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Cdc2: a monopotent or pluripotent CDK?

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Abstract

Cell cycle progression is controlled by both extracellular and intracellular signalling molecules. It has been generally believed that cdc2/CDK1 only control G_2-M transition in mammalian and many other higher eukaryotic cells. Accumulating evidence shows that cdc2 not only promotes G_2-M transition but is also capable of regulating G_1 progress and G_1-S transition *via* association with multiple interphase cyclins; cdc2 activity can be inhibited by p21 and p27, two traditional G_1 CDK inhibitors. In addition, cdc2-cyclin B controls pronuclear union in interphase fertilized eggs. These data suggest that cdc2 may be a pluripotent CDK. Although mechanisms responsible for the multiple functions of cdc2 remain to be further investigated, interactions of cdc2 with pRb and with several important transcription factors may provide a clue to the pluripotent role of cdc2.

Introduction

The critical role of cdc2 (CDK1) in cell cycle control has been well documented. In fission yeast, both G_2-M and G_1-S transitions are triggered by activity of a single protein kinase, cdc2 (or cdc28p in budding yeast). In higher eukaryotes, multiple cyclin-dependent kinases (CDKs; more than 11) have been identified, and these play different roles in the cell cycle. In mammalian cells, it has long been believed that CDK2, CDK4 and CDK6 drive cells through interphase, whereas cdc2 has been primarily implicated only in G_2-M transition, mainly in association with cyclin B. However, recent studies suggest that cdc2 is able to drive G_1/S transition (1,2). This new concept challenges the traditional model and suggests that cdc2 may be a pluripotent protein kinase acting globally in cell cycle control. In this mini-review, we will first briefly discuss cell cycle control and the role of cdc2 in G_2-M transition. Then, we will focus on recent progress of cdc2 in G_1-S transition and its interactions with several important transcription factors. Both terms (cdc2 and CDK1) will be used throughout this mini-review depending on usage described in the original publications.

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Overview of cell cycle control

In mammalian cells, cell cycle progression is regulated by a group of CDKs and their regulatory subunits in sequential order: cyclin D-CDK4/CDK6 and cyclin E-CDK2 complexes act on G_1 and G_1-S transition, respectively, followed by cyclin A-CDK2 on S and cyclin B-cdc2 at G_2-M transition. G_1 cyclin-CDK complexes have been reported to modify pRb by phosphorylation, thereby promoting cell cycle progression towards DNA replication. Dephosphorylation of pRb negatively regulates cell cycle progression through interactions with the E2F family of transcription factors. In contrast, phosphorylation of pRb results in loss of its replication suppression property (3,4). However, in some types of cells, pRb may be not a major player in cell cycle control (5,6).

Cell cycle progression controlled by cyclin-dependent kinases is counterbalanced by CDK inhibitors (CDKIs). There are two families of such inhibitors, one of which is INK4A-D, including $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$. The INK4 inhibitors negatively regulate cyclin D-CDK4/6 kinase activity. The second family of CDKIs, termed Cip/Kip, includes $p21^{Cip}$, $p27^{kip1}$ and $p57^{kip2}$, which are efficient inhibitors of cyclin E-CDK2 and cyclin D-CDK4/CDK6. CDK regulation by CDK inhibitors is an important step in linking anti-mitogenic signals to cell cycle progression.

Identification of G_2 protein kinase(s) is directly related to discovery of maturation-promoting factor (MPF) (7). For several years in the late 1960s, it had been suspected that some cytoplasmic factors might regulate nuclear

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activity during cell division. However, evidence for the existence of cytoplasmic factors that initiate M phase was not clear until 1971 during which year, Masui and Markert published their discovery of MPF (now termed M-phase promoting factor). In their studies, protein factors in cytoplasm from mature unfertilized eggs could promote *oocytes* to be released from G_2 and proceed through the first meiotic division. Subsequently, it was found that similar mitotic cell extracts from all eukaryotic cells tested including starfish, frog, sea urchin, yeast and human can function as MPF, demonstrating that MPF not only promotes meiosis but also triggers mitosis. Meanwhile, CDK, the protein that controls cell division, was first identified in yeast (8,9) then in a broad range of cell types. By the late 1980s, several key experiments led to realization that *cdc2* gene product, cdc2, controls entry into mitosis and is a catalytic subunit of MPF (10,11). In 1987, the human gene that encodes the protein corresponding to cdc2 of Xenopus MPF was already found to induce cell division in cdc2-deficient yeast mutants (12).

cdc2 interacts primarily with cyclin B or homologous B type cyclins to regulate G_2-M transition (13,14). Expression of cyclin B has periodic behaviour, which is parallel to expression of MPF activity (15). During interphase, concentration of cyclin B gradually increases following G_1, S and G_2, and reaches a critical threshold at the end of G_2, which promotes activation of cdc2 and triggers onset of mitosis. Activity of cdc2 is also regulated by phosphorylation and dephosphorylation and changes in subcellular localization (16,17). Before reaching their threshold, cdc2-cyclin B complexes are kept inactive through phosphorylation at Thr14 and Tyr15 of cdc2 by Myt1 and Wee1 (18,19). Myt1 is a cell membrane-associated protein kinase that is able to bind and phosphorylate cdc2 at both Thr14 and Tyr15, preventing its nuclear translocation. Different from Myt1, Wee1 suppresses cdc2 kinase activity by phosphorylation at Tyr15 in the nucleus. By the end of G_2, Myt1 and Wee1 are inactivated and a specific dual-phosphatase, cdc25, is activated. Activated cdc25 dephosphorylates two residues (Thr14 and Tyr15) in cdc2, leading to activation of cdc2 (20). cdc25 has three isoforms: cdc25A, cdc25B and cdc25C. cdc25A is thought to regulate G_1-S transition, cdc25B both G_1-S and G_2-M transition, and cdc25c only G_2-M transition (21). However, one recent report reveals that overexpression of cdc25A and cdc25B, but not cdc25c, promotes activation of cdc2 (22). Both Wee1 and cdc25 are regulated by Chk1 and 14-3-3 *via* phosphorylation during interphase (23–25).

Cdc2 drives G_1-S transition

As described above, cyclin D-CDK4/CDK6 complexes are thought to be essential for G_1 progress, whereas cyclin E-CDK2 and cyclin A-CDK2 regulate G_1-S transition and S phase progress, respectively. However, several recent reports have challenged this traditional model. One group has generated two strains of mice, one of which lacks CDK4 expression and the other expresses a CDK4 molecule with an inactivating mutation. Although loss of CDK4 causes insulin-deficient diabetes and partial sterility, embryonic fibroblasts proliferate normally and the mice lacking CDK4 are viable (26). Consistent with this report, CDK4 deficiency does not affect normal keratinocyte proliferation (27). In a further report, disruption of CDK4 delayed cell cycle entry of mouse fibroblasts; however, $CDK4^{-/-}$ and $p27^{-/-}$ cells showed partial recovery of G_0-S transition (28), suggesting that delay of cell cycle entry was not simply caused by deletion of CDK4. A more recent study, published in 2004, presented evidence that mouse embryos defective in CDK4 and CDK6 initially displayed normal organogenesis and most cell types proliferated normally, although the mice died during late stages of embryonic development due to severe anaemia. Additional supporting evidence is that quiescent CDK4/CDK6-null cells were able to enter S phase in response to serum stimulation (29). These data demonstrated that CDK4 and CDK6 are not as critical for cell cycle progression as had previously been believed. As D-type cyclins are the regulatory subunits of CDK4 and CDK6, 'normal' cell cycle progression in $CDK4^{-/-}$ and $CDK6^{-/-}$ cells may be due to presence of D type-cyclins, although these cyclins, in theory, would not be able to compensate for CDK4 or CDK6. To test this possibility, mice lacking all D-cyclins were generated and cell proliferation and mouse development were investigated. As expected, D-type cyclin (D1, D2 and D3)-deficient fibroblasts of these mice still proliferated almost normally, although with increased requirement for mitogenic stimulation (30).

A further important protein kinase involved in interphase progression in mammalian cells is CDK2, which targets many substrates that are important in DNA replication and transcription (31–33). Is it possible that CDK2 regulates the entire cell cycle and/or compensates for CDK4 or CDK6 in the absence of these molecules? Recently, two groups have reported their findings. Both studies generated CDK2 knockout mice to determine whether deletion of CDK2 could prevent cell progression. Surprisingly, CDK2 knockout mice survived and developed normally (34,35). Moreover, mouse embryonic fibroblasts (MEFs) lacking CDK2 proliferated normally in culture and re-entered the cell cycle without significant delay after stimulation with serum. Cyclin E is the major regulatory subunit of CDK2. Two research teams have tested requirement of cyclin E in mouse development. They found that cyclin E1- or cyclin E2-deficient animals

developed normally (36,37), but double-knock out cyclin $E1^{-/-}$ and cyclin $E2^{-/-}$ genotypes were embryonically lethal (36). One possible explanation for results caused by E1 and E2 double-knock outs is that cyclin Es, as regulatory subunits, not only associate with CDK2, but also bind to other CDK(s), whose functions are critical or important for cell cycle progression.

All data from gene knockout mice described above have demonstrated that deletion of interphase CDKs is not lethal to the mice and that their cells still proliferated in a 'normal' way, suggesting that some other molecules may compensate for CDK2, CDK4 and CDK6. By generating $CDK2^{-/-}$ $p27^{-/-}$ double knockout mice, Aleem *et al.* detected high numbers of cells in S and M phases in thymus and spleen, parallel to high levels of cyclin E activity (2). As cyclin E is a regulatory subunit of CDK2 and CDK2 is deleted here, high cyclin E activity must relate to (an)other associated protein kinase(s). Subsequently, this group demonstrated that in $CDK2^{-/-}$ cells, cyclin E was associated with cdc2 as an active complex. If cdc2 in wild type and $CDK2^{-/-}$ mice was silenced by shRNA, cells progression through S phase was slow and cell proliferation was significantly reduced, suggesting that cdc2 was able to drive G_1-S transition in the presence or absence of CDK2. However, deletion of CDK2 greatly enhanced efficiency of cdc2 in promoting G_1-S transition. Consistent with this observation, Satyanarayana *et al.* observed that CDK1 was translocated to the nucleus in $cdk2^{-/-}$ MEFs after serum stimulation, while wild type cells expressing cdk2 had delayed and weak nuclear translocation of CDK1 (38). The role of cdc2 in interphase progression was confirmed by application of Roscovitine, a highly potent inhibitor of CDK (especially cdc2 and CDK2) activity (39). Addition of Roscovitine caused significant growth inhibition of cells of several human cancer cell lines in culture (40). In human HeLa cells, CDK1 was shown to be required for establishment of G_1 phase, as cells expressing mutant CDK1 (CDK1AF) were able to enter and exit M phase, but were not able to carry out cytokinesis or karyokinesis compared to wild type cells (41). In fibroblasts, it has been shown that cdc2 mRNA is inducible in response to fresh culture medium; during quiescence, cdc2 mRNA is almost undetectable. Stimulation of cells with medium induces cdc2 expression, beginning at G_1-S transition and reaching maximum levels during late S and G_2 phases (42). Supplementary to these findings, experiments with TGFβ (43) and Cks1 (44) also support a pluripotent role for CDK1 in the presence of interphase CDKs; TGFβ is well known as a G_1 protein kinase inhibitor. We have found, in our previous studies on human myeloid leukaemia cells, that TGFβ not only inhibits several G_1 checkpoint kinases but also strongly downregulates expression of cdc2 without causing accumulation of the cells in G_2-M. As cdc2 is associated with pRb during S phase, our data suggest that cdc2 may participate in G_1-S regulation (43). Cks1 is a small protein component of CDK complexes, which regulates CDK1 activity. Deletion of Cks1 by siRNA in MCF-derived cells blocks oestrogen- and further growth factor-induced signalling pathways, slowed progression of cells through G_1-S and blocked their entry into M phase. Protein analysis demonstrated that deletion of Cks1 causes significant reduction of CDK1 and accumulation of hypophosphorylated pRb (44). More surprising results came from Santamaria's group's studies in which cdc2 was shown to execute all the events that are required to drive cell division, suggesting that cdc2 alone is sufficient to drive mammalian cell cycle progression (1). First, they demonstrated that CDK1 was able to interact with D-type cyclins in lysates extracted from embryos lacking CDK4 and it interacted with cyclin E in embryos lacking CDK2 and CDK4. CDK1-cylin D and CDK1-cyclin E complexes were able to phosphorylate pRb proteins *in vitro*. Second, although knockdown of CDK1 had no effect on interphase progression of primary MEFs induced by CDK4 and CDK2, CDK1 deletion completely abrogated S phase entry in embryonic cells lacking CDK4 (−/−),CDK6 (−/−) and CDK2 (−/−). Finally, by generating mutant mice at *cdc2a*, the locus encoding CDK1, they demonstrated that CDK1 is essential for early stages of embryonic development. Additional information on cdc2 came from a report published in 2008 according to which CDK1-cyclin B controls pronuclear union by regulating formation of sperm astral microtubules in interphase in fertilized eggs of starfish (45). Briefly, these data suggest that CDK1 is a pluripotent CDK that is able to promote cells' entry into S phase, as well as G_2-M transition, not only in somatic cells but also in gonad cells. The pluripotent role of cdc2 is shown in Fig. 1.

Interactions between cdc2 and cell cycle regulatory molecules/transcription factors

pRb and E2F

If cdc2 is a pluripotent CDK, what are the fundamental mechanisms responsible for the multiple functions of cdc2 described above? Interactions of cdc2 with several key molecules that control cell growth may provide a clue. It is well known that *Rb* is a tumour suppressor gene. The gene product, pRb, has the ability to suppress cell proliferation, which is regulated by G_1 cyclin-CDK complexes (3,46,47). Subsequently, studies of several groups have demonstrated that not only G_1 CDKs but also cdc2 have ability to phosphorylate pRb in various species including

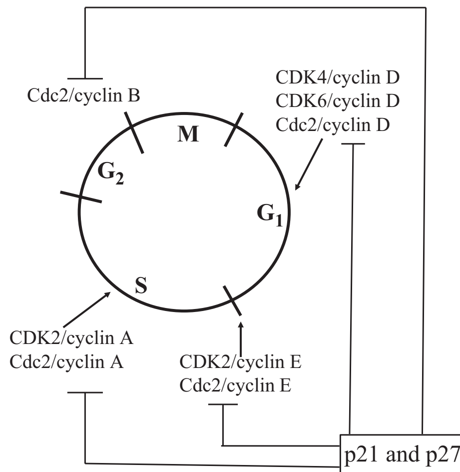

Figure 1. Cell cycle control by cdks and cdk inhibitors: cdc2 versus CDK2 and CDK4/6.

humans (43,48–50). As early as 1998, Taieb *et al.* observed, by microinjection of human *Rb* gene into Xenopus oocytes, elevated activities of cdc2-cyclin B and MAPK, but not cyclin D-CDK4/6 complexes, accompanying pRb phosphorylation. As inactivation or overexpression of cyclin D-CDK4/6 complexes does not affect Rb kinase activities, elevated cdc2 activity might be responsible for pRb phosphorylation (48). Soon, physical association of pRb with cdc2 and its phosphorylation by cdc2 in human cells have been observed (49). By using human myeloid leukaemia cell lines as research tools for cell cycle study, we have detected that in proliferating human myeloid leukaemia cells, there was a significant formation of cdc2-pRb complexes. Immunoprecipitates of cdc2 extracted from these cells had significant kinase activity upon pRb phosphorylation and inhibition of cdc2 contributes to transforming growth factor-beta-induced G_1-arrest (43). pRb may also directly or indirectly regulate cdc2 activity, as increased CDK1 (cdc2) activity has been detected in Rb-deficient mouse fibroblasts (51).

It has been reported that dephosphorylated pRb suppresses cell replication partly by turning off transcription of genes required for cell cycle progression, through interaction with E2Fs (50). The pRb family has three members: pRb, p107 and p130, which are collectively called ‘pocket proteins’. Each member can bind E2Fs and inhibit E2F activity. In contrast, free E2Fs bind to target DNA and activate DNA transcription. A number of reports have revealed that cdc2 is one of E2F’s targets. In quiescent human fibroblasts, p130-E2F4 complexes were found to bind to the cdc2 promoter, resulting in inhibition of cdc2 transcription (42). The binding site was detected at the R box of the cdc2 promoter, which is located downstream of AP1 or SP1 sites (52). In contrast, E2F1, E2F2 and E2F3 bind to positive-acting site in the cdc2 promoter and induces cdc2 expression (53). Thus, cdc2-pRb-E2F forms a positive feedback loop, which may amplify cdc2-induced proliferation of cells (Fig. 2), whereas inhibition of cdc2 gene may contribute to pocket protein-E2F complex-induced replication inhibition.

p21^{cip} and p27^{kip1}

As a negative regulator of cell cycle progression, p21^{cip} is thought to bind and inhibit CDK2/cyclin E and/or CDK4/cyclin D complexes, thereby arresting cells in G_1 phase. Induction of p21^{cip} is regulated by tumour suppressor gene, *p53*, in response to DNA damage. If cdc2 drives interphase progression, one would expect that p21^{cip} might be able to bind and inhibit cdc2, as it does with other G_1 CDKs, in response to *p53* activation. In 2001, one group found that overexpression of *p53* suppressed cell proliferation and cdc2 activity in TR9-7 cells; in contrast, deletion of p21^{cip} substantially impaired ability of p53 to repress the cdc2 promoter, suggesting that p21^{cip} is required for p53-induced inhibition of cdc2 activity (52). As there was no evidence for direct interaction between p21^{cip} and cdc2, the reasonable explanation for p21^{cip}-induced inhibition of cdc2 is that overexpression of *p53* activates transcription of p21^{cip} (p53-p21^{cip} pathway), which in turn inhibits interphase CDK activity. As a result, CDKs lose ability to phosphorylate pRb family proteins. In the dephosporylated form, p130-E2F4 complexes bind to the cdc2 promoter and inhibit cdc2 transcription. Apparently, the explanation described above is indirect

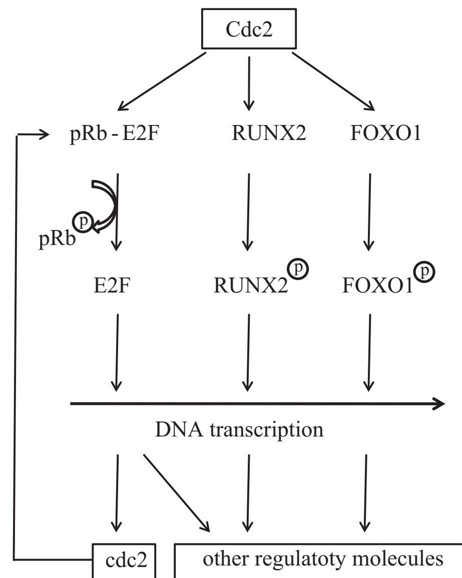

Figure 2. DNA transcription induced by Cdc2.

and crucial information is missing. Direct physical association between cdc2 and p21^{cip} has been reported recently. In CDK2−/− MEFs, but not in wild-type cells, both p21^{cip} and CDK1 were predominantly found in the nucleolus at about the same time, after cells were irradiated and serum stimulated. Immunoprecipitation revealed elevated levels of p21^{cip}-CDK1 complexes between 6 and 24 h in cells lacking CDK2 after stimulation (38). As time points typically represented G_1 progression and G_1/S transition after receiving a stimulating signal, this suggests that formation of a p21^{cip}-cdc2 complex is responsible for cell arrest at G_1/S transition in the absence of cdk2.

p27 ^{kip1} was first identified as an inhibitor of cyclin E-CDK2. Subsequently, it was found that p27 ^{kip1} can target cyclin-CDK4 complexes. Since then, p27 ^{kip1} as an interphase CDK inhibitor has been widely accepted; however, some recent progress in the field has challenged this model. In work performed by Martin *et al.*, forced expression of p27^{kip1} and p21^{cip} inhibited replication of mouse fibroblasts expressing CDK2 and cells without CDK2. In contrast, p27 ^{kip1} and p21^{cip} double-knockouts caused similar proliferation in both CDK$^{+/+}$ and CDK$^{-/-}$ cells. In agreement with these results, mice lacking p27^{kip1} have about the same size and body weight in presence or absence of CDK2. As p27^{kip1} and p21^{cip} had no any significant effect on expression or activity of CDK4, the data described above suggest that CDK2 is not a primary target of p27^{kip1} and p21^{cip} and these inhibitor-induced cell cycle arrests could be a result of their interaction with molecules other than cdk2 or CDK4 (54). In the same year, another group reported that cdc2 was able to interact directly with p27^{kip1} (2). In CDK2−/− MEFs p27^{kip1} was found to bind and inhibit cdc2 activity. In contrast, deletion of p27 ^{kip1} significantly upregulated cdc2 activity and promoted G_1-S transition. Clearly, these new findings advanced our knowledge on the roles of CDK inhibitors and cdc2 in cell cycle control. Most likely, p27^{kip1}- or p21^{cip}-induced growth inhibition, which has been well reported in the literature, is partially due to negative regulation of cdc2 activity (Fig. 1).

FOXO and RUNX

Forkhead box O (FOXO) is a group of transcription factors that belong to the FOX superfamily (55,56). These transcription factors possess tumour suppressor functions by regulating expression of genes involved in cell death and proliferation. For example, upregulation of FOXO activates proapoptotic genes encoding for Fas ligand, Bim and TRAIL (57–59). FOXO proteins also arrest cells in G_1 by upregulating CDK inhibitors, p27 (60,61), or p130-E2F4 complexes (62). The FOXO subfamily includes FOXO1, FOXO3a, FOXO4 and FOX6 in humans. The role of FOXO1 in regulating cell proliferation and cell cycle regulation has received particular attention recently (63,64). Both CDK1-induced apoptosis and proliferation through interaction with FOXO1 have been reported. In neurons, CDK1 phosphorylates FOXO1 at Ser249 *in vitro* and *in vivo*. Phosphorylated FOXO1 disrupts FOXO1 binding to 14-3-3 proteins and thereby causes nuclear accumulation of FOXO1, followed by activation of FOXO1-dependent transcription and cell death (65). In prostate cancer (Pca) cells, experiments performed by Liu *et al.* have shown that ectopically expressed CDK1 forms a complex with FOXO1 and inhibits FOXO1-induced apoptosis (66). As CDK1 and cyclin B1 are often overexpressed in human cancers, the authors believed that their findings suggested that aberrant activation of CDK1 may contribute to tumourigenesis by promoting cell proliferation *via* phosphorylation of FOXO1.

The RUNX transcriptional regulators have been reported to be essential for haematopoiesis, bone formation and gastric development. The family has three members, RUNX1, RUNX2 and RUNX3. Both RUNX1 and RUNX2 are able to promote bone marrow cell proliferation and cell cycle progression by up-regulation of D-type cyclins and down-regulation of CDK inhibitor, p21 (67–69). One of the mechanisms responsible for RUNX2-mediated cell proliferation involves interaction between RUNX2 and cdc2. Qian *et al.* reported that in proliferating endothelial cells, RUNX2 DNA binding activity is high and RUNX2 is associated with cyclin B1. In culture, cdc2 inhibitor, Roscovitine, dose-dependently inhibits RUNX2 DNA binding activity. *In vitro* protein kinase assay has shown that cdc2 phosphorylated RUNX2 at the Ser451 residue (69). Thus, phosphorylation of RUNX2 by cdc2 is linked to RUNX2-mediated cell cycle progression in bone marrow endothelial cells.

Conclusion

Taken together, recent findings have clearly demonstrated that cdc2, a traditional G_2-M regulator, not only promotes G_2/M transition but also regulates interphase progression and some other biological processes in mammalian and other higher eukaryotic cells. In the absence of interphase CDKs, cdc2 is able to associate itself with various interphase cyclins. Multiple functions of cdc2 may link to its interactions with the pRb-E2F and several other transcription factors (Fig. 2). Although much has been learnt from these new findings, many fundamental questions remain for future studies. First, as most of the multiple functions of cdc2 have been detected in the absence of interphase CDKs by approaches of gene deletion or gene knock-mice, the pluripotent role of cdc2 and its regulation in the presence of interphase CDKs, that is, in intact cells, are

less clear. Although interactions between cdc2 and interphase cyclins are required for the role of cdc2 in driving G_1-S transition, it is not ruled out that cdc2 may regulate interphase progression *via* association with some yet unidentified cyclins. Second, the loops and pathways for interactions between cdc2 and the multiple transcription factors described in this review are far from understood. If cdc2 is indeed a pluripotent CDK, this knowledge may have a profound impact on our understanding of tumourigenesis and therapeutic applications.

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