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The cell cycle is regulated by the action of a family of cyclin dependent kinases (Cdks) which catalyze particular cell cycle transitions. Cdks are positively regulated through interaction with cyclins and are negatively regulated through phosphorylation and through association with inhibitory proteins of the CIP/KIP and INK4 families. Our research has focused on the role of p57KIP2 in development and cancer. We have found that p57KIP2 is expressed in a highly cell type specific manner during embryonic development and in adult tussues, being most highly expressed in terminally differentiated cells. Through analysis of p57KIP2 deficient mice, however, we have found that p57KIP2 is required for normal development of several tissues including the kidney, lens, muscle, and bone. We also discovered the p57 is involved in the human overgrowth and cancer predisposition disease Bechwith-Weidemann Syndroem, BWS. Current studies are aimed at understanding in greater detail the function of p57KIP2 and its possible role in human cancers.

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FOREWORD

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Introduction

The eukaryotic cell cycle is regulated primarily at two points, in G1 prior to entry into S-phase and in G2 prior to entry into mitosis. The commitment to a round of cell division is made at a point in G1, referred to as the restriction point in mammalian cells[1-4] or START in yeast[5]. Passage through the restriction point depends critically on mitogen signals, but once this point is passed, cells are committed to S-phase and the remainder of the cycle in a mitogen independent manner[4]. Passage through the restriction point is thought to be the primary event controlling cell proliferation. Therefore, elucidating how positively and negatively acting genes function to regulate the G1/S transition and how mutations in these genes disrupt normal cell cycle control has been a primary focus of cancer research. Central to this focus has been the investigation of the role of cyclin-dependent kinases (Cdk) in the control of cell proliferation.

Cyclins, Cdks, and positive growth control. Cdks are protein kinases that require association with cyclins and phosphorylation for activity[5-8]. Cyclins promote cell cycle transitions via their ability to associate with and activate their cognate Cdks[5-12]. Cyclins D and E function in G1[6, 10, 13-16], and overexpression of cyclin D1 or cyclin E shortens G1 and accelerates entry into S-phase[1, 3, 17, 18]. Amplification of cyclins, D1, D2 and E have been identified in several tumors[19-23]. Cyclin D1 was identified as the PRAD1 oncogene [24]. Cyclin A was identified as the site of integration of HBV in a hepatocellular carcinoma[25]. Taken together, these observations suggest that inappropriate activation of Cdks is a mechanism that cells frequently use to reach the oncogenic state.

Cyclins D1,D2, and D3 bind Cdk4 and Cdk6 kinases and can phosphorylate and inactivate Rb[6, 26-29]. Because D-type cyclins are required for proliferation only if cells have an intact Rb gene, it is thought that Rb inactivation is their primary role. Cyclin E binds to and activates Cdk2 and considerable evidence has accumulated indicating that cyclin E/Cdk2 is the primary kinase involved in the G1/S transition[14, 15, 30-33]. In addition, a close homolog of Cdk2 - Cdk3 - is also thought to play a unique role in the G1/S transition[31]. Cyclin A binds Cdk2 and Cdc2 and is required for both S-phase and the G2/M transition [34-36], while cyclin B/Cdc2 complexes appear to be specific for control of mitotic entry.

Although Cdks are thought to be the critical regulators of cell proliferation, little is known about how cyclin/Cdk complexes regulate cell proliferation during development. In this regard, we have performed an analysis of the expression of the major cyclins during mouse embryonic development and in adult tissues. We have discovered that general cyclins E, A, B, and F are expressed in all proliferating tissues while the D cyclins are distributed in a pattern distinct for each cyclin but which is a subset of the general cyclins (Parker, Harper and Elledge, unpublished results). This is consistent with the notion that D cyclins are the primary initiators of cell cycle entry and orchestrate development. We have recently observed that cyclin D1 is the only D-type cyclin induced when breast cells proliferate during pregnancy. We and our collaborators in the Weinberg laboratory discovered that development of the breast during pregnancy is dependent upon cyclin D1[37]. As mentioned above, amplification of D-type cyclins is frequently observed in breast cancer. This provides a link between development and cancer and indicates that the developmental history of the breast is relevant to its susceptibility to tumorigenisis.

Since the controls utilized during development to regulate cell proliferation are similar to those utilized in maintenance of the non-proliferative state in differentiated tissues, it is likely that these controls are reactivated or overcome in cancer. Another example of this comes from our observation that cyclin D1 is expressed at extremely high levels in the retina and is required for its development [37]. Presumably the inability to properly develop the retina in cyclin D1 mutants reflects an inability to overcome Rb. In what is clearly more than a coincidence, the retina is the same tissue in which high frequency tumors arise in Rb mutant humans. It is therefore likely that the Rb protein is important in both development of that tissue and its maintenance in the non-proliferative state. Our understanding

of the links between development and cancer is in its infancy and is an area in which there is a great need to increase our knowledge base.

Tumor suppressor proteins and negative growth control. Rb and p53[29, 38] are the most well understood tumor suppressors. Mutations in these are found frequently in many human cancers[39,40], and reintroduction of wild-type genes into $p53^-$ or Rb^- tumor cells can suppress the neoplastic phenotype suggesting that loss of function of these genes contributes to tumorigenesis[28, 41, 42].

Mutations in p53 are the most common lesions observed in human malignancies, occurring in greater than 50% of all tumors[39] including those of the breast. The percentage is much higher if loss of p53 function via association with viral oncoproteins (E1B of adenovirus and E6 of papilloma virus) or amplification of the p53 binding protein MDM2 are included[43]. p53 deficient mice are prone to the spontaneous development of a variety of tumor types[44]. Cellular responses to DNA damage such as apoptosis and the G1 checkpoint are dependent on p53[45-53]. p53 also controls a spindle checkpoint and prevents genetic alterations such as gene amplification[54, 55]. p53 regulates the expression of $p21^{CIP1}$, an inhibitor of G1-cyclin/Cdks, in response to DNA damage[56-60]. Using a p21 knockout mouse, we have determined that p21 is required for full function of the G1 checkpoint in response to γ-irradiation, although there is residual checkpoint function[56]. Furthermore, these mice do not show the high rate of spontaneous tumor formation seen in p53-deficient mice. It is not clear whether p53's role in oncogenesis is through its checkpoint or apoptotic deficiencies, or a combination of these.

The current view of the role of Rb in the cell ccyle is that hypo-phosphorylated Rb functions during G1 in part to block the activity of E2F and related transcription factors that are required for the expression of genes involved in S-phase[29]. Hyper-phosphorylation of Rb or association with DNA tumor virus oncoproteins such as E1A results in release of E2F and is correlated with passage into S-phase.

The above observations are consistent with a model in which increased cyclin/Cdk activity in tumors, whether by increased cyclin expression or decreased negative regulation, can overcome the cell cycle repression function of Rb via direct phosphorylation and inactivation of its growth inhibitory function. Rb therefore acts as a potential energy barrier in the pathway that cyclin/Cdks must overcome to activate cell cycle entry. Removal of the barrier (Rb) may reduce the levels of kinase activity required, but some Cdk kinase activity is still required for the process of DNA replication and can therefore act as a target of further negative regulation. In this model, p53 acts to reduce the frequency of mutations that lead to altered growth control and to kill cells that have undergone extensive damage or are inappropriately growing. To fully understand this aspect of cancer, cell cycle dysfunction, it is imperative that we have a complete understanding of the regulation of cyclin dependent kinases and their regulators in the tissues of interest.

The cell cycle and development: potential roles for Cdk inhibitors. Once proliferation and morphogenesis have constructed a particular structure, it is of paramount importance that the proliferative state cease and be replaced with a homeostatic state. While much attention has been focused on how cells enter the cell cycle, little is known concerning the strategies organisms employ to exit the cycle and maintain the non-proliferative state. This state is of great importance to an organism because the vast majority of its cells exist in a non-proliferative state throughout adult life. The inability to appropriately halt growth can lead to malformation during development, and to cancer. Thus, equally important in the execution of developmental programs is the arrest of growth once the program is complete. While the control of terminal differentiation promises to be complex, cell cycle arrest via inactivation of Cdks is likely to be a central feature. Recently a new class of Cdk regulatory molecules have emerged that are potential mediators of cell cycle exit and maintenance of the non-proliferative state. These are the inhibitors of cyclin-dependent kinases, CKIs. Currently two

structurally defined classes of CKIs exist in mammals that are exemplified by $p21^{CIP1}$ (57-60) and $p16^{INK4/MTS1}$ (61-65).

Cyclin-dependent kinase inhibitors: mediators of negative cell cycle control. Cdk inhibitory proteins are a group of proteins that associate with and inhibit Cdks. These versatile molecules have potential roles in cell cycle arrest, checkpoint function and development and are likely to cooperate with Rb, p53, and other negative regulators in maintaining the non-proliferative state throughout adult life. At the time of submission of this grant in December 1993, the first mammalian Cdk inhibitors $p21^{CIP1/WAF1}$ (57-60) and $p16^{INK4a}$ (61) had only recently been identified. Subsequently, we and others identified additional inhibitors including p27, p57, p15, p18, and p19 (refs 61-69). We identified $p21^{CIP1}$ in a two-hybrid screen designed to identify proteins that associate with Cdk2 (57). Importantly, this protein was simultaneously cloned by several other laboratories. p21 was cloned as a p53 activated gene by the Vogelstein laboratory (59), as a Cdk associated protein by the Beach laboratory (58), and as an S-phase inhibitory cDNA in senescent cells (60). Since then we and others have identified two other members of the p21 family, p27 and p57. p57, also known as KIP2 has been the focus of this study. It is expressed in the breast and is localized to 11p15.5m a locus involved in breast cancer (see below).

Involvement of 11p15.5, the location of KIP2, in human cancers including cancer of the breast. Several chromosomal regions show frequent loss of heterozygosity(LOH) in breast tumors including but not exclusively 3p, 7q31, 11p15, 11q13 and 17p(reviewed in 96). The chromosomal location of KIP2, 11p15.5, marks it as a candidate tumor suppressor gene of the breast. The involvement of 11p15 in the breast is well documented(113-119). 35% of breast tumors show LOH at 11p15.5(119) and this LOH is associated with poor prognosis(119). Furthermore, 11p15 LOH has been associated with metastasis(116) and there is evidence that 2 distinct breast tumor suppressor genes may reside at this locus(118). 11p15 has also been intensively investigated because of frequent LOH at this locus in a number of other human cancers including bladder, lung, ovarian, kidney, and testicular carcinomas (reviewed in 70). Several childhood tumors including Wilms' tumor, adrenocortical carcinoma, rhabdomyosarcoma, and hepatocellular carcinoma show specific loss of maternal 11p15 alleles, suggesting a role for genomic imprinting. Chromosome transfer experiments have also indicated a tumor suppressor gene resides at this locus, the WT2 gene involved in Wilms' tumor and possibly rhabdomyosarcoma (reviewed in 71), either of which could be due to loss of a Cdk inhibitor. In addition, rearrangements in the 11p15 region are found in Beckwith-Wiedemann Syndrome (BWS) which is characterized by numerous growth abnormalities, including macroglossia (enlarged tongue), gigantism, visceromegely (enlarged organs) and an increased risk (7.5%) of childhood tumors (72). BWS occurs with an incidence of 1 in 13,700 births, 85% of which are sporadic and 15% familial(73). Genetic analysis indicates maternal carriers, also suggesting a role for genomic imprinting(reviewed in 74). Several features of KIP2 make it a reasonable candidate as a mediator of some phenotypes of BWS. First, a Cdk inhibitor could explain both overgrowth and tumorigenesis phenotypes. Furthermore, the expression pattern of KIP2 in mouse correlates with areas known to be affected in BWS including the tongue, kidney, muscle, and the eye. Third, KIP2 is imprinted and maternally expressed. Furthermore, LOH at 11p15 in Wilms' tumors are exclusively maternal, offering further support for the possibility that KIP2 might be the WT2 gene. LOH of the breast has not yet been examined for parental specificity of LOH. However, the potential for the existence of two tightly linked tumor suppressors(75) affecting the breast at 11p15 might complicate the analysis of parentally biased LOH depending on the relative frequency of the two events. Nevertheless, the biochemical properties of KIP2, its physical location and expression patterns suggest that it may be the tumor suppressor at 11p15.

The goals of our work were: 1) to determine whether p57 is imprinted in the breast, 2) to construct mice lacking p57, 3) to analyze the phenotype of mice lacking p57, 4) to analyze the role of the QT domain in p57 function by looking for binding proteins, and 5) to characterize the regulation of p57 and 6) to look for additional CKIs in the breast. To date, we have made significant progress on these initial goals. Our progress in these areas is summarized below.

Body

Aim 1: Determination of p57 imprinting status in the breast.
We have completed this Aim and described this in last years report..

Aim 2: Construction of mice deficient in KIP2. We completed this aim and discussed it in last years report.

Aim 3: Analysis of p57 mutants animals.
We have completed an exhaustive analysis of the mutant phenotypes present in the p57 mutant and p57/27 double mutant animals in last two years. In the past year we have investigated the possible overlap between CKIs by making mice mutant for both p57 and p21. p21 has been implicated in p53 mediated DNA damage checkpoint control and possibly cancer. The phenotypes of mice lacking these two inhibitors are discussed below in the order in which we detected them.

A. Generation of mice lacking both $p21^{CIP1}$ and $p57^{KIP2}$. To generate mice lacking p21 and p57, we crossed $p21^{+/-}$ $p57^{-p/+}$ 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 [76] , 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.

B. $p21^{-/-}p57^{-m/+}$ double mutants show altered lung development. Histopathological examination of $p21^{-/-}p57^{-m/+}$ mice revealed all of the phenotypes caused by $p57^{-p/+}$ loss alone [76, 77] 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 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 WT embryos display substantial formation of primitive alveoli manifested as "open spaces" on H&E stained sections (Figure 1A, a). In contrast, lungs from $p21^{-/-}p57^{+/-m}$ animals are virtually devoid of "open spaces" (Figure 1A, c). Under high magnification, it is evident that primitive alveoli do not develop in the double mutants (Figure 1 A, compare d and e). This defect persists until birth (Figure 1A, compare f and g). Furthermore, there is a decrease in the size of the lumenal space of the bronchi and bronchioles in the double mutants. $p21^{+/-}$ $p57^{+/-m}$ lungs exhibit an intermediate phenotype between the WT and the double mutant with some primitive alveoli but fewer than in the WT (Figure 1A, compare a, b and c), indicating 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 (Figure 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, B and C are expressed normally in the double mutants (data not shown). Although the absence of air sac lumenal 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 are 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 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.

C. 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 [76, 77]. However, as shown in Figure 2, $p21^{-/-}$ $p57^{-m/+}$ double mutant embryos display a posture clearly distinct from those of WT and $p57^{-m/+}$ mutants (Figure 2A-C). Skeleton staining revealed that double mutants (Figure 2E) lack the spinal curvature seen in WT (Figure 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 (Figure 2, compare D and E). Bifurcation of ribs was observed in double mutants, usually of the 9th rib (Figure 2F) although occasionally the 7th rib is also affected (Figure 2J). The femurs of double mutant lack a cartilage outgrowth seen in either p21 or p57 single mutants or WT littermates (Figure 2G and data not shown). The double mutants exhibited sternum fusion defects similar to those seen in p57 single mutants [76], but the sternum of double mutants is shorter than that of p57 single mutants (Figure 2H). The ribs of double mutants join the sternum at an angle of 90°(Figure 2J), while the ribs of WT or p57 single mutants join at an angle much less than 90° (Figure 2H). Both p21 and p57 have been found highly expressed in developing ribs (ref. [76], and data not shown). However, it is difficult to distinguish autonomous vs. nonautonomous roles of these two inhibitors in ribs, especially considering the fact that similar defects in the attachment of ribs to sternum are observed in mice lacking myogenin [78, 79].

D. $p21^{-/-}p57^{-m/+}$ 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 [56, 78, 79]. However, $p21^{-/-}p57^{-m/+}$ double mutants exhibit profound defects in skeletal muscle development. We have found no significant difference in skeletal muscle development between $p21^{+/+}$ $p57^{+/-m}$ 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 hemotoxylin and eosin staining of transverse sections of E18.5 embryos, the intercostal muscle is greatly reduced in double mutants (Figure 3, compare A and B), and the head muscle is diminished (Figure 3, C and D). In the hind limb, numerous long myotubes are observed in $p21^{-/-}p57^{+/+}$ embryos (Figure 3E), but many fewer and shorter myotubes are present in double mutants (Figure 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 (Figure 3, compare 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 WT control (Figure 4, A and B). MHC staining was diminished in the diaphragm of double mutants when compared to the WT (Figure 4, C and D). In the body wall, WT

embryos display three layers of skeletal muscle (Figure 4E) each of which is diminished in the double mutants (Figure 4F).

It is possible that the skeletal muscle defects observed in the 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 [80]. At E13.5, a time when primary myogenesis is well underway, however, we observed similarly patterned skeletal muscle groups in the double mutant when compared to a WT embryo (Figure 3, I and J). In addition, no difference in the morphology of somites are detected between double mutants and WT 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 [78, 79, 81].

E. Absence of both p21 and p57 lead to overproliferation, endoreplication, and apoptosis. Given the biochemical function of p21 and p57 as Cdk inhibitors, 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 2-fold 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 (Figure 5, A-C). We have also noticed incorporation of BrdU in the nuclei of residual myotubes in double mutants (Figure 5 D, arrow), indicative of endoreplication. This is never observed in WT or single mutants (data not shown). As a result, double mutants frequently display enlarged and unusually-shaped nuclei in the residual myotubes (Figure 5, compare E and F). In agreement with the observed elevation in proliferation rates in the double mutants, a three-fold higher Cdk2 kinase activity towards its physiological substrate Rb protein was detected in the muscle extracts made from the $p21^{-/-}p57^{+/-m}$ animals relative to $p21^{+/-}p57^{+/-m}$ animals (Figure 5 I, J), indicating that p21 and p57 are functioning as Cdk inhibitors 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 the 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 (Figure 5 H) but not in the WT (Figure 5 G) or single mutants (not shown), explaining the apparent discrepancy. Together, these data indicate that in the absence of both p21 and p57, myoblasts can not properly withdraw from the cell cycle in response to differentiation signals, leading to overproliferation, endoreplication and apoptosis.

F. 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 WT and $p21^{-/-}p57^{-m/+}$ double mutant animals (Figure 6 A and B and 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 the littermates with various genotypes (Figure 6C). To examine 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 WT control (Figure 6 D and 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, together these data demonstrate that the skeletal muscle phenotypes we observed in $p21^{-/-}p57^{-m/+}$ 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).

G. 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 Cdk inhibitors 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 (Figure 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 [82], 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 Figure 7E and F).

H. p21 and p57 are co-expressed in the same cells. The redundancy observed between p21 and p57 could be explained by redundant activities within each individual myocytes. Alternatively, p21 and p57 could each be individually required 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 [76]. To explore this we sought to determine whether p57 and p21 showed co-localization. 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 circumstance p21 and p57 were found to be completely co-localized in each nucleus in the myotubes formed (Figure 7C). This indicates that the redundancy between p21 and p57 is within an individual cell, 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 possible that the absence of p57 observed in 50% of nuclei in vivo reflects nuclei that have already reduced p57 expression.

Aim 4. Analysis of the QT domain.

CKIs of the p21 family have multiple domains. For example, in addition to Cdks, p21 can bind PCNA and this interaction inhibits PCNA-dependent DNA replication[67, 68] making p21 a dual specificity inhibitor. Importantly, p21 can associate with PCNA and Cdks simultaneously and may serve to target active kinases to particular substrates. Human p57 also has multiple domains: An N-terminal Cdk-binding domain, a proline alanine-rich central domain called the PAPA repeat, and a C-terminal sequence, the QT domain, displaying 50% identity with the C-terminus of p27. In the mouse p57 gene, the central region has both a proline rich region and an acidic repeat region. With the exception of the inhibitory domain, the function of these additional domains are not known. However, the finding of strong conservation in the QT-domain with p27 suggests that the it has roles independent of Cdk binding or inhibition. One possible function for this domain is to recruit proteins to the cyclin/Cdk complex. We are planning to look for proteins that bind to C-terminus of p57 by the two hybrid system. We are also interested in over expressing that domain of p57 in transgenic animals. We have not initiated these studies yet.

Aim 5. Transcriptional control of $p57^{KIP2}$.

Through in situ analysis and immunohistochemistry we know where p57 is expressed and we described that data in last years report. We propose to perform an analysis of the regulatory sites in the p57 promoter that controls its expression. Toward this goal, we have generated transgeninc mice in which the promoter of p57 is fused to the lacZ gene. We found that 1kb of p57 promoter region is required to recapitulate the expression pattern in muscle and the lens. We are now in the process of further characterizing this piece of DNA and other regions of the promoter.

Aim 6. Identification of new CKIs and other potential regulators of Cdks from normal breast. This aim proposes to look for additional Cdk binding proteins in the breast using the two hybrid system and breast cDNA libraries. We have the cDNA libraries but have not begun the screens yet because we have been so busy analyzing the mice. Hopefully be can begin these interesting experiments during the next funding period.

Conclusion:

The last year was a very productive one for our lab and the cell cycle field in general. Our work funded under this grant allowed us to establish the role of $p57^{KIP2}$ in mouse development and the human cancer and overgrowth syndrome BWS. $p57^{KIP2}$ clearly acts as a regulator of cell proliferation in the adrenal gland, the lens epithelia, and certain chondrocytes. The partial dependency on $p57^{KIP2}$ for reducing cell proliferation reveals the redundant mechanisms used to limit tissue growth. A similar situation is observed in cell culture where agents that induce cell cycle arrest immediately increase levels of certain CKIs and subsequently reduce the levels of the cyclins and Cdks. While undergoing the process of reducing Cdk activity during differentiation, the absence of CKIs may allow additional cell cycles to occur before Cdk activity is sufficiently reduced to block cell cycle entry. In addition, other CKIs may provide Cdk inhibitory functions in the absence of $p57^{KIP2}$, as we have shown here in the lens development of p27/p57 double mutant mice.

CKIs are the ultimate effectors of signal transduction pathway intended to bring about cell cycle arrest and the patterns of expression during embryonic development suggest that particular *CKIs* play important roles in terminal differentiation in a tissue specific manner. However, the fact that mice lacking single *CKIs* display surprisingly few developmental phenotypes has brought into question the essential nature of *CKIs* for cell cycle arrest and differentiation. Our studies **funded by this grant demonstrate** that two *CKIs*, *p57* and *p21* cooperate to control proliferation and differentiation in multiple tissues and reiterates the critical importance of *CKIs* to cell cycle control during development. The use of multiple *CKIs*, each controlled through distinct signaling pathways, provides a flexible mechanism to control proliferation in a cell type specific manner. It is likely that the combinatorial use of *CKIs* will emerge as one of the principal means through which cell cycle arrest and differentiation are integrated during development.

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Appendix

Figures1-7, Legends 1-7, Table 1

Figure Legends

Figure 1. A block in the formation of primitive alveoli in the absence of both p21 and p57. (A) Hematoxylin and eosin-stained transverse sections of lungs derived from E16.5 (a, b, c , d and e) and of E18.5 embryos (f and 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 μm.

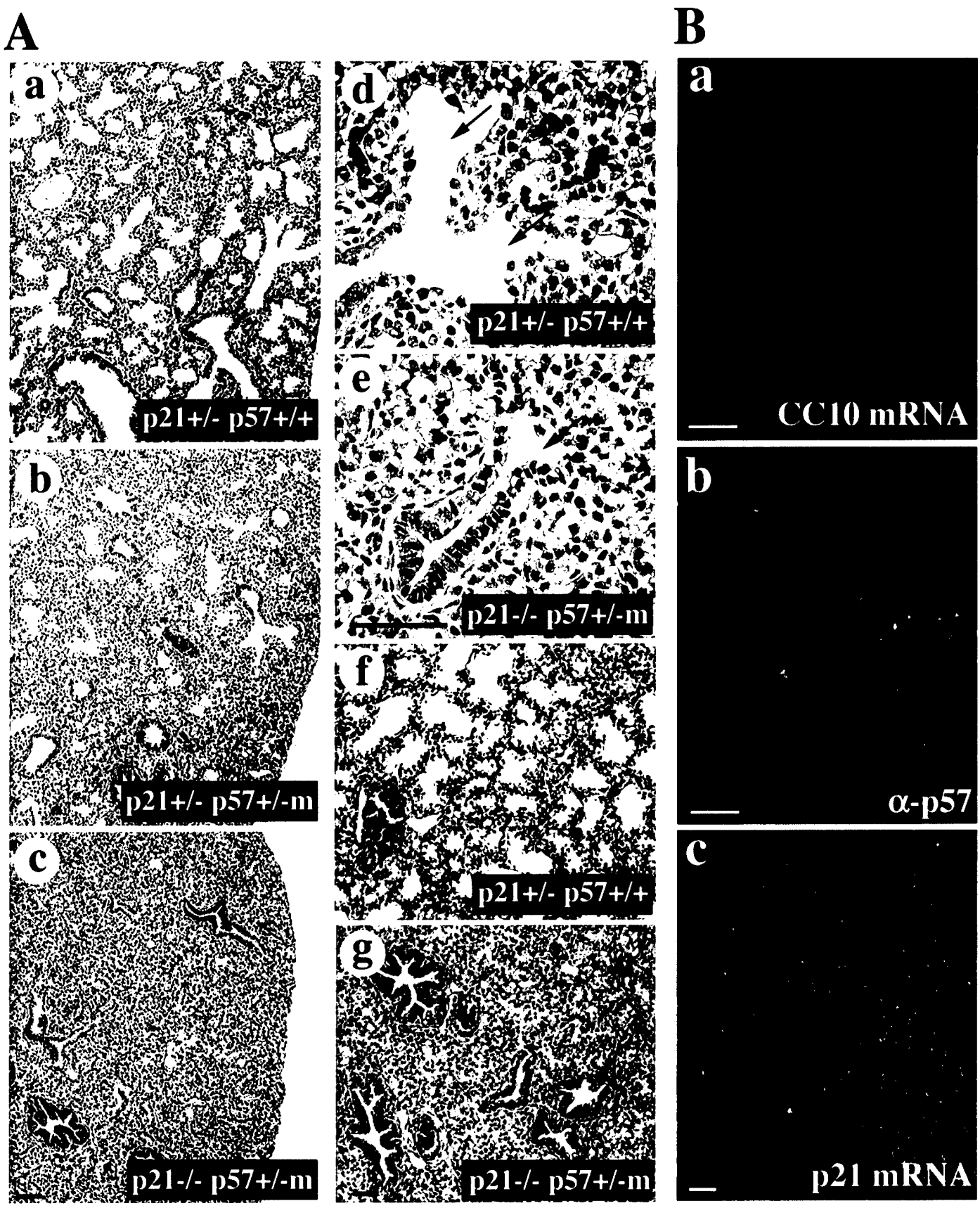

A
a
p21+/- p57+/+
b
p21+/- p57+/-m
c
p21-/- p57+/-m
d
p21+/- p57+/+
e
p21-/- p57+/-m
f
p21+/- p57+/+
g
p21-/- p57+/-m
B
a
CC10 mRNA
b
α-p57
c
p21 mRNA

Figure 2. Skeletal defects in $p21^{-/-}p57^{-m/+}$ mutants. (A, B, 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 9th rib (arrow) is observed in $p21^{-/-}p57^{-m/+}$ embryos. (G) The femur of E18.5 embryos stained with alcian blue and alizarin red. Arrow indicates the cartilage outgrowth. (H, I, J) Sternia and ribs of E18.5 embryos stained with alcian blue and alizarin red. Only 7 of the 13 ribs attach to the sternum. Note a bifurcation in the 7th rib in the double mutant (arrow in J).

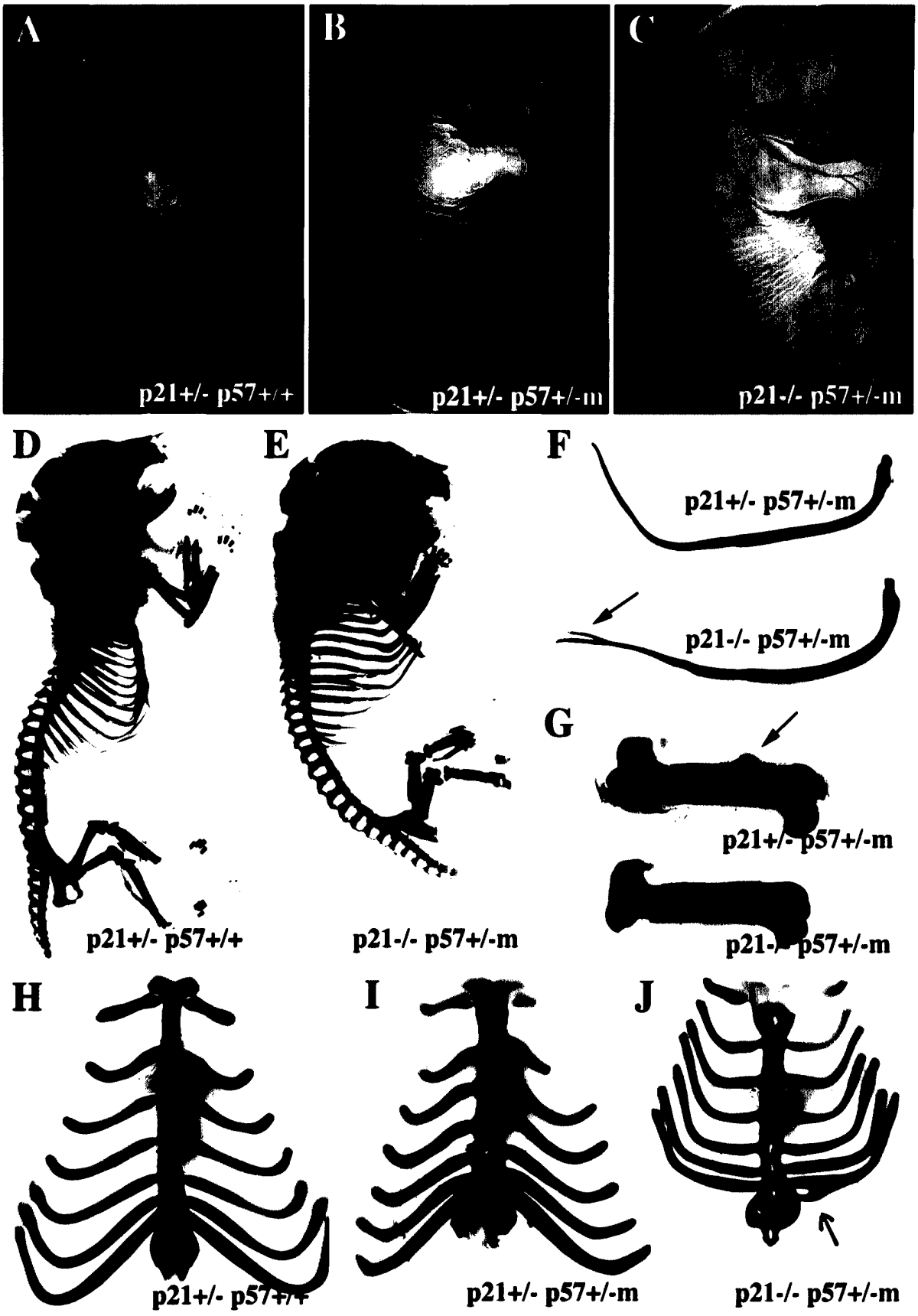

A
p21+/- p57+/+
B
p21+/- p57+/-m
C
p21-/- p57+/-m
D
p21+/- p57+/+
E
p21-/- p57+/-m
F
p21+/- p57+/-m
p21-/- p57+/-m
G
p21+/- p57+/-m
p21-/- p57+/-m
H
p21+/- p57+/+
I
p21+/- p57+/-m
J
p21-/- p57+/-m

Figure 3. $p21^{-/-}p57^{m/+}$ double mutant mice display defects in skeletal muscle development. (A-H) Hematoxylin and eosin-stained transverse sections of E18.5 embryos. Arrows in (A) and (B) indicate intercostal skeletal muscles. (I, J) Hematoxylin and eosin-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 μm.

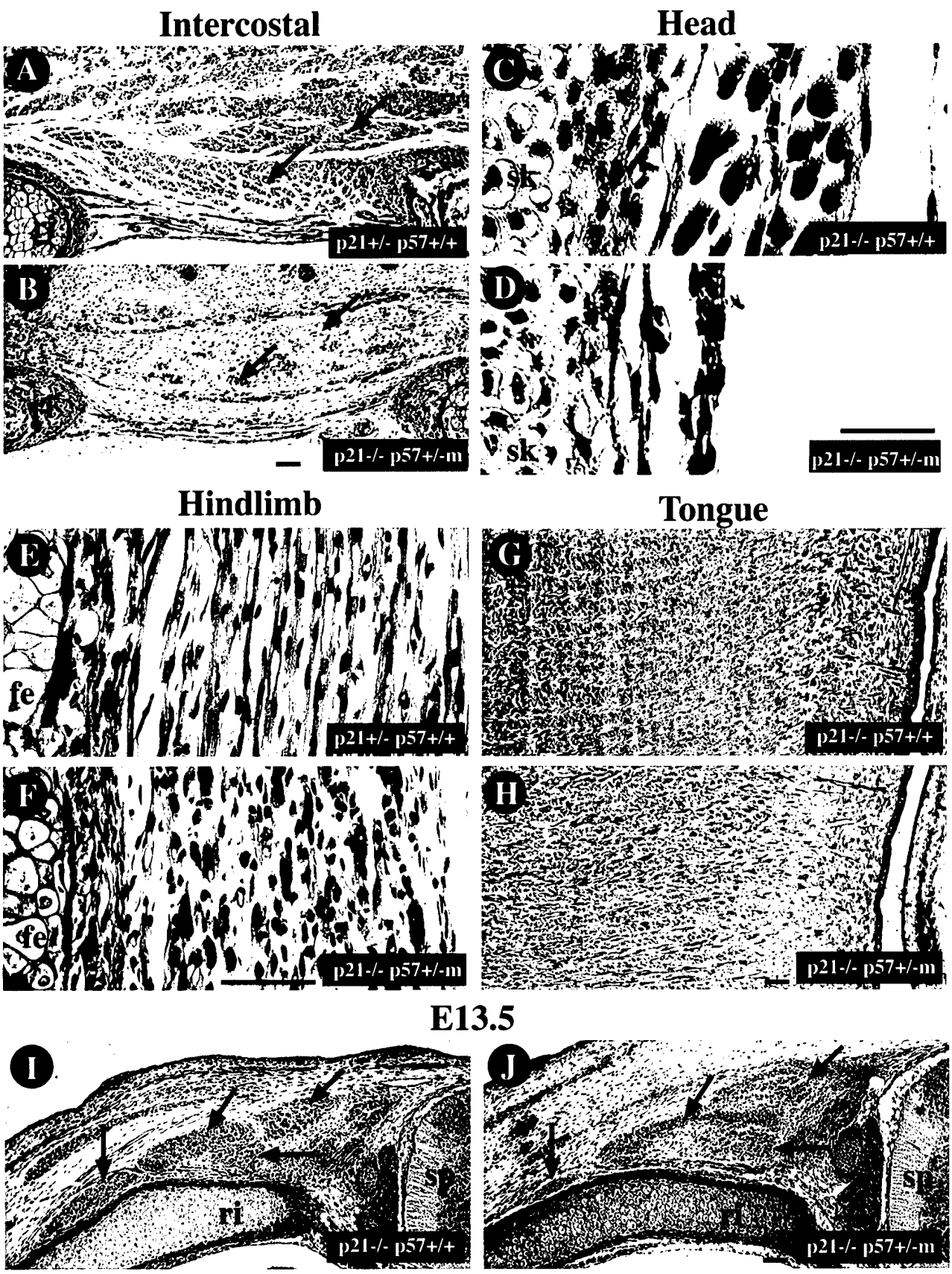

Intercostal
Head
A
C
sk
p21+/- p57+/+
p21-/- p57+/+
B
D
sk
p21-/- p57+/-m
p21-/- p57+/-m
Hindlimb
Tongue
E
G
fe
p21+/- p57+/+
p21-/- p57+/+
F
H
fe
p21-/- p57+/-m
p21-/- p57+/-m
E13.5
I
J
sp
ri
sp
ri
p21-/- p57+/+
p21-/- p57+/-m

Figure 4. Diminished expression of myosin heavy chain (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. Scale bars, 200 μm

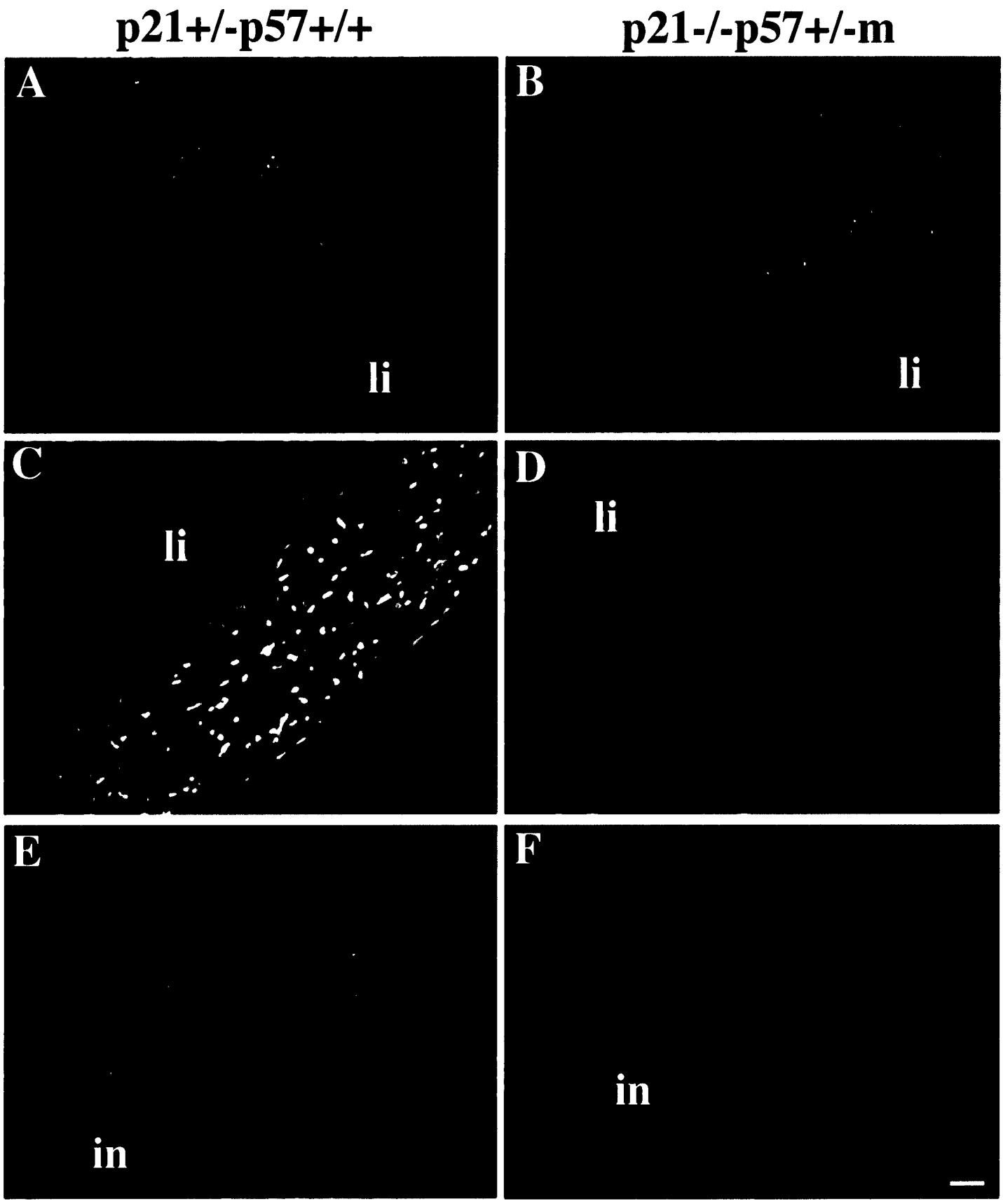
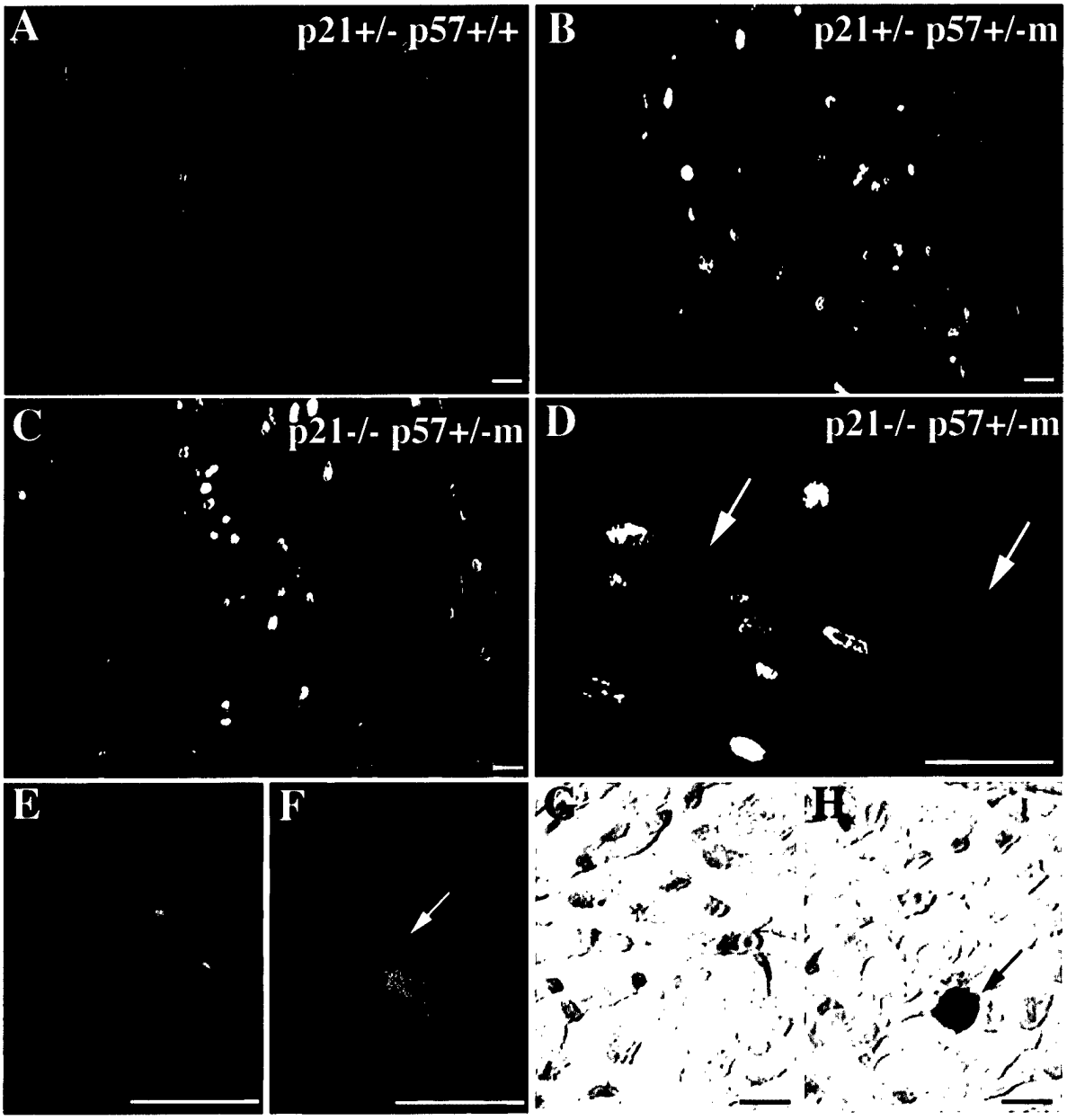
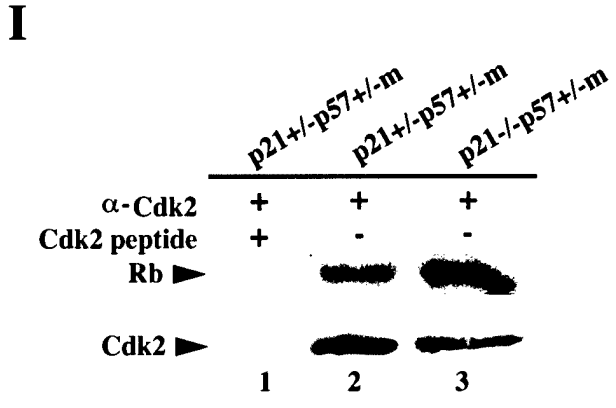

p21+/-p57+/+
p21-/-p57+/-m
A
li
B
li
C
li
D
li
E
in
F
in

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 $p21^{-/-}$ $p57^{+/-m}$ (F) embryos as in Figure 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 $p21^{-/-}$ $p57^{+/-m}$ (H) embryos. Arrow indicates a apoptotic nucleus in the intercostal muscle. (I) The activity of CDK2 kinse immunoprecipitated from muscle extracts was assayed using Rb as a substrate by measuring the incorporation of γ-^{32}P-ATP (upper panel). The amount of CDK2 protein present in the immunoprecipitates monitored by Western blotting (lower panel). Lane 1, immunoprecipitation from $p21^{+/-}$ $p57^{+/-m}$ muscle extracts using anti-CDK2 antibody neutralized with excess competing peptide. Lane2, immunoprecipitation from $p21^{+/-}p57^{+/-m}$ muscle extracts. Lane 3, immunopricipitation from $p21^{-/-}p57^{+/-m}$ muscle extracts. (J) Quantitation of assays in (I) by PhosphoImaging. Scale bars, 200 μm.


A
p21+/- p57+/+
B
p21+/- p57+/-m
C
p21-/- p57+/-m
D
p21-/- p57+/-m
E
F
G
H
I


I
p21+/-p57+/-m
p21+/-p57+/-m
p21-/-p57+/-m
α-Cdk2
Cdk2 peptide
Rb
Cdk2
1
2
3

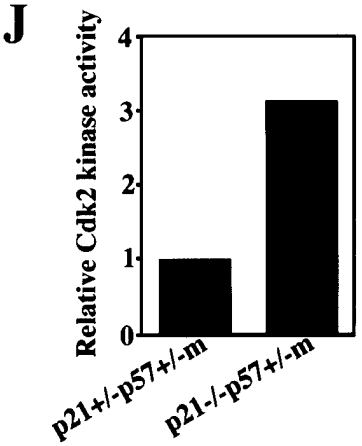
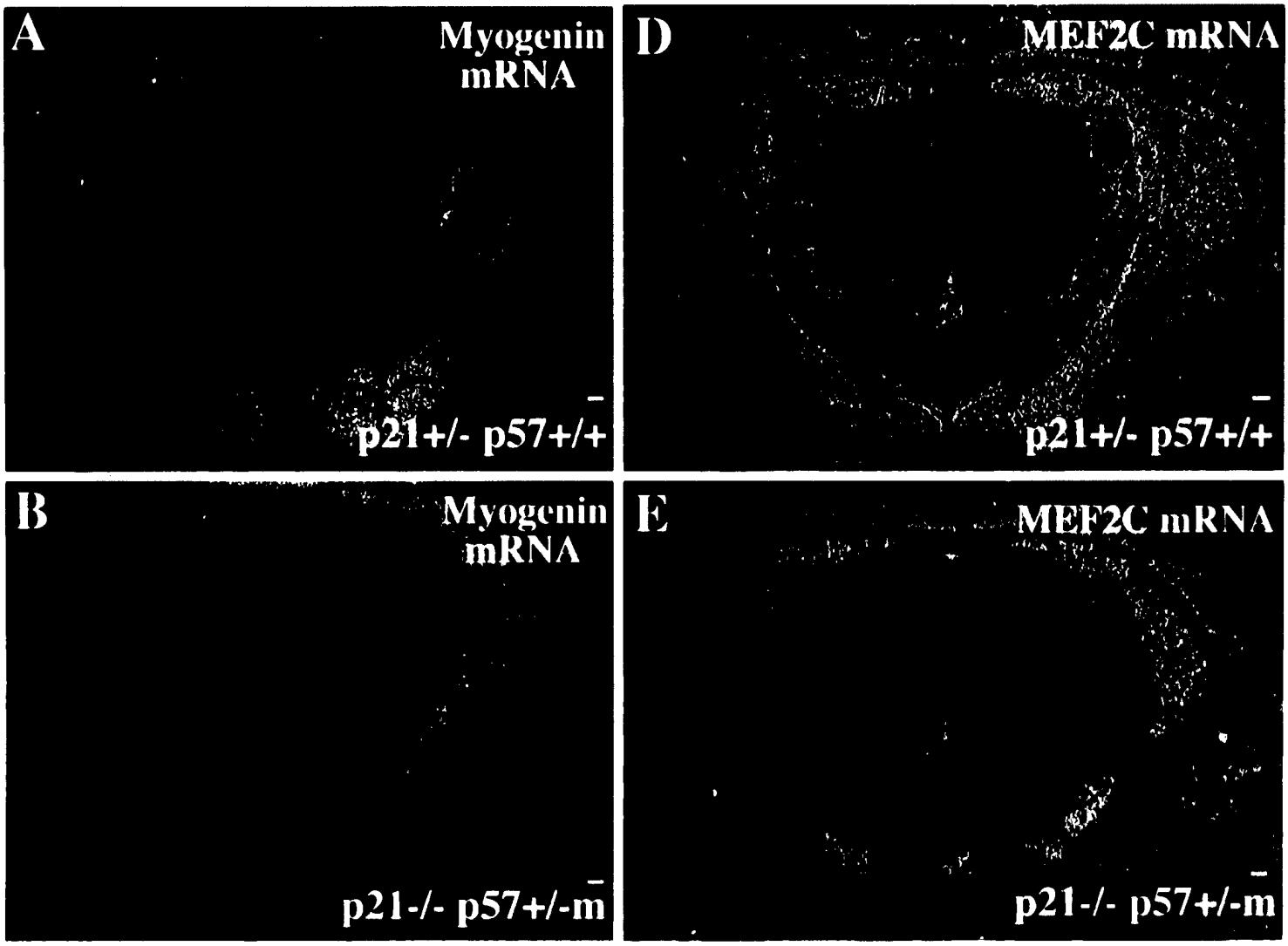

J
Relative Cdk2 kinase activity
0
1
2
3
4
p21+/-p57+/-m
p21-/-p57+/-m

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.


A
Myogenin mRNA
p21+/- p57+/+
D
MEF2C mRNA
p21+/- p57+/+
B
Myogenin mRNA
p21-/- p57+/-m
E
MEF2C mRNA
p21-/- p57+/-m

C

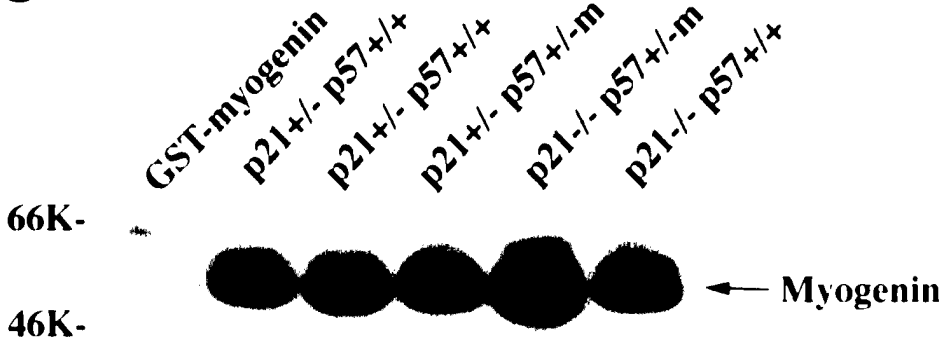
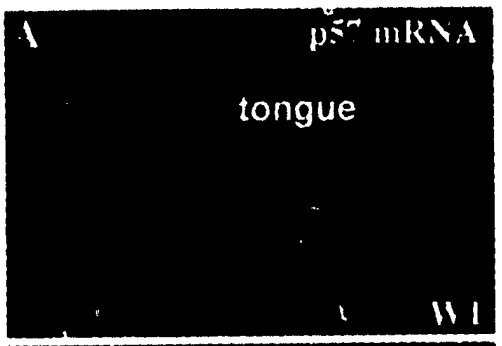
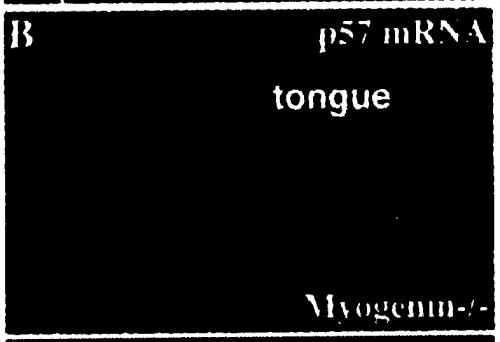
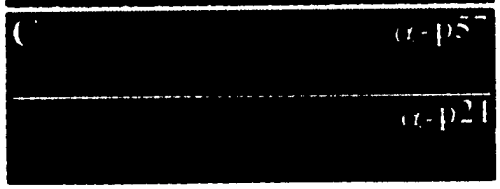
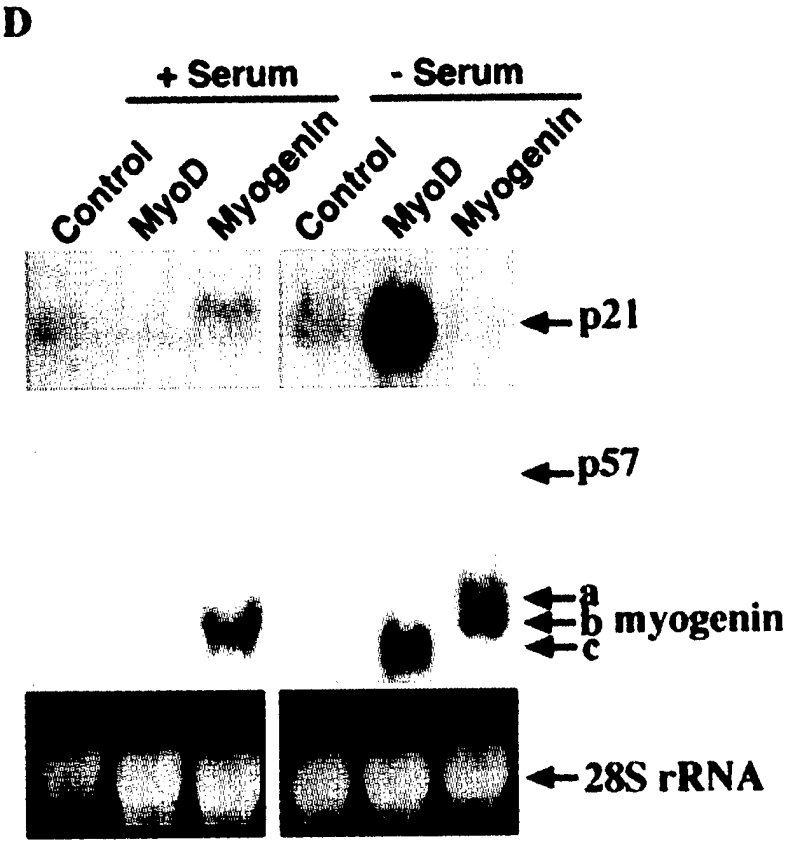

GST-myogenin
p21+/- p57+/+
p21+/- p57+/+
p21+/- p57+/-m
p21-/- p57+/-m
p21-/- p57+/+
66K-
46K-
Myogenin

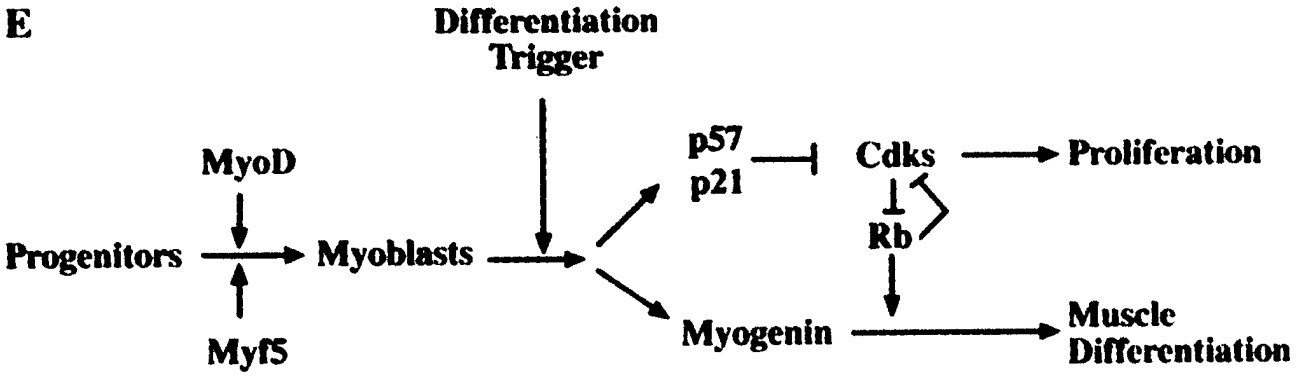
Figure 7. Myogenin is neither required nor sufficient for the expression of p21 and p57. (A, B) p57 expression detected with in situ hybridization on coronal sections of E15.5 embryos. (C) Colocalization of p21 and p57. Myotubes formed in vitro from primary embryonic myoblasts were stained with rabbit 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 are the critical events that trigger muscle cell differentiation. See text for details.


A
p57 mRNA
tongue
WT


B
p57 mRNA
tongue
Myogenin-/-


C
α-p57
α-p21


D
+ Serum
- Serum
Control
MyoD
Myogenin
Control
MyoD
Myogenin
p21
p57
a
b
c
myogenin
28S rRNA


E
Differentiation Trigger
MyoD
Progenitors
Myoblasts
Myf5
p57
p21
Cdks
Proliferation
Rb
Myogenin
Muscle Differentiation

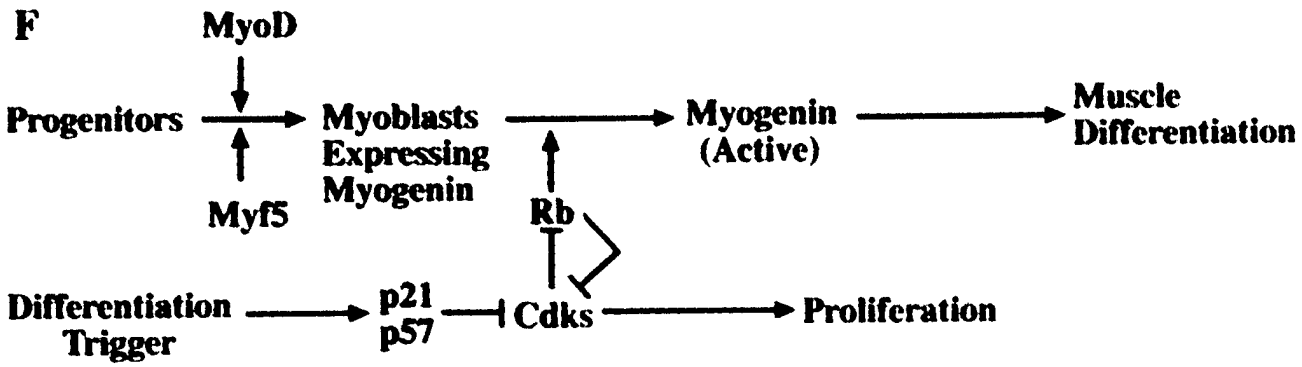

F
MyoD
Progenitors
Myoblasts Expressing Myogenin
Myf5
Myogenin (Active)
Muscle Differentiation
Rb
Differentiation Trigger
p21
p57
Cdks
Proliferation

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			

[a] Number in parentheses indicates the number of embryos that were already dead at the time of harvesting.

Personnel

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J. Wade Harper
Pumin Zhang
Calvin Wong

Publications

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