

# Critical requirement for cell cycle inhibitors in sustaining nonproliferative states

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In adult vertebrates, most cells are not in the cell cycle at any one time. Physiological nonproliferation states encompass reversible quiescence and permanent postmitotic conditions such as terminal differentiation and replicative senescence. Although these states appear to be attained and maintained quite differently, they might share a core proliferation-restricting mechanism. Unexpectedly, we found that all sorts of nonproliferating cells can be mitotically reactivated by the sole suppression of histotype-specific cyclin-dependent kinase (cdk) inhibitors (CKIs) in the absence of exogenous mitogens. RNA interference-mediated suppression of appropriate CKIs efficiently

triggered DNA synthesis and mitosis in established and primary terminally differentiated skeletal muscle cells (myotubes), quiescent human fibroblasts, and senescent human embryo kidney cells. In serum-starved fibroblasts and myotubes alike, cell cycle reactivation was critically mediated by the derepression of cyclin D-cdk4/6 complexes. Thus, both temporary and permanent growth arrest must be actively maintained by the constant expression of CKIs, whereas the cell cycle-driving cyclins are always present or can be readily elicited. In principle, our findings could find wide application in biotechnology and tissue repair whenever cell proliferation is limiting.

## Introduction

The cell cycle is orchestrated by the coordinated actions of several kinases whose activity is regulated positively by cyclins (Murray, 2004) and negatively by cyclin-dependent kinase (cdk) inhibitors (CKIs; Harper, 1997). Entry into the cell cycle from previous quiescence depends on the activation of G1-phase kinases. These chiefly include cdk4 and cdk6 kinases (cdk4/6) activated by D-type cyclins during early to mid-G1 phase and the cdk2 kinase, whose activation at the G1/S boundary depends on cyclins E and A (Sherr, 1994; Sherr and Roberts, 2004). The single most important substrate of these cdks is the retinoblastoma protein (pRb), whose phosphorylation is a prerequisite for S-phase initiation (Weinberg, 1995). Recent results have shown that cell cycle reentry is facilitated by the activity of the cyclin C-cdk3 complex, which is also a pRb kinase (Ren and Rollins, 2004).

CKIs belong to two families known as INK4 and Cip/Kip. The INK4 family comprises four members that are indicated according to their approximate molecular size as p15, p16, p18,

and p19. These inhibitors specifically bind cdk4/6, preventing heteroduplex formation with D cyclins. Cip/Kip inhibitors include p21, p27, and p57. These molecules show much wider binding specificity, as they are able to bind essentially all cyclin-cdk complexes and, albeit with lower affinity, free cyclins (Harper, 1997). In addition to their inhibitory role on cell cycle kinases, Cip/Kip family molecules facilitate cyclin-cdk complex formation (LaBaer et al., 1997); the precise balance between these two opposite activities is still debated.

The vast majority of the cells that make up a vertebrate's body spend most of their time in different nonproliferating states, which are collectively labeled as G0 phase of the cell cycle. Physiologically nonproliferating cells can be found in at least three distinguishable conditions, including reversible quiescence, replicative senescence, and the postmitotic state that characterizes and defines terminal differentiation. These three states are quite disparate with respect to both their phenomenology and the molecular mechanisms responsible for proliferative arrest.

Quiescence is defined as a temporary, reversible absence of proliferation. This state can be induced by a variety of conditions including, among others, growth factor deprivation, contact inhibition, and loss of anchorage (Coller et al., 2006). Quiescence can be usually readily reverted by removing the conditions that determined it. Indeed, the succession of events

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Abbreviations used in this paper: cdk, cyclin-dependent kinase; CKI, cdk inhibitor; HEK, human embryo kidney; MEF, mouse embryo fibroblast; MSC, mouse satellite cell; MyHC, myosin heavy chain; pRb, retinoblastoma protein; TD, terminally differentiated.

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that follow exit from quiescence has long served as the principal experimental paradigm for cell cycle studies. Quiescence is usually associated with very low levels of cyclins, and it is generally believed that such low levels are its main determinants (Ekholm and Reed, 2000; Sherr and Roberts, 2004). By way of example, serum-starved quiescent fibroblasts express very low amounts of cyclins associated with any phase of the cell cycle. Serum refeeding triggers exit from quiescence by bringing about a surge of cyclin D expression followed by cyclins E, A, and B in a coordinated succession (Kerkhoff and Rapp, 1997). Unlike cyclins, G1-controlling cdk's are not primarily regulated at the protein expression level, thus making their cognate cyclins the limiting factors for cell cycle reentry (Ekholm and Reed, 2000).

Replicative senescence, which is also called cell aging, is normally a permanent state of proliferation arrest. It was originally described as an intrinsic limit to the number of duplications that cells can undergo in vitro (Hayflick and Moorhead, 1961). More recently, replicative senescence has come to be viewed as the result of telomere shortening, damage at the molecular level, or both (Herbig and Sedivy, 2006). Indeed, preventing telomere attrition (Bodnar et al., 1998) and/or DNA damage accumulation (Parrinello et al., 2003) often results in cell immortalization. Unlike their reversibly quiescent counterparts, aging cells can express high levels of G1 cyclins, which are nonetheless devoid of associated kinase activity (Dulich et al., 1993). Removal of p53 (Bischoff et al., 1990) or pocket (pRb family) proteins (Shay et al., 1991; Sage et al., 2000) has been shown to weaken replicative senescence or prevent it altogether. In most cases, both pathways must be disrupted to produce full immortalization (Shay et al., 1991). The involvement of CKIs in establishing cell aging has long been recognized. p21 and/or p16 expression has been shown to associate with senescence in a variety of cell types (Herbig and Sedivy, 2006), whereas the role of p27 is somewhat more restricted (Bringold and Serrano, 2000). Ablation of relevant CKIs such as p21 (Brown et al., 1997) or p16 (Itahana et al., 2003) before senescence takes place can delay or prevent its establishment or reduce resistance to immortalization. p21 is a transcriptional target and is one of the most important effectors of the DNA damage response mediated by p53. p16 prevents the formation of cyclin D-cdk4/6 kinase complexes, thus interfering with pRb phosphorylation (Harper, 1997). In addition, it has been shown that injection of anti-p53 antibodies into senescent fibroblasts induces cell cycle reentry along with a reduction of p21 expression (Gire and Wynford-Thomas, 1998). Thus, CKIs belong to regulatory pathways that are essential for the onset of senescent arrest; surprisingly, however, whether or not they are absolutely required for the maintenance of senescence as opposed to its establishment is not known (Beausejour et al., 2003).

Terminal differentiation defines cells that permanently exit the cell cycle in the course of acquiring functional specialization. In extreme cases, terminally differentiated (TD) cells such as keratinocytes and erythrocytes lose their nuclei altogether, thus irreversibly relinquishing their ability to divide. However, most TD cells keep a full replicative apparatus while nonetheless becoming extremely refractory to spontaneous or induced cell cycle reactivation (Tiainen et al., 1996a). The molecular

basis of such refractoriness is incompletely understood. It has been shown that pRb is absolutely necessary for the establishment of terminal differentiation in several histotypes, including skeletal muscle myotubes (Schneider et al., 1994). However, pRb is not required for maintenance of the postmitotic state (Camarda et al., 2004; Huh et al., 2004). Although several cyclins are down-regulated early upon skeletal muscle differentiation (Ohkubo et al., 1994; Tiainen et al., 1996b), the up-regulation of several CKIs has been shown to correlate with myotube formation. p18 (Myers et al., 2004), p21 (Halevy et al., 1995; Parker et al., 1995), p27 (Zabludoff et al., 1998; Messina et al., 2005), and p57 (CDKN2C, CDKN1A, 1B, and 1C, respectively; Zhang et al., 1999) have all been indicated to participate in control of the cell cycle in muscle differentiation, making it difficult to sort their respective functions. Most conclusions concerning the role played by CKIs in muscle differentiation rely on knockout animals (Myers et al., 2004), whose deficiencies may have been compensated for in the course of ontogenesis. Although useful to investigate the significance of CKIs in establishing the postmitotic state, such animals are ill suited to assess their relevance in the maintenance of terminal differentiation. Mice lacking one or more CKIs before the onset of terminal differentiation cannot help distinguish between continuing proliferation during differentiation and cell cycle reentry after the establishment of the postmitotic state.

The three aforementioned proliferation arrest states behave differently at the molecular level. For instance, the forced expression of cyclin E-associated kinase activity can induce cell cycle reentry in quiescent cells (Lukas et al., 1997; Connell-Crowley et al., 1998) but not TD myotubes (Latella et al., 2001). Similarly, the functional suppression of p53 can reactivate senescent fibroblasts (Gire and Wynford-Thomas, 1998) but not TD cells (Crescenzi et al., 1995). The postmitotic state cannot be established in myotubes in the absence of pRb (Schneider et al., 1994), whereas this protein is not essential for initiating either quiescence or cell senescence (Sage et al., 2000). Conversely, the removal of pRb reactivates the cell cycle in quiescent and senescent cells (Sage et al., 2003) but not in TD myotubes (Camarda et al., 2004; Huh et al., 2004).

Despite their conspicuous differences, conditions sharing nonproliferation as an endpoint might also share a core mechanism responsible for their common phenotype. In this study, we suppress the expression of various CKIs in cells representative of all three states. Unexpectedly, CKI removal led to widespread cell cycle reentry in all cases even in the absence of exogenous growth factors. Thus, all states of nonproliferation depend critically on the constant expression of cell cycle inhibitors. We conclude that even the deepest, genuinely permanent growth arrest states are the result of a decision that must be continuously reasserted by the cell.

## Results

### CKI suppression reactivates the cell cycle in TD myotubes

We have previously shown that the cell cycle can be reactivated in postmitotic TD skeletal muscle cells, adipocytes, and neurons

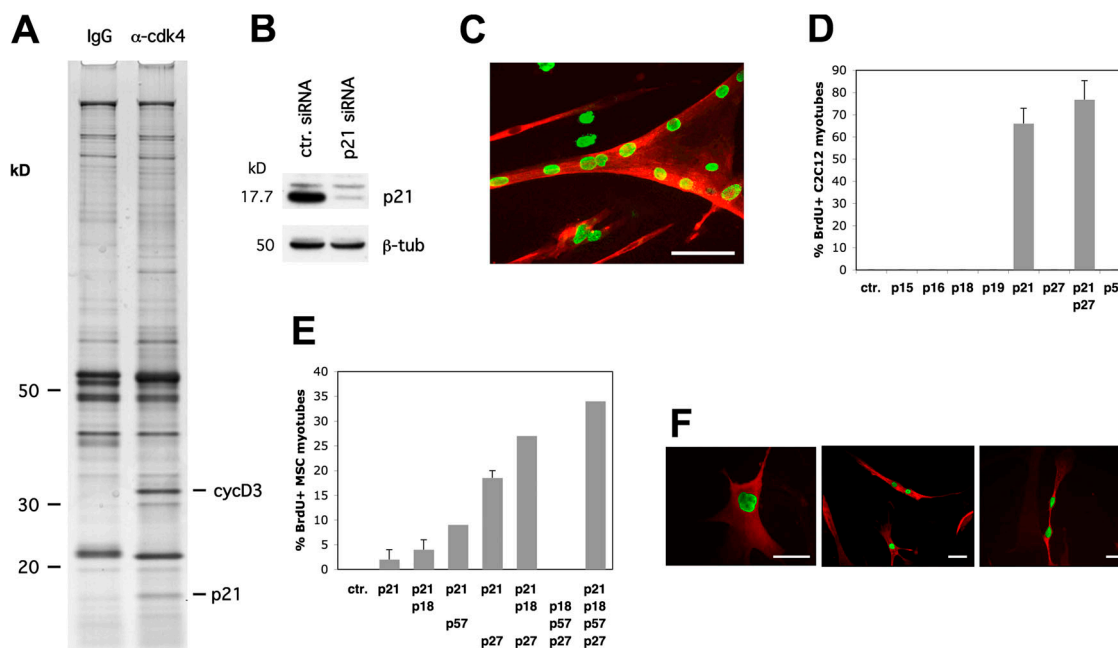
by restoring physiological levels of cdk4/6 activity. However, such restoration requires gross overexpression of both cyclin D1 and cdk4, suggesting the need to overwhelm preponderant levels of CKIs (Latella et al., 2001). We sought to alleviate the requirement for cyclin D1–cdk4 overexpression by identifying and removing functionally significant CKIs in myotubes, which is a prototypic TD model system.

To determine which CKIs are most relevant in our experimental settings, mouse C2C12 myoblasts were induced to terminally differentiate into myotubes that were then coinfecting with recombinant adenoviruses carrying cyclin D1 and cdk4. Infection multiplicities were chosen so as to achieve a moderate overexpression of both moieties in order to maximize capture of available inhibitory molecules without causing cell cycle reentry. Immunoprecipitations were performed with antibodies to cdk4 that were equally capable of precipitating free cdk4 and its cyclin D complexes. The coprecipitated molecules were electrophoretically resolved (Fig. 1 A). Several specifically precipitated molecules were identified by liquid chromatography/tandem mass spectrometry. Most relevant among these were mouse cyclin D3 and, as the only representative of CKIs, p21 (Fig. 1 A).

In an effort to reduce the load of CKIs in myotubes to facilitate cell cycle reactivation, we suppressed p21 expression by RNAi (Fig. 1 B). Unexpectedly, the sole suppression of p21 brought about a massive reentry of myotubes into the cell cycle, as shown by immunofluorescent detection of incorporated BrdU (Fig. 1 C). DNA synthesis took place equally well in the

presence of 5% FBS and in the absence of exogenous growth factors. Frequent mitotic figures were noted in the reactivated myotubes, although mitoses were nearly always morphologically aberrant. Such mitoses were quickly followed by apoptotic death as detected by TUNEL staining 48 h after RNAi: 40% of the p21-depleted myotubes were TUNEL positive versus 14.3% of the control cells. Both abnormal mitoses and cell death resemble those described in the case of viral oncogene–reactivated muscle cells (Latella et al., 2000). The specificity of p21 RNAi was ascertained by comparing the ability of four different double-stranded RNA oligonucleotides (siRNAs) for p21 to lower p21 protein levels and induce DNA synthesis in C2C12 myotubes (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200608109/DC1>).

The role played by the other known CKIs was systematically investigated by interfering with each of them singularly or in selected combinations (Fig. 1 D). The effectiveness of RNAi in reducing the expression of the different CKIs is shown in Fig. S1 (B–D). Ablation of no other single CKI stimulated BrdU incorporation in myotubes. Interference with p27 by itself also did not trigger DNA synthesis but reproducibly induced a small increase in the percentage of myotubes that were reactivated by interfering with p21 (Fig. 1 D). Fig. S2 (A–C; available at <http://www.jcb.org/cgi/content/full/jcb.200608109/DC1>) shows the association of three CKIs with cyclin D1, cyclin D3, or cdk4 in both myoblasts and myotubes. Total and cdk4-bound p21 and p27 were absolutely quantitated (Fig. S2 D). The relevance of



**Figure 1. Identification of relevant CKIs in myotubes and effects of their suppression.** (A) Electrophoretic gel separation of proteins immunoprecipitated with an anti-cdk4 ( $\alpha$ -cdk4) antibody or control Igs (IgG) from C2C12 myotubes. A silver-stained gel is shown here. Mass spectrometry was performed on bands excised from a Coomassie-stained gel loaded with 50 times as much total protein. Bands that appeared to be uniquely present in the cdk4 lane were processed for mass spectrometry identification. Corresponding portions of the control lane were processed identically. Bands containing p21 and cyclin D3 are indicated. (B) Western blot analysis of p21 in whole-cell lysates from C2C12 myotubes 48 h after transfection with a pool of four p21 siRNAs or control siRNA. (C) Immunofluorescence staining of C2C12 myotubes for BrdU (green) and MyHC (red). (D and E) Percentages of BrdU-positive C2C12 (D) and MSC (E) myotubes 48 h after interference for single or multiple CKIs as indicated. Data are presented as means and SDs (error bars) of up to five experiments. (F) Primary human myotubes were triple transfected with siRNAs for p21, p27, and p18 and were double immunostained for BrdU (green) and MyHC (red) 48 h later. Ctr, control;  $\beta$ -tub,  $\beta$ -tubulin, which was used as a loading control. Bars, 50  $\mu$ m.

Table I. Abrogation of BrdU incorporation in C2C12 myotubes by cyclin D3 suppression or dnK4 expression

Experiment	PT	PT only	PT + cyclin D3 siRNA	PT + dnK4
Percent BrdU <sup>+</sup> at 24 h	Control RNA	0	0	0
	p21 siRNA	30	<1	<1
Percent BrdU <sup>+</sup> at 48 h	Control RNA	0	0	0
	p21 siRNA	68	<1	<1
dnK4 added 24 h after RNAi	Control RNA	0	ND	0
	p21 siRNA	50	ND	50

PT, primary transfection. Values are percentages of BrdU-positive myotubes.

p21 does not descend from its abundance, at least in comparison with p27. Thus, the pivotal role of p21 in maintenance of the postmitotic state in C2C12 myotubes is dictated by variables other than mere cellular concentration.

CKI-RNAi experiments similar to those performed with C2C12 cells were performed with primary myoblasts (mouse satellite cells [MSCs]) freshly explanted from FVB mice. Fig. 1 E shows that in myotubes derived from these cells, multiple CKIs are involved in maintenance of the postmitotic state. Although in primary myotubes, ablation of p21 alone elicits very little BrdU incorporation, successive suppression of additional CKIs progressively increases the percentage of reactivated cells. However, interference with single CKIs other than p21 elicited no reactivation (negative data not depicted); even the combined suppression of p18, p27, and p57 was completely ineffective unless p21 was also suppressed (Fig. 1 E, right two histograms). These results suggest that key cdk complexes in MSC myotubes are controlled primarily by p21, whereas other CKIs take charge of suppressing cdk activity upon the loss of p21. Similarly, primary human myotubes incorporated BrdU (Fig. 1 F) and went through aberrant mitoses (not depicted) after the simultaneous ablation of p21, p18, and p27. Altogether, the results described thus far show that postmitotic myotubes can be reactivated by the sole removal of critical CKIs even in the absence of exogenous mitogenic stimuli. In turn, this suggests that fully competent cyclin-cdk complexes are present, although normally inhibited, even in permanently proliferation-arrested cells.

#### The activity of cyclin D3-containing complexes is responsible for cell cycle reactivation in myotubes

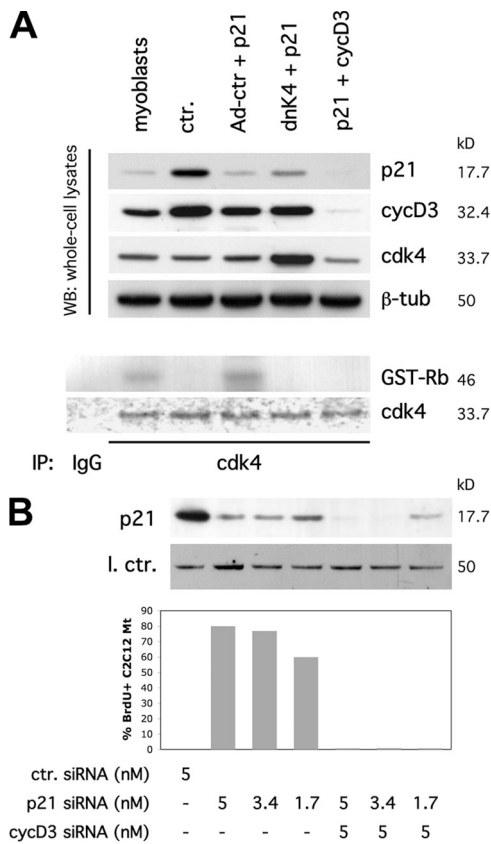
Reactivation of the cell cycle in the absence of exogenous growth factors presented an apparent paradox. Growth factors are required for cyclin D1 synthesis and assembly of cyclin D-cdk4/6 complexes (Sherr, 1995), which should be necessary to ignite a new cell cycle. Because the immunoprecipitation experiments have shown that sizable amounts of endogenous cyclin D3 associate with cdk4 (Figs. 1 A and S2 E), we hypothesized that preformed complexes containing at least cyclin D3, cdk4, and p21 are present in TD muscle cells. Indeed, similar complexes have been described in C2C7 myotubes (Cenciarelli et al., 1999). Such preexisting complexes would require neither de novo synthesis nor assembly, thus being growth factor independent. The subtraction of p21 would allow cell cycle-promoting kinase activity to be expressed. This model finds strong support

in a published quantitative appraisal of cell cycle-regulatory molecules in C2C12 myotubes showing low combined levels of D-type cyclins with a 3:1 prevalence of cyclin D3 over cyclin D1 (Wang and Walsh, 1996). Western blot analyses shown in Fig. S2 E agree qualitatively with this previous study.

Our model made verifiable predictions: impairment of either cyclin D3 or cdk4/6 should abrogate the effects of p21 removal. Indeed, in C2C12 myotubes, suppression of cyclin D3 or infection with a recombinant adenovirus carrying a dominant-negative mutant of Cdk4 (dnK4) abolished BrdU incorporation stimulated by interference with p21 (Table I). To assess the specificity of these results, similar experiments were performed with double p21/cyclin D1 RNAi. Interference with cyclin D1 determined only a modest reduction in myotube DNA synthesis in comparison with p21 RNAi alone (unpublished data). The specificity of dnK4 activity was verified by expressing it 24 h after p21 RNAi. In this case, there was no reduction in BrdU incorporation, as the newly activated cell cycle had already progressed past the point where cdk4/6 activity is required (Table I).

As also entailed by our model, interference with p21 should bring about substantial cdk4 kinase activity, which should be abrogated by the functional ablation of either cdk4 or cyclin D3. To test this prediction, C2C12 myotubes were subjected to RNAi for p21 either alone or in combination with cyclin D3 RNAi or infection with dnK4. Fig. 2 A shows that p21 protein levels were reduced in all p21-interfered cultures compared with myotubes transfected with control siRNA. Cyclin D3 protein levels were also profoundly decreased by the corresponding siRNA. In agreement with the model, cdk4 activity, which is absent in control-transfected TD muscle cells, was readily measured in p21-interfered myotubes at levels at least comparable with those found in proliferating myoblasts (Fig. 2 A). Such activity became undetectable upon either dnK4 infection or cyclin D3 RNAi. The specificity of cyclin D3 interference was verified by Western blot and cell cycle reentry analyses (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200608109/DC1>), as already performed for p21. We noticed that p21 protein levels, which were already much reduced by p21 RNAi alone, were further lowered by simultaneous transfection of siRNA for cyclin D3 (Fig. 2 A and see Fig. 4 A). Because p21 is known to facilitate cyclin-cdk complex assembly (LaBaer et al., 1997), it was conceivable that exceedingly low levels of p21 rather than cyclin D3 suppression might be responsible for impairing cell cycle reactivation. To exclude this

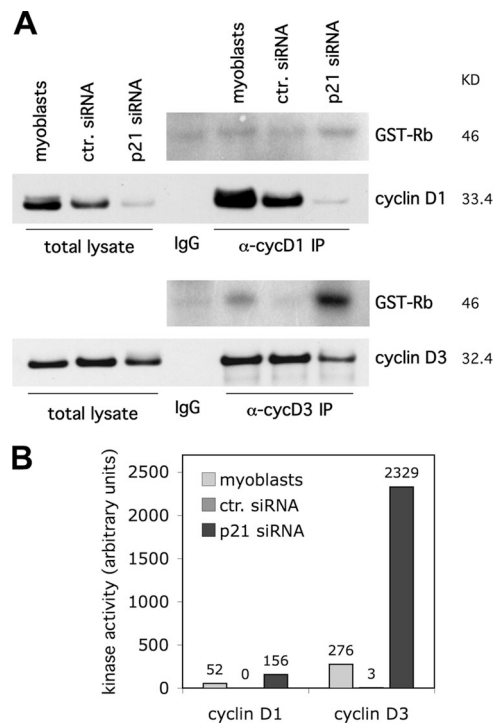




**Figure 2. cdk4-associated kinase activity in CKI-interfered C2C12 myotubes.** (A) C2C12 myotubes were infected with an adenovirus carrying dnK4 or a control virus (Ad-ctr) and/or were transfected 6 h later with siRNAs for the indicated molecules. (top) Western blot (WB) analyses of the indicated proteins in whole-cell lysates prepared 24 h after transfection. Proliferating myoblasts are shown for reference. (bottom) cdk4-associated kinase activity measured using GST-Rb as the substrate in immunoprecipitates obtained with an anti-cdk4 antibody from the same lysates as in the top panel. cdk4 was quantitated by Western blotting in the immunoprecipitates as a control for precipitation efficiency. (B) C2C12 myotubes (Mt) were transfected with p21 siRNA #4 (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200608109/DC1>) at the indicated concentrations in the presence or absence of 5 nM siRNA to cyclin D3. An identical culture was transfected with control siRNA. (top) Western blot analysis of p21 in whole-cell lysates collected 48 h after transfection. l ctr, loading control. (bottom) Percentages of BrdU-positive myotubes.

possibility, we interfered with cyclin D3 and cotransfected progressively decreasing amounts of p21 siRNA in C2C12 myotubes (Fig. 2 B). Double transfection with cyclin D3 and 1.7 nM p21 RNA oligonucleotides yielded p21 levels comparable with those obtained with p21 siRNA alone, yet BrdU incorporation was still entirely suppressed. Thus, the suppression effect can be ascribed entirely to cyclin D3 interference rather than to its modest indirect effect on p21 protein levels.

To explore the reasons why cyclin D3 plays a decisive role in the reactivation of C2C12 myotubes upon p21 removal, we measured cyclin D1- and cyclin D3-associated kinase activities in C2C12 myoblasts and in myotubes transfected with p21 or control siRNA. Fig. 3 shows that in myoblasts, the kinase activity associated with cyclin D3 is three times as high as that of cyclin D1. In control myotubes, there is virtually no measurable cyclin D-linked kinase activity. p21 RNAi in myotubes brought about a



**Figure 3. Cyclin D1- and cyclin D3-associated kinase activities in p21-interfered myotubes.** C2C12 myotubes were transfected with control or p21 siRNA, and immunoprecipitations for both cyclins were performed side by side 22 h later. Proliferating myoblasts synchronized in mid-G1 phase (see Materials and methods) are shown for reference. (A, top) Cyclin D1-associated kinase activity was measured using recombinant GST-Rb as the substrate. Western blot analysis of cyclin D1 in whole-cell lysates and immunoprecipitations are shown. (bottom) Cyclin D3-associated kinase activity measured as in the top panel. IgG, immunoprecipitation of myotube lysates with normal Igs.  $\beta$ -tubulin loading controls for whole-cell lysates are shown in Fig. S2 (A and B, available at <http://www.jcb.org/cgi/content/full/jcb.200608109/DC1>). (B) Densitometry-assisted quantitation of cyclin D1- and cyclin D3-associated kinase activities shown in A. See Materials and methods for details. Ctr, control.

marked decrease in cyclin D1 levels and a much more modest reduction of cyclin D3, which is consistent with the stabilizing role of p21 (Bagui et al., 2003). Although in p21-interfered myotubes the activities associated with both cyclin D1 and D3 raise, the latter is eightfold higher than that of cyclin D1. Thus, the vast majority of cyclin D-associated kinase activity in p21-depleted myotubes is carried by cyclin D3, which is the most abundant D-type cyclin in these cells (Wang and Walsh, 1996), explaining its pivotal role in myotube reactivation.

Altogether, these results lead us to conclude that in myotubes, preassembled, potentially competent cyclin D3-cdk4/6 complexes are held inactive chiefly by p21. CKI removal activates the kinase, allowing myotubes to reenter the cell cycle. To our knowledge, this is the first instance of reactivation of TD cells by subtraction of an endogenous inhibitor as opposed to the forced expression of exogenous activators.

#### Cdk4/6 is the direct target of CKI removal in myotubes

Cip/Kip family CKIs have the ability to bind most cyclins and cdk4s (Sherr and Roberts, 1999). As a consequence, removal of

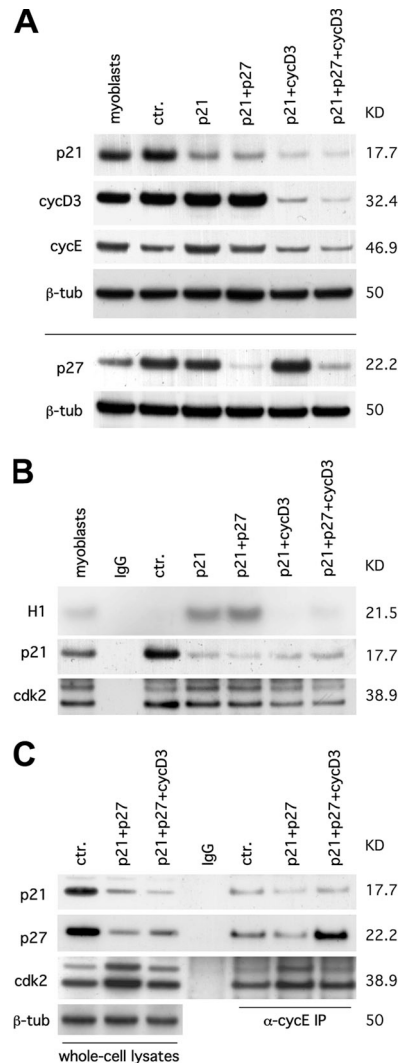
p21 or other Kip-type CKIs might derepress G1 kinases other than cdk4/6, most importantly the kinases associated with cyclin E. To investigate this possibility, C2C12 myotubes were transfected with control siRNA or various combinations of siRNAs for p21, p27, and cyclin D3. Fig. 4 A shows that the corresponding protein levels were effectively reduced. Substantial activity was found in cyclin E immunoprecipitates from myotubes interfered for p21 or p21 and p27. However, such activity was essentially abrogated by cyclin D3 RNAi (Fig. 4 B), indicating that the cyclin E-associated kinase is triggered as a result of the previous activation of cyclin D3-containing complexes. This result is in keeping with the notion that ordinarily, the activation of cdk2 is preceded by and is partially dependent on previous cdk4/6 firing (Sherr and Roberts, 2004). In the present case, the removal of CKIs might be supposed to free cyclin E-cdk2 complexes, but cyclin E-associated kinase activity still depends on previous cdk4/6 activation. Fig. 4 C shows that upon cyclin D3 RNAi, the association of residual p27 with cyclin E is enhanced in spite of the strong reduction in p27 total levels, possibly as a result of diminished competition from cyclin D3-containing complexes. This finding provides a plausible mechanistic explanation for the lack of cdk2 activation upon simultaneous removal of CKIs and cyclin D3. Altogether, these results indicate that in myotubes, the primary target of CKI-mediated inhibition is not a cyclin E-driven kinase but rather a cyclin D3-cdk4/6 complex. This conclusion is also in agreement with our previous results showing that TD myotubes can be reactivated by physiological levels of cdk4 activity but not by the cdk2 kinase even if hyperactive (Latella et al., 2001).

#### CKIs can substitute for one another in preserving the postmitotic state

We wished to investigate whether different CKIs could substitute for one another upon the selective suppression of one of them. We selected a well-studied model system: mouse embryo fibroblasts (MEFs) converted into muscle cells by the expression of MyoD (Figliola and Maione, 2004; Vaccarello et al., 2006). Wild-type and p21<sup>-/-</sup> MEFs were infected with a MyoD-carrying adenovirus and were induced to differentiate into myotubes (myotubes from MyoD-converted MEFs [MEF-Mts]). TD myotubes thus obtained were subjected to RNAi for multiple CKIs. Similar to C2C12- and MSC-derived myotubes, wild-type MEF-Mts were reactivated only if the suppressed CKIs included p21. In particular, the suppression of p27 or p57 singularly or in various combinations could not elicit DNA synthesis (negative data not depicted). On the contrary, a meaningful fraction of p21<sup>-/-</sup> MEF-Mts were reactivated by the subtraction of either p27 or p57 (Fig. 5). Combinations involving more than one CKI allowed even higher percentages of myotubes to reenter the cell cycle. Thus, as suggested by multiple RNAi experiments (Fig. 1, D and E), diverse CKIs can functionally replace p21 in maintenance of the postmitotic state.

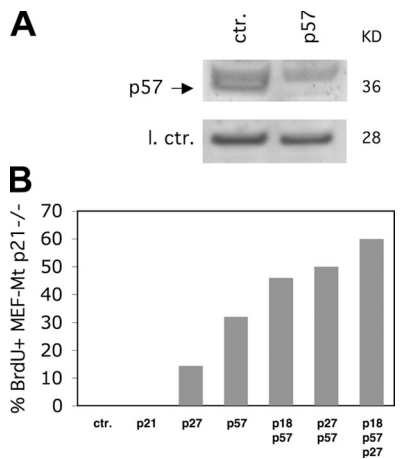
#### Quiescent human fibroblasts are reactivated by CKI removal

Prompted by the results obtained with TD myotubes, we asked whether other physiological nonproliferative states also need



**Figure 4. Kinase activity and CKIs associated with cyclin E in CKI-interfered C2C12 myotubes.** (A) Western blot analysis of specific proteins in C2C12 myotubes subjected to single or multiple RNAi. Cells were transfected with siRNAs for the indicated molecules, and whole-cell lysates were prepared 30 h later. These lysates are the same as those used to immunoprecipitate cyclin E and measure cyclin E-associated kinase activity in B. Proliferating myoblasts are shown for reference.  $\beta$ -tubulin ( $\beta$ -tub) is shown as a loading control in each of two separate blots. (B) C2C12 myotubes were transfected with siRNAs for the indicated molecules. Cyclin E-associated kinase activity on histone H1 was measured 30 h after transfection in anti-cyclin E immunoprecipitates. Immunoprecipitates were analyzed for p21 and cdk2. (C) C2C12 myotubes were treated as in A, and Western blot analyses of the whole-cell lysates and immunoprecipitates were performed.  $\beta$ -tubulin is shown as a loading control; cdk2 verifies immunoprecipitation efficiency. IgG, immunoprecipitation performed on myotube lysates with normal Igs.

constant maintenance by CKIs. RNAi experiments were performed with low passage primary mouse (not depicted) and human fibroblasts (Fig. 6, A and B) that were made quiescent by 48-h serum starvation. Interference with p21 alone or in association with p27 and/or p18 in the presence of 0.1% FBS was up to 82% as efficient in inducing cell cycle reentry as refeeding with 10% serum (Fig. 6 B). Mitotic figures were much more frequent among the CKI-interfered cells than in those transfected with control siRNA (unpublished data). To assess whether

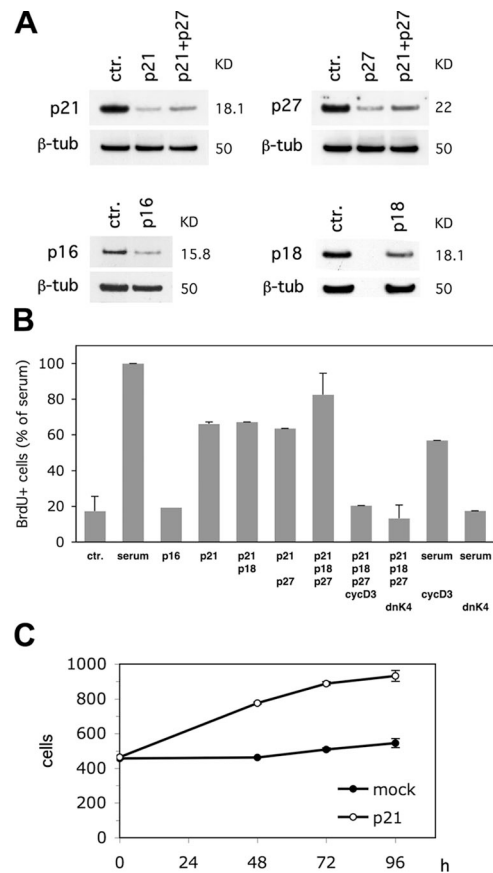


**Figure 5. CKIs can substitute for one another in preserving the postmitotic state.** MEF-Mts p21<sup>-/-</sup> were transfected with siRNAs for the indicated CKIs. (A) Western blot analysis of p57 performed 48 h after transfection with p57 siRNA. l. ctr, loading control. (B) Percentages of BrdU-positive MEF-Mt p21<sup>-/-</sup> measured 48 h after interference for single or multiple CKIs as indicated.

the reactivated cells are capable of substantial proliferation, serum-starved p21-interfered fibroblasts were kept in culture medium containing 0.1% FBS and were counted in the 4 d after RNAi. Fig. 6 C shows that p21-depleted fibroblasts doubled in four days despite the absence of added growth factors, whereas control cells proliferated negligibly. Cell division was not accompanied by an appreciable increase in cell death: the percentage of TUNEL-positive p21-interfered cells never exceeded that of control fibroblasts (unpublished data).

Importantly, cell cycle reentry driven by CKI RNAi was again completely abrogated by either cyclin D3 RNAi or dnK4 expression (Fig. 6 B). As a specificity control, although dnK4 also abolished serum-mediated cell cycle reentry, cyclin D3 RNAi reduced serum-promoted reactivation only partially, which is consistent with the notion that in human fibroblasts, serum acts mainly via cyclin D1 (Baldin et al., 1993). Immunoprecipitation and Western blot analyses show that low levels of all three D cyclins are present in quiescent fibroblasts along with levels of cdk4 and cdk6 as high as those found in proliferating cells (Fig. S4, A and B; available at <http://www.jcb.org/cgi/content/full/jcb.200608109/DC1>). However, only cyclins D1 and D3 are found associated with cdk4 (Fig. S4 C). Although the suppression of cyclin D3 abolishes the p21 RNAi-mediated reactivation of these cells (Fig. 6 B), we cannot exclude a proliferative role for cyclin D1-containing complexes.

A quantitative evaluation of p21, p27, and p16 (Fig. S4, D–F) suggests that, as in the case of myotubes, the absolute expression level of a cell cycle inhibitor is not the primary determinant of its relevance in the maintenance of growth arrest. These results agree with and expand upon a previous study showing that the reduction of p21 levels induces mitotic reactivation of serum-starved fibroblasts (Nakanishi et al., 1995). Our data indicate that in fibroblasts as well as myotubes, cyclin D3–cdk4/6 complexes are present and potentially functional even during quiescence. Furthermore, they show that multiple CKIs are involved in silencing their activity.

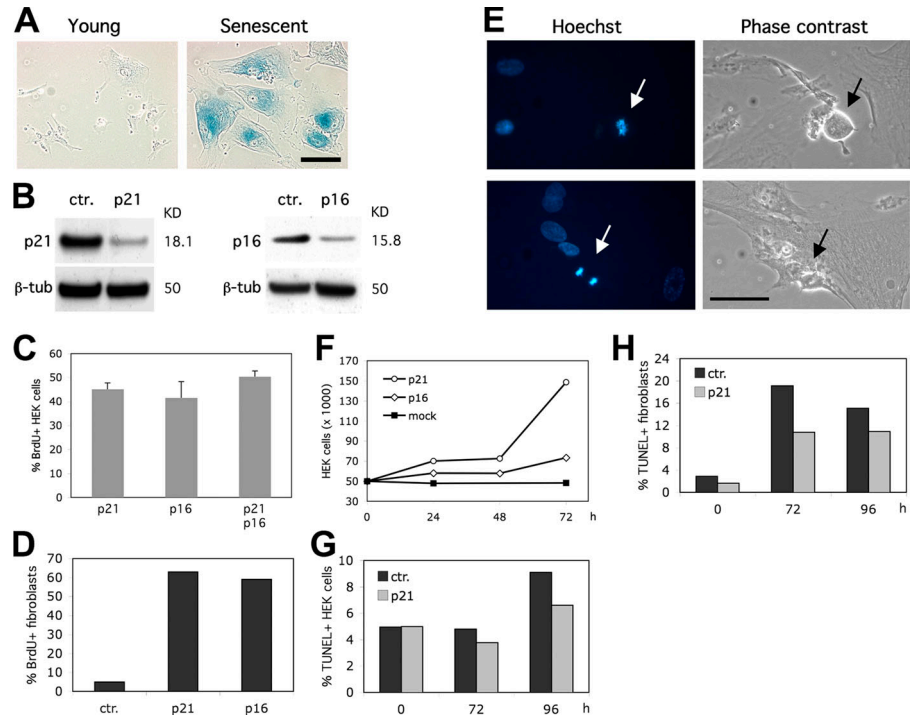


**Figure 6. Effects of CKI RNAi in serum-starved primary human foreskin fibroblasts.** (A) Western blot analyses of p21, p27, p16, and p18 in cells transfected with siRNAs for the indicated CKIs. Lysates prepared 48 h after transfection. (B) BrdU-positive cells in cultures subjected to RNAi for the indicated molecules. BrdU was added to culture medium 24 h after transfection, and the cells were fixed and stained 24 h later. Values are shown as means of up to three independent experiments with SDs (error bars) and are expressed relative to 10% serum-reactivated cells, which was set to 100. (C) Growth curves of cells subjected to p21 interference. Cells were transfected with siRNA to p21 or were mock-transfected and counted at the indicated times after RNAi. Ctr, control siRNA; serum, cells re-fed with 10% FBS.

### CKI suppression triggers proliferation of senescent human cells

Finally, we investigated the role of CKIs in the maintenance of proliferative senescence. Primary human embryo kidney (HEK) cells have a limited proliferative life span in vitro (Counter et al., 1992). Young cells are small and elongated, but, after a short term in culture, they become large, flat, irregularly shaped, and express acidic  $\beta$ -galactosidase activity, a marker of cellular senescence (Fig. 7 A; Dimri et al., 1995). Aging cells become unresponsive to growth factors and cease proliferation (unpublished data). Senescent HEK cultures (2% BrdU incorporation in 24 h) were subjected to RNAi for p21 and/or p16 (Fig. 7 B). Interference with p21 induced reactivation of the cells, bringing almost half of the population into the mitotic cycle. In these cells, p16 RNAi was almost equally effective, which is consistent with the known role of this inhibitor in establishing the replicative senescence of human cells (Fig. 7 C; Kiyono et al., 1998). Combined interference for the two CKIs demonstrated almost no

**Figure 7. Effects of CKI RNAi in senescent HEK cells and human foreskin fibroblasts.** (A) Acidic  $\beta$ -galactosidase staining of young and senescent HEK cells. (B) Western blot analyses of p21 and p16 in HEK cells transfected with siRNAs for the indicated CKIs. Lysates prepared 48 h after RNAi. (C) Senescent HEK cells were subjected to RNAi for the indicated molecules. BrdU incorporation assay as in Fig. 6 B. Percentages of BrdU-positive cells are shown after the subtraction of background. Error bars represent SD. (D) BrdU incorporation in senescent fibroblasts. Assay performed as in Fig. 6 B. (E) Mitotic figures in senescent HEK cells transfected with p21 siRNA. Arrows point to mitotic figures. (F) Growth curves of HEK cell populations transfected with siRNAs for the indicated molecules. The cells were transfected twice, at 0 and 48 h. (G and H) Percentages of TUNEL-positive HEK cells and human fibroblasts in cultures transfected with p21 siRNA. Ctr, control siRNA. Bars, 50  $\mu$ m.



synergy, suggesting that in this experimental system, unlocking either pathway is sufficient to induce cell cycle reentry. Similar results were obtained with senescent human foreskin fibroblasts. The depletion of p21 or p16 brought the majority of these cells back into the cell cycle (Fig. 7 D).

Although senescent cells are known to express high levels of inactive G1 cyclin-cdk complexes (Dulic et al., 1993), the reversal of senescence by direct removal of CKIs has not been shown before. Both HEK cells (Fig. 7 E) and fibroblasts (not depicted) displayed abundant mitotic activity upon RNAi-mediated reactivation, which suggested that they could actually proliferate. To test this possibility, senescent HEK cells were transfected with p21 or p16 siRNA twice at a 48-h distance. Daily counts showed that the number of control-transfected cells did not increase in 3 d, whereas the CKI-interfered populations grew (Fig. 7 F). In particular, the cells transfected with p21 siRNA increased more than threefold over a 72-h period, showing that interference with p21 brings about not only cell cycle reentry but actual population growth. Cell cycle reactivation in either HEK cells or fibroblasts did not cause any increase in the spontaneous rate of cell death (Fig. 7, G and H). Altogether, these data allow us to conclude that senescent as well as TD and quiescent cells rely on the continuous presence of cell cycle inhibitors to maintain their nonproliferative state.

## Discussion

In this study, we show that cells in such diverse nonproliferation states as terminal differentiation, quiescence, and replicative senescence can be allowed to reenter the cell cycle with comparable high efficiency simply by removing appropriate CKIs even in the absence of exogenous mitogenic stimuli. To de-

termine which molecules are most important in preventing cdk activation in TD myotubes, we selected an unbiased mass spectrometry-based approach. Such a strategy would be effective in uncovering both known and potentially unrecognized molecular brakes in our system. Immunoprecipitation/mass spectrometry experiments pointed at p21 as the main inhibitor in the C2C12 experimental system. Accordingly, interference with p21 was sufficient to reactivate the cell cycle in C2C12 myotubes. The unexpected discovery that removal of CKIs allows full cell cycle reactivation in notoriously refractory TD cells led us to suspect that more pliable cell types might be reactivated by the same strategy. This hypothesis was fully confirmed by results obtained with quiescent as well as senescent cells.

Absolute quantitation of some of the CKIs present in myotubes and in quiescent fibroblasts showed, somewhat surprisingly, that the inhibitor playing the most substantial role in suppressing proliferation was not the most abundantly expressed. In both mouse myotubes and resting human fibroblasts, p27 accumulates at much higher levels than p21 (Figs. S2 D and S4 D). However, the suppression of p21 is much more momentous than that of p27. Likewise, although in quiescent fibroblasts p16 protein levels are comparable with those of p21, the suppression of p16 is essentially inconsequential. There might be trivial explanations for these unexpected findings. For example, variable effectiveness in interfering with the different CKIs might skew the results. However, the large difference in the molar concentrations of p27 versus p21 (60-fold) strongly suggests the existence of partially unrecognized functional differences between the two molecules.

Several points concerning specific nonproliferation states are worth discussing. Myotubes are prototypic TD cells that are capable of resisting the mitogenic pressure of growth factors,



retroviral oncogenes, and many positive cell cycle regulators that act as powerful proliferative stimuli in non-TD cells (Tiainen et al., 1996a). The discovery of CKIs was quickly followed by reports of the high expression of a variety of them in numerous TD histotypes (Parker et al., 1995; Gill et al., 1998; Zabludoff et al., 1998). However, they are generally viewed as an aid in cell cycle exit at the inception of terminal differentiation (Missero et al., 1995) and as a safety feature against accidental cell cycle reactivation after the establishment of the postmitotic state. Indeed, there is no previous demonstration that CKIs are essential for maintenance of the postmitotic state in TD cells. As already discussed above (see Introduction), knockout animals cannot address this point, as they already lack CKIs before the onset of terminal differentiation. Thus, the appropriate means to investigate the role of inhibitory molecules in maintenance of the postmitotic state is to acutely ablate them after the establishment of this condition (Sage et al., 2003). Our results are all the more unexpected, as the functional suppression of p21 has been reported to be insufficient to reactivate C2C12 myotubes (Mal et al., 2000). However, this conclusion was reached indirectly by suppressing p21 with an E1A mutant whose other functions are incompletely understood; moreover, the activity of the essential cdk4/6 kinase was not investigated.

The present results are consistent with our previous conclusion that the cdk4/6 kinase must be suppressed to conserve the postmitotic state. Conversely, activation of this specific kinase (but not other cdk) is sufficient to drive TD cells into S phase (Latella et al., 2001). Indeed, we show here that CKI removal results in the reactivation of cdk4 and cell cycle re-entry. For the first time, however, TD cells have been reactivated not through the forced expression of exogenous molecules but by the ablation of endogenous inhibitors, highlighting the existence of latent kinase complexes, whose presence had previously drawn little attention. Furthermore, in light of potential applications of our findings, it is certainly desirable to achieve cell cycle reactivation with a comparatively mild, reversible manipulation of endogenous molecules.

Although the reactivation of quiescent fibroblasts by antisense suppression of p21 has been described previously (Nakanishi et al., 1995), we show here that D cyclins are the main players in such reactivation, which is similar to our finding in the myotubes. By analogy with TD muscle cells, resting fibroblasts possess considerable levels of preformed cyclin D-cdk4 complexes, whose activity is counteracted by specific CKIs.

At least two pathways are deeply involved in determining cell senescence: those of p53 and pRb. These two pathways concur to establish senescence proliferation arrest, at least in part, via p21 and p16. Although the roles of these two CKIs in initiating cell senescence are firmly established, their contribution to the maintenance of this state is far less clear (Beausejour et al., 2003). Our data show that even after its attainment, replicative senescence must be actively maintained by expressing high levels of cell cycle inhibitors. Removal of these CKIs brings about not only cell cycle reactivation but, at least in the short term, true cell proliferation. Whether CKI suppression is sufficient to overcome both the senescence and crisis barriers to immortalization remains to be investigated.

The absence of proliferation is usually thought of as a passive state deriving from the unavailability of key components of the cell cycle engine, with particular emphasis on G1 cyclins (Ekholm and Reed, 2000; Sherr and Roberts, 2004). In the present study, we show that TD, quiescent, and senescent cells can be reactivated by the sole removal of key CKIs. Thus, both temporary and permanent growth arrest, far from being negatively determined states, entail a relentless commitment by the cell, which must actively maintain such states through the continuous, critical expression of inhibitors. As a corollary, these results imply that even cells in permanent, physiologically irreversible arrested states harbor functionally relevant levels of key cyclins whose unchecked activity is sufficient to reactivate the cell cycle. The biological role of these cyclins in apparently incongruous cellular environments remains to be investigated.

An ever-growing number of biotechnology applications requires large quantities of specific cell types to be grown in the shortest possible time, provided that this goal can be achieved without introducing or causing harmful alterations in the cells themselves. An ideal proliferation-inducing/accelerating protocol should leave the cell's genome and differentiative properties intact. A relatively gentle intervention such as reversible CKI removal might fulfill these requirements, although its suitability for these purposes must be thoroughly investigated. We believe that the results summarized in this study have the potential to allow, facilitate, and/or accelerate wound healing, tissue repair, and *in vitro* culture of fastidious cell types. As CKIs are pivotal inhibitors of proliferation in all of the cases assessed, their suppression could be exploited to induce the proliferation of cell types that cannot be currently cultured.

## Materials and methods

### Cells and adenoviruses

The mouse C2C12 myoblast cell line (Blau et al., 1985) was cultured as previously described (Latella et al., 2001). Differentiation was induced by plating the cells in DME containing 2% horse serum and 50  $\mu$ M 1- $\beta$ -D-arabinofuranosylcytosine (Latella et al., 2001). Myoblasts used as a reference were synchronized in mid-G1 phase to approximate myotubes synchronously reactivated by CKI knockdown. Synchronization was obtained by serum starvation (2% horse serum) for 16 h followed by refeeding with 20% FBS for 8 h. Primary MSCs were isolated and cultured as previously described (Rando and Blau, 1994; Tiainen et al., 1996a). Primary human satellite cells were cultured in growth medium (PromoCell) and induced to differentiate by plating them onto gelatin-coated dishes in DME containing 5  $\mu$ g/ml human insulin, 5  $\mu$ g/ml human holotransferrin, and 50  $\mu$ M 1- $\beta$ -D-arabinofuranosylcytosine for 6 d. Wild-type and p21<sup>-/-</sup> MEFs were cultured in DME supplemented with 10% FBS (growth medium). MEF conversion into muscle cells was obtained by infecting them with a recombinant adenovirus carrying MyoD at an infection multiplicity of 400. 4 d later, terminal differentiation was induced following the same protocol used for C2C12 myoblasts. Low passage human primary foreskin fibroblasts (FB1329) were cultured in growth medium; quiescence was induced by switching confluent cultures to 0.1% FBS for 48 h. Synchronization in mid-G1 phase, which was attained for the same reasons described for C2C12 cells, was achieved by serum starving nonconfluent cultures in 0.1% FBS for 48 h followed by refeeding with 20% FBS for 8 h. Fibroblasts became senescent after prolonged culturing (passage >40). Primary HEK cells (a gift from S. Bacchetti, McMaster University, Hamilton, Ontario, Canada) were grown in  $\alpha$ -MEM supplemented with 10% FBS and 2 mM L-glutamine. The overexpression of human cyclin D1 and cdk4 was obtained by coinfecting C2C12 myotubes with the corresponding recombinant adenoviruses (Latella et al., 2001) at infection multiplicities of 15 and 150, respectively. Cultures were collected 24 h after infection and processed for immunoprecipitation and mass spectrometry identification. The expression

of dnK4 was obtained by infecting C2C12 myotubes or FB1329 cells with a recombinant adenovirus [Latella et al., 2001] at a multiplicity of infection of 800 or 150, respectively.

### RNAi experiments

Target cells were transfected with the HiPerfect transfection reagent (QIAGEN) complexed with siRNAs. We optimized the HiPerfect/siRNA ratio to obtain efficient suppression of the target molecule with the smallest possible amounts of both reagents. RNAi for a single molecule in C2C12, MSC, and MEFs converted into myotubes was performed by complexing 6  $\mu$ l HiPerfect and 5 nM siRNA per 35-mm petri dish. Human satellite cell myotubes, FB1329 fibroblasts, and HEK cells were interfered with 12  $\mu$ l HiPerfect and 10 nM siRNA per 35-mm petri dish. RNAi for multiple molecules was performed by adding each siRNA at a final 5- or 10-nM concentration, depending on the cell type, to the same volume of HiPerfect used in single transfections. Transfection complexes were kept in the culture medium until the end of the experiment.

### Immunoprecipitation, mass spectrometry, and kinase activity

To identify cdk4-associated molecules, C2C12 myotubes infected with human cyclin D1- and cdk4-carrying adenoviruses were lysed in cdk4 immunoprecipitation buffer containing 20 mM Hepes, pH 7.5, 250 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 0.25% NP-40, 5 mM ATP, 1 mM DTT, 0.5 mM PMSF, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml chymostatin, and 20  $\mu$ g/ml leupeptin. Cell lysates were briefly sonicated and centrifuged. The small-scale analytical SDS-PAGE shown in Fig. 1 A was performed by immunoprecipitating cdk4 from 1 mg of whole-cell protein extracts, whereas large-scale mass spectrometry sample preparation was performed as follows. 50 mg of protein extract was subjected to 4 h of immunoprecipitation with a mixture of antibodies to cdk4: mouse mAb-1 and rabbit pAb-5 antibodies to human and mouse proteins, respectively (NeoMarkers), or control IgGs (Bioscience) bound to magnetic beads (Dynal). After extensive washes, the precipitates were detached from the antibodies by incubating the beads in 0.1 M glycine buffer, pH 3, supplemented with 0.1% Triton X-100 for 15 min; the suspension was then neutralized with 0.75 M Tris, pH 8, boiled, and separated on a 4–12% polyacrylamide gel (NuPAGE; Invitrogen). Selected Coomassie-stained bands were removed and processed for mass spectrometry identification. In brief, gel slices were excised, treated essentially as described previously [Shevchenko et al., 1996], and digested with modified sequencing grade trypsin (Promega). Liquid chromatography/mass spectrometry/mass spectrometry analyses were performed on an ion trap instrument (LCQ-DECA XP; Thermo Electron) equipped with a  $\mu$ -ESI probe. Peptide mixtures were separated on a C<sub>18</sub> BioBasic column (100  $\times$  0.18 mm; 5  $\mu$ m; Thermo Electron) at a 2  $\mu$ l/min flow rate using a 60-min linear gradient from 5 to 60% acetonitrile in 0.1% formic acid. Protein identification was obtained using Bioworks Browser 3.1 software (Thermo Electron) and searching against a human protein database from the National Center for Biotechnology Information.

To evaluate cdk4 activity in C2C12 cells, myoblasts and myotubes were lysed in cdk4 immunoprecipitation buffer and treated as described for cdk4 immunoprecipitation. 1.8 mg of protein extract was subjected to 4 h of immunoprecipitation with the anti-cdk4 antibody Ab-5 or control IgG bound to magnetic beads. After extensive washes, immunoprecipitates were resuspended in kinase buffer [Latella et al., 2001], and the kinase assay was performed on a GST-Rb fragment substrate (Santa Cruz Biotechnology, Inc.) as previously described [Latella et al., 2001]. Labeled proteins were resolved on a 4–12% polyacrylamide gel and detected by autoradiography. To measure cyclin D1- and cyclin D3-associated kinase activities in C2C12 cells, myoblasts and myotubes were lysed in cdk4 immunoprecipitation buffer and treated as described for cdk4 immunoprecipitation. 350  $\mu$ g of protein extract was subjected to 3-h immunoprecipitation with anti-cyclin D1 (72-13G; Santa Cruz Biotechnology, Inc.) or -cyclin D3 (C-16; Santa Cruz Biotechnology, Inc.) antibodies or control IgG bound to protein G-Sepharose beads (GE Healthcare). Kinase assay and autoradiography were performed as previously described [Latella et al., 2001]. To assess cyclin E-associated kinase activity, C2C12 myotubes were lysed in immunoprecipitation buffer, briefly sonicated, and centrifuged [Pajalunga and Crescenzi, 2004]. 3 mg of protein extracts were immunoprecipitated for 3 h with an anti-cyclin E antibody (M-20; Santa Cruz Biotechnology, Inc.) or control IgG bound to magnetic beads. Immunoprecipitations were performed as previously described [Pajalunga and Crescenzi, 2004]. Labeled proteins were resolved on a 4–12% polyacrylamide gel and detected by autoradiography. To characterize cdk4 and cdk6 complexes in human fibroblasts, proliferating and quiescent FB1329 cells were lysed in cdk4 immunoprecipitation buffer and treated as described for cdk4

immunoprecipitation. 1 mg of whole-cell lysates were subjected to 3 h of immunoprecipitation with anti-cdk4 or -cdk6 antibodies or control IgG bound to protein G-Sepharose beads.

### Microscopy, immunofluorescence, and acidic $\beta$ -galactosidase assays

Pictures were obtained using a fluorescence microscope (Axioskop 2; Carl Zeiss Microimaging, Inc.) with a 20 $\times$  NA 0.50 or a 40 $\times$  NA 0.75 plan Neofluar objective. Images were digitized with a camera (AxioCam; Carl Zeiss Microimaging, Inc.) and acquired with AxioVision 3.1 software (Carl Zeiss Microimaging, Inc.). Images were contrast enhanced by applying the brightness/contrast regulation of Photoshop 7.0 software (Macintosh version) to the whole image. Double immunofluorescence images were obtained by superimposing two single-color pictures with Photoshop (Adobe). In immunofluorescence procedures, mAbs to BrdU (Bu20a clone; Dako-Cytomation) and myosin heavy chain (MyHC; MF20; Bader et al., 1982) were used. AlexaFluor488- or 594-conjugated antisera to mouse IgG were obtained from Invitrogen. Nuclei were counterstained with Hoechst 33258. HEK cultures were stained for acidic  $\beta$ -galactosidase activity as described previously [Dimri et al., 1995].

### siRNAs

siGenome SMARTpool reagents (Dharmacon) composed of four siRNAs were used to interfere with the following human and mouse transcripts: CDKN1A (p21), CDKN1B (p27; human only), CDKN1C (p57), CDKN2A (p16), CDKN2B (p15), CDKN2C (p18), CDKN2D (p19), CCND3 (cyclin D3), and CCND1 (cyclin D1). The antisense sequence of the Individual siGenome duplex (Dharmacon) for mouse p27 was 5'-P.UAUCCGGCAGUGCUUCUCUU-3'. Individual siRNAs for mouse p21 and cyclin D3 (siGENOME SMARTpool Upgrades; Dharmacon) were used in some experiments; the antisense sequences are as follows: p21, #1 (5'-P.CAAAGUUCACCGUUCUCGUU-3'), #2 (5'-P.UUUCGGCCUGAGAUGUUCUU-3'), #3 (5'-P.CAACGGCACAUUGCUCCUU-3'), and #4 (5'-P.GAACAGGUCGGACAUCACCUU-3'); and cyclin D3, #1 (5'-P.AAUCACGGCAGCCAGGUCCUU-3'), #2 (5'-P.UAUAGAUGCAAAGCUUCUCUU-3'), #3 (5'-P.CUCGAGGCAGUCCAUUCUU-3'), and #4 (5'-P.UACCUAGAAGCUGCAAUUGUU-3'). The siCONTROL Non-Targeting siRNA #2 (Dharmacon) was transfected as a control.

### Western blot analyses

Proteins were separated on 4–12% polyacrylamide gels and analyzed by Western blotting with the following mAbs or pAbs: mAbs to cyclin D1 (72-13G; Santa Cruz Biotechnology, Inc.), cyclin D2 (DCS-3; Abcam), p16 and human cdk4 (both Ab-1; NeoMarkers), p27 (Transduction Laboratories), and  $\beta$ -tubulin (Sigma-Aldrich); pAbs to mouse cdk4 (Ab-5; NeoMarkers), p18 (Biosource International), and p16, p21, p57, cyclin D3, cdk2, cdk6, and cyclin E (Santa Cruz Biotechnology, Inc.). Peroxidase-conjugated antisera to mouse and rabbit IgG were purchased from Bio-Rad Laboratories. Western blots were developed using the SuperSignal kit (Pierce Chemical Co.). In immunofluorescence procedures, mAbs to BrdU (Bu20a clone; Dako-Cytomation) and MyHC (MF20; Bader et al., 1982) were used. AlexaFluor-conjugated antisera to mouse IgG were obtained from Invitrogen. Nuclei were counterstained with Hoechst 33258. HEK cultures were stained for acidic  $\beta$ -galactosidase activity as described previously [Dimri et al., 1995].

### RT-PCR

In semiquantitative RT-PCR procedures, total cellular RNA was extracted from MSC-derived myotubes with TRIzol reagent (Life Technologies). cDNA synthesis and PCRs were performed starting from 1  $\mu$ g of total cellular RNA using the GeneAmp Gold RNA PCR Reagent kit (Applied Biosystems). Individual CKIs were amplified along with mouse  $\beta$ -actin in the same test tube. PCRs proceeded for 25 or 30 cycles (p15 or p16 and p19, respectively) on a personal machine (Mastecycler; Eppendorf). The following primers were used: p15 forward, 5'-GCCCAATCCAGGTCATGATGATG-3'; p15 reverse, 5'-GATGGGGCTGGGGAGAAAGAAG-3'; p16 forward, 5'-GTGCGAGGTTCTTGGTCACTG-3'; p16 reverse, 5'-CGC-ACGATGCTTGATGTCCC-3'; p19 forward, 5'-GTCTGCGTGGGACCGTTG-3'; p19 reverse, 5'-CAGGAGCTAGGAAGCTGACCACG-3';  $\beta$ -actin forward, 5'-TGTTACCAACTGGGACGACA-3'; and  $\beta$ -actin reverse, 5'-CTTTTACGGTTGGCCTAG-3'.

### Recombinant proteins and absolute quantifications

For absolute quantification of CKIs in C2C12 and human fibroblasts, the following recombinant-tagged proteins were used as standards: mouse GST-p21, poly-His-p27, and human GST-p16, -p21, and -p27. A dilution curve

of each reference protein was run on an SDS-PAGE gel along with the cell extracts or immunoprecipitates to be quantitated, and the proteins were analyzed by Western blotting. Developed films were computer scanned and subjected to densitometry. The data thus acquired and the unknown samples (lysates or immunoprecipitates) were plotted as exemplified in Fig. S4 F, yielding raw quantity estimates that were corrected to take into account the difference in the molecular weight of natural proteins and recombinant standards.

#### Densitometric evaluation of kinase assays

To directly compare the kinase activities associated with cyclin D1 and D3 (Fig. 3), the densitometric measurements of pertinent bands (GST-Rb) were adjusted by subtracting their respective backgrounds (IgG). The adjusted values were then normalized for immunoprecipitation efficiency, which was calculated as follows:  $OD_{IP}/OD_{TL} \times \mu g_{TL}/\mu g_{IP} \times 100$ , where  $OD_{IP}$  = OD of the immunoprecipitated protein (arbitrary units),  $OD_{TL}$  = OD of the protein band in the total lysate (arbitrary units),  $\mu g_{TL}$  = total lysate proteins loaded (micrograms), and  $\mu g_{IP}$  = amount of total lysate subjected to immunoprecipitation (micrograms).

#### Online supplemental material

Fig. S1 shows p21 RNAi specificity and the knockdown efficiencies of other CKIs in mouse myotubes. Fig. S2 shows a characterization of cyclin-cdk-CKI complexes and quantification of total and cdk4-bound p21 and p27 in mouse C2C12 cells. Fig. S3 shows cyclin D3 RNAi specificity. Fig. S4 shows the expression of D-type cyclins, cdk4/6, p21, p27, and p16 and cyclin D-cdk4 complexes in proliferating and quiescent human fibroblasts. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200608109/DC1>.

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