

# MyoD induces apoptosis in the absence of RB function through a p21<sup>WAF1</sup>-dependent re-localization of cyclin/cdk complexes to the nucleus

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**During differentiation of skeletal myoblasts, MyoD promotes growth arrest through the induction of the cdk inhibitor p21 and the accumulation of hypophosphorylated RB protein. Myoblasts lacking RB function fail to accomplish full differentiation and undergo apoptosis. Here we show that exogenous MyoD induces apoptosis in several cell backgrounds sharing RB inactivation. This process is associated with increased levels of cell cycle-driving proteins and aberrant cell cycle progression. The inability of MyoD to induce apoptosis in a p21-null background, highlights a requirement of p21 in RB-regulated apoptosis during myogenesis. This pro-apoptotic function of p21 cannot be exerted by simple p21 over-expression, but requires the co-operation of MyoD. We also suggest that the essential aspect of p21 activity involved in such a process is related to its ability to induce the nuclear accumulation and aberrant activity of cyclin/cdk complexes. These results establish a novel link between MyoD, p21 and RB during myogenesis, providing new insights into the antagonism between muscle differentiation and loss of RB function.**

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## Introduction

Differentiation of skeletal muscle cells is regulated by the MyoD family of bHLH transcription factors, which includes MyoD, myf5, myogenin and MRF4 (Weintraub, 1993). Among myogenic factors, MyoD plays a central role in the co-ordination between the onset of muscle-specific gene expression and the inhibition of cell proliferation during terminal differentiation (Maione

and Amati, 1997). In growing myoblasts MyoD is kept inactive by several components of growth factor signal transduction pathways (Puri and Sartorelli, 2000; Wei and Paterson, 2001). Removal of these constraints promotes the activation of MyoD and its ability to induce myogenesis. MyoD is capable of starting the entire differentiation program, even when ectopically expressed in several non-muscle cell types (Davis *et al.*, 1987; Weintraub *et al.*, 1989). Several reports have shown that the tissue-specific and the growth arrest functions of MyoD can be independently regulated (Crescenzi *et al.*, 1990; Sorrentino *et al.*, 1990; de La Serna *et al.*, 2001). The muscle-specific activity of MyoD involves the transcriptional activation of downstream muscle specific regulators, such as myogenin as well as members of the MEF2 family, which act in concert to induce the expression of 'late' muscle structural genes (Naya and Olson, 1999). With respect to the cell cycle withdrawal functions, a massive amount of evidence points to the transcriptional activation of the cyclin dependent kinase (cdk) inhibitor p21 as the mechanism by which MyoD induces cell cycle arrest (Guo *et al.*, 1995; Halevy *et al.*, 1995; Parker *et al.*, 1995). p21<sup>WAF1/CIP1</sup> belongs to the Cip/Kip family of cdk inhibitors, which also includes p27<sup>Kip1</sup> and p57<sup>Kip2</sup> (Sherr and Roberts, 1999). p21 has been also implicated in growth arrest associated with terminal differentiation in other cell types, such as keratinocytes and hemopoietic cells (Missero *et al.*, 1996; Cheng *et al.*, 2000). Although it has been recently suggested that the function of p21 in regulating differentiation can be more complex than simply inducing cell cycle arrest (Dotto, 2000), the best understood role of this protein during myogenesis involves the inhibition of G1 cyclin/cdk complexes (Guo *et al.*, 1995; Cenciarelli *et al.*, 1999), which co-operate in the induction of the G1-S phase transition through the phosphorylation of the retinoblastoma protein, RB (Sherr and Roberts, 1999). MyoD has also been implicated in the *Rb* gene transcriptional activation (Martelli *et al.*, 1994) so that, at the onset of differentiation, both the induction of RB and that of p21 lead to the accumulation of high levels of the hypophosphorylated, active form of the RB protein, able to repress E2F-dependent cell cycle progression (Guo and Walsh, 1997).

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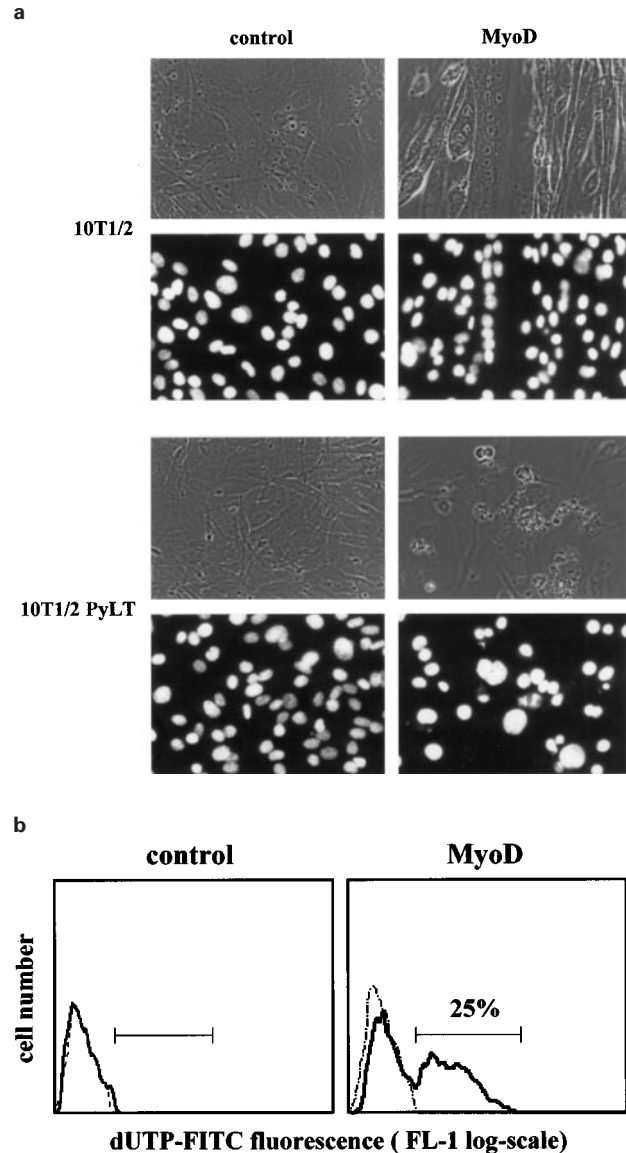
A central role of RB in muscle differentiation was initially suggested by the effects of viral oncoproteins, that were found to interfere with myogenesis through their ability to bind (and inactivate) RB family proteins (Gu *et al.*, 1993; see also Maione and Amati, 1997, and references therein). Moreover, myoblast cells defective for *Rb* show several abnormalities in the differentiation process, characterized both by the failure to arrest in G0 and by a reduced expression of muscle differentiation markers (Gu *et al.*, 1993; Novitch *et al.*, 1996; Zacksenhaus *et al.*, 1996). The role of RB in myogenesis has been clearly defined with regard to the cell cycle exit at the onset of myoblast differentiation and to the maintenance of the post-mitotic state in terminally differentiated myotubes. Moreover, it has been shown that RB co-operates with myogenic factors in the induction of late muscle-specific gene expression (Novitch *et al.*, 1999). On the other hand, several reports agree that RB function is not required for the expression of early differentiation genes, such as myogenin and p21 (Novitch *et al.*, 1996; Fimia *et al.*, 1998), directly induced by MyoD through a mechanism requiring the recruitment of the p300/CBP coactivator (Puri *et al.*, 1997; Cenciarelli *et al.*, 1999).

Another level of complexity in the role of RB in the onset of myogenesis is provided by the requirement of RB for cell survival in the muscle system. It has been previously shown that myoblast cells lacking RB function, because of either oncoprotein binding or gene mutation, undergo apoptosis at high frequency both *in vivo* and *in vitro* when they attempt to accomplish differentiation (Zacksenhaus *et al.*, 1996; Wang *et al.*, 1997; Fimia *et al.*, 1998). Here we show that the expression of exogenous MyoD induces apoptosis in several cell backgrounds sharing RB inactivation and that this apoptosis occurs through a p21-dependent pathway, that causes the nuclear accumulation and aberrant activity of cyclin/cdk complexes.

## Results

### *MyoD induces apoptosis in the absence of RB function*

We have previously reported that the induction of apoptosis in myoblast cells expressing the RB-inactivating oncoprotein Polyomavirus Large T (PyLT) is associated with the simultaneous expression of proliferation and differentiation markers and depends on the activity of myogenic factors (Fimia *et al.*, 1998). To confirm that both the activation of MyoD and the inactivation of RB are involved in the onset of myoblast apoptosis, we tested the effects of exogenous MyoD expression in a series of fibroblast cell lines, bearing different oncogene alterations. Cells were infected with a retrovirus encoding MyoD and analysed for the occurrence of apoptosis. As expected, MyoD expression in 10T1/2 fibroblasts leads to myogenic conversion and terminal differentiation, revealed by the fusion into multinucleated myotubes (Figure 1a). On the contrary, MyoD expression in a



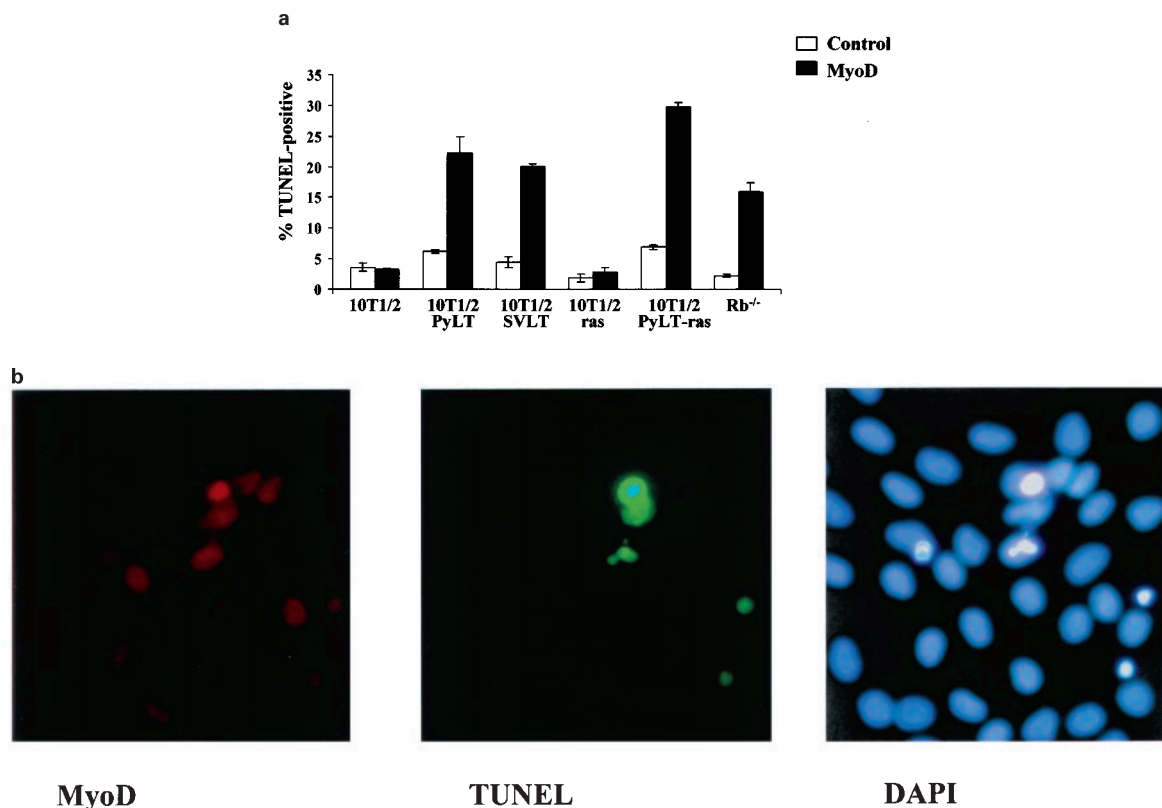
**Figure 1** MyoD induces apoptosis in PyLT-expressing fibroblasts. (a) 10T1/2 and 10T1/2 PyLT cells were infected with the empty vector (control) or with pBabeMyoD (MyoD). Forty-eight hours later cells were shifted to low-serum medium and, after further 24 h, fixed and DAPI-stained. For each cell line the same fields were photographed under phase contrast and fluorescence illumination. (b) 10T1/2PyLT were infected as above and the percentage of TUNEL-positive nuclei were calculated by flow cytometry, as described in Materials and methods. Hatched lines: negative controls, incubated with label solution, in the absence of terminal transferase. Black lines: test samples, incubated with TUNEL reaction mixture

PyLT-derived cell clone (10T1/2PyLT) results in the appearance of rounded, detached cells, containing condensed nuclei as revealed by DAPI staining (Figure 1a). TUNEL staining confirms that apoptotic cell death occurs in a high percentage of 10T1/2PyLT cells after infection with MyoD but not with the empty vector (Figure 1b). Remarkably, MyoD induces apoptosis also in 10T1/2 fibroblasts transformed by either SV40 Large T or the combination of PyLT and oncogenic ras (v-

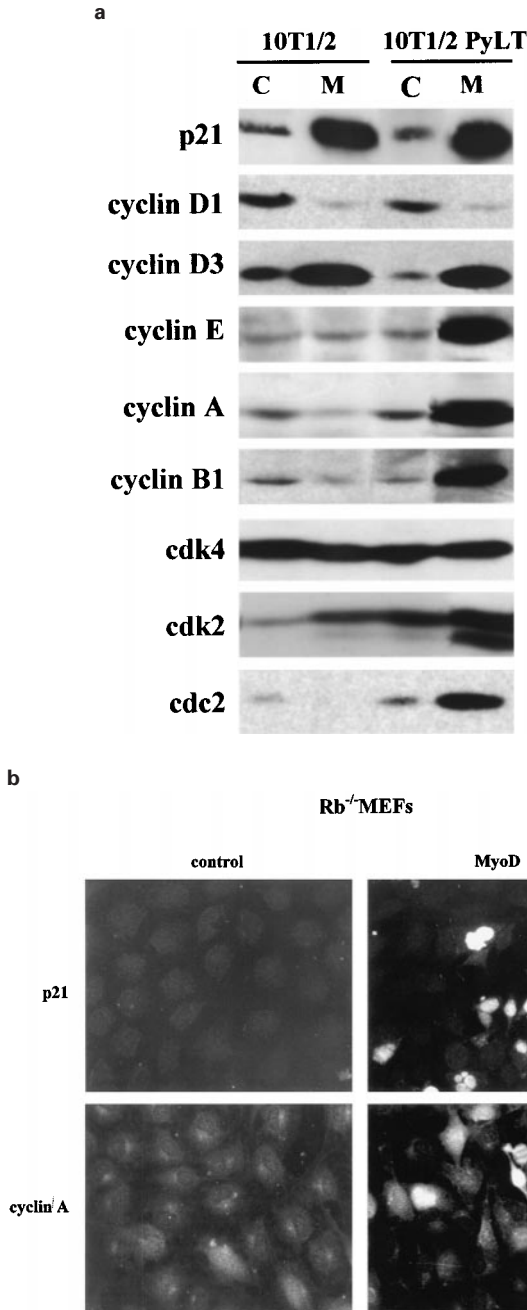
Ha-ras), while it fails to do so in 10T1/2 transformed by ras alone (Figure 2a). Interestingly, we have found that MyoD also induces apoptosis in Hela tumor cells (data not shown) where RB is inactive as a consequence of Papillomavirus E7 expression. These findings clearly indicated a strong correlation between the induction of apoptosis and RB-inactivation. Since our model involves the use of the viral oncoprotein PyLT, our first concern was to exclude that some oncoprotein-activated pathway, other than RB inactivation, could be involved in this apoptosis. We had already shown that PyLT mutants, defective for the binding to RB family proteins, fail to affect differentiation and survival in the muscle system (Maione *et al.*, 1994; Fimia *et al.*, 1998). Now, to clarify whether RB is the only member of its family with a role in MyoD-induced apoptosis, we analysed the effect of the myogenic factor in MEFs derived from RB 'knock-out' mice. As shown in Figure 2a,b, the loss of RB function is sufficient for MyoD to induce apoptotic cell death. In this case TUNEL-positive cells appear to localize on discrete areas of the dishes, most likely as a consequence of a non-homogeneous pattern of MyoD retroviral infection (Figure 2b). These data confirm that the activation of MyoD in the absence of a functional Rb pathway is interpreted by the cell as an apoptotic signal.

*Apoptosis induced by MyoD in the absence of RB function is associated with aberrant cell cycle progression*

Myoblasts expressing PyLT or lacking RB, in addition to activate an incomplete differentiation process, also exhibit a defective growth arrest (Zacksenhaus *et al.*, 1996; Fimia *et al.*, 1998). This finding, together with our observation that in some of the cell types analysed MyoD can induce apoptosis without inducing muscle-specific markers, prompted us to focus on the role of abnormal cell cycle regulation in this apoptosis. To this aim, we first determined the expression levels of some cell cycle regulatory proteins. In agreement with previous findings, the expression of exogenous MyoD in 10T1/2 fibroblasts induces a growth arrest pathway, associated with the induction of p21. In contrast with cyclin D3, that is up-regulated by MyoD to take part in the growth arrest mechanism (Cenciarelli *et al.*, 1999), several cell cycle-driving proteins, in particular cyclin D1, cyclin A, cyclin B1 and cdc2, appear to be down-regulated (Figure 3a). A different outcome was observed in 10T1/2PyLT. In line with previous observations, we found that MyoD induces high levels of p21 even in the absence of RB function. Unexpectedly, however, in these cells MyoD also leads to the accumulation of cell cycle-driving proteins, in



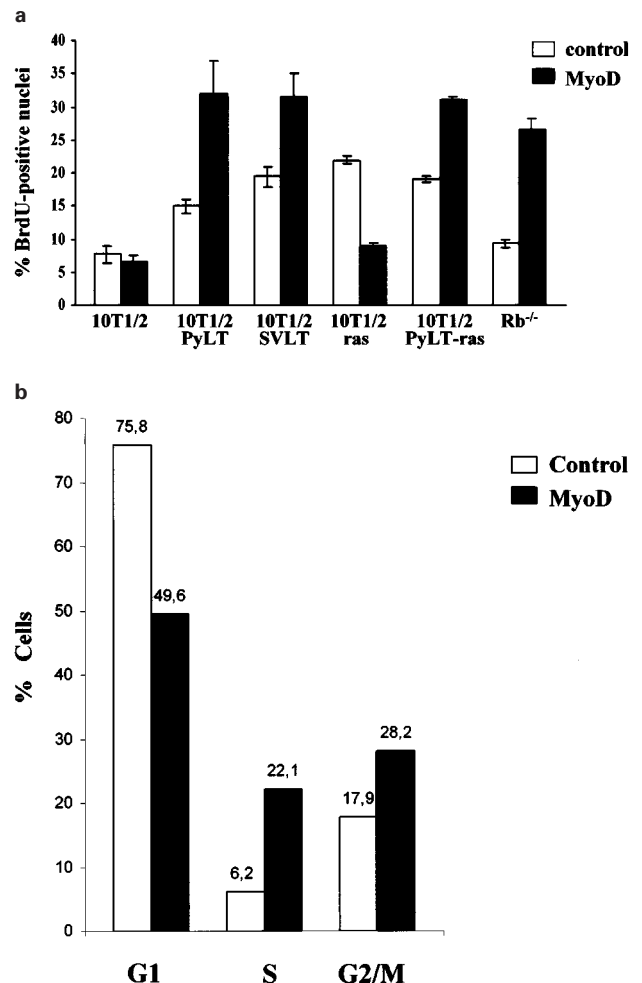
**Figure 2** MyoD-induced apoptosis is related to RB inactivation. (a) The indicated cell lines were infected as in Figure 1. The presence of TUNEL-positive cells was determined by fluorescence microscopy and the percentages were calculated with respect to total nuclei, visualized by DAPI staining. At least 1500 cells were counted for each sample and the results are the mean of three independent experiments. (b) MyoD-infected RB<sup>-/-</sup> MEFs were doubly stained for MyoD and TUNEL. Nuclei were counter-stained with DAPI and the same field was photographed under specific filters



**Figure 3** Expression of cell cycle markers during MyoD-induced apoptosis. (a) 10T1/2 and 10T1/2PyLT cells were infected with either the empty vector (C) or with MyoD (M). Cell lysates were prepared 24 h after the shift to low-serum medium and analysed by Western blot for the indicated proteins. (b) Fluorescence micrographs of Rb<sup>-/-</sup> MEFs, infected as in (a) and analysed by immunostaining with either p21 or cyclin A antibodies 24 h after the shift to low-serum medium

particular cyclin E, cyclin A, cyclin B1, cdk2 and cdc2 (Figure 3a). A similar effect on cell cycle regulators was observed in 10T1/2 SVLT cells and Rb<sup>-/-</sup> MEFs (Figure 3b and data not shown). Immunofluorescence staining in Figure 3b shows that MyoD leads to increased expression of both p21 and cyclin A in

Rb<sup>-/-</sup> MEFs. To determine whether the up-regulation of cyclins and cdk2 was associated with cell cycle progression, we also determined the occurrence of DNA synthesis. 10T1/2 fibroblasts, 10T1/2 oncogene-expressing subclones and Rb<sup>-/-</sup> MEFs were infected with MyoD or mock-infected with the empty vector and analyzed for BrdU incorporation (Figure 4a). As expected, the percentage of BrdU-positive cells is higher in the oncogene-expressing cell lines when compared to parental 10T1/2. Intriguingly, whereas MyoD markedly reduces BrdU incorporation, not only in the parental 10T1/2 cells, but also in the ras-transformed derivative, the opposite is observed in the absence of RB function. In this case, the percentage of DNA synthesizing cells is further increased following MyoD infection. Kinetic analysis of BrdU labeling



**Figure 4** MyoD causes S and G2/M accumulation in the absence of RB function. (a) The indicated cell lines were infected with either pBabe (control) or with pBabe-MyoD (MyoD) and analysed by immunofluorescence staining for BrdU incorporation after 24 h in low-serum medium. The percentages of BrdU positive cells were calculated with respect to total nuclei, visualized by DAPI staining. At least 1500 cells were counted for each sample and the results are the mean of three independent experiments (b) Flow cytometry analysis of cell cycle distribution of 10T1/2 and 10T1/2 PyLT cells infected as in (a)

suggests that this accumulation can result from a cell cycle delay (data not shown). To confirm that BrdU incorporation corresponded to S phase entry, we performed a flow cytometric analysis to assess the DNA content of MyoD-expressing cells. In this case, to prevent MyoD-induced cell fusion, already poor in the absence of RB, cells were cultured in the presence of EGTA. This condition does not impair biochemical differentiation and growth arrest of differentiating myoblasts (Hu and Olson, 1990), nor affects the ability of MyoD to induce increased levels of cyclins and BrdU incorporation in cells lacking RB function (our unpublished observations). As shown in Figure 4b, MyoD infection of 10T1/2PyLT cells causes their accumulation not only in S but also in G<sub>2</sub>/M phase. This effect is in contrast with the well described accumulation in G<sub>1</sub> induced by the myogenic factor in several cell types, also proved in our hands in the parental 10T1/2 cells (data not shown). These results suggest that MyoD activity, although unable to induce G<sub>1</sub> arrest in the absence of RB function, can still cause a cell cycle delay in other phases. They also suggest that the abnormal cell cycle distribution associated with apoptosis could result from the activity of a growth arrest pathway, affecting cells escaping from G<sub>1</sub>.

#### *Association of p21 with cdk2 and cdc2-containing complexes and alteration of their activity*

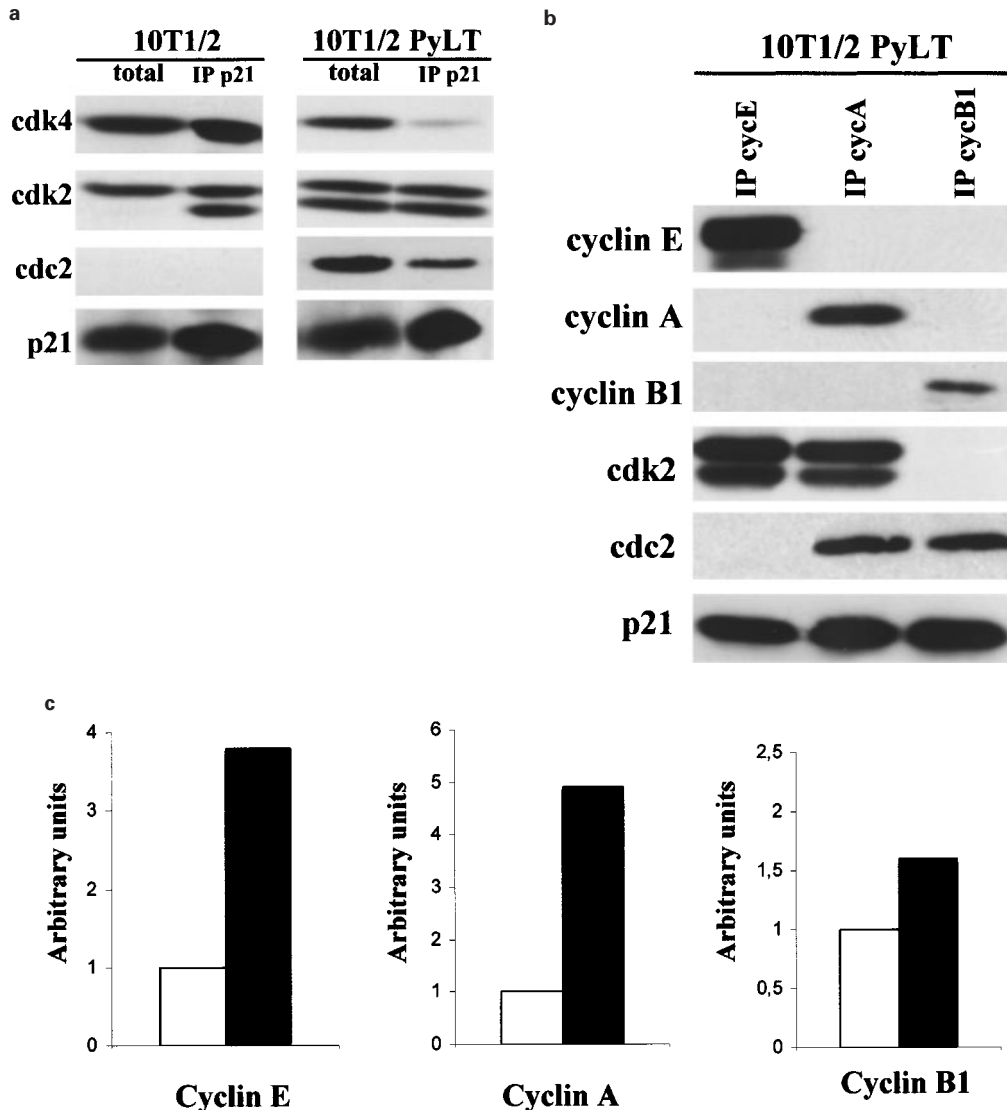
A well established growth arrest pathway activated by MyoD during differentiation involves the up-regulation of p21 and the inhibition of G<sub>1</sub> cdks. Thus, it was conceivable that the cdk inhibitor could play a role in the cell cycle effects exerted by the myogenic factor in the absence of RB function. To analyse the interaction of MyoD-induced p21 with cyclin-cdk complexes in PyLT-expressing fibroblasts, we performed a series of immunoprecipitation-Western blot experiments. As shown in Figure 5a, equivalent amounts of p21 are immunoprecipitated from 10T1/2 and 10T1/2PyLT cells after MyoD infection. According to previous reports (Guo *et al.*, 1995; Cenciarelli *et al.*, 1999), p21 is found associated with cdk4 and cdk2 in parental cells. A somewhat different situation is observed in 10T1/2PyLT cells. In this case, although p21 interacts with cdk2 to the same extent as in parental cells, it does not significantly associate with cdk4 but, rather, with cdc2. Western blot analysis of cyclin E, cyclin A and cyclin B<sub>1</sub> immune complexes (Figure 5b) confirms these cyclin/cdk associations. They also indicate that, in PyLT-expressing fibroblasts, p21 is present in cyclin E/cdk2-, cyclin A/cdk2-, cyclin A/cdc2- and cyclin B<sub>1</sub>/cdc2-containing complexes. We proceeded to explore the possible impact of the above-described changes in the pattern of cyclin-cdk-p21 associations on cdk activities. To this end we performed *in vitro* H1 kinase assays of cyclin/cdk complexes immunoprecipitated from 10T1/2PyLT cells infected with MyoD or with the empty vector. During normal differentiation, both the

decrease of cyclin levels and the incorporation of MyoD-induced p21 in cyclin/cdk complexes result in a strong inhibition of their kinase activity (Guo *et al.*, 1995 and our unpublished observations). Remarkably, MyoD infection of 10T1/2PyLT cells results in significantly increased kinase activities associated with cyclin E, cyclin A and cyclin B<sub>1</sub> (Figure 5c). However, it should not be overlooked that MyoD expression, in LT-expressing cells is accompanied by a prolonged permanence of these cells in S and G<sub>2</sub> phases. More importantly, these cell cycle alterations are linked to the accumulation of high levels of cyclins. It has been well established that cyclins are limiting in the formation of active cyclin/cdk complexes. Thus the observed changes in kinase activities could result from an increased abundance of cyclin-containing complexes. For this reason we considered that a useful information could be obtained from the cyclin-associated-specific kinase activity. If the absolute kinase activities associated with cyclin E, cyclin A and cyclin B<sub>1</sub> are normalized to the respective cyclin levels, as determined by densitometry scanning of Western blots, both cyclin A- and cyclin B<sub>1</sub>-containing complexes appear to be inhibited by about 70% when compared to mock infected controls. In contrast, cyclin E-associated specific activity is only inhibited by about 35–40%. This altered pattern of cyclin/cdk associated activities could play a role in the observed effects on cell cycle progression. In particular, cyclin A and cyclin B<sub>1</sub> are clearly involved in regulating S phase progression and G<sub>2</sub>/M transition (Sherr, 1993), so that the alteration of their associated kinase activities can easily account for both S and G<sub>2</sub>/M delay (see also discussion).

#### *p21 is necessary for MyoD to induce apoptosis, but dispensable to increase cyclin levels*

To investigate the possible role of p21 in MyoD-induced apoptosis, we determined not only whether p21 was required for MyoD to interfere with cell survival and cell cycle progression but also whether p21 over-expression was sufficient to reproduce the effects exerted by MyoD. To this end, fibroblasts derived from p21 knock-out mice were stably transfected with PyLT, in order to inactivate RB, and analysed for the response to MyoD or p21 retroviral infection. As shown in Figure 6, MyoD does not increase the percentage of TUNEL positive cells in p21<sup>-/-</sup> PyLT cells. Strikingly, although the re-introduction of p21 alone does not affect survival, the co-expression of p21 and MyoD results in a significant induction of apoptosis. This finding highlights a requirement of p21 activity in the mechanism by which MyoD induces apoptosis in cells lacking RB function. However, this pro-apoptotic function of p21 cannot be exerted by simple p21 over-expression, but requires the concomitant MyoD activity.

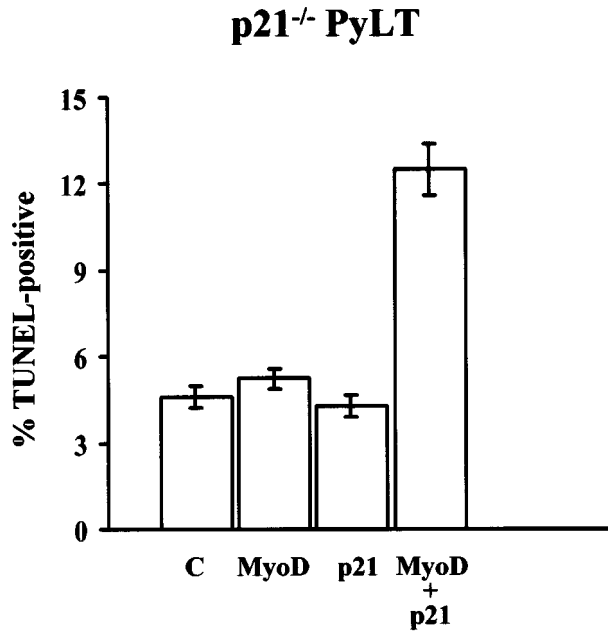
To verify whether the failure to induce apoptosis in the absence of p21 was determined by the inability of MyoD to affect some cell cycle event, we investigated



**Figure 5** Effects of MyoD on the composition and activity of cyclin-cdk complexes. (a) 10T1/2 and 10T1/2PyLT cells were infected with MyoD and, 24 h after the shift to low-serum medium, total proteins were extracted and immunoprecipitated with antibodies against p21. Total extracts (total) and immune complexes (IP p21) were resolved on SDS-polyacrylamide gels and then analysed by Western blotting using antibodies specific for the indicated proteins. (b) 10T1/2 PyLT cells were infected as above and total extracts were immunoprecipitated with antibodies against cyclin E (IP cycE), cyclin A (IP cycA) or cyclin B1 (IP cycB1). Immune complexes were resolved on SDS-polyacrylamide gels analysed by Western blotting with antibodies specific for the indicated proteins. (c) Cells extracts from 10T1/2PyLT cells, infected with the empty vector (white bars) or pBabeMyoD (black bars), were immunoprecipitated with antibodies against cyclin E, cyclin A or cyclin B1. The associated kinase activities were assayed using histone H1 as a substrate. The results of a representative experiment are reported as quantitation of radioactivity

the expression of cell cycle regulators in this condition. As shown in Figure 7a, the expression of MyoD, in p21<sup>-/-</sup> PyLT cells, induces increased levels of cyclin E, cyclin A, cyclin B1, cdc2 and cdk2, similarly to what is observed in their p21-positive counterparts. In contrast, the exogenous expression of p21 does not significantly increase the levels of cyclins and cdks, with the exception of cyclin E. This finding cannot be ascribed to a low level of p21 expression, since similar results were observed in 10T1/2PyLT cells, in which p21 levels were comparable after p21 and MyoD retrovirus infections (Figure 7b). In agreement with Western blot

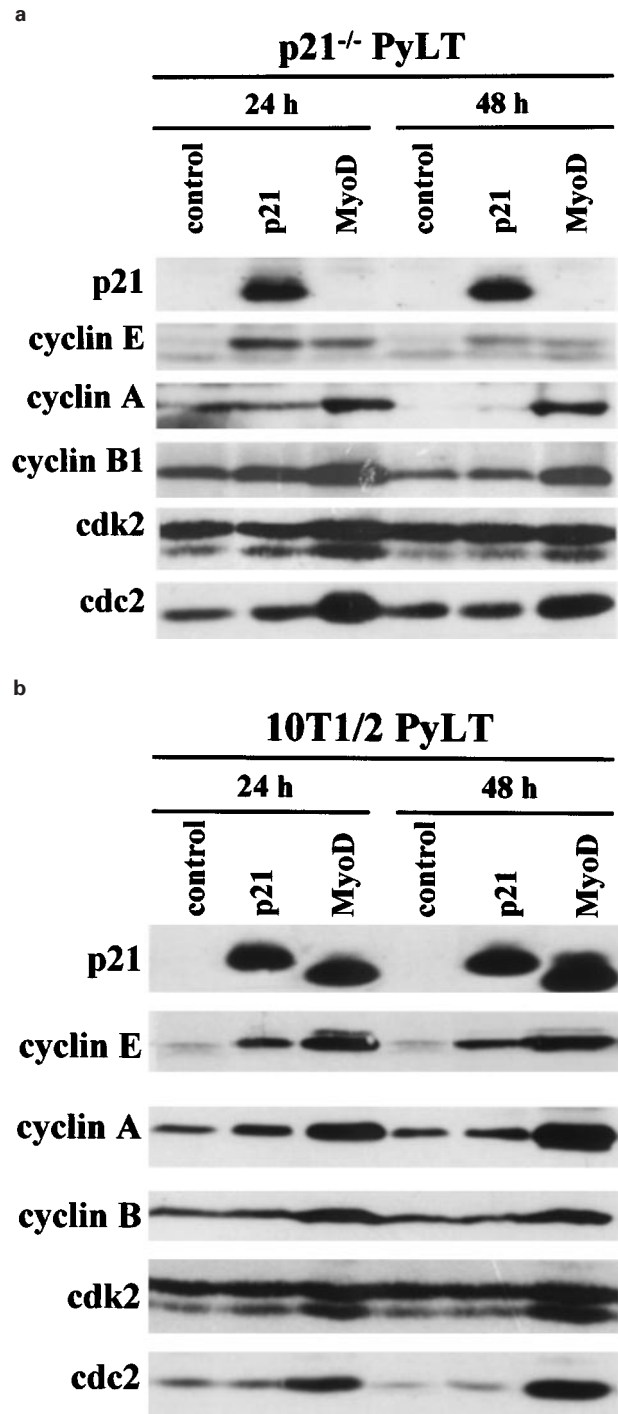
analysis of cell cycle markers, BrdU analysis of MyoD and p21 infected cells shows not only that p21 is unable to induce the accumulation DNA synthesizing cells, but also that it is dispensable for MyoD to induce this effect (data not shown). Two main conclusions can be drawn from these results. The first one is that MyoD can still cause an aberrant cell cycle progression, in cells lacking RB function, even in the absence of p21. The second one is that both the accumulation of p21 and that of cell cycle-driving proteins are necessary for MyoD-induced apoptosis, thus suggesting the occurrence of a cooperation between two pathways.



**Figure 6** p21 is necessary but not sufficient for MyoD to induce apoptosis in PyLT-expressing fibroblasts. p21<sup>-/-</sup> PyLT cells were infected with the empty pBabe retroviral vector (C), with pBabe-MyoD (MyoD), with pBabe-p21 (p21), or coinfecting with both pBabe-MyoD and pBabe-p21 (MyoD+p21). The percentages of TUNEL-positive cells were determined 48 h after the shift to low-serum medium

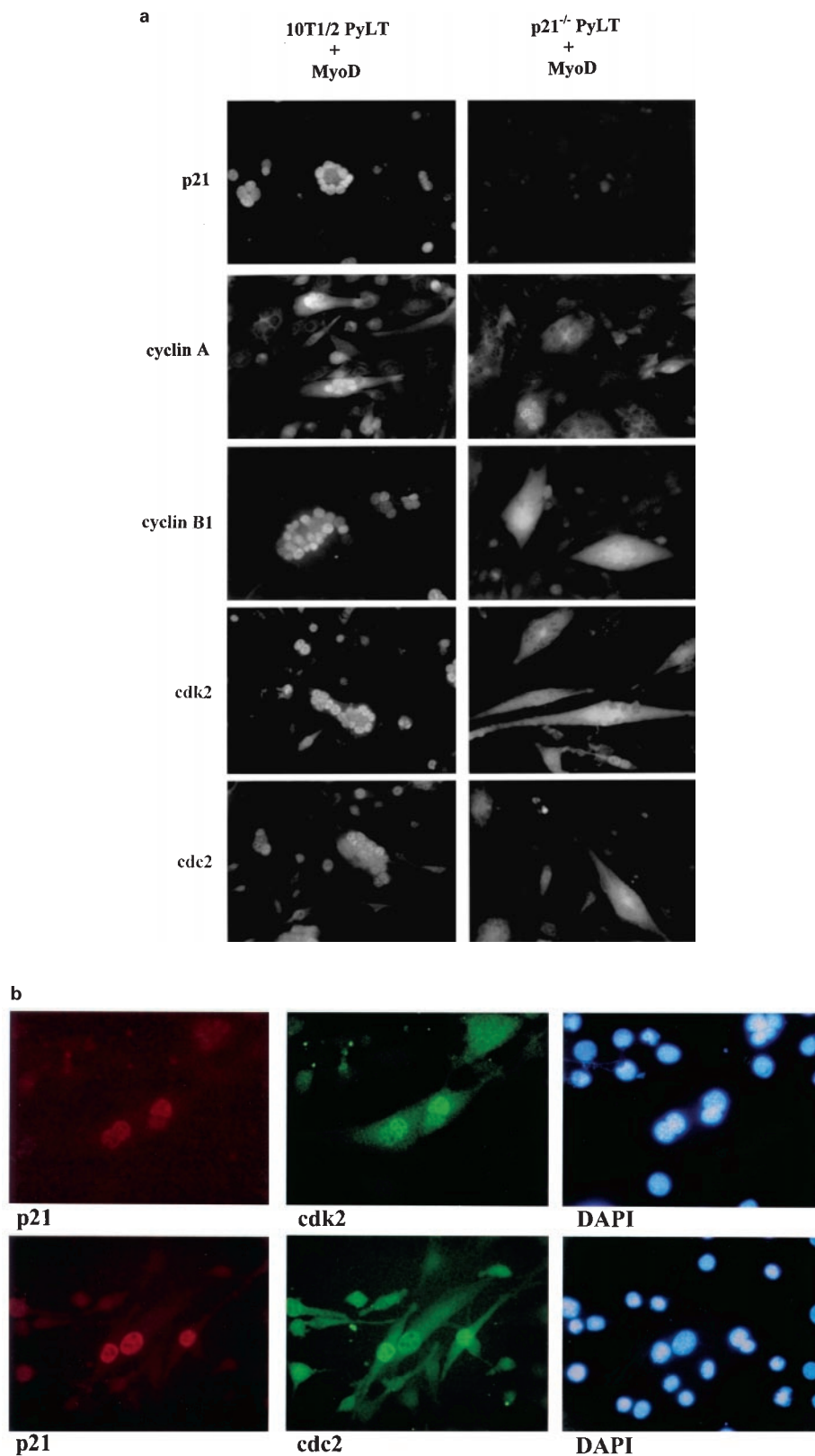
*p21 is required for MyoD to induce nuclear accumulation and activity of cyclin/cdk complexes*

It has been previously described that p21 is capable of inducing nuclear assembly of active cycD/cdk4 complexes (Labaer *et al.*, 1997). Moreover, it has been recently reported a strong impact of subcellular trafficking of cyclins and cdks in the regulation of several aspects of the cell cycle (Yang and Kornbluth, 1999). This prompted us to compare the subcellular localization of the cell cycle-related molecules up-regulated by MyoD, in the presence and in the absence of p21. To this end, 10T1/2PyLT and p21<sup>-/-</sup>PyLT cells, infected with MyoD, were fixed and immunostained for some cyclins and cdks (Figure 8a). In the presence of p21, cyclin A, cyclin B1, cdk2 and cdc2 accumulate in the nuclei of abnormally differentiated myotubes. In contrast, in the absence of p21, both cyclins and both cdks are prevalently detected in the cytoplasm of similar multinucleated structures. A similar behavior was observed for cyclin E (data not shown). Significantly, re-introduction of p21 by a retroviral vector, used in co-infection with MyoD, restores nuclear localization of a significant amount of cdk2 and cdc2 (Figure 8b). The co-localization of p21 with each of the kinases in all the cdk-positive nuclei, as determined by double immunofluorescence staining, strengthens the direct involvement of p21 in this process. In light of these observations, we reasoned that the role of p21 in this system could be that of redirecting an aberrant activity of cyclin/cdk complexes to the nuclear compartment. To clarify this point, we

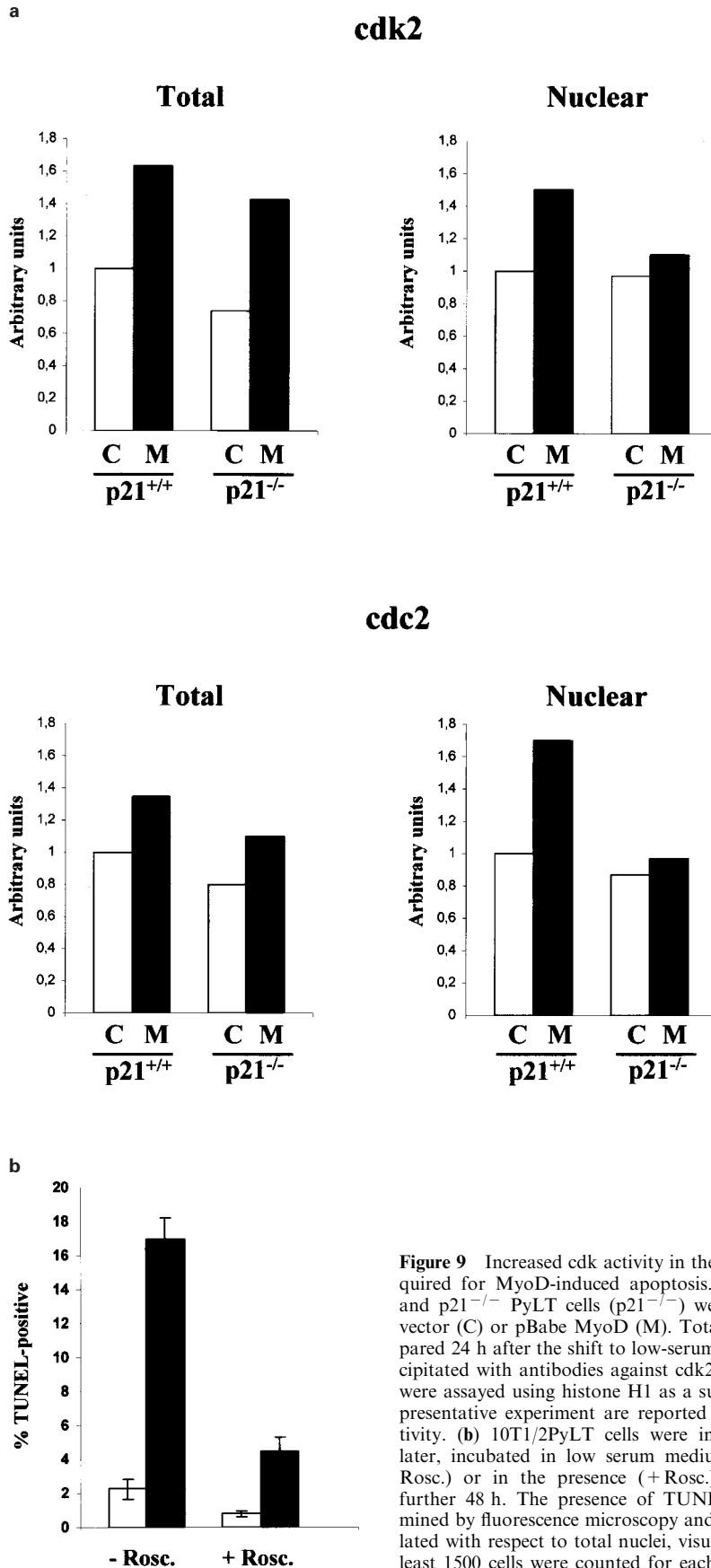


**Figure 7** p21 is not required for MyoD to induce the accumulation of cell cycle proteins. (a) p21<sup>-/-</sup> PyLT cells were infected with the empty vector (C), with pBabe-MyoD (M) or with pBabe-p21 (p21). Cell lysates were analysed by Western blot with antibodies specific for the indicated proteins, 24 and 48 h after the shift to low-serum medium. (b) 10T1/2PyLT cells were infected and analysed as in (a). The slightly different electrophoretic mobility of exogenous and endogenous p21 proteins reflects some minor differences in the aminoacid sequences of human and murine p21

compared the effects of MyoD on nuclear cdk activities in the presence and in the absence of p21. To this end cdk2- and cdc2-containing complexes were immuno-



**Figure 8** p21 is necessary for MyoD to induce nuclear accumulation of cyclin/cdk complexes. **(a)** 10T1/2PyLT and p21<sup>-/-</sup> PyLT cells, infected with pBabeMyoD, were analysed by immunofluorescence staining for the indicated markers 24 h after the shift to low-serum medium. **(b)** p21<sup>-/-</sup> PyLT cells, coinfecting with both pBabe-MyoD and pBabe-p21 were doubly immunostained either for p21 and cdk2 (upper micrographs) or for p21 and cdc2 (lower micrographs). Nuclei were counter-stained with DAPI. The same field, for each double immunofluorescence, was photographed under specific filters



**Figure 9** Increased cdk activity in the nuclear compartment is required for MyoD-induced apoptosis. **(a)** 10T1/2PyLT (p21<sup>+/+</sup>) and p21<sup>-/-</sup> PyLT cells (p21<sup>-/-</sup>) were infected with the empty vector (C) or pBabe MyoD (M). Total and nuclear extracts, prepared 24 h after the shift to low-serum medium, were immunoprecipitated with antibodies against cdk2 and cdc2. Kinase activities were assayed using histone H1 as a substrate. The results of a representative experiment are reported as quantitation of radioactivity. **(b)** 10T1/2PyLT cells were infected as above and, 48 h later, incubated in low serum medium either in the absence (-Rosc.) or in the presence (+Rosc.) of 25  $\mu$ M roscovitine for further 48 h. The presence of TUNEL-positive cells was determined by fluorescence microscopy and the percentages were calculated with respect to total nuclei, visualized by DAPI staining. At least 1500 cells were counted for each sample and the results are the mean of three independent experiments

precipitated from either total or nuclear extracts from p21<sup>-/-</sup> and p21<sup>+/+</sup> cells expressing PyLT, after infection with MyoD or with the empty vector. The low level of cytoplasmic protein contamination of nuclear fractions was checked by Western blot with antibodies to the cytoplasmic signalling molecule Grb-2 (data not shown). As shown in Figure 9a, increased cdk2 and cdc2 kinase activities are detected in total extracts after MyoD infection, both in the presence and in the absence of p21. In contrast, when cdk activities are measured in nuclear extracts, a MyoD-induced increase of both cdk2 and cdc2 is only detected in the presence of p21. This finding indicates that the increased abundance of cyclin-cdk complexes, albeit partially inhibited (see above), results in an increased kinase activity in the nuclear compartment. To determine whether this increased activity was required for MyoD-induced apoptosis, we examined whether roscovitine, a specific inhibitor of both cdk2 and cdc2 (Meijer *et al.*, 1997), could prevent this apoptosis. 10T1/2 PyLT cells (p21<sup>+/+</sup>), infected with MyoD or with the empty vector, were shifted to low-serum medium in the presence or in the absence of the inhibitor, and analysed for the occurrence of apoptotic cell death. As shown in Figure 9b, treatment with roscovitine significantly reduces the percentage of TUNEL-positive cells, further supporting the role of nuclear cdk activity in MyoD-induced apoptosis.

All these data not only provide evidence for a pivotal role of p21 in apoptosis induced by MyoD activation in the absence of RB, but also reveal that the essential aspect of p21 activity in such a process is related to the inappropriate nuclear re-localization and aberrant activity of cyclin/cdk complexes.

## Discussion

Myoblasts lacking a correct RB function exhibit incomplete differentiation and undergo apoptosis both *in vitro* and *in vivo* (Zacksenhaus *et al.*, 1996; Wang *et al.*, 1997; Fimia *et al.*, 1998). We have previously reported that the onset of this apoptotic process is triggered by the activity of the muscle-regulatory factor MyoD (Fimia *et al.*, 1998). Here we show that apoptosis induced by MyoD in the absence of RB function requires the activity of the cdk inhibitor p21.

We first observed that ectopic MyoD expression induces apoptosis in different cell types sharing the inactivation of the RB pathway, regardless of their transformation properties, as found in fibroblasts expressing SV40LT or oncogenic ras. In agreement with our previous reports (Gottifredi *et al.*, 1999) this apoptosis does not require p53 activation since it takes place in cells expressing p53-inactivating oncoproteins, such as SV40 Large T (in fibroblasts) or papillomavirus E6 (in HeLa tumor cells). Moreover, this apoptosis seems not to require the previously reported E2F1 pro-apoptotic activity (Phillips *et al.*, 1999), since we found that MyoD causes cell death even in E2F1<sup>-/-</sup> MEFs (our unpublished observations).

A common feature of cells undergoing MyoD-induced apoptosis is their inability to perform growth arrest, most likely involving the deregulated activity of more than one of the E2F family members, that results from RB inactivation. Cell cycle progression, however, does not proceed normally, as revealed by the accumulation of cells in S and G2 phases. This finding is in agreement with previously reported results (Novitch *et al.*, 1996), showing a similar pattern of cell cycle distribution in Rb-deficient myocytes. The authors, however, do not report apoptosis to occur in their system, probably due to the selection of an apoptosis-resistant phenotype following stable MyoD expression in Rb<sup>-/-</sup> fibroblasts. In most of our experiments we used 10T1/2PyLT fibroblasts as a model of RB inactivation, since these cells can be infected with MyoD retroviral vectors with higher efficiency than RB<sup>-/-</sup> MEFs. Our confidence in this model is based on several observations indicating that RB inactivation is the main mechanism by which PyLT, unlike other viral oncoproteins, affects myogenesis. Although it has been recently reported that PyLT can bind p300/CBP coactivators, this interaction has been suggested to be mainly involved in Polyomavirus growth *in vivo* (Cho *et al.*, 2001). More importantly, we have previously demonstrated that PyLT mutants, unable to inactivate RB family proteins, fail to affect differentiation and survival of myoblast cells (Maione *et al.*, 1994; Fimia *et al.*, 1998). In light of the observation that, among the RB family members, also including p107 and p130, RB plays a preponderant role in MyoD-induced differentiation (Novitch *et al.*, 1996), we exclude that some other mechanism, besides RB inactivation, can significantly contribute to the effects exerted by PyLT in myoblast cells. Accordingly, in some of the experiments reported in this work, the results obtained with RB<sup>-/-</sup> MEFs were comparable to those with 10T1/2 PyLT, thus confirming the reliability of our model.

The abnormal cell cycle progression, associated with MyoD-induced apoptosis, correlates with the induction of p21. It has been well established that the major mechanism of cell cycle inhibition by p21 consists, in RB-positive cells, in the prevention of RB phosphorylation by cyclin D-cdk4/cdk6, resulting in G1 arrest (Sherr and Roberts, 1999 and references therein). Another independent function, by which p21 participates in growth arrest, involves the binding of PCNA, and inhibition of DNA replication (Luo *et al.*, 1995). More recently, p21 has been reported also to regulate the G2/M transition during cell cycle (Dulic *et al.*, 1998) and to participate in the G2 checkpoint (Bunz *et al.*, 1998), through several mechanisms, not yet completely clarified, directly or indirectly converging on cdc2 (Taylor and Stark, 2001 and references therein). We found that the ability of MyoD to induce S and G2 phase accumulation was correlated with the incorporation of p21 into cdk2- and cdc2-containing complexes and with the inhibition of their specific kinase activities. Although we cannot exclude a possible role of inhibitory phosphorylations of cdk2,

it is likely that the binding and direct inhibition by p21 can participate in this regulation. A possible mechanism for S phase delay, through cdk inhibition, could involve the function of cyclinA-cdk2 complexes. In fact, it is believed that S phase exit requires the inactivation of E2F through cyclinA-cdk2 mediated phosphorylation (Krek *et al.*, 1995), so that the inhibition of these complexes could cause a prolonged E2F activity. It is currently believed that cyclin A/cdk2 plays a role, not yet well defined, also in promoting G2-M transition (Furuno *et al.*, 1999). Although it has been reported that the inhibition of cdc2/cyclin B1 complexes and the prevention of M phase transition can be induced through indirect mechanisms involving the inhibition of cdk2 (Guadagno and Newport, 1996), we do not exclude that the direct binding of p21 to cyclin B/cdc2 complexes, can itself play a role in the observed G2 accumulation.

The lack of MyoD ability to induce apoptosis in p21<sup>-/-</sup>PyLT fibroblasts and its restoration after reintroduction of p21, strongly suggests that p21 plays an important role in the apoptosis of RB-deficient myoblasts. A pro-apoptotic role of p21 is in apparent contrast with previous reports showing that cdk inhibitors, especially p21, act as survival factors in several cell systems (Poluha *et al.*, 1996; Gorospe *et al.*, 1997) including myoblast cells (Wang and Walsh, 1996; Lawlor and Rotwein, 2000). However, in most cases, the survival effects of p21 have been observed in the context of a functional RB pathway, where the cdk inhibitor is able to reinforce G1 arrest. Recently, Zacksenhaus and coworkers (Jiang *et al.*, 2000) reported a moderate increase in the frequency of apoptotic nuclei in some muscle areas of double Rb/p21 mutant, respective to single Rb mutant embryos. It is likely that several factors, related to the muscle cell type, the stage of muscle development and/or the expression pattern of different MyoD family members, could account for the discrepancy of this observation with our results. Consistently with an apoptotic role of p21, several reports indicate that the increased expression of natural cdk inhibitors, or the introduction of synthetic cdk inhibitory peptides, can selectively induce apoptosis in transformed cells (Chen *et al.*, 1999; Schreiber *et al.*, 1999; Tsao *et al.*, 1999; d'Agnano *et al.*, 2001).

The expression of MyoD in a p21-null background allowed us to make several interesting observations. It was not unexpected that, even in the absence of p21, MyoD could activate a growth arrest pathway. In fact, the lack of p21 does not affect muscle development *in vivo*, due to the redundant role of other cdk inhibitors, in particular p57<sup>KIP2</sup> (Zhang *et al.*, 1999b). However, although p57 is highly expressed in skeletal muscle (Zhang *et al.*, 1997), the pathways involved in its induction, during myogenesis, have not been elucidated, nor it is clear whether p57 and p21 are expressed in the same or in different myogenic lineages. Interestingly, we have found that MyoD activity leads to increased levels of p57, in p21<sup>-/-</sup> but not in p21<sup>+/+</sup> fibroblasts (our unpublished results), suggest-

ing the existence of some compensatory mechanism. Whatever the mechanism involved, the most important finding emerging from our data is that the activation of this alternative growth arrest pathway does not cause apoptosis. This pathway, potentially involving the inhibition of cdks by p57, could account, at least in part, for the cell cycle delay induced by MyoD in p21<sup>-/-</sup>PyLT, but is unable to relocalize cyclins and cdks to the nucleus, albeit their high levels of expression. Remarkably, reintroduction of p21 restores both apoptosis and nuclear localization of cyclins and cdks, strongly suggesting that the mechanism of MyoD-induced apoptosis may involve the ability, unique to p21, of inducing nuclear assembly of cyclin/cdk complexes. It should not be overlooked that these complexes are not completely inhibited, thus suggesting that a residual cdk activity, once directed to the nuclear compartment, may play an important role in MyoD-induced apoptosis. In line with this hypothesis, we have found that treatment with the chemical cdk inhibitor roscovitine, highly specific for cdk2 and cdc2 (Meijer *et al.*, 1997), efficiently increases cell survival in this system. These data are consistent with previous reports showing that either cdk2 or cdc2 activation is associated, through pathways not yet well clarified, with several apoptotic processes (Shi *et al.*, 1994; Meikrantz and Schlegel, 1996; see also Guo and Hay, 1999 and references therein). More work is required to determine the relative contribution of the two cdks' aberrant activities to the apoptosis executing machinery.

We propose that p21 plays a dual role in the alterations induced by MyoD in the absence of RB function. The first role relates to a partial inhibition of cdk2 and cdc2, a function that can be substituted for by other cdk inhibitors or alternative mechanisms. This inhibition would cause a delay of S and G2 progression, resulting in a partial cell synchronization in those phases and consequent accumulation of cyclins and cdks. The second one regards a more complex regulatory function, related to the subcellular compartmentalization of cyclins and cdks, and is unique to p21. Although p21 seems to control and co-ordinate both kinds of cell cycle alterations in the presence of MyoD, the sole over-expression of p21 does not reproduce all the effects exerted by MyoD. Accordingly, the over-expression of p21, in other cell types lacking RB, causes a different growth arrest pattern, mainly characterized by G1 and G2, but not S phase accumulation (Bates *et al.*, 1998; Niculescu *et al.*, 1998). Our data suggest that the effects of MyoD on cell cycle, in the absence of RB, are mediated not only by the induction of p21 but also by the alteration of its functional properties. Several hypotheses can be advanced to explain this co-operation. For example, it has been shown that MyoD contributes to cell cycle arrest through additional mechanisms, such as the direct binding and inhibition of G1 cdks, in particular cdk4 (Zhang *et al.*, 1999a). We have found that, in PyLT-expressing cells, p21 preferentially associates with cdk4 when expressed alone, but with cdk2 and

cdc2 when expressed in the presence of MyoD (our unpublished observations). Thus it is possible to imagine that MyoD, by interacting with cdk4, redirects p21 to cdk2 and cdc2. Furthermore, as another mechanism involved in growth arrest during myogenesis, it has been shown that MyoD induces the expression of cyclin D3, that is found in inactive cdk4-containing complexes (Cenciarelli *et al.*, 1999). The occurrence of cyclin D3 up-regulation observed in PyLT-expressing fibroblasts, could as well alter the pattern of p21-cdk interaction and inhibition. We cannot exclude that MyoD, in the absence of RB function, could directly contribute to the increased expression of S and G2 cyclins, as recently suggested for cyclin E (Tedesco and Vesco, 2001), thus promoting the re-direction of p21 to cdk2 and cdc2 complexes, in a cyclin-dependent manner. In this regard, we have found that the MyoD mutant B2ProB3, defective in DNA binding and transcriptional activation, but still retaining a significant growth arrest ability (Crescenzi *et al.*, 1990), is unable to increase cyclin levels in the absence of RB (our unpublished observations). More work is required to understand how the different functional activities of MyoD cooperate in inducing the observed cell cycle effects.

Our data provide new insights into the mechanisms regulating the antagonism between muscle differentiation and loss of RB function. In particular they suggest that the activation of a differentiation-dependent growth arrest pathway involving p21, in cells escaping from G1, can cause apoptosis. This is of particular interest since many differentiation programs are strictly associated with growth inhibition. This is why we are confident that our findings, obtained in the muscle system, will apply and be relevant for other types of differentiation-associated programmed cell death.

## Materials and methods

### Cell lines, stable transfections and retroviral infections

Mouse fibroblast cells C3H10T1/2 (10T1/2), their subclone derivatives, mouse embryo fibroblasts (MEFs) and BOSC cells were all maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10–20% fetal calf serum (FCS). 10T1/2 PyLT and 10T1/2 SVLT cell clones were generated by stable transfection of 10T1/2 fibroblasts with plasmids coding either for Polyoma Large T or SV40, using Lipofectamine Plus Reagent (Gibco Life Technologies). Two representative cell clones, isolated after geneticin selection, were chosen on the basis of their high levels of Large T expression. 10T1/2-ras and 10T1/2 PyLT-ras cell lines were generated from 10T1/2 and 10T1/2 PyLT, respectively, through infection with pBabe v-Ha-ras retrovirus, followed by 1 week of puromycin selection. RB<sup>-/-</sup> MEFs, derived from RB knock-out mice (Jacks *et al.*, 1992), and p21<sup>-/-</sup> MEFs, derived from p21 knock-out mice (Brugarolas *et al.*, 1995), were kindly provided by Dr M Caruso (Istituto Biologia Cellulare CNR, Roma, Italy) and Dr M Crescenzi (Istituto Superiore di Sanità, Roma, Italy), respectively. The p21<sup>-/-</sup> PyLT cell line was obtained by pooling together four clones, expressing PyLT at levels

comparable to 10T1/2PyLT, isolated after stable transfection of p21<sup>-/-</sup> MEFs and hygromycin selection.

The pBabe retroviral vector coding for wild type MyoD has been previously described (Fimia *et al.*, 1998). The pBabe-p21, coding for wild type human p21, and pBabe-v-Ha-ras coding for oncogenic ras, were kindly provided by Dr B Amati (DNAX, Palo Alto, California). To obtain recombinant retroviruses, BOSC 23 ecotropic-packaging cells were transfected as described (Pear *et al.*, 1993). For retroviral infection, cells, plated 24 h before, were incubated with undiluted BOSC retroviral supernatant for 10 h and then re-fed with fresh medium. To promote MyoD activation, 48 h after retroviral infection, cells were shifted to low-serum medium (DMEM-0.5% FCS) and analysed after 24 or 48 h, as indicated. The chemical cdk inhibitor roscovitine (Calbiochem) was added to low serum-medium at the concentration of 25  $\mu$ M.

### Indirect immunofluorescence staining and in situ apoptosis detection

Cells were fixed in methanol/acetone (3:7, v/v) or, in the case of MyoD detection, in 4% paraformaldehyde followed by permeabilization in 0.1% Triton X-100. In some cases, prior to fixation, a pulse-labeling of 60 min with bromodeoxyuridine BrdU (Amersham) was performed.

The following primary antibodies, from Santa Cruz Biotechnology Inc, were used diluted 1:100 in 3% BSA/PBS: sc-596 for cyclin A, sc-245 for cyclin B1, sc-163 for cdk2, sc-54 for cdc2, either sc-397 or sc-6246 for p21 and sc-760 for MyoD. To detect bromodeoxyuridine (BrdU, Sigma), mouse monoclonal antibody BU-1 (Amersham Pharmacia Biotech.) was used undiluted (in this case a 30-minutes incubation with 1.5 N HCl at room temperature was performed before the incubation with the primary antibody). As secondary antibodies either a rhodamine-conjugated goat IgG fraction to mouse IgG or a fluorescein-conjugated IgG fraction to rabbit IgG (both from Cappel Immunochemical) were used diluted 1:100. Total nuclei were stained by incubation in a 1  $\mu$ g/ml solution of 4',6-diamidino-2-phenylindole (DAPI, Boehringer Mannheim, Germany).

Apoptotic cells were visualized following the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), according to the instructions for In Situ Detection Kit Fluorescein (Roche). The percentages of TUNEL-positive cells, in some experiments, were determined through microscopic inspection, by counting at least 1500 nuclei for each sample.

### Flow cytometry

Cell cycle analysis was performed by flow cytometry after Propidium Iodide-staining, as previously described (D'Agnao *et al.*, 2001). To avoid the consequences of MyoD-induced cell fusion, cells to be used for cytofluorimetric analysis of the DNA content were cultured, after retroviral infection, in the presence of 1.4 mM EGTA. The conditions for apoptosis detection by TUNEL and data acquisition have been described previously (D'Agnao *et al.*, 1998). Briefly, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and washed with PBS. Each sample was incubated in 50  $\mu$ l reaction mixture (deoxynucleotidyl transferase TdT and nucleotide mixture), washed in PBS and measured by FCM using FACScan cytofluorimeter (Becton Dickinson, Sunnyvale, CA, USA). To minimize MyoD-induced cell fusion, 10T1/2 PyLT cells to be used for

cytofluorimetric analysis of TUNEL staining were kept subconfluent throughout the experiment.

#### Immunoprecipitation and Western blot

Cells were lysed on ice in 50 mM Tris HCl pH 8.0, 150 mM NaCl, 5 mM EGTA pH 8.0, 50 mM NaF pH 8.0, 10% glycerol, 1.5 mM MgCl<sub>2</sub>, 1% Triton X-100 containing freshly added protease and phosphatase inhibitors (PMSF, leupeptin, aprotinin, Na<sub>3</sub>VO<sub>4</sub>, β-glycerophosphate). Lysates were clarified by centrifugation at 4°C and protein concentrations were determined by Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA).

To perform immunoprecipitations, cell lysates were precleared for 1 h with either protein G-agarose or protein A-agarose (Amersham Pharmacia biotech) and incubated for 2 h with protein G/A-agarose bound to the specific primary antibodies (Santa Cruz Biotechnology Inc). Immune complexes were washed with cold NET-gel buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.1% vol/vol Nonidet P-40, 0.25% gelatin, 1 mM Na<sub>3</sub>VO<sub>4</sub>), eluted and denatured in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol). Proteins from either immunoprecipitations or cell lysates were resolved on SDS-PAGE and transferred to PVDF filters (Millipore). Blots were probed with specific antibodies after blocking nonspecific reactivity with TBS-T plus 5% milk powder. Primary antibodies were diluted in TBS-T containing either 0.2% gelatin or 5% milk powder. Immune complexes were detected with horseradish peroxidase-conjugated species-specific secondary antiserum (Bio-Rad Laboratories, Hercules, CA, USA) followed by enhanced chemiluminescence reaction (Pierce). The following primary antibodies (Santa Cruz Biotechnology Inc) were used: for cyclin A, cyclin B1 and p21 a 1:1000 dilution of sc-596, sc-245 and sc-397, respectively; for cyclin E, cyclin D1, cyclin D3, cdk4, cdk2 and cdc2 a 1:500 dilution of sc-481, sc-450, sc-182, sc-260, sc-163 and sc-54 respectively.

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#### Immune complex kinase assays

Total extracts were prepared using a lysis buffer for kinase assay (50 mM HEPES pH 7.5, 250 mM NaCl, 0.1% Triton X-100, 1 mM DTT, 50 mM NaF, 5 mM EGTA, 1 mM EDTA). Nuclear fractions were prepared according to Pagano *et al.* (1993). Briefly, cells were incubated for 10 min in ice-cold hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 300 mM sucrose, 1 mM EDTA, 1 mM DTT, 2.5 mM NaF) containing freshly added protease and phosphatase inhibitors, and then disrupted by repeated aspiration through a 22-gauge needle. Nuclei were separated by centrifugation at 4°C at 960 g, washed once with hypotonic buffer and solubilized in lysis buffer for kinase assay.

Immune complexes were washed four times with cold NET gel Buffer, twice with kinase buffer (50 mM HEPES pH 7.5, 1 mM DTT, 10 mM MgCl<sub>2</sub>) and then resuspended in 30 μl of kinase buffer supplemented with 2 μg of histone H1, 2 μCi of [<sup>32</sup>P]ATP, 50 μM ATP, 10 mM β-glycerophosphate and 2.5 mM EGTA. After incubation for 10 min at room temperature samples were boiled in Laemmli buffer and separated by SDS-PAGE. Phosphorylated histone H1 was visualized by exposure to phosphorstorage screens.

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