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The RelA/p65 Subunit of NF-κB Specifically Regulates Cyclin D1 Protein Stability: Implications for Cell Cycle Withdrawal and Skeletal Myogenesis

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ABSTRACT

Studies support that NF-κB functions in cellular growth through the transcriptional regulation of cyclin D1, but whether such regulation is attributed to a single NF-κB subunit remains unclear. To address this issue we examined endogenous cyclin D1 levels during cell cycle re-entry in mouse embryonic fibroblasts (MEFs) lacking specific NF-κB signaling subunits. Results showed that each of these subunits were dispensable for regulating cyclin D1 transcription. However, we found that resulting cyclin D1 protein was severely reduced in MEFs lacking only RelA/p65. Cyclohexamide treatment revealed that this regulation was due to an increase in protein turnover. Similar downregulation of cyclin D1 protein, but not RNA, was observed in vivo in multiple tissues lacking RelA/p65. Co-immunoprecipitation analysis also showed that RelA/p65 and cyclin D1 were capable of interacting, thus providing a possible explanation for cyclin D1 protein stability. In addition, although the decrease in cyclin D1 in *RelA/p65*$^{-/-}$ MEFs was concomitant with lower CDK4 activity during cell cycle re-entry, this was not sufficient to affect S phase progression. Nevertheless, similar decreases in cyclin D1 protein in primary *RelA/p65*$^{-/-}$ myoblasts was adequate to accelerate cell cycle exit and differentiation of these cells. Based on these findings we conclude that RelA/p65 functions as a specific regulator of cyclin D1 protein stability, necessary for proper cell cycle withdrawal during skeletal myogenesis. J. Cell. Biochem. 106: 42–51, 2009.

KEY WORDS: NF-KAPPA B; CYCLIN D1; MYOGENESIS; CELL CYCLE; PROTEIN STABILITY; RelA; p65

NF-κB is a family of transcription factors composed of five subunits: RelA/p65, c-Rel, RelB, p50, and p52 [Verma et al., 1995; Baeuerle and Baltimore, 1996; Baldwin, 1996]. These members contain a Rel homology domain required for DNA binding, dimerization, and nuclear localization. However, only RelA/p65 (from here on referred to as only p65), c-Rel, and RelB possess additional carboxy terminal transactivation domains which regulate NF-κB dependent gene expression [Baldwin, 1996; Chen and Ghosh, 1999].

In most resting cells, NF-κB is localized predominately in the cytoplasm bound to its IκB inhibitor which masks the nuclear localization sequence of NF-κB and prevents its nuclear translocation and subsequent DNA binding [Baeuerle and Baltimore, 1996; Baldwin, 1996; Li and Nabel, 1997; Whiteside et al., 1997]. NF-κB activation occurs in response to a number of stimuli that signal through the IκB kinase (IKK) complex [Baeuerle and Henkel, 1994; Baldwin, 1996; DiDonato et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Zandi et al., 1997]. IKK stimulates the phosphorylation of IκB causing its ubiquitination and subsequent degradation which in turn allows NF-κB to translocate to the nucleus and activate transcription.

Aside from its role in immune response, cell survival, and differentiation, NF-κB is also considered to be an important regulator of cellular proliferation [Li and Verma, 2002; Hayden and Ghosh, 2004]. For instance, mammary glands deficient in IκBα have been shown to display an increase in ductal branching and epithelial

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expansion [Brantley et al., 2001]. NF-κB is also required for normal hepatocyte cellular proliferation as MLP29 hapatocytes expressing the degradation resistant IκBα super repressor (IκBα-SR) displayed significant defects in both cellular proliferation and tubular morphogenesis [Muller et al., 2002]. Another mechanism by which NF-κB is thought to regulate proliferation is by stimulating c-Myc expression and cyclin E/CDK2 activity [Duyao et al., 1990; Kessler et al., 1992; Feng et al., 2004], as well as other proliferation-associated genes such as platelet derived growth factor, colony stimulating factor, and cyclin D1 [Chen and Ghosh, 1999; Guttridge et al., 1999; Hinz et al., 1999; Zhou et al., 2003].

Cyclin D1 is a member of the D-type cyclins responsible for regulating the G1/S phase transition of the cell cycle. In response to mitogens, cyclin D1 is induced and dimerizes with CDK4 or CDK6 to phosphorylate the retinal blastoma (Rb) protein, which in turn derepresses E2F transcriptional activity for S-phase entry [Sherr, 1993; Weinberg, 1995; Beijersbergen and Bernards, 1996]. Use of the IκBα-SR revealed that cyclin D1 is a direct target of NF-κB [Guttridge et al., 1999; Hinz et al., 1999; Westerheide et al., 2001] and in vivo, mice expressing a catalytically inactive form of IKKα or IκBα-SR led to decreases in NF-κB activity and concomitant reduction in cyclin D1 expression [Cao et al., 2001; Demicco et al., 2005]. Regulation of cyclin D1 by NF-κB occurs through a binding site that, in the human promoter, is located 39 nucleotides upstream from the transcriptional start site [Guttridge et al., 1999; Hinz et al., 1999]. Earlier studies showed that this site can be occupied by the classical p50/p65 complex [Guttridge et al., 1999; Hinz et al., 1999], but later work demonstrated that cyclin D1 is preferentially regulated by a non-classical p52/Bcl-3 complex [Westerheide et al., 2001; Rocha et al., 2003].

To further address the specificity of NF-κB in regulating cyclin D1 transcription, we utilized a genetic approach whereby cyclin D1 induction was studied in serum-stimulated MEFs lacking individual NF-κB subunits. Consistent with previous findings, we show that multiple subunits are capable of controlling cyclin D1 transcriptional activity when cells re-enter cell cycle. Interestingly however, only p65 appears to have an additional function to stabilize cyclin D1 protein. This added function of p65 to regulate cyclin D1 post-translationally occurs both in vitro and in vivo, and is associated with CDK4 activity. Nevertheless, in p65 deficient cells, lower cyclin D1 protein and CDK4 activity is not sufficient to effect S phase entry, but does impact the rate at which primary myoblasts exit cell cycle during their differentiation process. Therefore, whereas NF-κB subunits are functionally redundant with regards to controlling endogenous cyclin D1 transcription, regulation of cyclin D1 protein stability is specific to p65, an activity that may be relevant in controlling cell cycle exit for proper skeletal myogenesis.

MATERIALS AND METHODS

MATERIALS

Antibodies to p50, c-Rel, Bcl-3, p52, RelB, CDK4, CDK2 and cyclin D3 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); p65 from Rockland Immunochemical Inc. (Gilbertsville, PA); phospho histone H3 (Ser10) from Cell Signaling (Boston, MA); histone H1 from Upstate (Lake Placid, NY); GST-Rb substrate kindly provided by Lawrence Kirschner; α-tubulin from Sigma (St. Louis, MO); cyclin D1 from BD Pharmigen (San Jose, CA); and secondary anti-mouse Rhodamine from Invitrogen (Carlsbad, CA). Cyclohexamide was obtained from Sigma and Hoescht stain from Sigma. S-phase northern probes were provided to us by Gustavo Leone. RT primers were from IDT (Coralville, IA); Specific sequences for cyclin D1 were, 5′-CCCTCCGTATCTTACTTCAAGTGC-3′, 5′-CACTTGAGCTTGTT CACCAGAAGC-3′, and GAPDH, 5′-GGAGATTGTTGCCATCAACGACC-3′, 5′-GGTCAT GAGCCCTTCCACAATG-3′. Control and p65 targeted siRNA was obtained from Dharmacon (Lafayette, CO). Both collagenase P and dispase (grade II) were obtained from Roche (Indianapolis, IN). Basic human FGF was purchased from Promega (Madison, WI). Geletin was obtained from Sigma–Aldrich (St. Louis, MO).

CELL CULTURE

Fibroblasts were cultured in high-glucose DMEM containing 10% fetal bovine serum and antibiotics. C2C12 myoblasts were cultured as previously described [Guttridge et al., 1999]. Primary myoblasts were prepared from 2-day-old neonates adopted from the described procedures [Rando and Blau, 1994] and cultured in 20% fetal bovine serum and antibiotics.

EMSA AND SUPER SHIFT ANALYSIS

Nuclear extracts were prepared from cultured cells and EMSA was performed as previously described [Cheshire and Baldwin, 1997; DiDonato et al., 1997]. For supershift assays, nuclear extracts were pre-incubated for 10 min with 1.5 μg of antibody prior to addition of the ^{32}P-labled oligonucleotide probe.

INFECTIONS, TRANSFECTIONS, AND REPORTER ASSAYS

IκBα-SR stably expressing cells was generated via a retroviral infection similar to what had been earlier described [Guttridge et al., 1999]. Subconfluent fibroblasts were transfected with expression and reporter plasmids in serum free DMEM using superfect from Qiagen (Valencia, CA) and used according to the manufacture's recommendations. For knockdown studies, myoblasts were transfected with control or p65 siRNA from Dharmacon (in low-serum Opti-MEM using Lipo2000 reagent from Invitrogen), according to the manufacture's recommendations. Cells utilized for reporter assay analysis were lysed in mammalian protein extraction reagent solution (Thermo Fischer Scientific) and luciferase assays were performed as previously reported [Guttridge et al., 1999].

WESTERN AND NORTHERN BLOTTING

Whole cell extracts were prepared from cultured cells and mouse tissues and immunoblotted as previously described [Guttridge et al., 1999; Hertlein et al., 2005]. Antibodies were used at the following dilutions: cyclin D1 (1:2,000), α-tubulin (1:2,000), p65 (1:10,000), cyclin D3 (1:500), CDK4 (1:500), and CDK2 (1:500). RNA extracts were prepared from cultured cells or mouse tissues using Trizol reagent (Invitrogen) according to the manufacture's recommendations and Northern blotting was performed as previously described [Guttridge et al., 1999, 2000].

MICE AND GENOTYPING

Animals were housed in the animal facility at The Ohio State University comprehensive cancer center under sterile conditions, with temperature and humidity kept constant, and were fed a standard diet. Treatment of the mice was in accordance with institutional guidelines of the Animal Care and Usage Committee. $p65^{-/-}$ *TNF*-$\alpha^{-/-}$ mice were generated as previously described [Doi et al., 1999]. Genotype of $p65^{+/+}$ *TNF*-$\alpha^{-/-}$ and $p65^{-/-}$ *TNF*-$\alpha^{-/-}$was confirmed using PCR analysis from prepared tail DNA. At approximately 4 weeks of age littermate $p65^{+/+}$ *TNF*-$\alpha^{-/-}$ and $p65^{-/-}$ *TNF*-$\alpha^{-/-}$were sacrificed using two forms of euthanasia and tissues were procured.

IMMUNOFLUORESCENCE

Paraffin tissue sections were prepared and immunostained with cyclin D1 (1:500) or α-tubulin (1:1,000) as described [Hertlein et al., 2005]. Rhodamine conjugated secondary antibody (1:250) was used to detect indirect immunofluorescence. Staining of primary myoblasts was performed by fixing cells in 2% formaldehyde for 30 min, then permeabilizing in 0.5% NP-40 for 5 min followed by a blocking step in 1:100 horse serum for 30 min. Antibodies were diluted in the 3% BSA in PBS at the following concentrations; phospho histone H3 (ser10) (1:200), anti-mouse rhodamine (1:250). Cells were then mounted with mounting medium containing DAPI for nuclear staining.

IMMUNOPRECIPITATIONS AND KINASE ASSAYS

Whole cell extracts were prepared and immunoprecipitations were performed as previously described [Hertlein et al., 2005]. CDK4 kinase assays were performed as described [Matsushime et al., 1994; Neuman et al., 1997] with the following modifications. Cell lysis was prepared in extract buffer (20mM Tris, 0.5 M NaCl, 0.25% TritonX-100, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 1 mM PMSF, 10 mg/ml protease inhibitors) and incubated for 2 h at 4°C, Immunoprecipitation was performed in pull down buffer (20 mM Tris, 250 mM NaCl, 0.05% NP-40, 3 mM EDTA, 3 mM EGTA, 10 mM β-glycerophosphate, 10 mM NaF, 10 mM PNPP, 300 μM Na_3VO_4, 1 mM benzamidine, 2 mM PMSF, 10 mg/ml protease inhibitors), over night at 4°C. GST-Rb was prepared accordingly [Neuman et al., 1997] with the following alteration; DH5α cells were transfected with pGEX-6p-2-Rb (791–928) and then induced with IPTM for 8 h. Histone H1 kinase assays were performed as described [Naderi and Blomhoff, 1999].

RESULTS

NF-κB SUBUNITS EXHIBIT FUNCTIONAL REDUNDANCY IN INDUCING CYCLIN D1 mRNA

Although NF-κB has been shown to regulate cyclin D1 expression during cell cycle re-entry [Guttridge et al., 1999; Hinz et al., 1999],

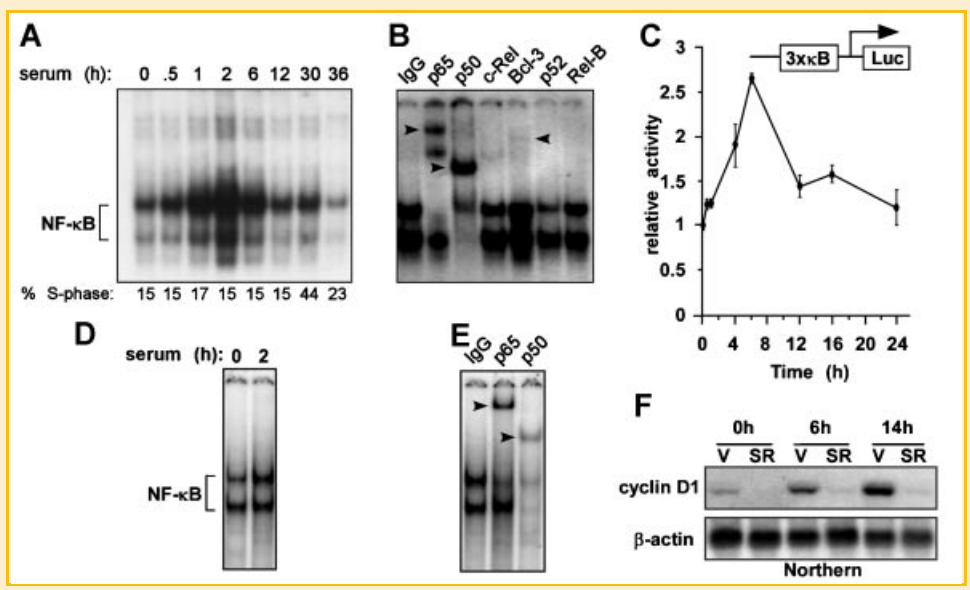

Fig. 1. NF-κB is activated and regulates cyclin D1 during cell cycle re-entry. A: Quiescent MEFs were stimulated to re-enter cell cycle with 10% serum. Cells were collected at indicated time points and either nuclear extracts were prepared for EMSA or cells were stained with propidium iodide (PI) to monitor S phase progression. B: EMSA super shifts were performed with anti sera specific to indicated NF-κB subunit (arrowheads denote super shifted complexes). C: MEFs cells were generated to stably express an NF-κB responsive reporter (3× κB-Luc), or as a control, a cell line containing a mutated version of the same NF-κB binding sites. Once clones were established, cells were induced into quiescence and then stimulated back into cell cycle where at indicated time points whole cell extracts were isolated and luciferase activity was measured, normalized to values obtained from cells containing mutant NF-κB binding sites. D: EMSA was performed with 0 and 2 h nuclear extracts from (A) with an oligonucleotide specific to the human cyclin D1 promoter. E: EMSA super shifts were repeated as in (B; arrowheads denote super shifted complexes). F: MEFs were generated to stably express either empty vector (V) or the IκBα-SR transgene (SR). These cells were then stimulated to re-enter cell cycle and at indicated times points total RNA was prepared and cyclin D1 expression was analyzed by Northern blotting. To account for equal loading, the blot was stripped and re-probed for β-actin.

specifically which subunits are required for this regulation remains unclear. To gain insight, we utilized quiescent MEFs stimulated to re-enter the cell cycle as a model to study the endogenous induction of cyclin D1 and its regulation by NF-κB. Previous work established that NF-κB becomes activated during cell cycle re-entry [Baldwin et al., 1991]. Consistent with these findings, EMSA analysis using a probe containing an NF-κB consensus binding site showed that DNA binding activity peaked in MEFs 2 h following serum stimulation (Fig. 1A) and returned to nearly basal levels after 12 h, a time when cells have yet to enter S phase. Supershift EMSAs also revealed that this binding activity derived predominantly from the classical NF-κB subunits, p50 and p65, and to a lesser degree from the NF-κB associated proto-oncogene, Bcl-3 (Fig. 1B). Using MEFs that stably express an NF-κB responsive reporter, we determined that NF-κB was maximally transcriptionally active after 4–6 h of serum stimulation, a time that closely followed NF-κB DNA binding activity (Fig. 1C). To characterize the regulation of cyclin D1 by NF-κB during cell cycle re-entry, EMSA analysis was repeated with a probe containing the described NF-κB site in the cyclin D1 promoter [Chen and Ghosh, 1999; Guttridge et al., 1999; Hinz et al., 1999; Zhou et al., 2003]. Similar to the consensus probe shown in Figure 1A, NF-κB binding to the cyclin D1 promoter increased in response to serum stimulation (Fig. 1D). Supershift EMSAs determined that NF-κB binding to the cyclin D1 promoter consisted of classical p65 and p50 subunits (Fig. 1E). To further examine the regulation of cyclin D1 mRNA by NF-κB, MEFs were generated to stably express the IκBα-SR inhibitor. Compared to vector control (V) cells, cyclin D1 induction was strongly diminished in IκBα-SR (SR) expressing MEFs (Fig. 1F), thus supporting earlier findings that NF-κB is a regulator of endogenous cyclin D1 transcription [Guttridge et al., 1999; Hinz et al., 1999].

Next, we utilized knockout MEFs to address the specificity of cyclin D1 regulation by different NF-κB subunits. We initiated this analysis with p65 since this was the first subunit described to regulate cyclin D1 transcription [Guttridge et al., 1999]. Interestingly, results indicated that the induction and overall levels of cyclin D1 mRNA were largely unaffected in serum-stimulated $p65^{-/-}$ MEFs compared to wild type cells (Fig. 2A). This implied that either p65 was not required for this regulation or that other NF-κB family members functionally compensated for its absence. Given that p52 and Bcl-3 subunits have also been identified as activators of cyclin D1 transcription [Westerheide et al., 2001; Rocha et al., 2003; Park et al., 2006], we performed a similar analysis in $p52^{-/-}$ and $Bcl\text{-}3^{-/-}$ MEFs. Results showed that equivalent to $p65^{-/-}$ cells, cyclin D1 mRNA expression was unaffected by the absence of p52 or Bcl-3 (Fig. 2B). Since we observed in Figure 1 that inhibition of NF-κB by IκBα-SR expression strongly blocked cyclin D1 induction as fibroblasts re-entered cell cycle, our results are consistent with the notion that transcriptional regulation of cyclin D1 is under the control of multiple NF-κB complexes. To test this notion, cyclin D1 promoter activity was monitored in fibroblasts transfected with varying combinations of NF-κB subunits. In line with past findings [Rocha et al., 2003], results showed that cyclin D1 transcription could be stimulated by various NF-κB complexes, the most responsive of which contain the p52 subunit (Fig. 2C).

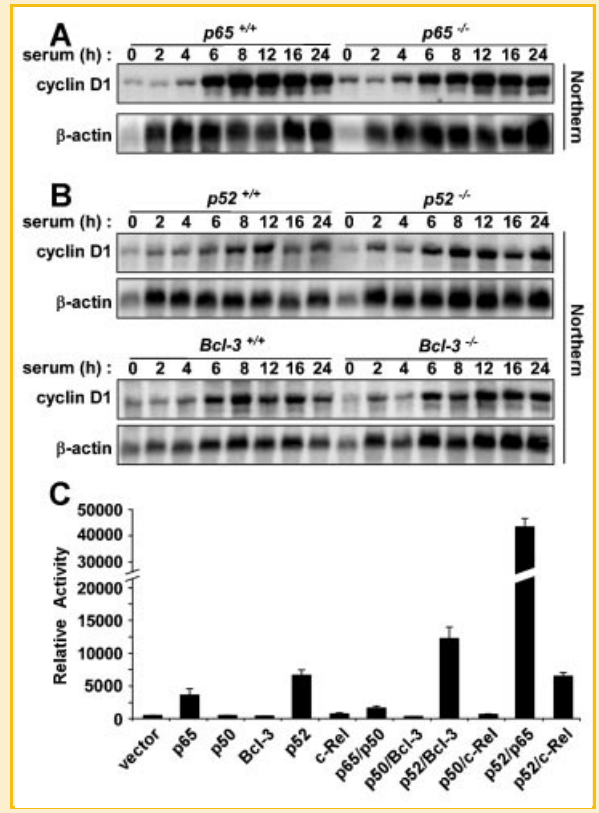

Fig. 2. Induction of cyclin D1 mRNA is regulated by multiple NF-κB subunits. A,B: Quiescent $p65^{-/-}$, $p52^{-/-}$, and $Bcl3^{-/-}$ MEFs compared to their wild type controls, were stimulated back into cell cycle and at the indicated times cyclin D1 mRNA levels were analyzed by Northern blot. β-actin was used as a loading control. C: Fibroblasts were transiently transfected with a cyclin D1 reporter plasmid [Guttridge et al., 1999; Hinz et al., 1999] along with empty vector or plasmids expressing indicated NF-κB subunits. Forty-eight hours post-transfection extracts were prepared and luciferase assays were performed.

LOSS OF p65 CAUSES CYCLIN D1 PROTEIN DESTABILIZATION

Although we were unable to detect changes in cyclin D1 mRNA in $p65^{-/-}$ MEFs, analysis of these same cells stimulated to re-enter cell cycle revealed significant differences in the levels of cyclin D1 protein (Fig. 3A). Even accounting for differences in α-tubulin levels, $p65^{-/-}$ MEFs could be seen to quantitatively express lower amounts of cyclin D1 protein (Fig. 3B), This regulation also appeared specific to p65 since similar effects were not observed in $p52^{-/-}$, $Bcl\text{-}3^{-/-}$, $p50^{-/-}$, or $c\text{-}Rel^{-/-}$ MEFs (Fig. 3C and data not shown). This suggested that cyclin D1 protein may be under specific control of p65.

To investigate the mechanism by which p65 regulates cyclin D1, asynchronously growing NF-κB subunit knockout MEFs were treated with cycloheximide and cyclin D1 protein turnover was analyzed over time. Compared to wild type cells, cyclin D1 turnover was considerably accelerated in $p65^{-/-}$ cells (Fig. 4A). FACS analysis confirmed that this regulation was not due to an unrelated event in cell cycle (Supplementary Fig. 1). Results also demonstrated that cyclin D1 regulation was specific to p65 since reconstitution of this subunit back into $p65^{-/-}$ MEFs restored cyclin D1 levels in response to cyclohexamide (Fig. 4B). However, similar to previous

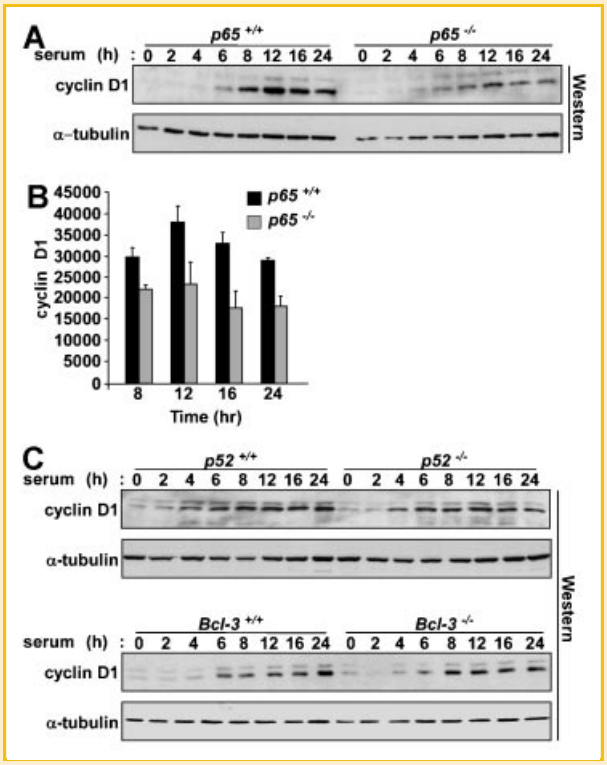

Fig. 3. p65 regulates cyclin D1 protein in MEFs re-entering cell cycle. A,B: Quiescent $p65^{+/+}$ and $p65^{-/-}$ MEFs were stimulated to re-enter cell cycle and at the indicated times whole cell lysates were prepared. Cyclin D1 protein was analyzed by Western blotting (A) and quantitated using Image Quant (B), normalized to α-tubulin, which was used as a loading control. C: Cyclin D1 Westerns from p52 and Bcl-3 wild type and null MEFs under similar conditions as explained in (A).

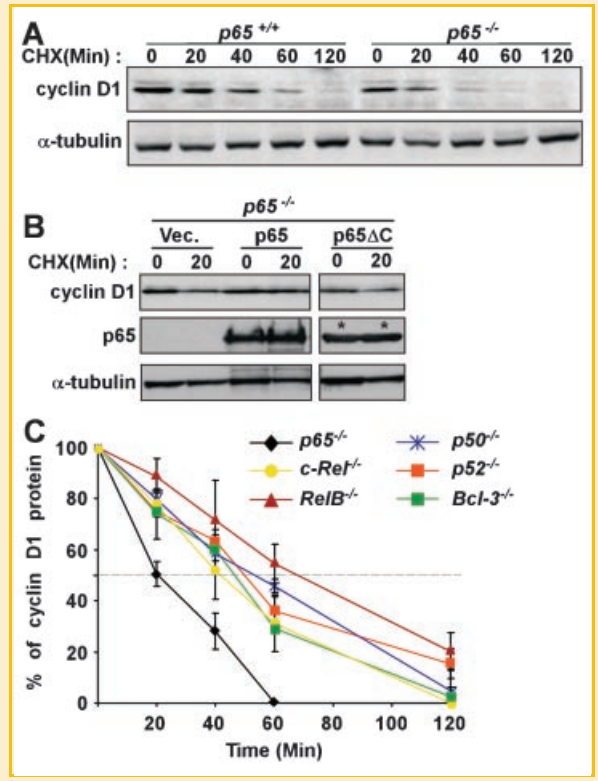

Fig. 4. Regulation of cyclin D1 protein is specific to the p65 subunit of NF-κB. A: Asynchronous $p65^{+/+}$ and $p65^{-/-}$ MEFs were treated with cycloheximide (10 μg/ml) and at indicated time points, cells were collected and cyclin D1 protein was analyzed by Western blotting. The blot was stripped and reprobed with α-tubulin used as a loading control. B: $p65^{-/-}$ MEFs were transfected with empty vector (Vec.), full length p65 (p65), or a deletion mutant lacking the carboxyl terminus of p65 (p65ΔC). The following day, MEFs were treated with cycloheximide and collected after 20 min. Cyclin D1 and p65 protein expression was analyzed by Western blotting and α-tubulin was used as an internal loading control. The asterisk (*) denotes the truncated form of p65 that migrates with an apparent motility of 37 kDa. C: Similar analysis as described in (A) was performed for individual NF-κB knockout MEFs. Cycloheximide experiments were performed in triplicate and levels of cyclin D1 protein from each cell line was recorded over time and normalized to levels of cyclin D1 in untreated cells. Quantitation was performed using Image Quant.

findings where p65 stabilization of IκBβ was found to depend on the carboxy terminus of p65 [Hertlein et al., 2005], here too, re-expression of a p65 truncation mutant lacking its carboxy end (p65ΔC) also led to an increase in cyclin D1 turnover (Fig. 4C). To further address specificity, we compared the half-life of cyclin D1 in $p65^{-/-}$ MEFs with that of other NF-κB subunit knockouts. Results showed that cyclin D1 turnover was reduced two to three times more in $p65^{-/-}$ MEFs compared to other NF-κB knockout cells (Fig. 4D). Together, these results demonstrate that p65 is specific for regulating cyclin D1 protein stability.

p65 REGULATION OF CYCLIN D1 PROTEIN OCCURS IN VIVO

To examine whether p65 stabilization of cyclin D1 protein was specific to MEFs and cultured cells, we probed for cyclin D1 in various tissues from p65 deficient mice. Although $p65^{-/-}$ mice die between E14.5 and E15.5 [Beg et al., 1995], this lethality can be rescued with the additional deletion of TNFα [Doi et al., 1999]. Thus, $p65^{-/-}$; $TNF\alpha^{-/-}$ double knockouts were generated and tissues were collected at approximately 4 weeks of age. Western results showed that cyclin D1 protein levels were strongly downregulated in multiple tissues lacking p65 (Fig. 5A), a finding that was corroborated by immunofluorescent staining (Fig. 5B). This showed that p65 regulation of cyclin D1 protein occurs in vivo. Our results also revealed that the magnitude of cyclin D1 regulation was not uniform among all tissues. Whereas absence of p65 in kidney, lung, and skin led to strong decreases in cyclin D1 expression, marginal differences were detected in heart and brain. Furthermore, analysis of cyclin D1 mRNA in $p65^{-/-}$ tissues confirmed that the regulation of cyclin D1 by p65 occurred specifically at the protein level (Fig. 5C).

p65 INTERACTS WITH CYCLIN D1

To gain insight into the mechanism by which p65 stabilizes cyclin D1, we examined the potential interaction of these two proteins. Cyclin D1 and p65 were over expressed either alone or together in COS cells and whole cell extracts were subsequently immunoprecipitated and probed for cyclin D1 or p65. Findings showed that a

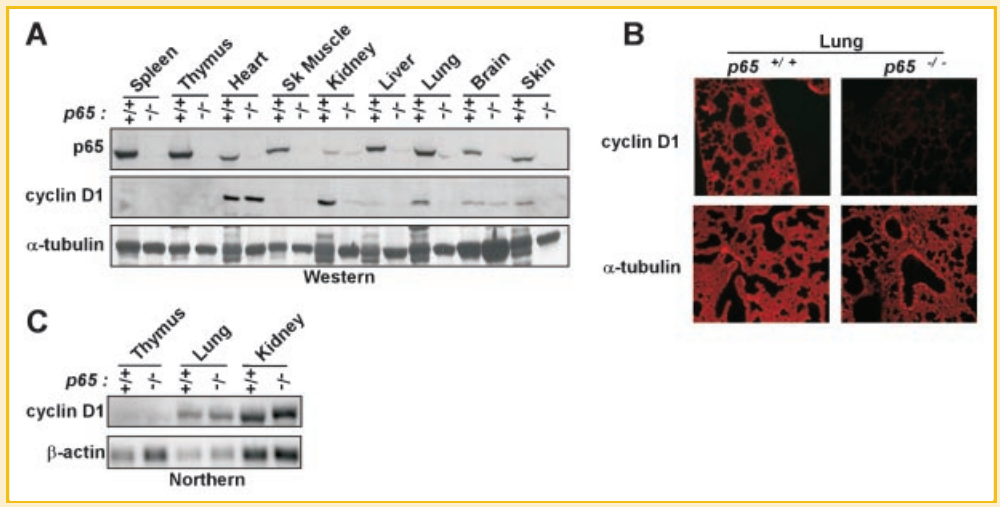

Fig. 5. p65 regulation of cyclin D1 protein occurs in vivo. Tissues were procured from 4-week-old $p65^{+/+}$ $TNF^{-/-}$ and $p65^{-/-}$ $TNF^{-/-}$ mice. A: Indicated tissues were homogenized followed by analysis of p65 and cyclin D1 protein levels via Western blotting. B: Immunofluorescence performed on paraffin sections of lung tissue were stained with either cyclin D1 or α-tubulin. C: Total RNA was isolated from indicated tissues and Northern blotting was performed probing for cyclin D1 and β-actin, used as a loading control. Note that thymus was used as a negative control since this tissue does not express cyclin D1 RNA.

band corresponding to cyclin D1 was detected only in cells transfected with both cyclin D1 and p65 plasmids, indicating that these proteins existed in complex (Fig. 6A). This binding appeared specific since similar results were obtained when the reverse co-immunoprecipitation were performed (Fig. 6B). To further address the specificity of this interaction, co-immunoprecipitation reactions were repeated with a Flag tagged version of p65 along with plasmids expressing cyclin D1 or cyclin D3. Whereas cyclin D1 was readily immunoprecipitated with p65, no interaction between p65 and cyclin D3 was observed (Fig. 6C). To determine whether a p65 and cyclin D1 complex occurred endogenously, we immunoprecipitated cyclin D1 in MEFs stimulated to re-enter cell cycle. As seen by Western analysis, cyclin D1 protein was induced between 4 and 8 h following serum addition (Fig. 6D). Consequentially, this was approximately the same time at which p65 could be found immunoprecipitated with cyclin D1, supporting the idea that a p65 and cyclin D1 complex occurs in vivo. We further asked whether such a complex could be mediated by direct contact between both proteins. However, repeated attempts using standard GST pull down assays with recombinant proteins failed to detect direct binding (data not shown). Therefore, it is likely that p65 mediates cyclin D1 stability indirectly by binding an intermediary protein.

ABSENCE OF p65 LEADS TO SPECIFIC REDUCTION OF CDK4 ACTIVITY WITHOUT EFFECTS IN S PHASE PROGRESSION

To examine the functional relevance of p65 regulation of cyclin D1, we asked whether cyclin dependent kinase (CDK) activity could be affected in p65 null MEFs re-entering cell cycle. Kinase assay results showed that CDK4 activity on the GST-Rb substrate was strongly diminished at 24 h post-serum addition in $p65^{-/-}$ compared to $p65^{+/+}$ MEFs (Fig. 7A). These results were repeatable at 12 h, when cells had not yet progressed into S phase (data not shown). This regulation was also not due to varying expression of CDK4 since immunoblots revealed no differences in the levels of this kinase (Fig. 7A). Furthermore, in contrast to $p65^{-/-}$ cells, CDK4 activity was unaltered in both $p52^{-/-}$ and $p52^{+/+}$ MEFs (Fig. 7A) reaffirming our earlier results in Figure 3 that regulation of cyclin D1 protein is specific to p65. Significantly, we also found that loss of p65 did not affect CDK2 activity (H1 phosphorylation), demonstrating that p65 is not a general regulator of S phase regulatory kinases (Fig. 7B). Together these results suggest that p65 regulation of cyclin D1 stability is important for CDK4 activity during cell cycle re-entry.

Next, we asked whether the decrease in CDK4 activity detected in $p65^{-/-}$ MEFs would be sufficient to affect progression into S-phase. To test this, quiescent $p65^{+/+}$ and $p65^{-/-}$ MEFs were stimulated to re-enter cell cycle and S phase genes, MCM3, cyclin E, and RR2 were analyzed at selected time points. Results showed that there were no obvious differences in the induction or overall expression of these genes (Fig. 7C), nor was a postponement in S phase progression between $p65^{+/+}$ and $p65^{-/-}$ MEFs observed (Supplementary Fig. 2). These results demonstrate that the decrease in cyclin D1 protein and CDK4 activity in cells lacking p65 is not sufficient to impact cell cycle progression. Although it remains to be tested, we suspect these findings are in line with the redundancy that has been described among G1/S phase regulatory proteins since MEFs lacking all three D type cyclins progress normally through cell cycle due to compensation from cyclin A and cyclin E [Kozar et al., 2004].

ABSENCE OF p65 LEADS TO REDUCED CYCLIN D1 PROTEIN AND ACCELERATED CELL CYCLE EXIT DURING MYOBLAST DIFFERENTIATION

Since NF-κB transcriptional control of cyclin D1 has been shown to function as a negative regulatory step in skeletal muscle differentiation by maintaining myoblasts in cell cycle [Guttridge et al., 1999; Hinz et al., 1999; Westerheide et al., 2001], we asked whether p65 contributed to this regulation through cyclin D1

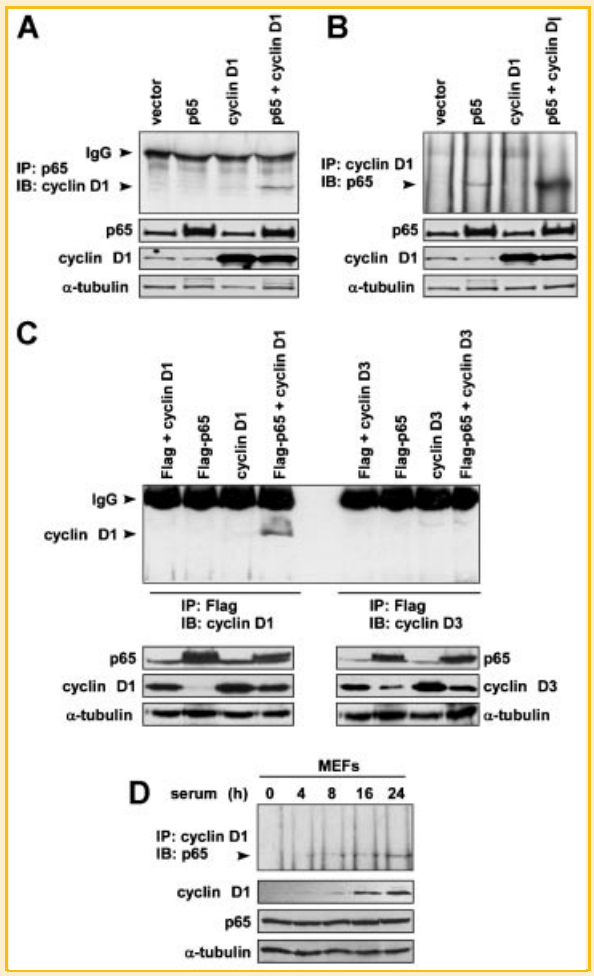

Fig. 6. p65 and cyclin D1 interact. A,B: COS cells were transfected with p65 and cyclin D1 constructs. Co-immunoprecipitations for p65 and cyclin D1 were performed followed by Western blotting for either cyclin D1 or p65. Arrows indicate the identity of p65 and cyclin D1 bands. Panels below represent Western blots probing for input proteins. Tubulin was used as a loading control. C: COS cells were transfected with a Flag tagged p65 plasmid along with cyclin D1 or D3 expression constructs. Immunoprecipitations were performed with a Flag antibody followed by Western blotting probing for cyclin D1 and D3 (arrows indicate IgG and cyclin D1 bands). Panels below are Westerns of input proteins similar to conditions in (A). D: Quiescent $p65^{+/+}$ MEFs were stimulated to re-enter cell cycle and collected at indicated time points. Collected cells were lysed and then immunoprecipitated for cyclin D1 followed by Western blotting for p65. Arrowhead indicates the identity of the p65 band.

protein stabilization. Consistent with our previous findings in MEFs, as well as in tissues lacking p65, knockdown of p65 in C2C12 myoblasts led to the concomitant reduction of cyclin D1 protein but not RNA (Fig. 8A). As expected, similar results were obtained when primary $p65^{+/+}$ and $p65^{-/-}$ myoblasts were anaylzed (Fig. 8B). Although absence of p65 did not impair the growth rate of primary myoblasts, it nevertheless led to their accelerated exit from cell cycle upon serum withdrawal (Fig. 8C,D), and as recently demonstrated, their enhanced myogenesis [Bakkar et al., 2008]. Based on these findings, we suggest that p65 regulation of cyclin D1 protein plays an important role in regulating cell cycle exit upon myogenic differentiation.

DISCUSSION

In this study we utilized a genetic approach to examine the specific subunit of NF-κB responsible for regulating cyclin D1 transcription upon mitogenic stimulation. We discovered that the individual deletion of any one of the previously identified transcriptionally relevant subunits [Guttridge et al., 1999; Hinz et al., 1999; Westerheide et al., 2001; Rocha et al., 2003; Park et al., 2006] does not effect the induction of cyclin D1 mRNA as quiescent fibroblasts are stimulated to re-enter cell cycle. This suggests that multiple NF-κB dimer complexes are capable of controlling cyclin D1 transcription. This is consistent with our results and others [Guttridge et al., 1999; Hinz et al., 1999; Westerheide et al., 2001; Rocha et al., 2003; Park et al., 2006], which showed that multiple NF-κB dimer complexes can regulate cyclin D1 promoter activity. In keeping with these findings, we observed that complexes containing the p52 subunit promoted the highest transcriptional activity of cyclin D1. Nevertheless, the absence of p52 in serum stimulated MEFs did not affect endogenous levels of cyclin D1. Since p52 binding activity was not detected in these cells, it suggests that this NF-κB subunit is not required for cyclin D1 induction during cell cycle re-entry in MEFs, and that this function may instead be carried out through binding complexes containing p65, p50, or Bcl-3. A possibility remains however that p52 regulation of cyclin D1 might be more relevant in non-fibroblast cell types or under pathophysiological conditions [Cogswell et al., 2000; Rocha et al., 2003; Park et al., 2006].

Although differences in cyclin D1 mRNA were not detected in NF-κB knockout MEFs, results revealed cyclin D1 protein stability could be controlled specifically by p65. Compared with the other knockout cells, the half-life of cyclin D1 protein was reduced two-three fold in asynchronously growing $p65^{-/-}$ MEFs. Importantly, our results showed that this regulation was not specific to fibroblasts since a similar effect on cyclin D1 protein was found in primary myoblasts lacking p65, as well as in selected tissues from p65 null mice. Why p65 regulation of cyclin D1 protein was not seen in all tissues is not clear, but one possibility is that other proteins may compensate for the absence of p65, or possibly kinases that promote cyclin D1 turnover such as GSK-3β might be less active in these tissues [Diehl et al., 1998; Benzeno and Diehl, 2004; Lin et al., 2006; Barbash et al., 2007; Barbash et al., 2008]. By co-immunoprecipitation, we detected that p65 and cyclin D1 are capable of interacting. However, after numerous attempts we were unable to demonstrate direct physical binding, suggesting that the interaction between cyclin D1 and p65 might occur through a bridging factor. Since cyclin D1 is less stable in the absence of p65, it is conceivable that such a factor may itself function as a stabilizer of cyclin D1 and be under NF-κB control. One potential candidate is the cyclin dependent kinase inhibitor p21. This cell cycle regulator has been shown to be both induced by c-Rel [Bash et al., 1997] and contribute to cyclin D1 protein stability [Cheng et al., 1999]. The ability of p65 to bind to cyclin D1 even indirectly is very much in line with our findings which showed that IκBβ protein stability is also under the specific regulation of p65 [Hertlein et al., 2005]. Based on these findings, we propose that p65 specifically regulates cyclin D1 through a protein stabilization mechanism, which is most likely mediated through p65's indirect interaction with cyclin D1.

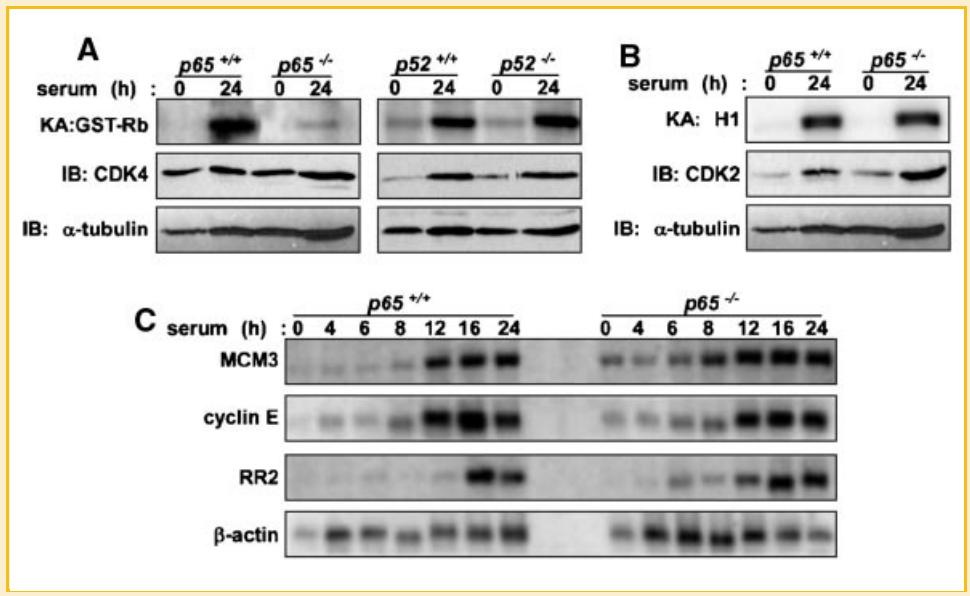

Fig. 7. Cyclin D1 stability is essential for Rb phosphorylation in vitro but dispensable in vivo. A: Quiescent MEFs were stimulated to re-enter cell cycle and whole cell lysates were isolated at the indicated time points. CDK4 kinase assays were performed as previously described [Matsushime et al., 1994]. CDK4 levels were analyzed by Western blotting and normalized to α-tubulin. B: CDK2 kinase assays were performed using similar conditions as described in (A) with the following changes: immunoprecipitations were performed using a CDK2 specific antibody followed by a kinase reaction that used recombinant histone H1 protein as the substrate. CDK2 levels were analyzed by Western blotting and normalized to α-tubulin. C: $p65^{+/+}$ and $p65^{-/-}$ MEFs were stimulated to re-enter the cell cycle and total RNA was prepared at the indicated time points. Northern analysis was performed to determine the expression profile of the indicated S-phase genes.

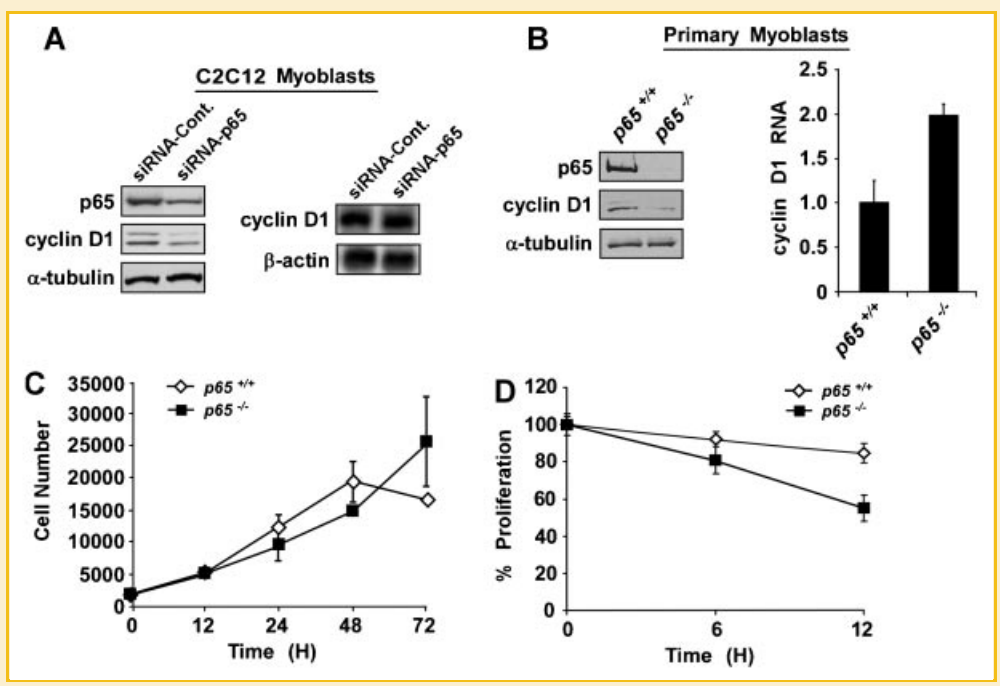

Fig. 8. Myoblasts lacking p65 exhibit reduced cyclin D1 protein expression and accelerated cell cycle exit upon differentiation. A: C2C12 myoblasts were transfected with siRNA control or siRNA targeted to p65. Forty-eight hours post-transfection, cyclin D1 protein expression levels were determined by Western and Northern blotting. α-tubulin and β-actin were used as respective loading controls. B: Cyclin D1 protein and RNA was examined in primary $p65^{+/+}$ and $p65^{-/-}$ myoblasts by Western and real time PCR analysis, respectively. C: Equal number of primary $p65^{+/+}$ and $p65^{-/-}$ myoblasts were plated on 35 mm dishes and proliferation assays were conducted by counting cells in duplicate at indicated times. D: Equivalent numbers of primary $p65^{+/+}$ and $p65^{-/-}$ myoblasts were seeded onto 35 mm dishes and switched to differentiation conditions. At indicated time points, cells were fixed and stained for phospho histone-H3. Proliferating cells were scored in triplicate by counting phospho histone-H3 staining cells. Results were normalized by taking the number of phospho histone-H3 positive cells and dividing by the number of total nuclei that were identified by DAPI staining. Quantitation was determined by scoring for phospho histone-H3 positive cells in 20 randomly selected fields from three independent experiments.

Since cyclin D1 function is tied to the G1 phase of the cell cycle, we speculated that p65 regulation of cyclin D1 would be relevant for cells re-entering cell cycle and progressing into S phase. Indeed p65 was seen to be required for CDK4 activity, suggesting that p65 regulation of cyclin D1 is important for cyclin D1 function. However, upon examination of S phase genes, we were unable to see a significant effect in the $p65^{-/-}$ MEFs, indicating that a decrease in cyclin D1 function is not sufficient to affect S phase progression. This notion is consistent with recent reports that demonstrated the loss of all the D-type cyclins or their respective CDKs do not affect cell cycle as a result of compensatory function from other G1 cyclin/CDK complexes [Kozar et al., 2004; Malumbres et al., 2004]. Analysis of Ki67 or other proliferation makers also did not reveal a difference between $p65^{+/+}$ and $p65^{-/-}$ tissues in mice (data not shown), further suggesting that reduction of cyclin D1 in those tissues most effected by the absence of p65 (skin, lung, kidney) is not sufficient to adversely affect cellular proliferation.

Although loss of cyclin D1 stability has no effect on cell cycle progression it is possible that stabilization of this protein is relevant in other cellular processes such as cell cycle exit and differentiation. Our data suggest that p65 functions in a myoblast to regulate cyclin D1 protein expression and cells lacking p65 exit the cell cycle at an accelerated rate upon their differentiation. Since one hallmark of myogenesis is the rapid loss of cyclin D1 expression [Rao and Kohtz, 1995; Guttridge et al., 1999] we speculate that in the absence of p65, cyclin D1 protein is rapidly turned over leading to early cell cycle exit and an accelerated myogenic program.

In addition to the regulation of skeletal myogenesis, it is possible that p65-mediated stabilization of cyclin D1 has implications in pathological conditions such as cancer. NF-κB activity and cyclin D1 overexpression has been linked to multiple cancers [Karin, 2006; Alao, 2007] and clinical studies utilizing breast and head and neck tumor samples have revealed that cyclin D1 overexpression is prevalent in a large percentage of tumors (50–70%). Interestingly, this over expression has been linked to genomic duplication of cyclin D1 in only a subset of these tumors (10–20%) [Masuda et al., 1996; Worsley et al., 1996; Monden et al., 1997; Simpson et al., 1997], leaving the possibility that p65-mediated regulation of cyclin D1 in this disease may occur at the post-translational level.

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