active throughout the cell cycle and

the destruction of its substrates depends on their phosphorylation, with different phosphate binding proteins (F box proteins) guiding different set of substrate destruction. The APC/C is activated at the onset of anaphase and degrades its substrate as the cell exit mitosis, two cofactor called Cdc20 and Cdh1 bind and activate the APC/C (see below for further details) .

In human, there are sixty-eight SCF ligases, each characterized by a different **F-box protein** subunit that provides specificity by directly recruiting the substrate to the rest of the ligase and, ultimately, to the ubiquitin conjugating enzyme (**UBC**)(Cardozo and Pagano 2004).

There is a high level of crosstalk between APC/C and different type of SCF and interdependency is required for the progression through the cell cycle. One example is SKP2 a leucine rich repeat F_box protein that target the cdk inhibitors $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$for degradation, thus promoting entry into S phase. The G1- S transition marks the commitment for another round of cell division and the level of Skp2 are tightly controlled trough autoubiquitination and ubiquitination by APC/C^{Cdh1}. The degradation of Skp2 during G1 keeps the level of inhibitors of Cdk high, thus preventing a premature entry into the cell cycle(Bashir, Dorrello et al. 2004).

The ubiquitin system is often the target of cancer-related deregulation and is involved in processes such as oncogenic transformation and tumor progression. Indeed, increased stability of positive regulators of proliferation (e.g. cyclins) can be achieved by lowering the activity or the levels of the specific enzymes necessary for their degradation. Thus, the ubiquitinating enzymes specific for proto-oncoproteins can act as tumor suppressors. Accordingly, other ubiquitinating enzymes could be oncogenic if their specific function is to target tumor suppressors (e.g., p53, pRb, p27). There are many established examples of both inactivation and

overactivation of the ubiquitin pathway (especially of ubiquitin ligases) in human tumors(Nakayama and Nakayama 2006).

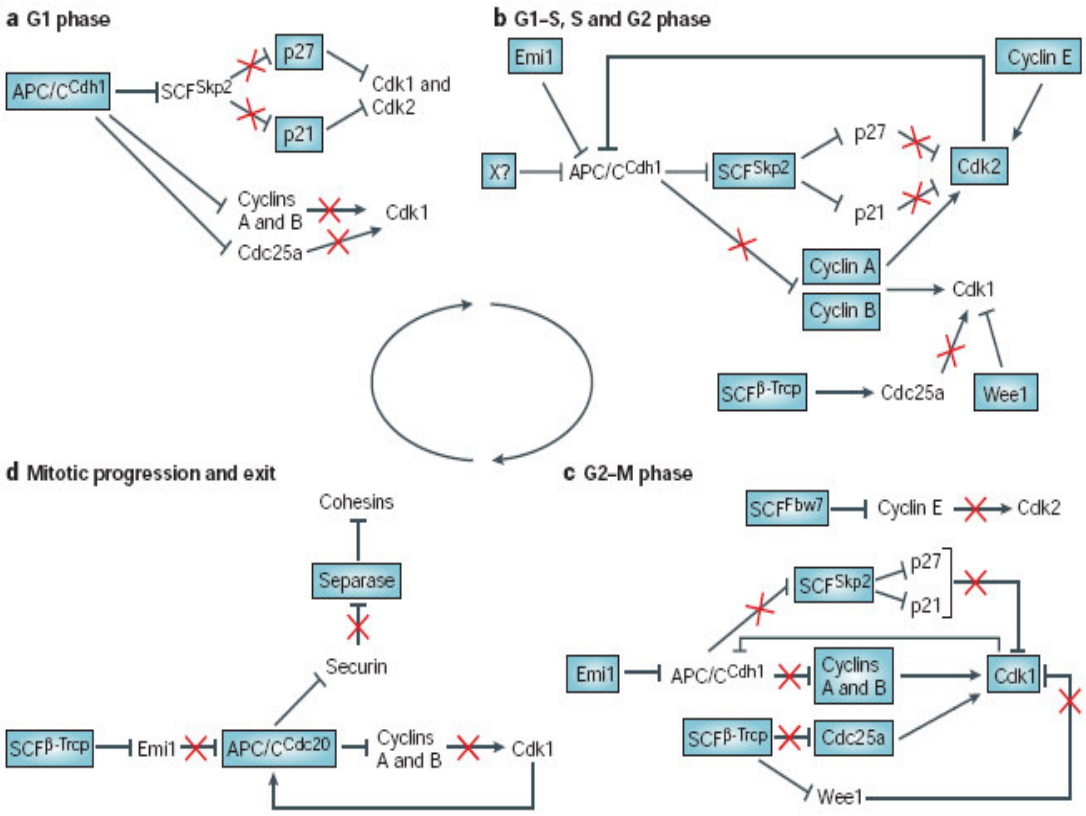

Fig.4 (kindly provided by Dr Pagano) the picture describes a simplified model emphasizing the contribution of ubiquitin ligases to the regulation of CDKs. There are, of course, crucial contributions from transcription, translation, translocation mechanisms, etc., which were omitted here to focus on how SCF and APC/C complexes work in concert to control and modulate CDK activity. In brief, SCF^{Skp2} is an activator of both Cdk1 and Cdk2; SCF^{Fbw7} is an inhibitor of Cdk2; and $SCF^{ß\text{-}Trcp}$ contributes to turning Cdk1 off during S and G2 phase, and turning it on at G2/M. With the exception of early M, APC/C is an inhibitor of CDKs. Notably, SCF ligases and APC/C reciprocally control each other: in G1, APC/C^{Cdh1} induces the degradation of Skp2, thereby maintaining the G1 state, and in early mitosis $SCF^{ß\text{-}Trcp}$ activates APC/C^{Cdc20} by inducing the degradation of Emi1. Thus, a picture emerges highlighting waves of different ubiquitin ligases that instantaneously modify components of the CDK regulatory network to maintain, activate or attenuate these drivers of the cell cycle. At the same time, these ubiquitin ligases are themselves subjected to direct or indirect control by CDKs.

Cyclin B proteolysis and mitosis exit

At the heart of cell cycle relies the APC/C that controls mitosis exit through the degradation of cyclin B and Securin. The degradation of Securin signs the beginning of anaphase. Securin inhibits the activity of the protease Separase, that cleaves the cohesin glue holding the sister chromatid together. When the APC/C is activated Securin disappears thus allowing the activation of Separase and sister chromatid separation. Also Cyc B is degraded upon metaphase –anaphase transition, thus allowing mitosis exit through the inactivation of the M–Cyclin wave. Cyclin B degradation is essential for sister chromatid separation, the reduction in Cdk activity is required to relieve the inhibitory phosphorylation by the latter on the Separase (Stemmann, Zou et al. 2001). During metaphase a complex signalling network called spindle checkpoint ensure that only when all chromosome are correctly aligned on the metaphase plate the APC/C is activated, and chromosomes are segregated to the daughter cells (Musacchio and Hardwick 2002). Fig.5 shows the different phases of mitosis highlighting the role of cdk1.

As cell proceed trough mitosis the APC/C is activated in two ways: its subunits are phosphorylated and its interaction with the activating protein Cdc20 increase. The phosphorylation of APC by cdk1 stimulates the activity of the latter towards the degradation of cyclin B while the interaction with Cdc20 is stimulated by a decrease in Cdk activity(Rudner, Hardwick et al. 2000; D'Angiolella, Mari et al. 2003). Thus the reduction in cdk activity is required to permit the activation of Separase and strengthen the interaction between Cdc20 and APC/C.

In human cells as cell leave mitosis, the APC/C remains active but switches from depending on Cdc20 to depending on a related protein (Hct1/Cdh1/

Fizzy related) for its activity. Cdh1 is inhibithed by Cdk1(Jaspersen et al.,1999; Zacharie et al.,1998), and it is unclear whether the APC/C must remain phosphoryated in order to remain active, and if so, which kinases modifies it. Finally, in late G1, the reactivation of Cdks leads to the phosphorylation and inactivation of Hct1, terminating the activity of the APC/C until cells reach mitosis.

Xenopus laevis relies only on the activity of Cdc20 during mitosis exit. Cdh1 is not expressed during the initial embryonic division. Furthermore, Cyclin B degradation during the early embryonic division is controlled by the activity of Protein Kinase A. PKA activity rises slightly after MPF inactivation and cyclin B degradation. By adding the regulatory subunits of PKA to cycling Xenopus laevis egg extract the degradation of cyclin B is prevented, while it can be reactivated with an excess of cAmp (Grieco, Porcellini et al. 1996). Many evidences suggested that PKA control Cdc25c and Wee1 by phosphorylation on Ser287(Ser216 in human) and Ser 549 respectively(see above for further details) (Grieco, Avvedimento et al. 1994; Grieco, Porcellini et al. 1996; Duckworth, Weaver et al. 2002; Stanford and Ruderman 2005). In lower eukaryotes like yeast the easiness of genetic manipulation has allowed a detailed dissection of the proteins implicated in mitosis exit. In fission yeast cdc25p (the homologue of the human Cdc25c) is inactivated during mitosis exit and this inactivation is required to control late mitotic events (Wolfe and Gould 2004). The observed phenomenon during mitosis exit may be a widely conserved mechanism that regulates the timing of Cyc B degradation and late mitotic events in higher eucariotes. It is interesting to note that in xenopus laevis egg extract the inhibitory phosphorylation on Ser549 of Wee1 increases during mid mitosis after Cdc2 inactivation, furthermore the addition of a non degradable form of Cyc B strongly stimulates Wee1 inhibitory phosphorylation on Ser549(Stanford and Ruderman 2005) . In the current

work we have investigated the role of Cdk1 inactivation (through tyr-15 phosphorylation) during mitosis exit and its effect on cyc B degradation. We hypothesize that PKA can contribute to MPF inactivation during mitosis exit through Wee1 or Cdc25c phosphorylation, thus allowing cyclin B degradation.

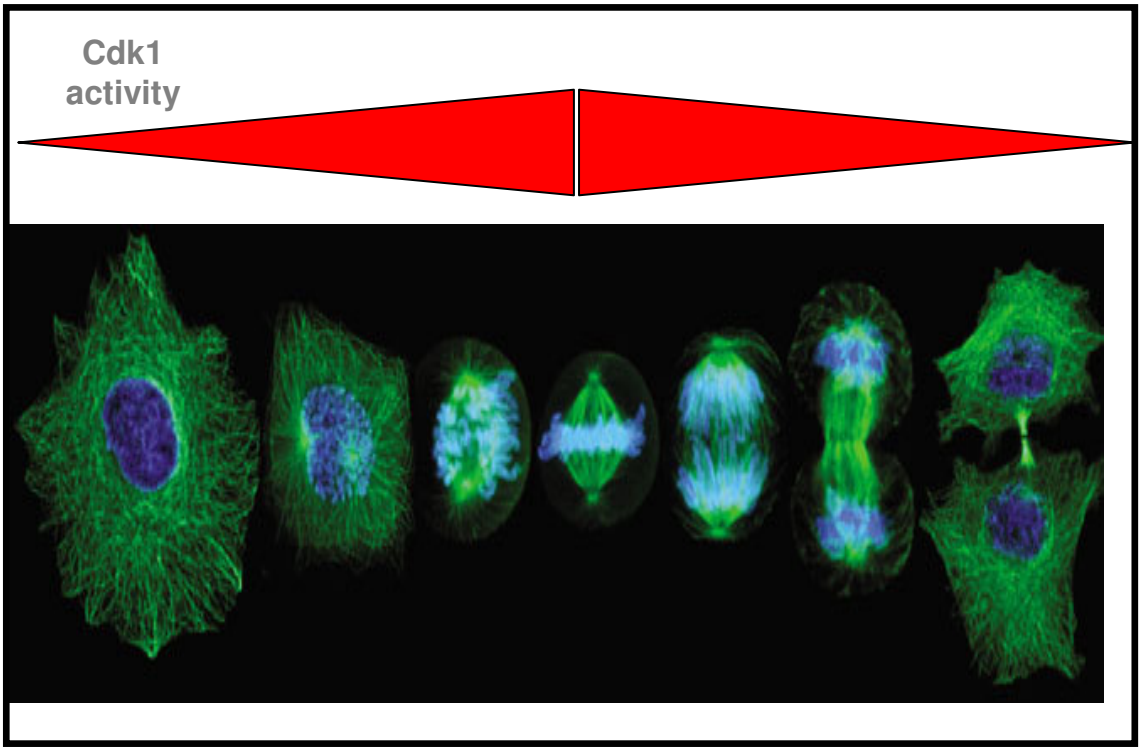

Fig.5 Mitosis is divided operationally into six phases. At prophase, chromosome condensation initiates, the duplicated centrosomes separate, and some mitotic checkpoint proteins are recruited to kinetochores. With nuclear envelope disassembly at entry to prometaphase , the chromosome spill into the cytoplasm, and the mitotic checkpoint is activated at every unattached kinetochore. Microtubule capture at both kinetochores of a duplicated chromatid pair result in checkpoint silencing and chromosome alignment to a midzone using a combination of microtubule-motor activities and microtubuledunamics. After capture and congression of the final sister chromatid pair (metaphase) and the turnover of the previously produced inhibitor that send a wait anaphase signal, anaphase is initiated. During anaphase A the duplicated chromosome pairs are pulled apart. Then, during the subsequent anaphase B; the spindle elongates ; further separating the sister chromatids; and invaginations of the plasma membrane aroud the spindle midzone become apparent. At the end of telophase, the chromatin decondenses and the nuclear envelope reforms, while cytokinesis is competed.

RESULTS

CDK1- tyr15 phosphorylation during mitosis exit

As discussed above M-phase exit relies on ubiquitin-dependent cyclin degradation initiated by activation of the ubiquitin ligase APC/C associated with its co-activator Cdc20 (APC/CCdc20)(King, Deshaies et al. 1996). Several APC/C subunits and Cdc20 undergo MPF dependent phosphorylation during M-phase(Peters 2002). Phosphorylation of APC/C stimulates its ubiquitin-ligase activity, however, phosphorylation of Cdc20 hampers binding to APC/C (Yudkovsky, Shteinberg et al. 2000; Peters 2002). Thus, MPF activity may play both positive and negative actions on APC/CCdc20 activation(Ciliberto, Lukacs et al. 2005). Evidence from Xenopus, yeast and human cell cycle systems, suggests that interruption of the MPF activation loop may also play a role for timing M-phase exit(Jin, Hardy et al. 1998; D'Angiolella, Costanzo et al. 2001; Tunquist and Maller 2003; Wolfe and Gould 2004). To date, however, no direct evidence that the MPF activation loop is interrupted at M-phase exit has been provided. We set out to gain insight into this matter using the *in vitro* cell cycle system derived from Xenopus eggs(Murray and Kirschner 1989). Cell cycle progression is marked by fluctuations in MPF activity and cyclin B concentration during incubation at 23° C of activated egg extracts. The cdk1 phopsho-tyr-15 content varied during cell cycle(Solomon, Glotzer et al. 1990) (Fig. 1a). In samples take at 10 min intervals, the cdk1-phopsho-tyr-15 signal decreased while MPF activity and cyclin content rose, reached

a minimum when cyclins were degraded and MPF inactivated and reappeared as cyclins begun re-accumulating (Fig. 1a). Cdk1-tyr-15 phopshorylation, hence, appeared to be progressively lost from the onset until the exit of M-phase. However, the analysis of samples taken at 2 min intervals revealed a strikingly different picture showing that cdk1 underwent tyr-15 phosphorylation twice during this period (Fig. 1b, c). After initial decrease, that accompanied MPF activation and reached a minimum at the MPF activity peak (Fig. 1 b, c; 26-28 min), the cdk1-phopsho-tyr-15 signal abruptly and transiently reappeared just prior to substantial cyclin degradation (Fig. 1b, c; 30, 32 min). Thereafter, when cyclin degradation was achieved, the signal rapidly decreased (Fig. 1b, c; 34, 36 min). The changes in cdk1-tyr-15 phosphorylation were also confirmed by detection of cdk1 in anti cdk1-tyr-15 immunoprecipitates (Fig. 1d). We, next, determined the amount of cyclin and of cyclin-associated cdk1, cdk1-phospho-tyr-15 and kinase activity in cyclin B1 and cyclin A immunoprecipitates from extracts aliquots taken across the mitosis-interphase transition. The cyclin B1-associated kinase activity decreased ahead of the cyclin B1 content (Fig. 1e, f). The decline in activity was accompanied by an increase in cyclin B1-associated cdk1-phospho-tyr-15 signal (Fig. 1h). Cyclin A-associated kinase activity and cyclin A content decreased with parallel kinetics and begun by the time cyclin B1-associated kinase activity started to decline (Fig. 1e, g). The cdk1-phospho-tyr15 content in cyclin A immunoprecipitates was undetectable (Fig. 1h).

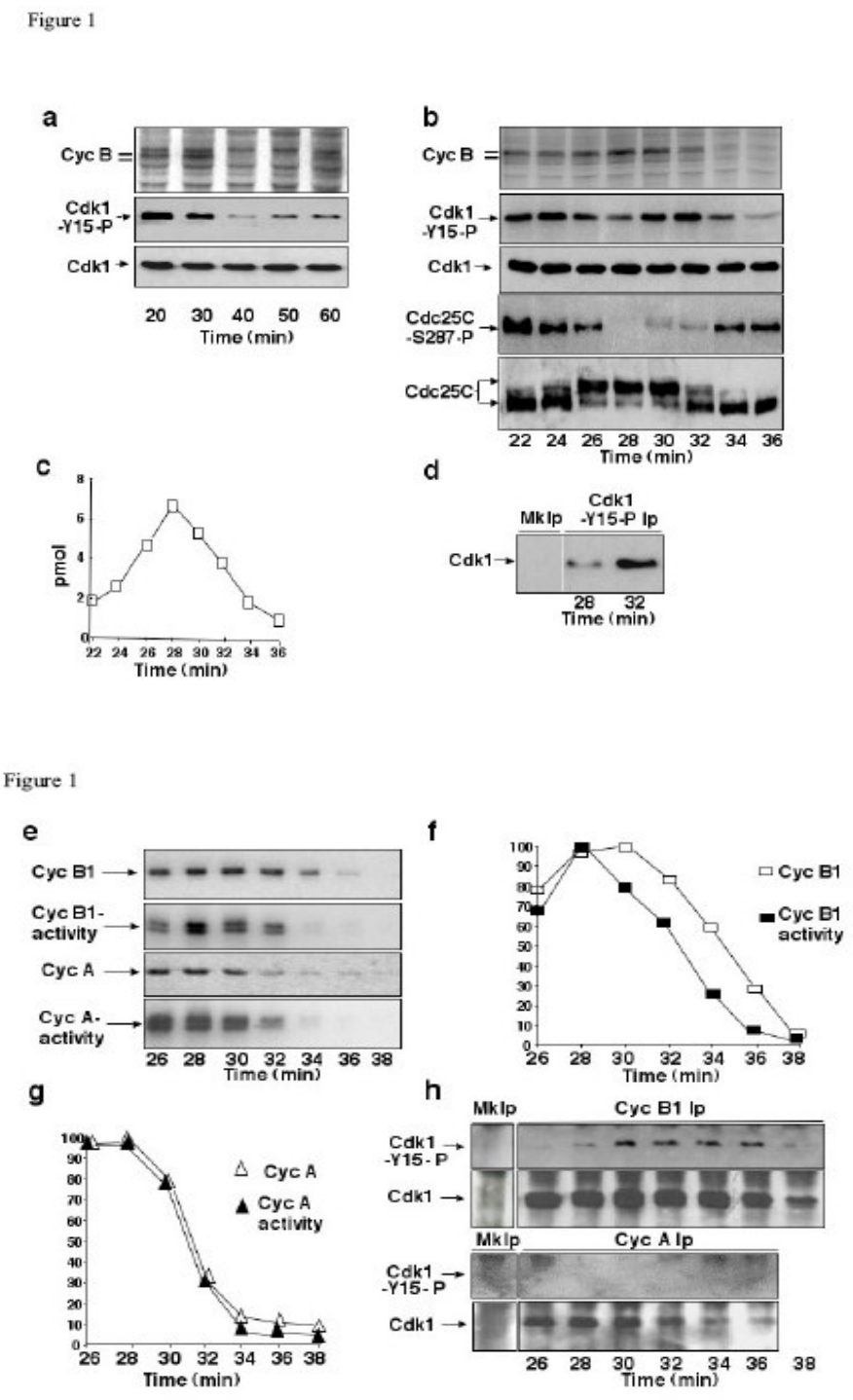

Figure 1 Cdk1-tyr-15 phosphorylation in cycling extracts. A cycling extract was incubated at 23 °C in the presence of [S^{35}]methionine. **a,** cyclin B abundance (detected by an autoradiograph of [S^{35}]labelled extracts proteins; the position of cyclin B is indicated), cdk1-phospho-tyr15 (Cdk1-Y15-P) and cdk1 (Cdk1) contents (detected by immunoblot) from samples taken at 10 min intervals during incubation. **b,** cyclin B abundance (the position of cyclin B is indicated), cdk1-phospho-tyr15 (Cdk1-Y15-P), cdk1 (Cdk1), cdc25c-phospho-ser-287 (Cdc25C-S287-P) and cdc25c (Cdc25C) contents (detected by immunoblot) from samples of a cycling extract taken at 2 min intervals during incubation at 23 oC, across the mitosis-interphase transition. **c**, histone H1 kinase activity in total extract from the samples described in **b. d,** cdk1 content in anti cdk1-phospho-tyr-15 immunoprecipitates from samples taken at the indicated time points from the extract described in **b**. **e,** cyclin B1 (Cyc B1), cyclin B1-associated kinase activity (an autoradiograph of phosphorylated histone H1; Cyc B1-activity), cyclin A (Cyc A) and cyclin A-associated kinase activity (an autoradiograph of phosphorylated histone H1; Cyc A-activity) in cyclin B1 and cyclin A immunoprecipitations from samples of the same extract described in **b**. **f,** quantization, expressed as percent of peak value, of cyclin B1 content (open squares), cyclin B1-associated kinase activity (filled squares), and, **g,** of cyclin A content (open triangles) and cyclin A-associated kinase activity (filled triangles), from immunoprecipitations described in **e**. **h,** cdk1-phospho-tyr-15 and cdk1 contents in the immunoprecipitates described in **e** (Cyc B1 Ip and Cyc A Ip).

Cdk1-tyr-15 phosphorylation in CSF-arrested extracts.

We determined whether similar changes in MPF activity, cdk1 phosphorylation and cyclin content were also found at meiotic metaphase II exit. Extracts derived from non-activated eggs preserve the calcium-sensitive Cytostatic Factor (CSF) activity that maintains metaphase II arrest(Tunquist and Maller 2003). In these extracts CSF is inactivated upon CaCl2 addition. Fig. 2a shows that, also during exit from metaphase II, cdk1 underwent a wave of tyr-15 phosphorylation that was maximal shortly after CaCl2 addition and decreased as cyclin degradation proceeded. Determination of the amount of cyclin B1 and of cyclin B1-associated cdk1, cdk1-phospho-tyr-15 and kinase activity in cyclin B1 immunoprecipitates showed that MPF activity loss coincided with increased cdk1-tyr-15 phosphorylation rather than cyclin degradation (Fig. 2b, c). These data suggest that the MPF activation loop must be interrupted at M-phase exit. Cdc25C inactivation correlates with its dephosphorylation at several, MPF-dependent, phosphorylayion sites and phosphorylation at the inhibitory site ser-287 (Hoffmann, Clarke et al. 1993; Hutchins, Dikovskaya et al. 2003). We analysed aliquots of cycling and CSF-arrested extracts for the presence of cdc25C-ser-287 phosphorylation. Cdc25C-ser-287 and cdk1-tyr-15 phosphorylations inversely correlated with MPF activity and ensued when MPF activity begun declining (Figs, 1b, c; 2a, c). Cdc25C ser-287 phosphorylation continued to accumulate as cell cycle progressed towards interphase, while the cdk1 phospho-tyr-15 signal was lost along with the cyclin signal (Figs. 1b; 2a). Cdc25C also underwent a shift in migration on SDS/PAGE indicative of loss of multiple activating

phosphorylations. Thus, interruption of the MPF-cdc25C activation loop marks M-phase exit.

Figure 2

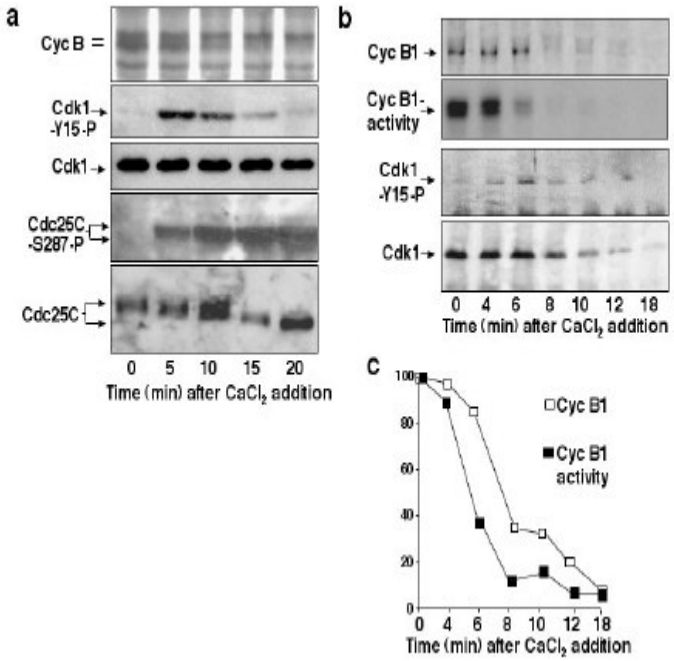

Figure 2 Cdk1-tyr-15 phosphorylation in CSF-arrested extracts. A CSF-arrested extract was pre incubated with[S^{35}]methionine to label extracts proteins. **a**, cyclin B abundance (the position of cyclin B is indicated), cdk1 phospho-tyr15(Cdk1-Y15 P), cdk1 (Cdk1) , cdc25c-phospho-ser-287 (Cdc25C-S287-P) and cdc25c (Cdc25C) contents (detected by immunoblot) from samples of a CSF-arrested extract taken at the indicated time points during incubation at 23 oC after CaCl2 addition. **b**, cyclin B1 (Cyc B1), cyclin B1-associated kinase activity (Cyc B1-activity) and cdk1-phosphotyr15 (Cdk1-Y15-P) and cdk1 (Cdk1) contents in cyclin B1 immunoprecipitations from samples of a CSF-arrested extract taken at the indicated time points after CaCl2
addition. **c**, quantisation, expressed as percent of peak value, of cyclin B1 content(open squares), cyclin B1-associated kinase activity (filled squares) from immunoprecipitations described in **b.**

Preventing MPF activation loop interruption inhibits Cdc20-APC/C interaction in cycling extracts

Relevant for direct cdc25C-ser-287 phosphorylation, during unperturbed extract's cell cycle, appears the regulated activities of CaMKII and PKA, CaMKII for the first mitotic interphase following CSF inactivation, while PKA for subsequent mitotic cycles(Grieco, Avvedimento et al. 1994; Grieco, Porcellini et al. 1996; Duckworth, Weaver et al. 2002; Hutchins, Dikovskaya et al. 2003). It has been shown that PKA also stimulates a phosphatase activity, in egg extracts, that removes multiple activating

cdc25C phosphorylations(Grieco, Avvedimento et al. 1994). In addition, blocking PKA fluctuations prevents the mitosis-interphase transition while blocking CaMKII activation prevents the metaphase II-first interphase transition upon egg activation(Lorca, Cruzalegui et al. 1993; Grieco, Porcellini et al. 1996). We set out to determine the role for these kinases in the interruption of the MPF activation loop at M-phase exit. In cycling extract, PKA inhibition (by addition of excess recombinant, glutathione-S-tranferase (GST)-fused, PKA regulatory subunit RIIb; GST-RII;) prevented cdc25C-ser-287 and cdk1-tyr-15 phosphorylations while cdc25c remained in its mitotic hyperphosphorylated state and cyclin stable (Fig. 3a, b). Reactivation of PKA was shortly followed by loss of multiple cdc25C activating phosphorylations, as indicated by appearance of faster migrating cdc25C forms, reappearance of cdc25C-ser-287, cdk1-tyr-15 phosphorylations and cyclin degradation (Fig. 3a; - + cAMP; and supplementary Fig. S1). Thus, PKA inhibition prevented interruption of the MPF activation loop and blocked mitosis exit. To determine whether the block to mitosis exit was due to sustained MPF activity, we lowered cdk1 activity by adding the cdk inhibitor roscovitine in PKA-inhibited extracts. Cdk inhibition rapidly restored cyclin degradation (Fig. 3b). Thus, interruption of the MPF activation loop appears the crucial function for PKA in mitosis exit. It is worth to note that also the kinase that phosphorylates cdk1-tyr-15, wee1, has been recently shown to undergo activating, PKA-inducible, phosphorylation just around the time of cyclin degradation(Stanford and Ruderman 2005). We asked why interruption of the MPF activation loop would be required for cyclin degradation. In this system, degradation tightly relies on binding of APC/C to its coactivator Cdc20 (Lorca, Castro et al. 1998; Peters 2002). We immunoprecipitated Cdc27, an APC/C subunit, and determined the amount of bound Cdc20 from control extract samples, taken around the time of MPF inactivation,

and from PKA-inhibited extract samples, taken at the same time points. In the control extract, very little Cdc20 co-precipitated with Cdc27 when MPF was at peak activity (Fig. 3 c). Binding increased during the time of MPF inactivation and was lost thereafter (Fig. 3 c). In the PKA-inhibited extract, APC/Ccdc20 binding was always poorly detectable (Fig. 3 b). APC/C-cdc20 interaction was, however, restored in the PKA-inhibited extract after roscovitine addition (Fig. 3 c). These data indicate that interruption of the MPF activation loop is required for binding of APC/C to Cdc20. It is worth to note that it has been recently published that cycling extracts forced to use a mutant cdk1AF, refractory to inhibitory phosphorylation, enter mitosis, albeit with different kinetics than control extracts, but fail to activate the cyclin degradation pathway(Pomerening, Kim et al. 2005).

Figure 3

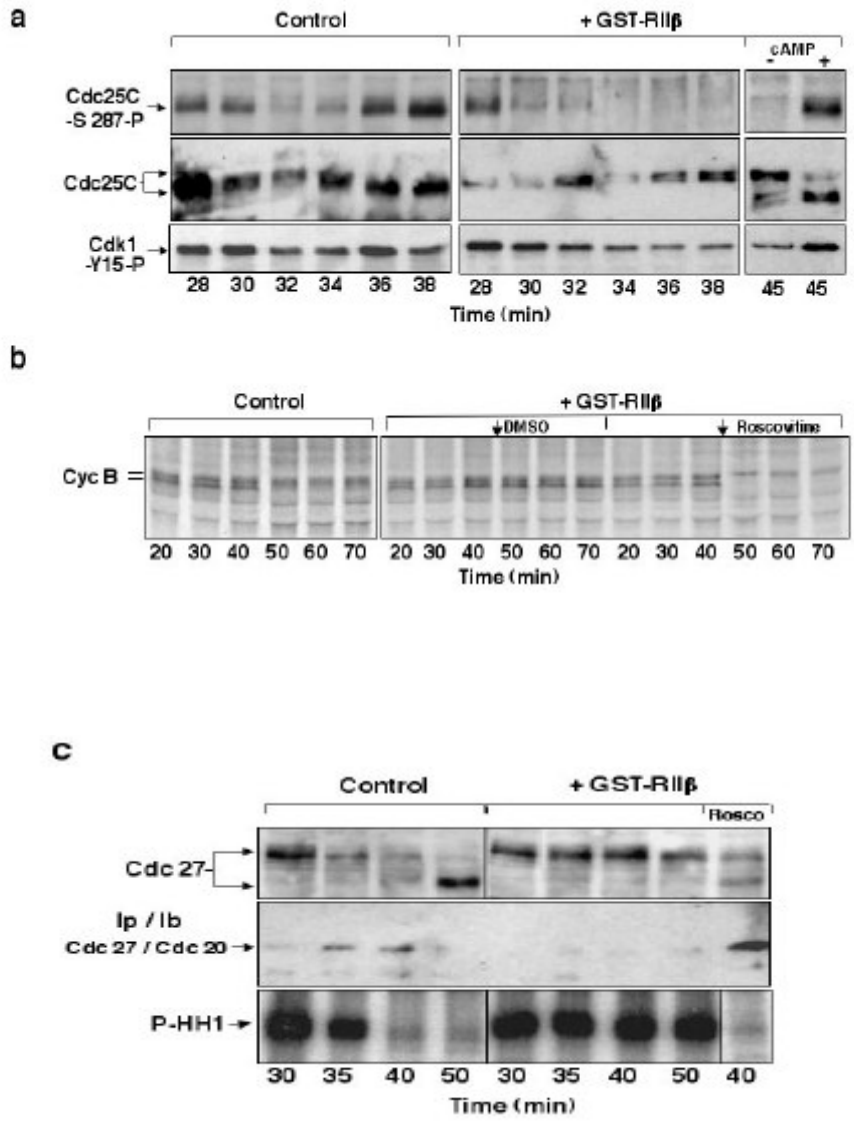

Figure 3 Preventing MPF activation loop interruption inhibits Cdc20-APC/C interaction in cycling extracts. **a**, portions of a cycling extract were treated with GST, as control (Control), and with GST-RIIb (+ GST-RIIb) to inhibit PKA. Samples were taken at the indicated time points during incubation at 23° C. To portion of the GST-RIIb, cAMP (4 mM) was added at 40 min of incubation, to reactivate PKA, and samples taken at 45 min (- + cAMP; 4 mM, 1/30 extract volume). The Cdc25c phospho-ser-287 (Cdc25C-S287-P), cdc25c (Cdc25C) and cdk1-phospho-tyr15 (Cdk1-Y15-P), contents were determined by immunoblot. **b**, cyclin B stability (the position of cyclin B is indicated) during incubation at 23° C in samples, taken at the indicated time points, from a control extract and portions of the same extract that weretreated with GST-RIIb and to which either roscovitine (2 mM; 1/30 extract volume), to inhibit cdk activity, or DMSO (1/30 extract volume), as control, were added. **c**, total Cdc27, cdc20 bound to Cdc27 (IpCdc27/IbCdc20) and total histone H1 kinase activity (detected by phsophorylated histone H1 autoradiograph; P-HH1) from samples of control, PKA-inhibited (+ GST-RIIb) and PKA-inhibited (+ GST-RIIb) plus roscovitine (added at 32 min; Rosco) extract portions, taken at the indicated time points during incubation at 23° C.

MPF activity inhibits APC/CCdc20 activation through cdc20 phopshorylation.

Interruption of the MPF activation loop is required also for metaphase II exit. In CSF-arrested extracts, cdc25C ser-287 and cdk1-tyr-15 phosphorylations were induced very rapidly after calcium addition (Fig. 4a). Cdc25C-ser-287 and cdk1-tyr-15 phosphorylations, as well as cyclin degradation, were prevented by treatment with the CaMKII inhibitor peptide CaMKII Ntide (Fig. 4a, b). However, lowering cdk1 activity with roscovitine was unable to restore cyclin degradation in CaMKII-inhibited extracts (Fig. 3b). Thus, in the absence of other CaMKII-dependent events, loss of MPF activity is insufficient to activate cyclin degradation in CSF-arrested extracts, in agreement with recently published observations(Liu and Maller 2005; Rauh, Schmidt et al. 2005). Nevertheless, if interruption of the MPF activation loop was required, although not sufficient, for cyclin degradation, supplying CSF-arrested extracts with a mutant cyclin B-cdk1 complex, resistant to inhibitory phosphorylation (cdk1AF; thr 14 > ala, and tyr 15 > phe), should affect cyclin degradation upon calcium addition. We tested this hypothesis by treating CSF-arrested extract portions with recombinant human cyclin B1-cdk1 (cyclin B1-cdk1wt) or cyclin B1-cdk1AF. Fig 4c shows that cyclin degradation was significantly delayed in the cyclin B1-cdk1AF-treated compared with the cyclin B1-cdk1-treated extract. Cyclin B1-cdk1wt treatment did not significantly affect cyclin degradation relatively to untreated extracts (possibly it induced a slight acceleration; supplementary Fig. S2). Cyclin degradation was, however, restored in the cyclin B1-cdk1AF-treated extract by lowering cdk activity

with roscovitine (Fig. 4c). CSF-arrested extracts already contained some APC/C-Cdc20 complex, however, APC/C-Cdc20 interaction was significantly stimulated shortly after calcium addition (supplementary Fig. S3). Treatment with cyclin B1-cdk1AF, but not with cyclin B1-cdk1wt, significantly inhibited induction of APC/C-cdc20 interaction after calcium addition (Fig. 4d). In addition, in the absence of calcium, the sole addition of roscovitine was able to strongly stimulate APC/C-Cdc20 binding (Fig 4e).

APC/C and Cdc20 undergo phosphorylation in M-phase. By *in vitro* studies, Hershko and co-workers have shown that cdk-dependent phosphorylation of Cdc20 inhibits binding to APC/C, thus blocking APC/CCdc20 activation(Yudkovsky, Shteinberg et al. 2000). We asked whether Cdc20 phosphorylation affected binding to APC/C in egg extracts. CSF extracts were incubated with *in vitro* translated wild type human Cdc20 and a mutant Cdc20 version, non-phosphorylatable at 7 cdk1 consensus sites, for 40 min, to induce Cdc20 phosphorylation (that caused retarded mobility on SDS/PAGE; Fig. 4f). After incubation, more phosphorylation-resistant mutant Cdc20 than wild type Cdc20 could be recovered in Cdc27 immunoprecipitates (Fig. 4 f), indicating that Cdc20 phosphorylation hampered APC/C-Cdc20 interaction.

Figure 4

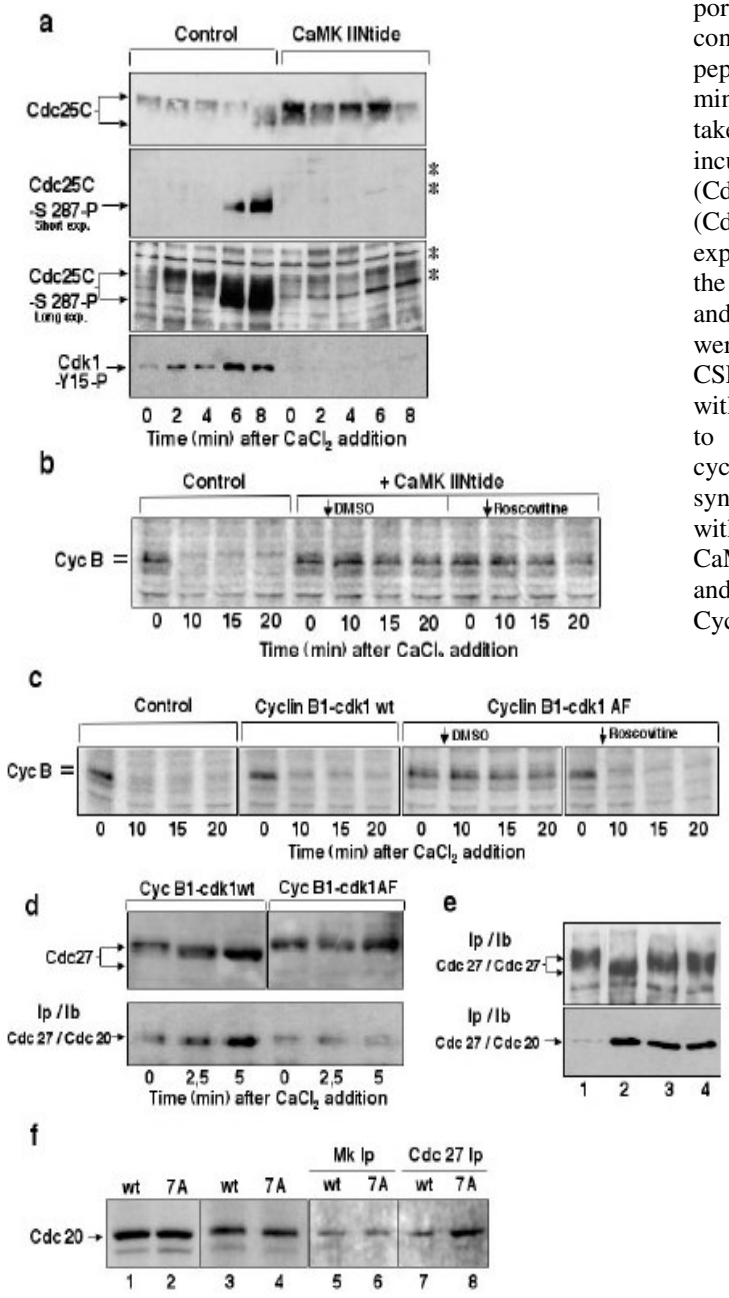

Figure 4 MPF activity inhibits APC/CCdc20 activation through cdc20 phopshorylation. a, CSF-arrested extract portions were pre-incubated with buffer, as control, or with the CaMKII inhbitor peptide CaMKII Ntide (0.5 mM) for 20 min at 23° C. Then, extract samples were taken at the indicated time points of further incubation after CaCl2 addition. Cdc25c (Cdc25C), cdc25c-phospho-ser-287 (Cdc25C-S287-P; short and long exposures of the immunoblot are shown; the asterisks mark non-specific signals) and cdk1-phospho-tyr15 (Cdk1-Y15-P), were visualised by immunoblotting. **b** , a CSF-arrested extract was pre-incubated with [S^{35}]methionine for 30 min at 30° C to label extracts proteins. Then, cycloheximide was added to block protein synthesis, and extract portions were treated with buffer, as control, or with the CaMKII inhbitor peptide CaMKII Ntide and further incubated for 20 min at 23° C. Cyclin B stability is shown by an autoradiograph of [S^{35}]labeled extracts proteins (the position of cyclin B is indicated) in total extracts samples, taken at the indicated time points after CaCl2 addition. To portions of the CaMKII Ntide treated extract either roscovitine (2 mM; 1/30 extract volume), or DMSO (1/30 extract volume) as control, were added 1 min after CaCl2. **c**, a CSF-arrested extract was preincubated with [S]methionine for 30 min at 30° C to label extracts proteins. Then, Cloheximide was added to block protein synthesis, and extract portions were treated wth buffer, as control, recombinant cyclin B1-cdk1wt and cyclin B1-AF complexes nd incubation prolonged for further 20 min. Cyclin B stability is shown by an utoradiograph of [S^{35}] labelled extracts proteins (the position of cyclin B is indicated)in total extracts samples, taken at the indicated time points after CaCl2 addition. To portions of the cyclin B1-AF -treated extract, either roscovitine (2 mM; 1/30 extract volume), or DMSO (1/30 extract volume) as control, were added 1 min after CaCl2. **d**,total Cdc27 and Cdc20 bound to Cdc27 (IpCdc27/IbCdc20) from CSF-arrested samples, pre-incubated for 20 min at 23° C with recombinant cyclin B1-cdk1wt and cyclin B1-AF complexes, taken at the indicated time points after CaCl2 addition (the time 0 sample received no CaCl2). **e**, Cdc27 (IpCdc27/IbCdc27) and cdc20 bound to Cdc27 (IpCdc27/IbCdc20) in Cdc27 immunoprecipitates from CSF-arrested samples taken 5 min after the following additions, lane 1, DMSO, lane 2, 80 mM roscovitine, lane 3, 40 mM roscovitine, lane 4, 20 mM roscovitine (each addition = 1/30 extract volume). **f**, [S^{35}]labelled Cdc20 wild type (wt) and a 7 phosphorylation sites mutant version (7A) were produced in reticulocyte lysates (lanes 1, 2). Labeled proteins were incubated with portions of a CSF-arrested extract containing cycloheximide for 30 min at 23 °C (lanes 3, 4). Cdc27 was, then, immunoprecipitated (Cdc 27 Ip) and the amount of bound wt (lane 7) and 7A (lane 8) Cdc20 detected by autoradiography. Lanes 5, 6, mock immunoprecipitations (Mk Ip).

Wee1 and cdk1 physically associate in M-phase.

By our analysis it cannot be excluded that the pathways leading to MPF inactivation via inhibitory phosphorylation take the upper hand only after initial degradation of small cyclin B amounts. In addition, the fact that cyclin degradation is activated when cdk1 activity is sustained by non-degradable cyclin B suggests that a reduction in MPF activity is not absolutely required to initiate cyclin degradation. To gain further insight, we assayed cdk1 activity, in cdk1 immunoprecipitates from samples taken at 5 min intervals from the time of recombinant non-degradable sea urchin cyclin B (DB; at 100 nM) addition to interphase extracts. After the already described lag of 10-20 min (Solomon, Glotzer et al. 1990), we found that cdk1 activity did not increase continuously during incubation (Fig. 5a). Shortly after reaching M-phase activity levels (Fig. 5a; around 35-40 min after DB addition), activity underwent fluctuations with decrements up to 20-25% relatively to M-phase activity (Fig. 5a; 40 to 60 min). Later, it further increased with relatively steady increments (Fig. 5a; from 60 min on). As previously demonstrated (Solomon, Glotzer et al. 1990), cdk1-tyr-15 phosphorylation was markedly stimulated shortly after DB addition and decreased as cdk1 activity reached M-phase activity levels (Fig. 5b, 10 to 40 min). However, the subsequent decrease in cdk1 activity was accompanied by a relative increase in cdk1-tyr-15 phosphorylation (albeit lower than that observed at early time points; Fig. 5b, 45-50-55 min). Cdc25C has been reported to be hyperphosphorylated at activating sites and non-phosphorylated at ser287 (Stanford and Ruderman 2005) under DB-induced M-phase arrest, making unlikely its involvement in the control of cdk1 phosphorylation under these conditions. Wee1 activity is believed

to be down-regulated in M-phase by cdk1-dependent phosphorylations . Recent evidence indicated that wee1 undergoes phosphorylation at ser-549 in M-phase of unperturbed cell cycle as well as under DB conditions (Stanford and Ruderman 2005). However, by *in vitro* kinase assays, the authors showed that wee1 ser-549 phosphorylation did not increase wee1 activity and that wee1 isolated from interphase was slightly more active than that isolated from M-phase. What mechanism could, then, lead to mitotic cdk1-tyr-15 phosphorylation under DB conditions? We investigated whether wee1 and MPF could physically interact in M phase, as recently shown for the budding yeast wee1 homolog swe1 (Harvey, Charlet et al. 2005), providing a possible mechanism for mitotic cdk1-tyr-15 phosphorylation. Indeed, cdk1 could be detected in wee1 immunoprecipitates from CSF-arrested extracts, while very little cdk1 co-precipitated with wee1 from interphase samples (Fig 5c). Analysis of wee1-cdk1 interaction under DB conditions revealed that it was easily detectable during the first 10-20 min after DB addition to an interphase extract, when cdk1 activity was still low (Fig. 5c). During the subsequent cdk1 activation period the complex significantly dissociated (Fig. 5c; 20-35 min). After cdk1 regained M-phase activity levels and then started to fluctuate, wee1-cdk1 interaction was regained (Fig. 5c; 35-45 min). Analysis of phospho-tyr-15 content in wee1-bound cdk1 showed that cdk1-phospho-tyr-15 signal was detectable at early (interphase; Fig. 5d; 10 min) and, more pronouncedly, at later (M-phase; Fig. 5d; 40-45 min) time points after DB addition, further indicating that wee1-cdk1 interaction is accompanied by cdk1-tyr-15 phosphorylation. Upon prolonged incubation, when cdk1 activity further increased, cdk1 appeared to dissociate from wee1 (Fig. 5d; 90 min). Under our experimental conditions, degradation of full-length cyclin initiated 40-45 min from addition of nondegradable cyclin B (Fig. 5e). To determine whether DB-cdk1 activity was affecting the timing of

cyclin degradation, we inhibited cdk1 activity after significant cdk1 activity had been reached following DB addition and monitored full-length cyclin B degradation. Cdk1 inhibition slightly but consistently accelerated initiation of degradation (Figs. 5e, f). We asked whether wee1-cdk1 interaction was detected during unperturbed M-phase exit in cycling extracts. Fig. 5g shows that across the mitosis-to-interphase transition, wee1 interacted with cdk1. Substantial interaction was detected at the peak of MPF activity and shortly after. Soon after cyclin B1 was degraded, wee1-cdk1 interaction was still maintained (Fig. 4d; 50 min) to be lost thereafter, possibly after significant wee1 dephosphorylation. Tyr-15-phosphorylated cdk1 was also detected in wee1 immunoprecipitates, increasing as degradation of cyclin proceeded and disappearing thereafter (Fig. 4d; 45-50 min). Although we know that PKA inhibition delays cyclin degradation under DB conditions, further investigation will be required to establish the role for phosphorylations in the wee1-cdk1 interaction and in wee1 activity towards wee1-bound cdk1, the domains involved in binding as well as other potential other M-phase aspects for which interaction is relevant. Our data show that timely execution of M-phase exit in a vertebrate system relies on a proteolysis-independent control over MPF activity.

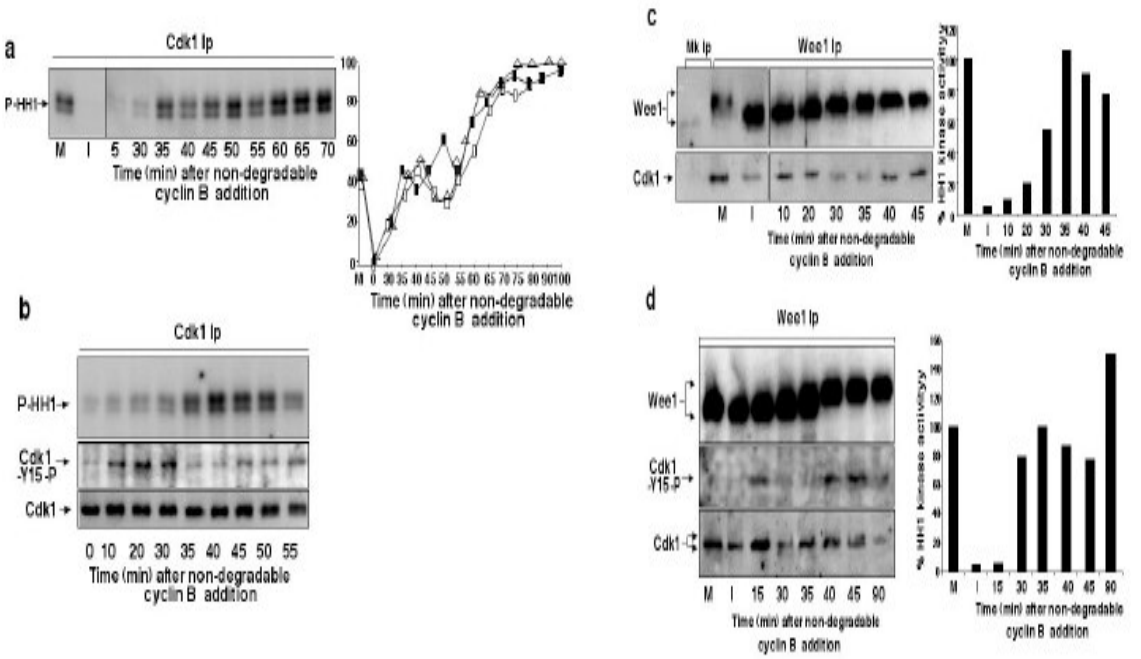

Figure 5 Wee1 and cdk1 physically associate in M-phase. a, histone H1 kinase activity in cdk1 Ips from samples of a CSF-arrested extract (M), an interphase extract (I; 40 min after CaCl2 and CHX additions) and from samples taken at the indicated time points after sea urchin DB (150nM) addition to an interphase extract. Left panel, shown is an autoradiograph of phosphorylated histone H1 (P-HH1)**.** Right panel, quantisation of cdk1 activity, expressed as percent of maximum, from three similar but independent experiments. **b**, Cdk1 activity, cdk1-phospho-tyr15 and cdk1 content in cdk1 Ips from samples taken at the indicated time points after DB addition to an interphase extract**. c, d,** wee1 and cdk1 were immunoprecipitated from samples of CSF-arrested (M), interphase (I) extracts and samples taken at the indicated time points after DB addition to an interphase extract. Left panels, wee1 and wee1-associated cdk1 in wee1 Ips. Right panel, quantisation of histone H1 kinase activity, expressed as percent of maximum, in cdk1 Ips. In **d**, was also detected the cdk1-phospho-tyr15 content in wee1 Ips. **e, f,** Full-length, [S^{35}]labelled, xenopus cyclin B1 was added to two independent interphase extracts along with DB. The extracts were spilt into two portions and DMSO, as control, or roscovitine (10 mM) were added after 33 min incubation, samples were, then, taken at the indicated time points after DB addition. Shown are autoradiographs and quantisations, as percent, of remaining [S^{35}]labelled, full-length, cyclin B1. **g**, Cyclin B1 content (by immunoblot) and cyclin B1 associated activity from a cycling extract across the mitosis-to interphase transition. From the same samples, wee1 was immunoprecipitated (Wee1 Ip) and wee1, wee1-associated cdk1-phospho-tyr15 and cdk1 were visualised by immunobloting

Discussion

In the current work we show that, at M-phase exit, cyclin degradation requires loss of MPF activity by inhibitory cdk1 phosphorylation. Phosphorylation depends on activation of pathways, like those that relay on CaMKII and PKA activities, that antagonize the MPF activation loop. Nevertheless, M-phase exit strictly relies on proteolysis(King, Deshaies et al. 1996). Thus, the action of inhibitory pathways is insufficient to lower MPF activity to the levels required to exit M-phase if proteolysis cannot take over. We propose that proteolysis and inhibitory cdk1 phosphorylation are interconnected in a MPF inactivation loop for M-phase exit. Initial, phosphorylation-dependent, loss of MPF activity stimulates APC/CCdc20 activation. MPF degradation further weakens the activation loop promoting inhibitory phosphorylation. Consequent additional MPF activity loss further helps APC/CCdc20 activation. We cannot exclude that degradation of a small pool of cyclin initiates the loop. However, full activation of the degradation pathway requires further reduction of MPF activity by inhibitory phosphorylation, otherwise M-phase exit is prevented.

The inhibitory phosphorylation on cdk1 can take place by the interaction of cdk1 with wee1 during mitosis it is interesting to note that the same mechanism is functional in yeast where cdk1 form a complex with wee1 thus blocking the autoamplification loop.

During initial embryonic division the dynamic of Xenopus laevis system ensure the rapid oscillation of MPF. At the onset of mitosis MPF phosphorylates cdc20 thus preventing cyc B degradation. Slowly, APC phosphorylated by cdk1 builds up until it overcomes phosphorylated Cdc20, APC/C Cdc20 is formed, cyclin B is degraded.

Initial loss of cdk1 activity through degradation is rapidly amplified by MPF inactivation that further increases the pool of active and dephosphorylated cdc20.

We have initial evidences that the same mechanism of cdk inactivation during mitosis exit operates also in human cells. We have followed cyclin B accumulation tyr 15 phosphorylation of cdk1 for 12 hours after release from a double thymidine block in hela cells. We found that tyr-15 is high during S-phase until cell reach mitosis where the tyr-15 signal completely disappear, tyr-15 phosphorylated cdk1 increases sharply after cyclin B degradation to disappear thereafter(D' angiolella and grieco submitted). We are investiganting the functional relevance of this phosphorylation in human cells.

In xenopus laevis egg extract the absence of checkpoints allow the rapid alternation of S-phase and M-phase. What happens during spindle checkpoint? How cdk activity is controlled during checkpoint activation?

We have previously shown that cdk activity is required to sustain the spindle checkpoint, we speculate that a branch of the complex signaling network present during an active spindle checkpoint is deputed to the control of cdk activity.

Cells undergo rapid adaptation upon spindle checkpoint if Cdc25c activity is weakened (Grieco and D' Angiolella in preparation), thus it can be possible that a localized control of Cdk activity (on the mitotic spindle for example) is important for the timing of cyclin B degradation and the fidelity of sister chromatid disjunction.

We speculate that the inactivation of cdk1 by tyr 15 phosphorylation can be a primeval mechanism to ensue mitosis exit. During evolution, degradation take over cdk activity control, to ensure the irreversibility of mitosis exit.

Given the reduced wee1 function between meiosis I and II(only single cdk1-thr-14 inhibitory phosphorylation could be performed by myt1 at this

developmental stage)(Minshull, Murray et al. 1991; Iwabuchi, Ohsumi et al. 2000; Nakajo, Yoshitome et al. 2000) our observations may also explain why the cyclin degradation pathway is only partially activated during this period, helping the transition to proceed without intervening S-phase and loss of sister chromatid cohesion.

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