

p21^{CIP1} and p57^{KIP2} control muscle differentiation at the myogenin step

Pumin Zhang,^{1,2,5} Calvin Wong,^{1,2} Dou Liu,^{1,2} Milton Finegold,⁴ J. Wade Harper,² and Stephen J. Elledge^{1-3,6}

¹Howard Hughes Medical Institute, ²Verna and Marrs McLean Department of Biochemistry, ³Department of Molecular and Human Genetics, ⁴Department of Pathology, Baylor College of Medicine, Houston, Texas 77030 USA

Cell-cycle arrest is thought to be required for differentiation of muscle cells. However, the molecules controlling cell-cycle exit and the differentiation step(s) dependent on cell-cycle arrest are poorly understood. Here we show that two Cdk inhibitors, p21^{CIP1} and p57^{KIP2}, redundantly control differentiation of skeletal muscle and alveoli in the lungs. Mice lacking both p21 and p57 fail to form myotubes, display increased proliferation and apoptotic rates of myoblasts, and display endoreplication in residual myotubes. This point of arrest during muscle development is identical to that of mice lacking the myogenic transcription factor myogenin, indicating a role for cell-cycle exit in *myogenin* function. Expression of myogenin, p21, and p57 is parallel but independent, and in response to differentiation signals, these proteins are coordinately regulated to trigger both cell-cycle exit and a dependent muscle-specific program of gene expression to initiate myoblast terminal differentiation and muscle formation.

[Key Words: Muscle cell differentiation; cell-cycle arrest; myogenin; Cdk inhibitors]

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Embryonic development is a complex process that requires precise spatial and temporal control of cell proliferation coordinated together with differentiation, morphogenesis, and pattern formation. Cell proliferation in the embryo is controlled by an intricate network of signal transduction pathways that integrate growth regulatory signals through regulation of cyclin-dependent kinases (Cdks), a family of enzymes that catalyze events required for cell-cycle transitions. Primary targets for this regulation are the G₁ cyclin/Cdk complexes cyclin D/Cdk4 and cyclin E/Cdk2, which cooperate to control the G₁ → S transition through phosphorylation and inactivation of the retinoblastoma (Rb) protein. Among other functions, Rb acts as a transcriptional repressor of E2F-regulated genes important for cell proliferation (Weinberg 1995). A large number of regulatory mechanisms exist to modulate Cdk activity, reflecting the complexity of the signaling pathways involved and the necessity to precisely control proliferation for the development of an organism. A particularly versatile mechanism for developmental control is the inhibition of Cdks by cyclin-dependent kinase inhibitors (CKIs), of which there are two families: the p16^{INK4a} family, including p15, p16, p18, and p19; and the p21^{CIP1/WAF1} family, including p21, p27, and p57 (Harper and Elledge 1996). The

p16 family specifically inhibits Cdk4 and Cdk6, whereas the p21 family inhibits all Cdks involved in G₁/S transition. The roles of these CKIs in development and in cancer have been revealed through targeted gene inactivation in mice. Although CKIs show striking tissue-specific patterns of expression during development, surprisingly only p57 loss has been shown to have a significant role in multiple tissues during embryonic development, suggesting that the other inhibitors are either not required or are redundant. Loss of p57 in humans results in the complex overgrowth and cancer predisposition disease Beckwith–Wiedemann syndrome (Zhang et al. 1997).

A variety of studies have indicated that cells must exit the cell cycle to terminally differentiate. The most revealing of these come from the analysis of skeletal muscle development. Skeletal muscle development is controlled by a group of basic helix–loop–helix (bHLH) transcription factors, including MyoD, Myf5, myogenin, and Mrf4, each of which is capable of forcing nonmuscle cells to adopt skeletal muscle phenotypes when expressed ectopically (Olson and Klein 1994). Analysis of mice lacking bHLH myogenic factors has revealed a genetic hierarchy in which MyoD and Myf5 play redundant roles in specifying muscle lineage (the generation of myoblasts); myogenin directly controls the differentiation process (the formation of myotubes); and Mrf4 is thought to be involved in the maturation of myotubes. Early studies using cultured myoblasts revealed that cell-cycle exit and differentiation are coupled (Bischoff and Holtzer 1969; Nadal-Ginard 1978; Clegg et al. 1987).

⁵Present address: Division of Basic Sciences, National Jewish Medical and Research Center, Denver, Colorado 80206 USA.

⁶Corresponding author.

E-MAIL selledge@bcm.tmc.edu; FAX (713) 798-8717.

Furthermore, MyoD ectopically expressed in fibroblasts fails to function if these cells are also provided proliferative stimulation in the form of additional expression of cyclin D (Rao et al. 1994; Skapek et al. 1995), cyclin A, or cyclin E (Skapek et al. 1996). Additional connections between cell-cycle exit and differentiation have been established through analysis of the role of Rb in skeletal muscle differentiation. These include observations that viral oncogenes capable of inactivating Rb, such as T antigen and E1A, can interfere with muscle differentiation (Fogel and Defendi 1967; Yaffe and Gershon 1967; Graessmann et al. 1973; Endo and Nadal-Ginard 1989; Taylor et al. 1993; Crescenzi et al. 1995) and that cells lacking Rb fail to properly differentiate in vitro (Gu et al. 1993; Novitsch et al. 1996). Although Rb-deficient mice display an apparently normal musculature (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992), the early lethality of these mice [before embryonic day 14.5 (E14.5)] has precluded analysis of the role of Rb in secondary myogenesis when the majority of skeletal muscles are formed (Kelly 1983). Recently, Zacksenhaus et al. (1996) reported that *Rb*^{-/-} embryos can be rescued to birth by the low-level expression of an *Rb* transgene, and these embryos show skeletal muscle defects. Taken together, this body of evidence points to an important role for Rb in control of cell-cycle exit and differentiation in myogenesis.

The Rb protein acts as a switch operated by cell-cycle Cdk machinery to control cell-cycle entry and exit and is likely to be a critical target of Cdk regulation relevant to differentiation. However, it is not at all clear which component of the myogenic regulatory hierarchy requires Rb for its function. Rb has been shown to interact with MyoD in vitro (Gu et al. 1993), although the significance of the interaction is brought into question by the fact that early targets of MyoD transcription appear to be fully induced in the absence of Rb (Novitsch et al. 1996). How Rb is activated to facilitate myogenic differentiation is currently not known. Although it is likely that Cdk inactivation is employed, other mechanisms such as increasing the activity of Rb specific phosphatases are also possible. Furthermore, assuming that Cdk inactivation is the mechanism, how this is achieved is not known.

Initial insights into how cell-cycle exit is achieved in vivo came from the observation that the CKI p21 is highly expressed in muscle and other terminally differentiating tissues in vivo (Parker et al. 1995) and in vitro (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995). Furthermore, cells ectopically expressing MyoD can induce p21 when stimulated to differentiate in vitro suggesting that p21 is a downstream target of MyoD. However, mice lacking p21 develop normally and fail to show muscle cell differentiation defects, bringing into question the role of p21 in muscle development (Deng et al. 1995). The absence of a role for p21 in skeletal muscle development could be explained by redundancy of the cell-cycle control mechanisms. We report such a redundant mechanism in this study by showing that mice lacking both p21^{CIP1} and p57^{KIP2} display severe defects

in skeletal muscle development (and other tissues including lung); this indicates that these proteins cooperate as terminal effectors of signaling pathways that impinge on cell-cycle control and differentiation. Furthermore, the stage in muscle cell differentiation at which these double mutants arrest reveals the point at which cell-cycle exit is required and implicates myogenin function as the critical step requiring cell-cycle exit.

Results

Generation of mice lacking both p21^{CIP1} and p57^{KIP2}

To generate mice lacking p21 and p57, we crossed p21^{+/-} p57^{+/-} females to p21^{-/-} males. Animals inheriting the mutant p57 allele from the mother have a p57 null phenotype because imprinting renders the paternally inherited allele silent. Consistent with our previous report (Zhang et al. 1997), there were no live-born mice lacking either p57 or both p21 and p57 functions (data not shown). However, E16.5 embryos of all genotypes were detected at Mendelian ratios. A substantial fraction of p57^{-m/+} single mutant (30%) and p21^{-/-} p57^{-m/+} double mutant (65%) embryos die in utero due to placental failure (Table 1). Thus, loss of p21 exacerbates the placental defects observed in p57^{-m/+} mutants. The following phenotypic analysis on p21^{-/-} p57^{-m/+} double mutants was based on animals that were not affected by placental failures.

p21^{-/-} p57^{-m/+} double mutants show altered lung development

Histopathological examination of p21^{-/-} p57^{-m/+} mice revealed all of the phenotypes caused by p57 loss alone (Yan et al. 1997; Zhang et al. 1997) and several novel phenotypes in tissues that are apparently unaffected in either of the single mutant animals. Unlike p21^{-/-} or p57^{+/-m} animals, the lungs of p21^{-/-} p57^{-m/+} animals were clearly defective, failing to fully differentiate distal air sacs, the ultimate functioning unit for gas exchange in lung tissue. The mammalian lung is composed of two types of tissues: an epithelium that lines all the airways from the trachea to alveoli, and a mesenchymal stroma that supports the epithelium. Lung development is divided into several periods. In the pseudoglandular period

Table 1. Distribution of genotypes among embryos derived from a cross between p21^{-/-} p57^{+/+} males and p21^{+/-} p57^{+/-p} females

Genotype	p21 ^{+/-} p57 ^{+/+}	p21 ^{-/-} p57 ^{+/+}	p21 ^{+/-} p57 ^{+/-m}	p21 ^{-/-} p57 ^{+/-m}
Number of embryos	26	24	20 (6) ^a	29 (19) ^a
Observed (%)	26	24	20	29
Expected (%)	25	25	25	25
Lethality (%)	0	0	30	65

^aNumber in parenthesis indicates the number of embryos that were already dead at the time of harvesting.

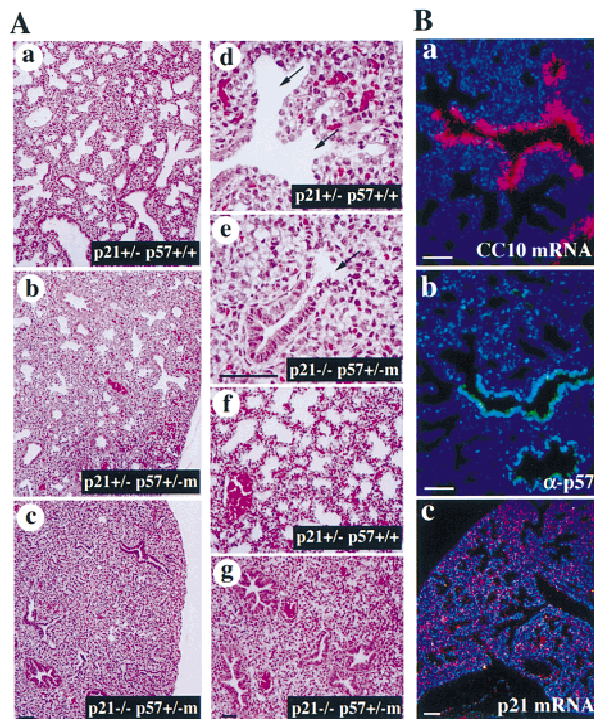


Figure 1. A block in the formation of primitive alveoli in the absence of both *p21* and *p57*. (A) H&E-stained transverse sections of lungs derived from E16.5 (a–e) and from E18.5 embryos (f,g). (B) Expression of *p21* and *p57* in the lung of E18.5 embryos. (a) CC10 expression detected with in situ hybridization; (b) immunofluorescence staining of *p57*; (c) *p21* expression detected with in situ hybridization. Scale bars, 200 μ m.

early during embryogenesis, the lung resembles an exocrine gland and consists of a complex of branching bronchial tubes that include the primary, secondary, segmental, and terminal bronchi and the bronchioles. This is followed by the canalicular period when respiratory bronchioles are formed. Each respiratory bronchiole is terminated in two or three thin-walled dilations termed terminal sacs or primitive alveoli. At E16.5, lungs from wild-type embryos display substantial formation of primitive alveoli manifested as open spaces on sections stained with hematoxylin and eosin (H&E) (Fig. 1A,a). In contrast, lungs from *p21*^{−/−} *p57*^{+/−m} animals are virtually devoid of open spaces (Fig. 1A,c). Under high magnification, it is evident that primitive alveoli do not develop in the double mutants (Fig. 1A, cf. d and e). This defect persists until birth (Fig. 1A, cf. f and g). Furthermore, there is a decrease in the size of the luminal space of the bronchi and bronchioles in the double mutants. *p21*^{+/−} *p57*^{+/−m} lungs exhibit an intermediate phenotype between the wild-type and the double mutant with some primitive alveoli but fewer than in the wild type (Fig. 1A, cf. a, b, and c), indicating that a single *p21* gene is insufficient in the absence of *p57*.

To explore the cause of the lung defect, we examined the expression of both *p57* and *p21* in the developing lung. *p57* is highly expressed in bronchiole epithelium,

mirroring that of CC10, a marker for that tissue. *p57* is expressed at lower levels in an undefined subset of lung mesenchymal cells and the epithelium lining of the terminal primitive alveoli (Fig. 1B). In contrast, *p21* is expressed throughout the lung. Despite high levels of expression of *p57* in the bronchiole epithelium, no significant abnormalities were detected in this tissue, and tissue-specific differentiation markers such as CC10 and SP-A, SP-B, and SP-C are expressed normally in double mutants (data not shown). Although the absence of air sac luminal space gives the appearance of increased cellularity in the mutants, this is not the case. This is due to the fact that the lungs of the double mutant mice are smaller than the wild-type lungs (data not shown); thus, the total number of cells is approximately the same. Furthermore, the overall proliferation rates in the double mutant lung were not elevated as judged by BrdU pulse labeling nor was there an increase in apoptosis (data not

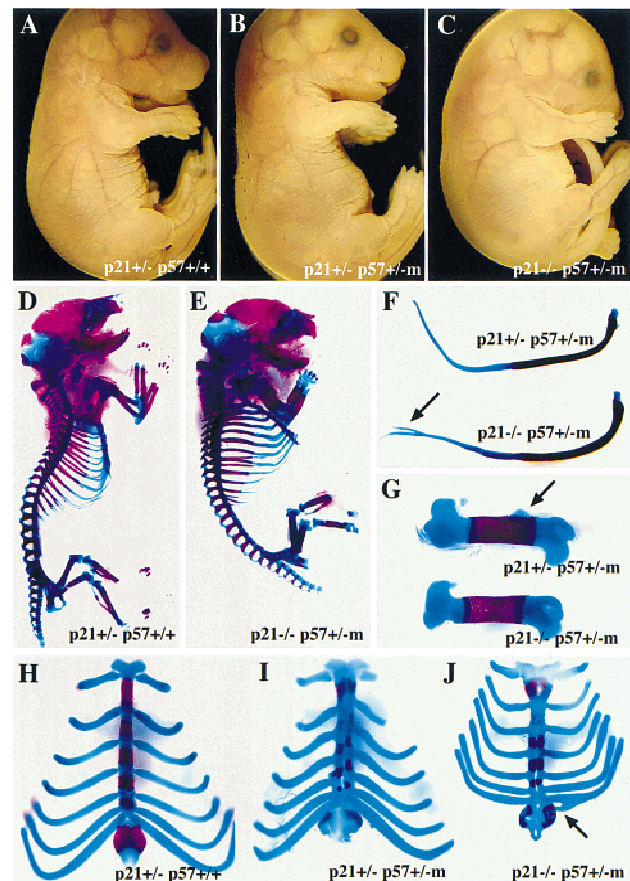


Figure 2. Skeletal defects in *p21*^{−/−} *p57*^{+/−m} mutants. (A–C) *p21*^{−/−} *p57*^{+/−m} embryos display altered posture. (D,E) Skeletons of E18.5 embryos stained with alcian blue to identify cartilage and alizarin red to identify ossified bone. (F) Bifurcation of the ninth rib (arrow) is observed in *p21*^{−/−} *p57*^{+/−m} embryos. (G) The femur of E18.5 embryos. The arrow indicates cartilage outgrowth. (H–J) Sternum and ribs of E18.5 embryos. Only 7 of the 13 ribs attach to the sternum. Note a bifurcation in the seventh rib in the double mutant (arrow in J). Embryos in G–J stained as in D and E.

shown). Thus, the defects in primitive alveoli formation in the absence of *p21* and *p57* is likely to result from subtle changes in the differentiation of either the epithelia or the mesenchymal stroma for which additional studies are required to delineate more precisely.

Skeleton defects in $p21^{-/-}$ $p57^{-m/+}$ double mutants

The only phenotype of $p57^{+/-m}$ mice that is enhanced by loss of *p21* is the skeletal phenotype. Deletion of *p57* alone causes delay in ossification and sternal fusion defects but no overall abnormality in the shape of the skeleton (Yan et al. 1997; Zhang et al. 1997). However, as shown in Figure 2, $p21^{-/-}$ $p57^{-m/+}$ double mutant embryos display a posture clearly distinct from those of wild-type and $p57^{-m/+}$ mutants (Fig. 2A–C). Skeleton staining revealed that double mutants (Fig. 2E) lack the spinal curvature seen in wild type (Fig. 2D) and $p57^{-m/+}$ single mutants (data not shown), which might stem from

defects in musculature (see below). Rib cage shape in double mutant embryos is also abnormal (Fig. 2, cf. D and E). Bifurcation of ribs was observed in double mutants, usually of the ninth rib (Fig. 2F), although occasionally the seventh rib is also affected (Fig. 2J). The femurs of double mutants lack a cartilage outgrowth seen in either *p21* or *p57* single mutants or wild-type littermates (Fig. 2G; data not shown). The double mutants exhibited sternum fusion defects similar to those seen in *p57* single mutants (Zhang et al. 1997), but the sternum of double mutants is shorter than that of *p57* single mutants (Fig. 2H). The ribs of double mutants join the sternum at an angle of 90°C (Fig. 2J), whereas the ribs of wild type or *p57* single mutants join at an angle much less than 90°C (Fig. 2H). Both *p21* and *p57* have been found highly expressed in developing ribs (Zhang et al. 1997; and data not shown). However, it is difficult to distinguish autonomous versus nonautonomous roles of these two inhibitors in ribs, especially considering the fact that similar defects in the attachment of ribs to sternum

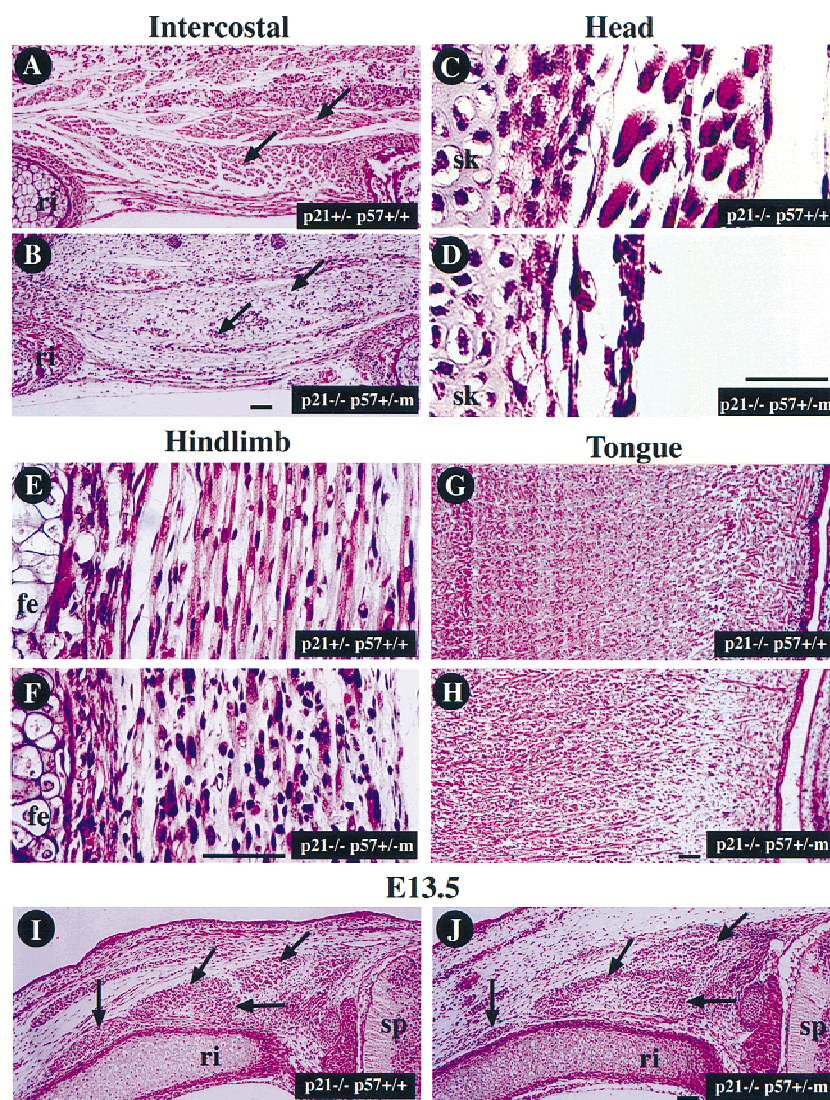


Figure 3. $p21^{-/-}$ $p57^{-m/+}$ double mutant mice display defects in skeletal muscle development. (A–H) H&E-stained transverse sections of E18.5 embryos. Arrows in A and B indicate intercostal skeletal muscles. (I, J) H&E-stained transverse sections of the chest region of E13.5 embryos. Arrows indicate various muscle groups. (fe) Femur; (ri) rib; (sk) skull; (sp) spinal cord. Scale bars, 200 μ m.

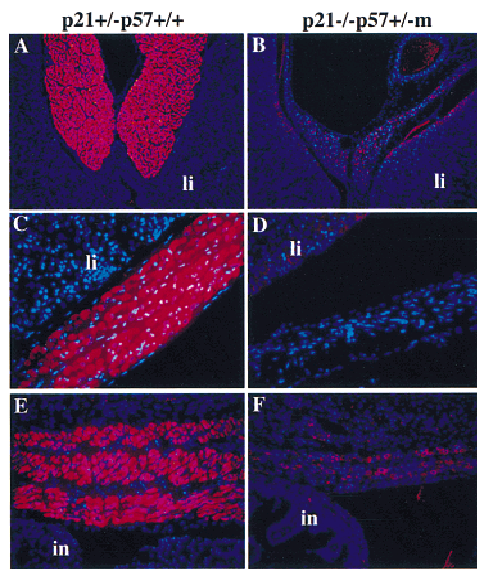


Figure 4. Diminished expression of MHC in the skeletal muscle of $p21^{-/-} p57^{-m/+}$ double mutant embryos. Immunofluorescence staining was performed on transverse sections of E18.5 embryos of indicated genotypes with a monoclonal antibody against MHC. MHC expression was visualized with a Texas red-conjugated secondary antibody, and nuclei were stained with DAPI. (A,B) The root of the diaphragm; (C,D) the diaphragm; (E,F) the body wall. Genotypes are indicated above for each column. (in) Intestine; (li) liver.

are observed in mice lacking myogenin (Hasty et al. 1993; Nabeshima et al. 1993).

$p21^{-/-} p57^{-m/+}$ double mutants exhibit a profound defect in skeletal muscle

Both $p21$ and $p57$ proteins are highly expressed in skeletal muscle, but neither single mutant animal showed significant muscle cell differentiation defects (Deng et al. 1995; Yan et al. 1997; Zhang et al. 1997). However, $p21^{-/-} p57^{-m/+}$ double mutants exhibit profound defects in skeletal muscle development. We have found no significant difference in skeletal muscle development between $p21^{+/+} p57^{+/+}$ and $p21^{+/+} p57^{+/-m}$ mice (data not shown), indicating that a single copy of the $p21$ gene can fully support skeletal muscle development. As shown by H&E staining of transverse sections of E18.5 embryos, the intercostal muscle is greatly reduced in double mutants (Fig. 3, cf. A and B), and the head muscle is diminished (Fig. 3C,D). In the hind limb, numerous long myotubes are observed in $p21^{-/-} p57^{+/+}$ embryos (Fig. 3E), but many fewer and shorter myotubes are present in double mutants (Fig. 3F). Defects in the tongue muscle were somewhat less severe, and double mutant animals exhibit slightly disorganized and less dense muscle mass when compared to $p21^{-/-} p57^{+/+}$ animals (Fig. 3, cf. G and H).

The diaphragm and body wall muscles of double mutants are also severely diminished as demonstrated by

immunofluorescence staining using a monoclonal antibody against myosin heavy chain (MHC). The root of the diaphragm in double mutants is much thinner and poorly stained by the antibody relative to the wild-type control (Fig. 4A,B). MHC staining was diminished in the

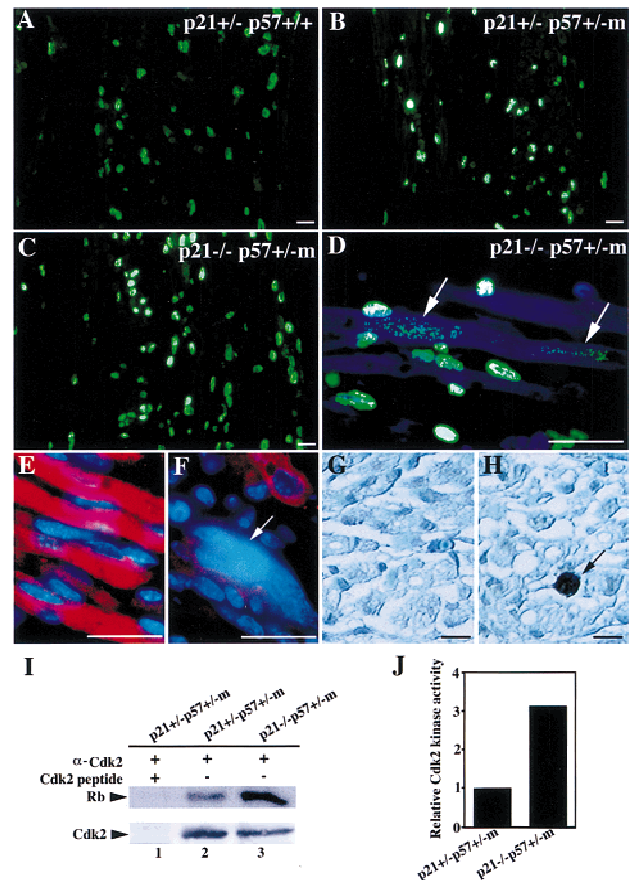


Figure 5. Increased rates of proliferation and apoptosis in $p21/p57$ double mutants. (A–D) BrdU pulse-labeled cells in the intercostal region of E16.5 embryos were visualized by immunofluorescence staining with a monoclonal antibody against BrdU that was subsequently detected with a FITC-conjugated secondary antibody. Arrows indicate BrdU-positive nuclei in the residual myotubes that were revealed by background DAPI (blue) staining. (E,F) MHC immunofluorescence staining of transverse sections of the abdomen region of E18.5 $p21^{+/+} p57^{+/+}$ (E) and $p21^{-/-} p57^{+/+}$ (F) embryos as in Fig. 4. Nuclei are visualized with DAPI staining (blue). Arrow indicates a giant nucleus. (G,H) TUNEL assays performed on transverse sections of the chest region of E16.5 $p21^{+/+} p57^{+/+}$ (G) and $p21^{-/-} p57^{+/+}$ (H) embryos. Arrow indicates an apoptotic nucleus in the intercostal muscle. (I) The activity of CDK2 kinase immunoprecipitated from muscle extracts was assayed using Rb as a substrate by measuring the incorporation of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (top). The amount of CDK2 protein present in the immunoprecipitates monitored by Western blotting (bottom). (Lane 1) Immunoprecipitation from $p21^{+/+} p57^{+/+}$ muscle extracts using anti-CDK2 antibody neutralized with excess competing peptide; (lane 2) immunoprecipitation from $p21^{+/+} p57^{+/-m}$ muscle extracts; (lane 3) immunoprecipitation from $p21^{-/-} p57^{+/+}$ muscle extracts. (J) Quantitation of assays in I by PhosphorImager. Scale bars, 200 μm .

diaphragm of double mutants when compared to the wild type (Fig. 4C,D). In the body wall, wild-type embryos display three layers of skeletal muscle (Fig. 4E), each of which is diminished in the double mutants (Fig. 4F).

It is possible that the skeletal muscle defects observed in double mutants arise from defects in primary myogenesis by which myoblasts are specified and migrate out of somites to various places in the embryo to form skeletal muscles later during secondary myogenesis (Kelley 1983). At E13.5, a time when primary myogenesis is well under way, however, we observed similarly patterned skeletal muscle groups in the double mutant when compared to a wild-type embryo (Fig. 3I,J). In addition, no difference in the morphology of somites are detected between double mutants and wild-type animals at E9.5 (data not shown). Therefore, we conclude that the skeletal muscle defects in the double mutants are a result of problems in secondary myogenesis, similar to the defects observed in mice lacking myogenin (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995).

Absence of both p21 and p57 lead to overproliferation, endoreplication, and apoptosis

Given the biochemical function of p21 and p57 as CKIs, proliferation rates and Cdk2 kinase activities in skeletal muscle from animals with different genotypes were examined. BrdU pulse labeling in E16.5 embryos demonstrated a greater than twofold increase in the number of cells undergoing DNA synthesis in the intercostal muscle region of double mutants when compared to those of either $p21^{+/-} p57^{+/+}$ or $p21^{+/-} p57^{+/-m}$ animals (Fig. 5A–C). We have also noticed incorporation of BrdU in the nuclei of residual myotubes in double mutants (Fig. 5D, arrows), indicative of endoreplication. This is never observed in wild type or single mutants (data not shown). As a result, double mutants frequently display enlarged and unusually shaped nuclei in the residual myotubes (Fig. 5, cf. E and F). In agreement with the observed elevation in proliferation rates in the double mutants, a threefold higher Cdk2 activity toward its physiological substrate Rb protein was detected in the muscle extracts made from $p21^{-/-} p57^{+/-m}$ relative to $p21^{+/-} p57^{+/-m}$ animals (Fig. 5I,J), indicating that p21 and p57 are functioning as CKIs in vivo.

The fact that double mutant animals exhibit greatly reduced skeletal muscle mass appears to contradict the fact that they also display increased proliferation. This apparent inconsistency could be explained by an increase in cell death by apoptosis in double mutants. To test that hypothesis, TUNEL assays were performed on transverse sections of E16.5 embryos. Apoptotic cells were readily detected in the double mutants (Fig. 5H) but not in the wild type (Fig. 5G) or single mutants (not shown), explaining the apparent discrepancy. Together, these data indicate that in the absence of both p21 and p57, myoblasts cannot properly withdraw from the cell cycle in response to differentiation signals, leading to overproliferation, endoreplication, and apoptosis.

The block to differentiation in $p21^{-/-} p57^{-m/+}$ muscle is after the myogenin expression step

The skeletal muscle and rib phenotypes of $p21^{-/-} p57^{-m/+}$ double mutants are nearly identical to those of mice lacking myogenin. This coincident phenotype could be explained if $p21^{-/-} p57^{-m/+}$ animals failed to make myogenin or if myogenin null animals failed to express p21 and p57. To address this, we examined myogenin expression in $p21^{-/-} p57^{-m/+}$ double mutants. In situ hybridization with a *myogenin* antisense probe revealed equivalent expression of *myogenin* mRNA in all skeletal muscles examined in both wild-type and $p21^{-/-} p57^{-m/+}$ double mutant animals (Fig. 6A,B; data not shown). Western blot analysis of hind limb muscle extracts using a monoclonal antibody against myogenin also demonstrated similar levels of protein expression and electrophoretic mobility of myogenin proteins among littermates with various genotypes (Fig. 6C). To test the functionality of the myogenin protein, we examined transcription of a gene thought to be downstream of *myogenin*, *MEF2C*, a MADS box-containing transcription factor involved in myogenesis. *MEF2C* is expressed at lower levels in double mutants than that in the wild-type control (Fig. 6D,E). It should be noted that we cannot distinguish between reduced *MEF2C* expression versus selective loss of *MEF2C*-expressing cells through apoptosis producing the appearance of reduced *MEF2C* expression. Nevertheless, these data together demonstrate that the skeletal muscle phenotypes observed in

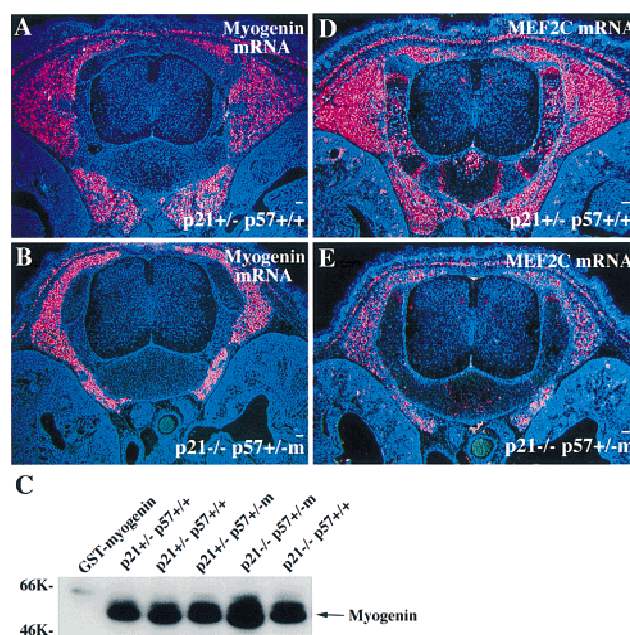


Figure 6. Expression of skeletal muscle differentiation factors in $p21^{-/-} p57^{-m/+}$ double mutants. (A,B) *myogenin* expression detected with in situ hybridization on transverse sections of E18.5 embryos. (C) A Western blot of hind limb muscle extracts probed with a monoclonal antibody against myogenin. (D,E) *MEF2C* expression detected by in situ hybridization on transverse sections of E18.5 embryos.

p21^{-/-} *p57*^{-m/+} double mutants are not due to impaired expression of myogenin, a major skeletal muscle differentiation transcription factor but may result from an inability of myogenin or a myogenin-controlled factor to properly function in the absence of proper cell-cycle exit (see Discussion).

p21 and p57 expression are parallel to myogenin expression in the myogenic pathway

The normal expression of myogenin in the *p21*^{-/-} *p57*^{-m/+} animals indicates that these two CKIs are not upstream of *myogenin* in the myogenic pathway. It is possible that the opposite is true, however—that *myogenin* actually controls the transcription of both *p21* and *p57*. To test that possibility, we investigated the ability of myogenin to induce *p21* and *p57* expression in myogenin-expressing 10T1/2 fibroblasts. Unlike MyoD-programmed 10T1/2 cells induced to differentiate, myogenin was incapable of inducing *p21* expression upon serum withdrawal although it could induce its own transcription and cause the formation of myotubes (Fig. 7D). We were unable to detect induction of *p57* in either MyoD- or myogenin-programmed cells in vitro, suggesting that *p57* may be controlled by a novel signal transduction pathway. To determine the dependency of inhibitor expression on the presence of myogenin in vivo, we examined *p57* and *p21* expression in *myogenin* null animals by in situ hybridization. As shown in Figure 7, A and B, *p57* is expressed at equivalent levels in *myogenin* null mice compared to that of wild-type controls. Taken together with our previous report showing normal *p21* expression in *myoD*/*myogenin* double mutant animals (Parker et al. 1995), we conclude that *myogenin* is not required for either *p21* or *p57* expression. Therefore, myogenin is neither necessary nor sufficient for the expression of the two inhibitors, resulting in the placement of *p21* and *p57* in parallel to *myogenin* in the myogenic pathway (see Fig. 7E,F).

p21 and p57 are coexpressed in the same cells

The redundancy observed between *p21* and *p57* can be explained by redundant activities within each individual myocyte. Alternatively, *p21* and *p57* can each be required individually in different cell types representing distinct but redundant myogenic lineages. Such lineages have been hypothesized to explain the apparent redundancy between MyoD and Myf5. Furthermore, previous analysis of *p57* expression in the nuclei within myotubes revealed that only half of the nuclei contained *p57* protein, consistent with a two lineage hypothesis (Zhang et al. 1997). To explore this we sought to determine whether *p57* and *p21* showed colocalization. For technical reasons we were unable to visualize *p21* protein in myotubes harvested from mice. To circumvent this, we harvested myoblasts from mice, differentiated them into myotubes in vitro, and analyzed *p21* and *p57* protein by indirect immunofluorescence. Under these circum-

stances *p21* and *p57* were found to be completely colocalized in each nucleus in the myotubes formed (Fig. 7C). This indicates that the redundancy between *p21* and *p57* is within an individual cell and not between cells. The difference in the number of nuclei expressing *p57* in vitro versus in vivo is probably due to the fact that these cells in vitro are synchronized in their differentiation process. After differentiation, *p57* levels drop, and it is

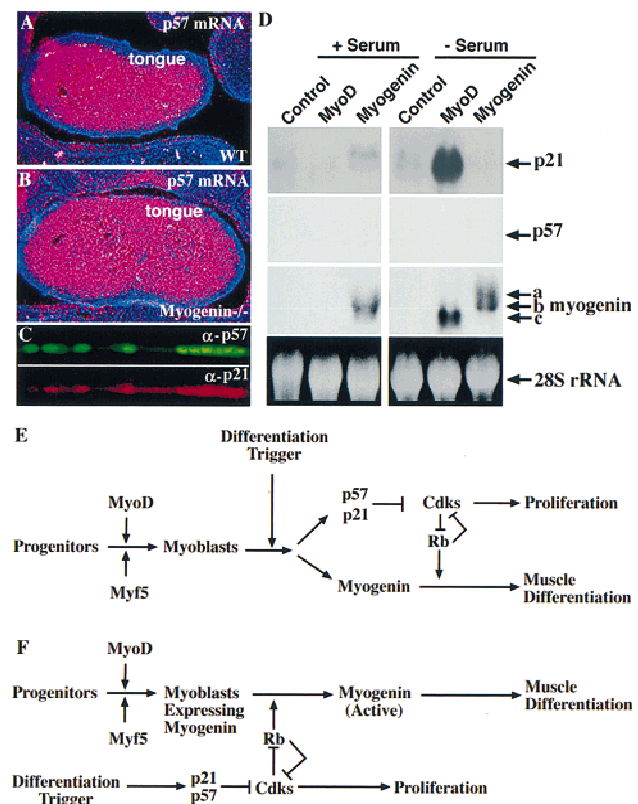


Figure 7. Myogenin is neither required nor sufficient for the expression of *p21* and *p57*. (A,B) *p57* expression detected with in situ hybridization on coronal sections of E15.5 embryos. (C) Colocalization of *p21* and *p57*. Myotubes formed in vitro from primary embryonic myoblasts were stained with rabbit anti-*p57* and goat anti-*p21* polyclonal antibodies. *p57* was visualized with a FITC-conjugated secondary antibody. *p21* was visualized with a biotin-conjugated secondary antibody followed by Texas red-linked streptavidin. (D) A Northern blot of total RNA isolated from proliferating (+ serum) and differentiated (- serum) 10T1/2, MyoD-10T1/2, and myogenin-10T1/2 cells was probed sequentially with *p21*, *p57*, and *myogenin*. EtBr-stained 28S rRNA was used as a loading control. Three different sizes of *myogenin* mRNA are observed; *a* corresponds to the endogenous *myogenin* mRNA induced by myogenin, *b* corresponds to the *myogenin* transgene, and *c* corresponds to the endogenous *myogenin* mRNA induced by MyoD. The endogenous *myogenin* transcripts induced by MyoD and myogenin are of a different size. (E) A model for myogenesis in which *myogenin*, *p21*, and *p57* are coordinately induced by a differentiation triggering signal to coordinate muscle cell differentiation. (F) A different model for myogenesis in which induction of *p21* and *p57* is the critical event that triggers muscle cell differentiation (see text for details).

possible that the absence of p57 observed in 50% of nuclei in vivo reflects nuclei that have already reduced p57 expression.

Discussion

Proliferation control is vital to a developing organism. CKIs are good candidates for molecules providing the controls on cellular proliferation required for embryonic developmental programs because of their biochemical properties and their patterns of expression. For example, p21 was found to be highly expressed in a number of terminally differentiating tissues (Parker et al. 1995). Surprisingly, however, mice lacking p16, p21, or p27 display normal embryonic development (Deng et al. 1995; Serrano et al. 1996; Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996), suggesting that other cell-cycle regulatory mechanisms might exist to compensate for their loss. The present work reveals such a redundancy and demonstrates that two CKIs, p21 and p57, cooperate to control proliferation and differentiation in multiple tissues in mice.

p21 and p57 control differentiation and morphogenesis but not the cell cycle in the lung

The lung develops through interactions between an epithelial tissue that lines airways and a mesenchymal tissue that surrounds the epithelium. It is likely that in the absence of p21 and p57, epithelium–mesenchyme interactions are somehow disrupted in the formation of primitive alveoli. This disruption is stage specific, as development of the bronchial tree is unaffected. Thus far, we have been unable to define more precisely the cell types defective in the lung of mutant animals because p21 and p57 are coexpressed in a number of different cell types and no morphological differences are observed among them.

The lung defects appear to be quite distinct from those observed in skeletal muscle. First, MyoD and myogenin do not have a role in lung development, so novel signal transduction pathways are likely to control p21 and p57 in lung differentiation pathways. Second, in the absence of p57, muscle can properly differentiate with a single copy of the *p21* gene, whereas the lung shows haploinsufficiency under these conditions. This is likely to reflect differences in the other differentiation information present in these cell types. Third, unlike skeletal muscle, the defects in the formation of primitive alveoli do not appear to be a result of overproliferation or apoptosis. It is possible that these two CKIs contribute directly to the differentiation of either epithelial or mesenchymal cells in a cell-cycle-independent way. Alternatively, increased Cdk activity caused by the lack of CKIs might be insufficient to drive the G₁/S transition but sufficient to interfere with the differentiation processes. Analysis of *Rb* mutants has shown that it is possible to separate the cell cycle and differentiation functions of *Rb* (Sellers et al. 1998), although whether it is possible

to separate these functions by differential Cdk-dependent phosphorylation is not known. Alternatively, there could exist Cdk-dependent pathways parallel to *Rb* that are required for differentiation. In support of this, Skapek et al. (1996) have shown that additional cyclin D1 could block myogenesis even in the presence of nonphosphorylatable and presumably constitutively active *Rb*. Clearly, however, different tissues can be dependent on the same two regulators in different ways, underscoring the complex relationship between the cell cycle and development.

p21 and p57 are required for skeletal muscle differentiation

Several studies have suggested a coupling between cell-cycle exit and differentiation of myoblasts (Bischoff and Holtzer 1969; Nadal-Ginard 1978; Clegg et al. 1987). Furthermore, a large body of evidence indicates that *Rb* is necessary for the permanent withdrawal of skeletal muscle cells from the cell cycle (Gu et al. 1993; Schneider et al. 1994; Novitsch et al. 1996; Mulligan and Jacks 1998). Because *Rb* is a Cdk substrate and is inactivated by Cdk phosphorylation, Cdk regulation is implicated as a key event in muscle development. How Cdks are regulated to initiate muscle differentiation has been a difficult issue to resolve because in vitro differentiation systems utilize nonphysiological stimuli such as serum deprivation to initiate muscle cell differentiation. Thus, the mechanistic details of the initial cell-cycle exit have remained unexplored. This work identifies an important part of the mechanism through which muscle cells exit the cell cycle and initiate differentiation in vivo. Mice lacking both p21 and p57 display severe skeletal muscle defects, manifested as a failure to form myotubes, increased proliferation and apoptosis rates of myoblasts, and endoreplication in the nuclei of residual myotubes. The skeletal muscle phenotypes are similar to those observed in *Rb*^{-/-} mice rescued to term by a *Rb* transgene expressed at low levels (Zacksenhaus et al. 1996). This strongly suggests that the primary role of p21 and p57 is to down-regulate Cdk activity and maintain *Rb* in an active (hypophosphorylated) form. Inactivation of both p21 and p57 leads to higher Cdk2 kinase activity toward *Rb* in the skeletal muscle (Fig. 5I,J). Other CKIs have also been implicated in the regulation of pocket proteins by virtue of phenotypic similarities of mutant mice; p27 and p57 have been implicated in *Rb* control in the pituitary and lens, respectively (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996; Zhang et al. 1997), and p57 has been implicated in p107/130 regulation in chondrocyte differentiation (Cobrinik et al. 1996; Yan et al. 1997; Zhang et al. 1997).

p21, p57, and Rb function in muscle development at the myogenin step

To fully differentiate, cells must not only cease proliferation but also initiate a differentiation program of cell

type-specific transcription. An increasing body of evidence suggests that in addition to its role in cell-cycle exit, hypophosphorylated Rb directly promotes differentiation by acting as a transcriptional coactivator of differentiation transcription factors. During adipocyte differentiation, an interaction between Rb and members of the C/EBP family of transcription factors occurs that potentiates C/EBP DNA binding and transcriptional activity (Chen et al. 1996). In terminally differentiating keratinocytes, Rb interacts with c-Jun and stimulates its transcriptional activity (Nead et al. 1998).

In vitro studies coupled with our in vivo genetic studies suggest that Rb may carry out a similar function in muscle cell differentiation. The skeletal muscle differentiation pathway is controlled by a group of bHLH myogenic transcription factors that form a transcriptional hierarchy in which MyoD and Myf5 are redundant and act upstream of myogenin, and myogenin is the major differentiation factor (for review, see Olson and Klein 1994). Gu et al. (1993) have shown that Rb interacts physically with myogenic transcription factors such as MyoD and myogenin. Furthermore, transcriptional activation of a skeletal muscle-specific promoter by MyoD requires the presence of a functional Rb protein (Novitch et al. 1996). If Rb were required for the function of a particular muscle transcription factor, one might expect the phenotype of *Rb*-deficient animals and *p21*^{-/-} *p57*^{-m/+} animals to resemble the phenotype of animals lacking that transcription factor. MyoD mutants have no phenotype unless combined with Myf5, in which case they are completely defective in formation of any muscle precursor cell types. This is clearly different from the *Rb*^{-/-} and *p21*^{-/-} *p57*^{-m/+} phenotypes. However, skeletal muscle defects in *p21*^{-/-} *p57*^{-m/+} double mutants and *Rb*^{-/-} mutants rescued by a hypomorphic Rb transgene bear strong similarity to the defects in *myogenin* null mice. This could be due to the failure of a part of the MyoD/Myf5 program, for example, the failure to express myogenin. However, myogenin is expressed normally in the double mutants, suggesting that myogenin, and not MyoD or Myf5, fails to function in the absence of proper Cdk regulation. Furthermore, expression of MEF2C, a myogenic transcription factor thought to be downstream of myogenin (Cserjesi et al. 1994; Edmondson et al. 1994; Molkentin et al. 1995), is reduced in *p21*^{-/-} *p57*^{-m/+} animals, consistent with a defect in myogenin activity or an earlier step. The involvement of myogenin in the Rb-dependent step is supported by Tedesco et al. (1995), who have shown that SV40 T antigen blocks myogenesis after myogenin has been expressed, and Novitch et al. (1996), who demonstrated that in the absence of Rb, MyoD induces the expression of both p21 and myogenin, but not later differentiation markers such as MHC, which depend on the function of myogenin (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995). Whereas myogenin has been shown to interact physically with Rb in vitro, our studies do not address whether this connection is direct. The failure to inactivate Cdks could block the function of *myogenin*, a parallel gene required together with *myogenin*, or a gene directly downstream of

myogenin, and any one of these could physically require hypophosphorylated Rb.

Muscle differentiation in vivo—what pulls the trigger?

A key issue in muscle development is what triggers the differentiation process. MyoD and Myf5 have been shown to specify the myoblast lineage. Once specified, myoblasts continue to proliferate until they receive a differentiation signal that has not yet been identified. The process initially affected by this signal is not known. However, in vitro cell culture experiments have demonstrated that serum deprivation can trigger differentiation and therefore substitute for the in vivo signal. The dependency on Rb for this process, coupled with the in vivo dependency on p21 and p57, suggests that the relevant function of serum deprivation is cell-cycle arrest through Cdk inactivation. p21 and p57 are essential targets of signal transduction pathways intended to control muscle differentiation in vivo. A critical question is whether p21 and p57 up-regulation in vivo is the trigger for the differentiation cascade or plays a critical downstream coordinating function, or both.

Combining our knowledge of the regulation of myogenic differentiation, together with the transcriptional regulation of *p21* and *p57*, sheds some light on these issues. First, both *p21* and *p57* are expressed normally in mice deficient for either MyoD or Myf5 (Parker et al. 1995; P. Zhang and S. Elledge, unpubl.), although they may require one of these. Second, MyoD in 10T1/2 cells or C2C12 cells can direct the expression of *p21* but, intriguingly, not *p57* (Fig. 7; data not shown), suggesting that *p57* is under the control of a novel signaling pathway that is not active in the in vitro model systems, although it is possible that expression of *p57* is inactivated in established cell lines in a manner that does not reflect its true in vivo regulation. Finally, it is clear that myogenin is neither necessary nor sufficient for the expression of p21 or p57 and vice versa, indicating that all three are expressed in parallel. In vitro model systems such as C2C12 cells show that myogenin expression is low initially and then highly induced in response to serum deprivation (Parker et al. 1995; Andres and Walsh 1996). If myogenin expression in vivo is activated after the differentiation trigger is pulled, the fact that myogenin is fully expressed in the absence of p21 and p57 indicates that these CKIs cannot be the event initially triggering myocyte differentiation. The major caveat in this analysis is whether or not myogenin expression in vivo follows the in vitro model or whether in vivo myogenin is expressed in an inactive form prior to the triggering event (see below). Alternatively, it is possible that both p21 and p57 are part of a forward feeding differentiation switch that once initiated, perhaps by transient cell-cycle exit, maintains the differentiation process, similar to the models put forth for MyoD and p21 (Halvay et al. 1995; Parker et al. 1995).

On the basis of these observations and information from in vitro muscle differentiation systems, we propose a myogenesis model as depicted in Figure 7E. MyoD and

Myf5 specify cells to adopt the myoblast fate. Myoblasts then migrate and proliferate. In response to differentiation signals of unknown origin, expression of myogenin, the driving force in myoblast differentiation, is activated. At the same time, both p21 and p57 expression are induced and act to inhibit Cdk activity, causing G₁ arrest and maintaining Rb in its hypophosphorylated and active form. Rb then works in conjunction with myogenin (or an unknown factor with a similar function) to activate a program of muscle-specific gene expression that executes the differentiation process. A candidate Rb-responsive alternative to myogenin might be MEF2C, a myogenic transcription factor thought to be downstream of myogenin. Although MEF2C mutant mice are embryonic lethal and the role of MEF2C in muscle development has not been assessed, it is likely to be required for muscle cell differentiation because its *Drosophila* homolog, Mef2, is required for muscle formation (Bour et al. 1995; Lilly et al. 1995). Mef2 is the only *Drosophila* myogenic homolog required for muscle development. In this model, CKIs could have additional nonessential roles besides activation of the myogenin-dependent step, such as enhancing MyoD function or non-cell-cycle roles in differentiation that are not indicated in the model. Both p21 and p57 have domains in addition to the Cdk inhibitory domain that are likely to have other functions, possibly involved in differentiation.

A second possible model for myogenesis is shown in Figure 7F in which p21 and p57 induction acts as the trigger for muscle differentiation. In this model, after specification, myoblasts express myogenin in an inactive form. The differentiation trigger functions by activating p21 and p57 expression, which inhibits Cdk activity causing G₁ arrest and Rb activation. Rb then functions together with myogenin to carry out differentiation. We favor the first model because it is consistent with the results from in vitro muscle differentiation systems. However, the second model makes two testable predictions. If true, myogenin will be expressed in proliferating myoblasts and will be expressed prior to p21 and p57 induction. Furthermore, ectopic activation of p21 or p57 expression in myoblasts should induce muscle differentiation.

Regardless of whether p21 and p57 are the initiating molecules for muscle development, the profound defects observed in p21/p57 double mutant mice demonstrate the pivotal importance of coordination between proliferation and differentiation in development and demonstrate that multiple layers of Cdk regulation have been selected during evolution to ensure this coordination.

Materials and methods

Genotyping animals

p21-deficient mice were kindly provided by Philip Leder (Harvard Medical School, Boston, MA). We have developed PCR protocols to identify wild-type and disrupted alleles of p21 and p57, using a set of four primers for p21 and three primers for p57. For p21, the sequences are primer 1, TCCTGGTGATGTCCGACCTG; primer 2, TCCGTTTTCGGCCCTGAG; primer 3,

GCGAGGATCTCGTCGTGAC, and primer 4, TCATCAATT-TATGCAGAC. For p57, the sequences are primer 1, CGTCCACAGGCCGAGTGC; primer 2, GCTGCGGAGGTACACGTCG; and primer 3, GCGAGGATCTCGTCGTGAC. Detailed protocols are available on request.

Gross and histological analysis

Embryos or placentas were fixed in 4% paraffin aldehyde dissolved in PBS for several hours to overnight at 4°C, depending on the size of the specimen. Fixed samples were dehydrated using ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. Embedded samples were sectioned at 3 µm. For histopathological evaluation, tissue sections were stained with H&E. Skeleton staining was performed as described (Ramirez-Solis et al. 1993).

Immunofluorescence analysis

MHC was detected using a monoclonal antibody (Sigma, clone MY-32) and visualized with Texas Red-conjugated secondary antibody (Amersham). p57 was stained as described (Zhang et al. 1997). For cell proliferation assays, pregnant mice were injected with BrdU (0.1 µg/gram body weight) 2 hr prior to delivery by cesarean section, and positive cells identified with an anti-BrdU mAb (Dako) and visualized with FITC-conjugated secondary antibody (Amersham). Apoptotic cells were detected with a kit from Trevigene, and the assay was performed as recommended by the manufacturer.

In situ hybridization

In situ hybridization analysis was performed as described (Parker et al. 1995). Sense and antisense probes were generated from linearized plasmid templates obtained from the following sources: myogenin, Eric Olson (University of Texas Southwestern Medical Center, Dallas); p21 and p57 (our laboratory); MEF2C, CC10, SP-A, SP-B, and SP-C EST clones from Genome Systems (St. Louis, MO).

Culture of primary myoblasts

Hind limb muscles dissected from E18.5 embryos were digested with 0.5 ml of dispase (Boehringer Mannheim, Indianapolis, IN.) at 37°C for 30 min, and tissues were dislodged by repeated pipetting. The resulting cell suspensions were mixed with DMEM (GIBCO) containing 20% FBS and appropriate antibiotics, and incubated in 10-cm tissue culture dishes for 30 min at 37°C to adhere fibroblasts onto the dish. The supernatants containing myoblasts (and fibroblasts) were plated in appropriate tissue culture vessels. After a 48-hr recovery period, myoblasts were induced to differentiate with DMEM containing 2% horse serum. Myotubes appear by 48 hr after induction. For immunofluorescence staining, cells were fixed in cold methanol (−20°C).

Protein and mRNA analysis

Hind limb skeletal muscle tissues dissected from E19 embryos were lysed in NP-40 buffer (Harper et al. 1993) and cleared by centrifugation. Thirty micrograms of protein was immunoblotted with a monoclonal anti-myogenin antibody available from Hybridoma Bank (University of Iowa, Iowa City) using ECL detection (Amersham). GST-myogenin was purchased from Santa

Cruz Biotechnology (Santa Cruz, CA). mRNA expression was analyzed by Northern blotting total RNA isolated from 10T1/2 fibroblasts ectopically expressing MyoD or myogenin (Parker et al. 1995). Cdk2 kinase was immunoprecipitated from muscle extracts using a polyclonal antibody against Cdk2 (Santa Cruz, CA) and its activity assayed using *Escherichia coli* expressed Rb protein (kindly provided by David Goodrich, University of Texas M.D. Anderson Cancer Center, Houston) as substrates.

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p21^{CIP1} and p57^{KIP2} control muscle differentiation at the myogenin step

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