p53 family members in myogenic differentiation and rhabdomyosarcoma development

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Summary

The p53 family comprises the tumor suppressor p53 and the structural homologs p63 and p73. How the three family members cooperate in tumor suppression remains unclear. Here, we report different but complementary functions of the individual members for regulating retinoblastoma protein (RB) function during myogenic differentiation. Whereas p53 transactivates the retinoblastoma gene, p63 and p73 induce the cyclin-dependent kinase inhibitor p57 to maintain RB in an active, hypophosphorylated state. ΔNp73 inhibits these functions of the p53 family in differentiation control, prevents myogenic differentiation, and enables cooperating oncogenes to transform myoblasts to tumorigenicity. ΔNp73 is frequently overexpressed in rhabdomyosarcoma and essential for tumor progression in vivo. These findings establish differentiation control as a key tumor suppressor activity of the p53 family.

Introduction

The tumor suppressor gene p53 is central to an intricate network of pathways that senses various types of cellular stress to coordinate cell fate decisions such as cell cycle arrest, senescence, or apoptosis (Vogelstein et al., 2000). p53 is a key regulator preventing genotoxic stress and thus cancer progression. However, the physiological role of p53 in normal (unstressed) cells is still a matter of debate.

It has been suggested for a long time that p53 might have important functions in the control of differentiation and development. p53 is highly expressed in the mouse embryo (Choi and Donehower, 1999), and p53 expression or activity is increased during differentiative processes such as hematopoiesis, spermatogenesis, and myogenesis (Almog and Rotter, 1997). In support of a critical role for p53 in cellular differentiation, alterations of p53 in tumors frequently correlate with undifferentiated cancer phenotypes, and reconstitution of wild-type p53 triggers differentiation in a variety of tumor cells (Almog and Rotter, 1997). Conversely, the developmental viability of p53 null mice proves the contrary (Choi and Donehower, 1999). Apart from neural tube closure defects and reduced fertility, the lack of further developmental abnormalities suggests that p53 function is dispensable for mammalian development (Choi and Donehower, 1999).

Besides p53, p63 and p73 are members of the p53 family that share >60% amino acid identity within the DNA-binding domain and regulate an overlapping set of target genes (Kaghad et al., 1997; Yang et al., 1998). In addition, p63 and p73 display essential, nonredundant functions during development. p63-deficient mice exhibit striking epithelial defects, including complete absence of skin, hair, and further ectodermal derivatives (Mills et al., 1999; Yang et al., 1999). In contrast, p73-deficient mice suffer from hippocampal dysgenesis, hydrocephalus, chronic infections, and inflammation, as well as abnormalities in pheromone sensory pathways (Yang et al., 2000). Simultaneous inhibition of all p53 family members by deregulated expression of the antagonistic p73 isoform ΔNp73 induces embryonic lethality

SIGNIFICANCE

The role of p53 as a tumor suppressor is generally attributed to its ability to induce cell cycle arrest or apoptosis in response to genotoxic and oncogenic stress. However, potential tumor suppressor activities of the structural p53 homologs p63 and p73 are still a matter of debate. Here, we demonstrate that in myoblasts all three p53 family members cooperate in differentiation control. Ablation of these p53 family functions blocks the differentiation program and promotes malignant transformation. Induction of cellular differentiation therefore contributes to the tumor suppressor activities of the p53 family and provides an explanation for the high frequency of p53 pathway alterations in rhabdomyosarcoma patients.

(Erster et al., 2006; Hüttinger-Kirchhof et al., 2006). This phenotype is more severe than the homozygous knockout of any single p53 family member, suggesting that there are overlapping functions that are only revealed in compound knockouts.

In *Xenopus*, p53 depletion results in embryonic lethality due to gastrulation failure and defects in mesendoderm development (Cordenonsi et al., 2003). A possible explanation for the difference between mice and frogs is that in early mouse embryos the p53 family members p63 and p73 compensate for p53 loss, whereas in *Xenopus* p73 is absent and p63 is only expressed at later stages of development (Cordenonsi et al., 2003).

Skeletal muscle differentiation (myogenesis) involves a cascade of muscle-specific gene expression that is coordinated with permanent withdrawal from the cell cycle. The commitment of cells to the myogenic lineage and progression through the myogenic differentiation process requires the bHLH transcription factor family of myogenic regulatory factors (MRFs), including MyoD, Myf5, Myogenin, and MRF4 (Puri and Sartorelli, 2000). Whereas early markers of the myogenic program, such as Myogenin, can be expressed in proliferating cells, late markers of the differentiation program, including muscle structural genes like myosin heavy chain (MHC), are induced only after cell cycle withdrawal. The retinoblastoma tumor suppressor protein (RB) plays a critical role in establishing the G0 arrest observed in differentiated myocytes, and muscle cells lacking RB fail to exit the cell cycle (Novitch et al., 1996; Zacksenhaus et al., 1996). In addition, RB is specifically required for execution of the later steps in skeletal myogenesis, and its absence molecularly uncouples the early and late phases of this differentiation program (Novitch et al., 1996). Although the last few years have revealed some of the molecular mechanisms underlying the cooperation of RB and differentiation-specific transcription factors in the execution of specific transcription programs during terminal differentiation, the mechanisms that activate RB during myogenesis and other cellular differentiation processes still remain unclear. Here, we report that the p53 family members have essential functions in the activation of RB during physiological myogenesis. These functions are commonly disabled in rhabdomyosarcoma (RMS) patients, indicating that differentiation control is a critical tumor suppressor activity of the p53 family.

Results

ΔNp73 interferes with myogenic differentiation

We have previously shown that ΔNp73, a p73 isoform that functions as a transdominant inhibitor of all other p53 family members, interferes with multiple developmental programs (Hüttinger-Kirchhof et al., 2006). To understand the mechanistic basis, we investigated the impact of ΔNp73 on myogenic differentiation in more detail. For these studies, we used C2C12 myoblasts that faithfully mimic skeletal muscle differentiation in vitro (Blau et al., 1985). Upon shift to differentiation medium, mock myoblasts stopped proliferating, elongated, aligned, and fused to multinuclear myotubes. In contrast, myoblasts expressing ΔNp73α within the physiological range following retroviral transduction continued to proliferate and failed to form differentiated myotubes (Figure 1A; Figure S1A in the Supplemental Data available with this article online). The absence of myotubes in the ΔNp73α population was further confirmed by immunofluorescence staining and immunoblotting for myosin heavy chain (MHC) as a marker of mature muscle fibers (Figure S1B; Figure 1B). Similarly, primary human skeletal muscle myoblasts transduced with a ΔNp73α-expressing lentivirus failed to form myotubes and express muscle creatine kinase (Figures 1C and 1D).

To test whether ΔNp73α can also override myogenic differentiation signals induced by a 3D tissue environment, the differentiation of C2C12 cells after incorporation into 3D fibrillar collagen was investigated in the presence of serum. Whereas mock myoblasts upregulated MHC and differentiated into multicellular muscle fiber-like structures, ΔNp73α prevented MHC upregulation and maintained a dispersed single-cell state (Figure 1E). Thus, ΔNp73α overrides synergistic growth factor and ECM-induced differentiation signals.

Defects of terminal cell cycle withdrawal in ΔNp73α myoblasts

In growth factor-rich medium, mock and ΔNp73α myoblasts showed no significant difference in their proliferation rate (Figure 1F). Under conditions of mitogen deprivation, mock myoblasts completely withdrew from the cell cycle within 1 day. In contrast, ΔNp73α myoblasts slowed down markedly but continued to proliferate, resulting in a progressive increase in cell number. Cell cycle profiles demonstrated that only few mock cells (6%) remained in S phase 2 days after differentiation induction compared to more than 22% of the ΔNp73α cells (Figure 1G). Similarly, expression of the proliferating cell nuclear antigen (PCNA) was completely repressed in mock but not in ΔNp73α cells (Figure 1H). Importantly, cell cycle withdrawal during myogenic differentiation is known to be permanent, and restimulation with growth factors did not cause cell cycle reentry (Figure 1I). All nuclei within multinuclear, MHC-expressing myotubes failed to incorporate BrdU. In contrast, ΔNp73α cells rapidly increased their proliferation rate and showed a BrdU incorporation index of more than 90% (Figures 1I and 1J). These data indicate that ΔNp73α myoblasts are sensitive to growth factor depletion and slow down their proliferation rate but fail to permanently withdraw from cell cycle progression.

Genome-wide expression analysis of differentiation in ΔNp73α myoblasts

The myogenic differentiation program is initiated by a limited set of transcription factors that progressively unfold a complex program of gene expression (Bergstrom et al., 2002; Delgado et al., 2003). ΔNp73α is a protein with sequence-specific DNA-binding properties, suggesting that it directly interferes with the expression of critical genes during differentiation (Grob et al., 2001; Kartasheva et al., 2002; Stiewe et al., 2002a). We monitored the differentiation process of mock and ΔNp73α myoblasts by gene expression profiling with Affymetrix GeneChip Mouse Genome 430 2.0 Arrays. We extracted total RNA from cells at three different time points (0, 6, and 24 hr) after they had been induced to differentiate. To distinguish differentiation-associated changes in gene expression from experimental noise, we extracted all genes that were induced or repressed by more than 3-fold at 6 or 24 hr compared to the 0 hr time point (Table 1). The expression levels of these 646 genes correlated between the two cell types both in proliferation medium and 6 hr after induction of differentiation. Only 23/646 genes (3.6%) were expressed differentially in the two cell types at the 0 hr and 19/646 (2.9%) at the 6 hr time point. In contrast, 24 hr after induction

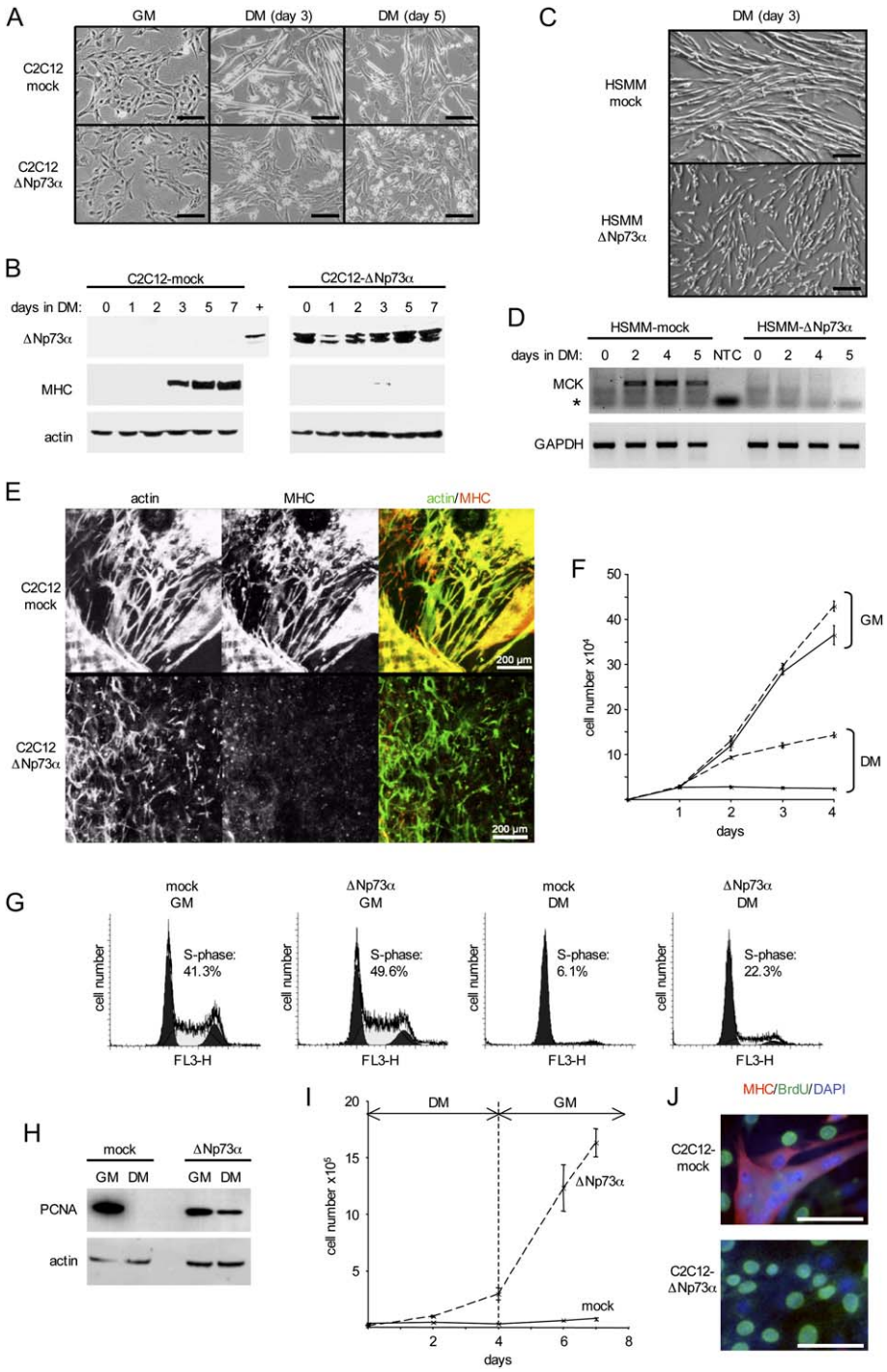

Figure 1. ΔNp73 interferes with cell cycle exit and differentiation of myoblasts

A: Phase-contrast micrographs of proliferating (GM) and differentiating (DM) mock and ΔNp73α-expressing C2C12 myoblasts. Scale bar, 100 μm.

B: Immunoblot for ΔNp73α, MHC, and actin.

C and D: Differentiation of mock and ΔNp73α-transduced primary human skeletal muscle myoblasts (HSMM). **C:** Phase-contrast micrographs on day 3 of differentiation. Scale bar, 200 μm. **D:** Semiquantitative RT-PCR for muscle creatine kinase (MCK) and GAPDH. The asterisk denotes a band of primer dimers.

E: Mock and ΔNp73α-expressing myoblasts were cultured in anchored 3D collagen lattices for 5 days, fixed, stained for F-actin (green) and MHC (red), and three-dimensionally reconstructed by confocal fluorescence microscopy. Images show a depth of 400 μm.

F: Proliferation curve of mock (solid line) or ΔNp73α (dashed line) myoblasts in proliferation (GM) and differentiation medium (DM).

G and H: Cell cycle profiles (**G**) and immunoblot for PCNA (**H**) of indicated myoblasts under proliferating and differentiating conditions.

I: Proliferation curve of mock (solid line) and ΔNp73α (dashed line) myoblasts during differentiation (DM) and subsequent restimulation with 10% FCS (DM).

J: Immunofluorescence staining for MHC (red), BrdU (green), and DAPI (blue) of differentiated myocytes 24 hr after restimulation with FCS. Scale bar, 50 μm.

Data are reported as the mean ± standard deviation.

of differentiation 261/646 genes (40.4%) were expressed differently (>3-fold) between the two cell types (Figure 2A). Furthermore, in mock cells 73/646 genes showed a more than 3-fold expression change during the first 6 hr. Of these 73 genes, 53 (72.6%) were also altered in the ΔNp73α myoblasts. Between 6 hr and 24 hr of differentiation, expression of 188/646 genes changed more than 3-fold in the mock cells. However, expression of only 5 (2.6%) of these 188 genes changed in ΔNp73α cells (Figure 2B). These data show that ΔNp73α-expressing myoblasts respond to differentiation stimuli with immediate-early gene expression changes but fail to show the profound changes observed at 24 hr associated with execution of the differentiation program.

Functional annotation of the list of 646 genes revealed the presence of several muscle-related genes, known p53 target genes, and genes related to cell proliferation (Figure 2C). In mock cells the muscle-related genes (e.g., *Acta1*, *Tncc*, *Myh1*) were induced or repressed most prominently at 24 hr of differentiation. Consistent with the observed differentiation defect, only a few changes were observed in ΔNp73α myoblasts.

Table 1. p73 modulates the tumorigenicity of myogenic cell lines

Cell line	Tumor frequency
C2C12-mock	0/20 (0%)
C2C12-ΔNp73α	1/20 (5%)
C2C12-IGF2	3/29 (10%)
C2C12-IGF2 + ΔNp73α	15/29 (52%)
C2C12-Pax3:Fkhr	0/15 (0%)
C2C12-Pax3:Fkhr + ΔNp73α	13/15 (87%)
Rh30-ns (bulk)	9/10 (90%)
Rh30-p73shRNA (bulk)	3/10 (30%)
Rh30-ns (clone)	23/25 (92%)
Rh30-p73shRNA (clone 1)	3/15 (20%)
Rh30-p73shRNA (clone 2)	2/10 (20%)
Rh30-p73shRNA (clone 3)	1/10 (10%)

Cells (5×10^6) of the indicated genotype were subcutaneously injected into nude mice that were monitored for tumor development for 3 months. Shown is the number of mice with a tumor, the total number of mice injected with each cell type, and the percentage of mice with tumors.

Furthermore, as expected from the function of ΔNp73 as a dominant-negative inhibitor of p53 family members, most of the p53 target genes (e.g., *p21*^{Cdkn1a}, *Gadd45a*) that showed expression changes in the mock cells were unchanged in the ΔNp73α cells. Finally, consistent with the defects in permanent cell cycle withdrawal, a number of cell proliferation-related genes (e.g., *Rfc3*, *Ccna2*, *Pole*, *Pold1*, *Mcm2*) failed to be silenced in the ΔNp73α myoblasts. The validity of the gene expression data was further verified by measuring the expression levels of selected genes by semiquantitative RT-PCR (Figure 2D).

ΔNp73 interferes with the activation of RB

To locate the differentiation block more precisely, we investigated the expression of typical muscle differentiation markers (Figure 3A). MyoD and Myf5, which play roles in specifying muscle lineage, were expressed at equivalent levels in proliferating and differentiating myoblasts irrespective of genotype. Myogenin, one of the first markers to be expressed after the onset of differentiation, was also similarly expressed in both mock and ΔNp73α myoblasts. In contrast, downstream targets of Myogenin (MEF2C, MHC, CKM) were absent in ΔNp73α-expressing cells. The block to differentiation therefore occurs primarily after the Myogenin expression step.

It has previously been shown that Myogenin activity is compromised in RB-deficient myoblasts (Novitch et al., 1996). In fact, in a direct comparison both *Rb*$^{-/-}$ and ΔNp73α-expressing fibroblasts stopped the MyoD-induced differentiation program at the Myogenin step (Figure 3B). The similar phenotype of RB loss and ΔNp73α expression suggested that RB function might be compromised by ΔNp73α. In differentiating wild-type myoblasts, we observed an increase in RB protein amount and in electrophoretic mobility, indicating a shift from inactive, hyperphosphorylated to active, hypophosphorylated RB species (Figure 3C). This activation of RB was significantly reduced in ΔNp73α myoblasts, and high levels of inactive, hyperphosphorylated RB were maintained over the differentiation time course. Whereas the reduced levels of RB protein can be explained by transactivation defects of the *Rb1* gene (Figure 3E), the phosphorylation state of RB is regulated by cyclin-dependent kinases. Whereas differentiating mock myoblasts downregulated CDK2- and CDK4-associated kinase activity, ΔNp73α cells displayed higher CDK activity under both proliferating and differentiating conditions (Figure 3D). In mock myoblasts, CDKs are typically inactivated by repression of cyclins and induction of CDK inhibitors (Figure 3C). In contrast, ΔNp73α cells maintained detectable levels of cyclins E and D and failed to induce expression of the CDK inhibitors p21 and p57 (Figures 3A

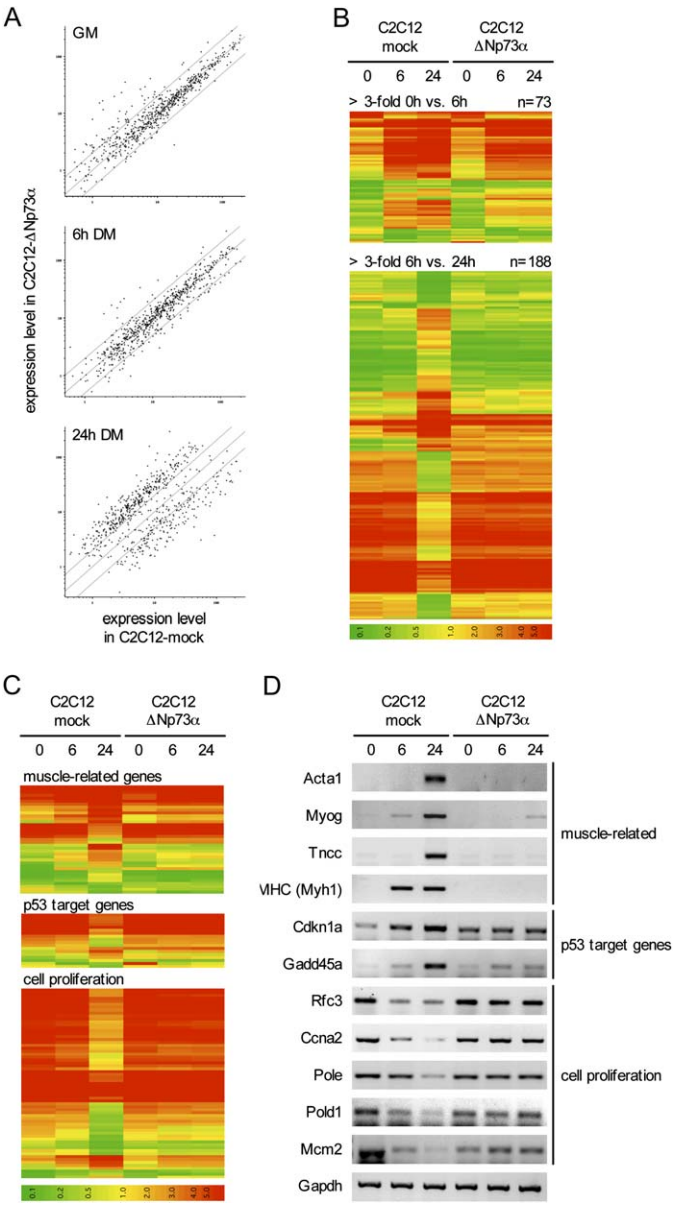

Figure 2. ΔNp73α prevents differentiation but not immediate-early gene expression changes in response to differentiation stimuli

A: Gene expression profiles of mock and ΔNp73α C2C12 myoblasts were analyzed under proliferating conditions (GM, 0 hr) or 6 and 24 hr after induction of differentiation. A list of 646 genes changed by more than 3-fold during the time course of differentiation was extracted (Table S1). Scatter plots comparing the expression levels of these selected genes between mock and ΔNp73α cells are shown.

B: Gene expression levels for a subset of genes that are altered within the first 6 hr of differentiation (n = 73, upper panel) or between the 6 and 24 hr time points of differentiation (n = 188, lower panel).

C: Gene expression levels for subsets of genes that are muscle related (upper panel), p53 target genes (middle panel), or related to cell proliferation (lower panel).

D: Semiquantitative RT-PCR analysis of differentiating C2C12 myoblasts.

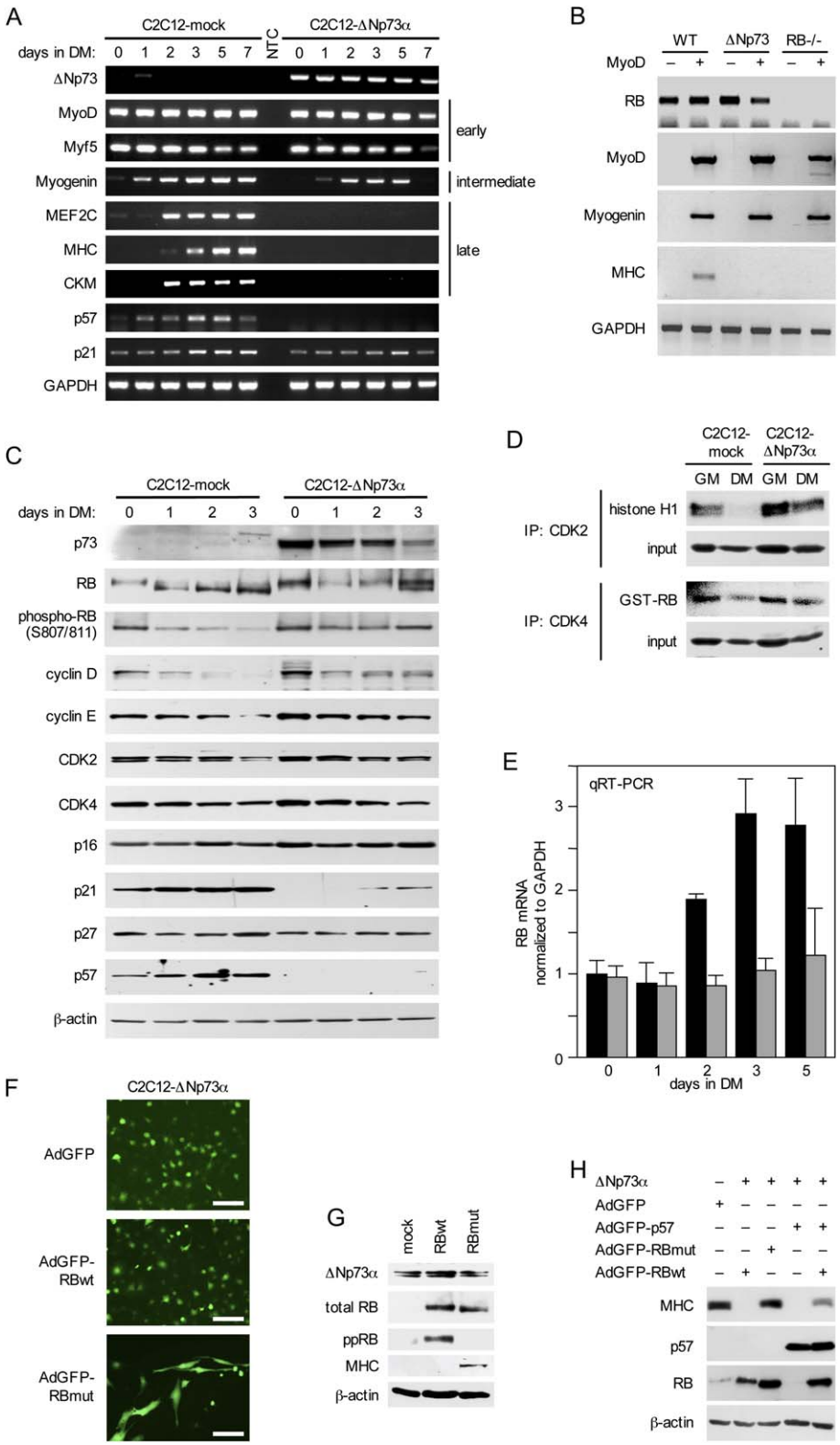

Figure 3. ΔNp73α interferes with RB activation in myoblasts

A and B: Semiquantitative RT-PCR of genes critically involved in myogenic differentiation of C2C12 myoblasts (**A**) and 3T3 fibroblasts (wild-type, ΔNp73α-transfected, $Rb^{-/-}$) after transfection with MyoD (**B**).

C: Immunoblot of mock and ΔNp73α myoblasts harvested at the indicated time points of differentiation.

D: CDK activity in proliferating (GM) and day 3 differentiated (DM) myoblasts. Kinase activity of CDK2 and CDK4 immunoprecipitates was determined with histone H1 and GST-RB as substrates.

E: qRT-PCR of RB expression in differentiating C2C12-mock (black bars) and C2C12-ΔNp73α cells (gray bars). Expression levels were normalized to GAPDH and expressed as the fold change compared to proliferating C2C12-mock cells.

F: Rescue experiment. C2C12-ΔNp73α myoblasts infected with the indicated adenoviruses were shifted to differentiation medium for 2 days. The morphology of the infected cells is shown by GFP fluorescence microscopy. Scale bar, 100 μm.

G and H: Immunoblot of C2C12-ΔNp73α cells differentiated after infection with indicated adenoviruses.

Data are reported as the mean ± standard deviation.

and 3C). These findings explain the inability to activate RB and the failure of ΔNp73α myoblasts to exit the cell cycle and induce muscle-specific gene expression.

To investigate if the observed defect in RB activation is causally related to the block in differentiation, we tried to rescue the differentiation defect with ectopic expression of RB (Figures 3F and 3G). For this, ΔNp73α myoblasts were infected with recombinant adenoviruses expressing wild-type RB (AdGFP-RBwt), a constitutively active phosphorylation site mutant of RB (AdGFP-RBmut), or an empty virus control (AdGFP). Only cells expressing the constitutively active form of RB stopped proliferating and exhibited morphological and biochemical signs

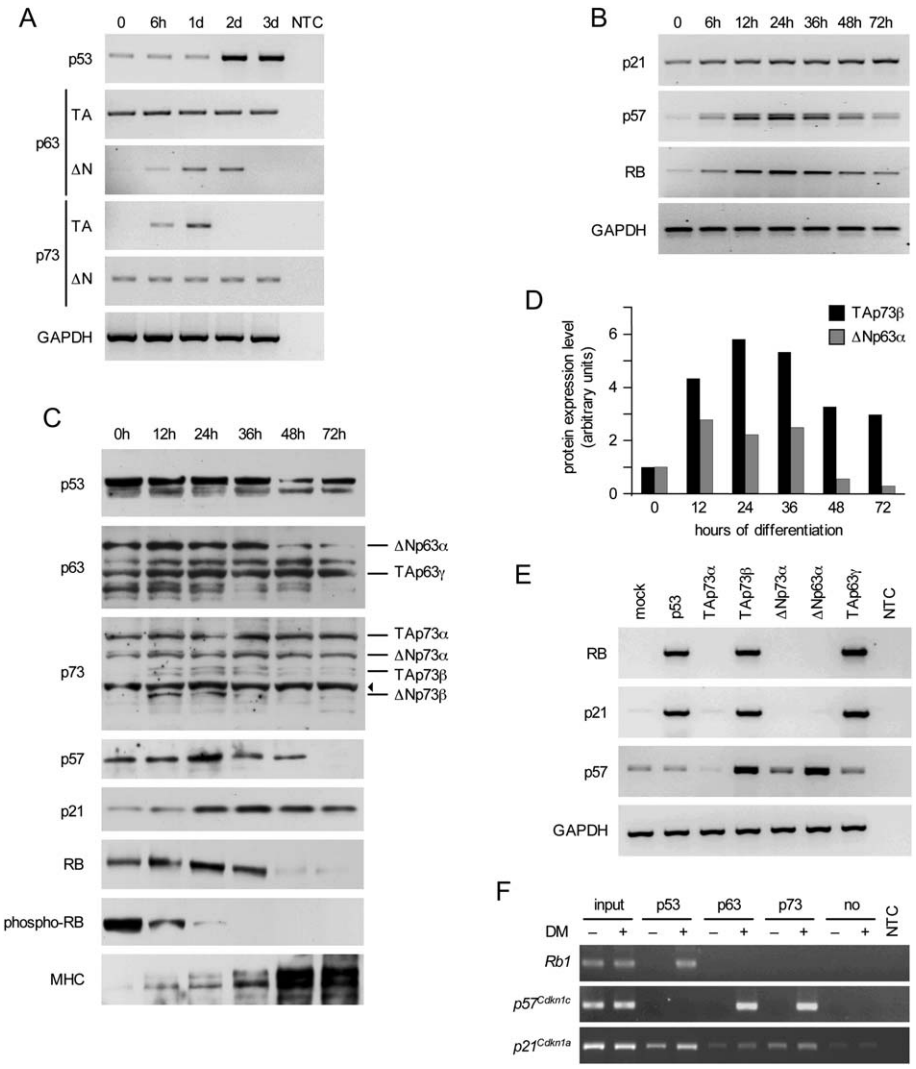

Figure 4. Role of p53 family members in myogenic differentiation

A–D: Expression of p53 family members and differentiation markers during in vitro differentiation of primary mouse myoblasts. **A and B:** Semiquantitative RT-PCR. **C:** Immunoblots. **D:** Quantification of ΔNp63α and TAp73β protein levels by densitometric analysis.

E: C2C12 myoblasts were transiently transfected with the indicated expression plasmids and analyzed by semiquantitative RT-PCR.

F: C2C12 myoblasts were harvested under proliferating conditions and 36 hr after induction of differentiation. Association of p53, p63, and TAp73 with the *Rb1*, *p57^{KIP2}*, and *p21^{Cdkn1a}* promoters was analyzed by chromatin immunoprecipitation. For *p57^{KIP2}* the fragment −966/−757 upstream of the first exon (Ensembl transcript ENSMUST00000037287) was amplified.

of differentiation (Figure 3F, lower panel; Figure 3G). Immunoblots showed that wild-type RB was inactivated by hyperphosphorylation and therefore unable to induce differentiation. However, in combination with p57 (AdGFP-p57), hyperphosphorylation of wild-type RB was prevented, resulting in expression of MHC (Figure 3H). Lower MHC levels induced by the adenoviral combination compared to the constitutively active RB mutant most likely reflect the low rate of coinfection with two different adenoviruses. p57 alone prevented RB phosphorylation but also decreased RB protein levels to barely detectable levels. Therefore, both p57 induction and an increase in RB expression are required to obtain levels of active RB that support completion of the differentiation program. In summary, active RB in the form of a constitutively active mutant or as a combination of wild-type RB with p57 rescued the ΔNp73α-induced differentiation block, proving that defects in activating RB are indeed the underlying cause.

Individual p53 family members in myogenic differentiation

Since ΔNp73α functions as a dominant-negative inhibitor of the transactivation-competent p53 family members, ΔNp73α might inhibit the activation of RB by interfering with essential functions of the individual p53 family members. In primary murine myoblasts (Figures 4A–4D) and C2C12 myoblasts (Figure S2A and S2B), all p53 family members were expressed. Although p53 mRNA levels increased progressively over 3 days, this was not accompanied by an increase in p53 protein levels. Considering the previously reported increase in p53 activity during myogenic differentiation (Porrello et al., 2000), our results suggest that the differentiation function of p53 might not be regulated on the level of protein stability.

On the mRNA level, expression of both ΔNp63 and TAp73 peaked around day 1. TAp63 and ΔNp73 mRNAs were detectable but unchanged over the time course of differentiation. The analysis of p73 (and p63) proteins is complicated by the multitude of different isoforms and the lack of isoform-specific antibodies that are suitable for detection of endogenous p73 in the mouse (Sayan et al., 2005). Here, we used a polyclonal p73 antibody raised against a common epitope and identified individual isoforms by comparison to myoblasts transfected with specific isoforms (Figure S2B). The most prominent changes are an increase in ΔNp63α and TAp73β expression at 12 to 36 hr (Figures 4C and 4D). In fact, TAp73β was the only p73 isoform detected by the monoclonal p73 antibody GC15 in primary myoblasts differentiated for 24 hr (Figure S2B). Importantly, the

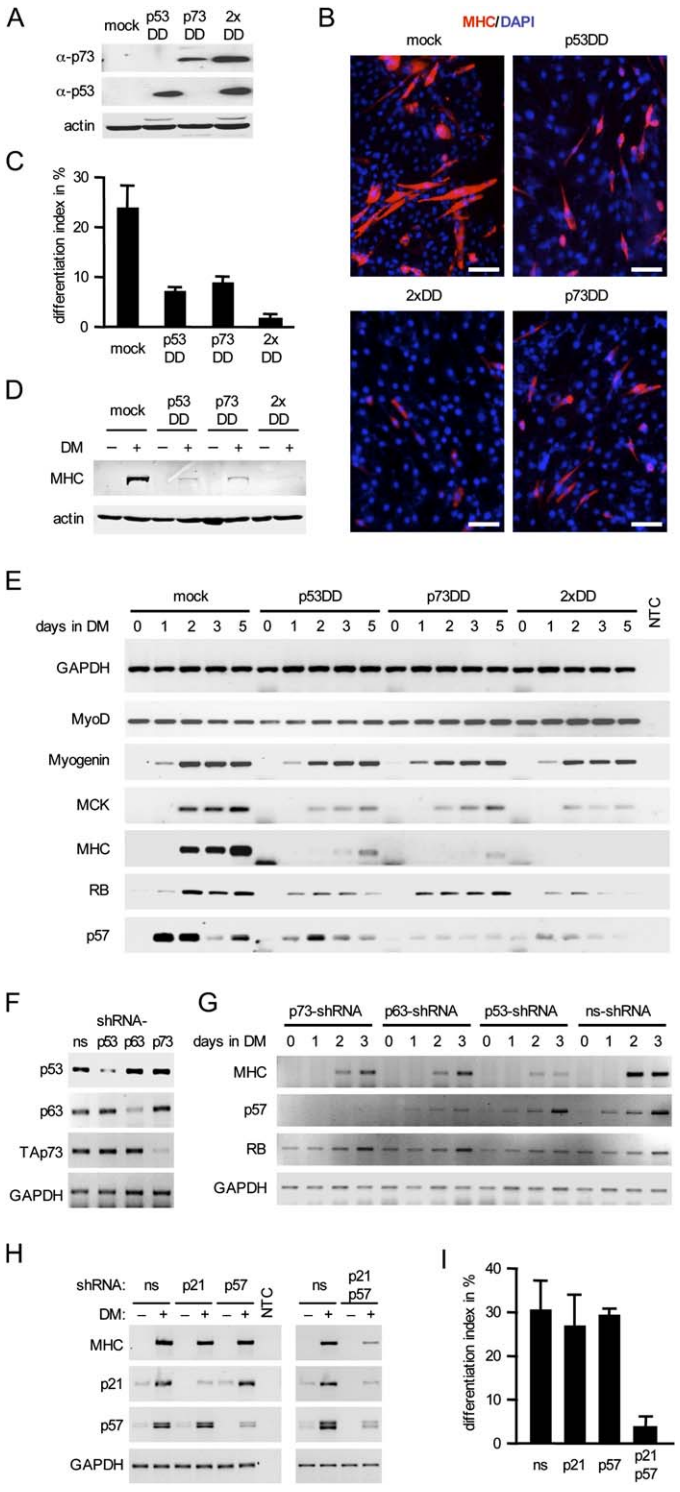

Figure 5. Specific functions for individual p53 family members in myogenic differentiation

A: C2C12 myoblasts were transduced with p53DD, p73DD, or both (2xDD). Following selection, expression of the constructs was verified by immunoblotting. The differentiation potential of these cells was analyzed 3 days after shift to differentiation medium by immunofluorescence staining for MHC (red) (**B**), calculation of the differentiation index (**C**), and immunoblotting for MHC (**D**). Scale bar, 100 μm. **E:** Expression of genes involved in muscle differentiation analyzed by RT-PCR. **F:** C2C12 myoblasts were transfected with shRNA constructs directed against p53, p63, and p73. A nonsilencing shRNA was used as a control. The knockdown efficiency was analyzed by RT-PCR. **G:** MHC, RB, and p57 expression was analyzed in these cell populations at the indicated time points of differentiation by RT-PCR. **H:** C2C12 myoblasts were transfected with shRNA constructs directed against p21 and p57 as indicated. The knockdown efficiency and MHC expression were analyzed by semiquantitative RT-PCR before and 3 days after induction of differentiation. **I:** Differentiation index of p21 and p57 knockdown cells on day 5 of differentiation. Data are reported as the mean ± standard deviation.

increase in p63/p73 expression coincides with induction of p21 and p57 and decreased phosphorylation of RB. Similar expression changes were observed in regenerating muscles in vivo (Figure S3).

When ectopically expressed, all the major transactivating proteins of the p53 family (p53, TAp73β, TAp63γ) induced the *Rb1* and $p21^{Cdkn1a}$ genes (Figure 4E), which have typical p53-binding sites in their promoter regions (Porrello et al., 2000). In contrast, consistent with recent reports, $p57^{KIP2}$ was transactivated only by TAp73β and ΔNp63α but not by p53 (Beretta et al., 2005; Balint et al., 2002; Vaccarello et al., 2005). The analysis of the sequences upstream of the $p57^{KIP2}$ transcriptional start or in the introns of $p57^{KIP2}$ failed to reveal any p53-binding sites arranged in tandem, as usually is seen in p53-responsive promoters. Considering the lack of p53 responsiveness, the absence of typical p53-binding sites is not surprising and suggests that other mechanisms of promoter targeting are employed by TAp73β and ΔNp63α. The experiment further revealed that, although both TAp73α and ΔNp73α failed to transactivate *Rb1*, $p21^{Cdkn1a}$, and $p57^{KIP2}$, only ΔNp73α had dominant-negative activity when coexpressed with p53 or TAp73β (Figure S2C).

We next analyzed if *Rb1*, $p21^{Cdkn1a}$, and $p57^{KIP2}$ are indeed regulated by the p53 family in differentiating myoblasts and investigated recruitment of p53, p63, and TAp73 to the respective promoter regions by chromatin immunoprecipitation (Figure 4F). Whereas none of the investigated proteins was associated with the *Rb1* and $p57^{KIP2}$ promoters in proliferating myoblasts, upon differentiation p53 bound to the *Rb1* promoter, and p63 and TAp73 were recruited to the $p57^{KIP2}$ promoter. The $p21^{Cdkn1a}$ promoter was bound most strongly by p53 and only weakly by p63/p73. Binding was already detected in proliferating myoblasts and increased slightly during differentiation.

Dominant-negative inhibitors or knockdown of p53 family members compromises myogenic differentiation

To test the requirement for individual p53 family members in myogenic differentiation, we used previously validated dominant-negative inhibitors, which allow us to specifically ablate the function of either p53 (p53DD) or p63/p73 (p73DD) (Irwin et al., 2000, 2003). Single inhibitors reduced the differentiation index from 24% in the mock myoblasts to 8% (p53DD) or 9% (p73DD), respectively (Figures 5A–5D). The combination of both inhibitors further reduced the differentiation index to less than 2%, supporting the hypothesis that p53 and p63/p73 have different but complementary functions in differentiation control. The effect of ΔNp73α could therefore be mimicked by the combination of two dominant-negative inhibitors, indicating that the dominant-negative function of ΔNp73α was sufficient to block myogenic differentiation. Similar to ΔNp73α, the two inhibitors (alone or in combination) had no significant effect on Myogenin expression but reduced expression of MHC and MCK cooperatively to barely detectable levels (Figure 5E). Furthermore, the analysis revealed that RB induction was primarily

inhibited by p53DD, whereas p57 induction was impaired by p73DD. This confirmed that the p53 family members coordinate progression to later stages of differentiation by regulating RB.

To rule out effects of p53DD and p73DD unrelated to their dominant-negative function, we also used RNA interference as an independent method. Specific knockdown of single p53 family members resulted in impaired differentiation using MHC expression as a read-out (Figures 5F and 5G). Inhibition of p53 compromised RB induction (see Figure S4A for qRTPCR), whereas shRNAs directed against either p63 or p73 interfered with p57 induction. Both p63 and p73 were essential for regulation of p57, as the knockdown of either factor blocked p57 expression. In support of these data, $p53^{-/-}$ myoblasts failed to induce RB, whereas $p73^{-/-}$ myoblasts showed impaired induction of p57 (Figures S4B–S4D). As p63-deficient mice die at birth with severe limb truncations, comparable differentiation studies could not be performed on $p63^{-/-}$ myoblasts.

As these data suggested regulation of CKIs as the critical activity of p63/p73 in differentiation, we investigated the effects of p21 and p57 depletion (Figures 5H and 5I). Whereas the knockdown of either CKI had no significant impact, the combined depletion significantly reduced differentiation and mimics a loss of p53 family functions. The data correlate with the chromatin immunoprecipitation results and support the hypothesis of a functional dichotomy in the p53 family with respect to differentiation control. p53 on the one side regulates RB expression, and p63 and p73 on the other side are in control of p57. The two p53 family functions are complementary and cooperate to activate RB, which is essential for efficient myogenic differentiation.

Malignant transformation of myoblasts by ΔNp73α

Differentiation failure is a characteristic feature of rhabdomyosarcoma (RMS), the most common soft tissue sarcoma in children. We therefore investigated whether ΔNp73α has oncogenic functions in RMS. However, C2C12-ΔNp73α myoblasts failed to form tumors in nude mice, indicating that inhibition of differentiation is not sufficient for tumor formation (Table 1). Since single oncogenes are rarely sufficient to induce transformation, we tested cooperation of ΔNp73α with the known RMS oncogenes IGF2 and Pax3:Fkhr (Merlino and Helman, 1999). ΔNp73α inhibited the differentiation of parental and Pax3:Fkhr myoblasts and also the accelerated and enhanced differentiation of IGF2 myoblasts (Figure 6A) (Stewart et al., 1996). Of these various myoblast populations, only IGF2+ΔNp73α and Pax3:Fkhr+ΔNp73α cells were tumorigenic in nude mice (Table 1). To understand why single oncogenes do not result in tumor formation whereas combinations together with ΔNp73α do, we injected the cells mixed with Matrigel Matrix into the subcutis of mice and explanted the Matrigel plugs after 1 week (Figure 6B). Matrigel plugs of Pax3:Fkhr myoblasts contained few cells in the center but were surrounded by a dense rim of MHC-positive cells that frequently fused into multinuclear myotubes. In contrast, matrigel plugs containing Pax3:Fkhr+ΔNp73α myoblasts showed no MHC staining and contained areas of active proliferation as shown by PCNA staining. These findings demonstrate that myoblasts injected into mice exit the cell cycle and differentiate into mature myotubes irrespective of Pax3:Fkhr expression. ΔNp73α interferes with cell cycle withdrawal and differentiation and thus unleashes the oncogenic activities of Pax3:Fkhr (and IGF2) and enables tumor growth.

Oncogenic function of ΔNp73 in human rhabdomyosarcoma

Inspired by the role of ΔNp73α as a cooperating oncogene in myoblasts, we investigated p73 expression in tumor samples from rhabdomyosarcoma patients. As long as no isoform-specific antibodies are available for the immunohistochemical detection of endogenous ΔNp73 proteins, RT-PCR remains the method of choice for quantification of p73 isoforms in limited amounts of tumor tissues (Sayan et al., 2005; Stiewe et al., 2002b, 2004; Zaika et al., 2002). Whereas no p73 expression was detectable in normal muscle tissues and undifferentiated human skeletal muscle myoblasts (HSMM), various p73 isoforms were expressed to high levels in the majority of patient samples (Figure 6C). High TAp73 levels were seen in 85.7% of patients and were always associated with overexpression of ΔNp73 generated by either alternative splicing of exon 2 (ΔN^{AS}) or alternative promoter usage (ΔN^{AP}). Further immunohistochemical analyses indicated that RB and p57 are inversely regulated. Whereas normal muscle tissue showed a strong p57 staining associated with undetectable RB staining, two rhabdomyosarcoma tissues showed barely detectable p57 staining combined with high levels of RB (Figure 6D). Importantly, a rhabdomyosarcoma with high ΔNp73α expression (sample #20) displayed a significantly higher phospho-RB staining index than a low ΔNp73-expressing tumor (sample #22), providing further support that ΔNp73 regulates RB activity by controlling its phosphorylation state (Figure 6D).

Similar to the primary tumor samples, three of five RMS cell lines (including the Rh30 cell line) showed concomitant overexpression of TAp73 and ΔNp73 on both the RNA and protein levels (Figures 7A and 7B). As ΔNp73α, which counteracts the antiproliferative activity of TAp73 (Figure S5A), is known to efficiently sequester TAp73 (and also p63) in transcriptionally inactive complexes, ΔNp73α is likely the dominant isoform (Grob et al., 2001; Slade et al., 2004; Stiewe et al., 2002a). To investigate if high p73 expression contributes to the differentiation defect and the tumorigenic phenotype, we depleted p73 in Rh30 cells using RNA interference (Figure 7C). Whereas cells transduced with a nonsilencing control shRNA induced tumors in 32 of 35 nude mice (91.4%), cells with silenced p73 expression formed tumors in only 9 of 45 mice (20.0%) (Table 1). Analysis of individual clones further demonstrated that the decrease in tumorigenicity directly correlated with the degree of p73 depletion. We further analyzed whether inhibition of p73 also affects the growth of established RMS tumors that developed 4 weeks after subcutaneous injection of parental Rh30 cells into nude mice. For in vivo gene delivery, we generated a recombinant adenovirus (AdGFP-p73shRNA) that efficiently reduced p73 expression in Rh30 cells by RNA interference (Figure 7E). Following an intratumoral injection of 5×10^7 PFU of AdGFP-p73shRNA but not the control adenovirus (AdGFP-ns) we observed a significant retardation of tumor growth ($p < 0.002$) over a period of 5 weeks (Figure 7D). To understand the mechanisms underlying this growth inhibition, we further investigated the effects of p73 knockdown in Rh30 cells in vitro. Depletion of p73 induced p57, providing evidence that ΔNp73α is indeed a negative regulator of p57 expression in RMS cells, as suggested by the initial overexpression studies in C2C12 myoblasts (Figure 7E).

However, p73 depletion did not result in differentiation but induced a cell cycle arrest instead (Figure 7F; Figure S5B). This effect on cell proliferation is also supported by a clonogenic

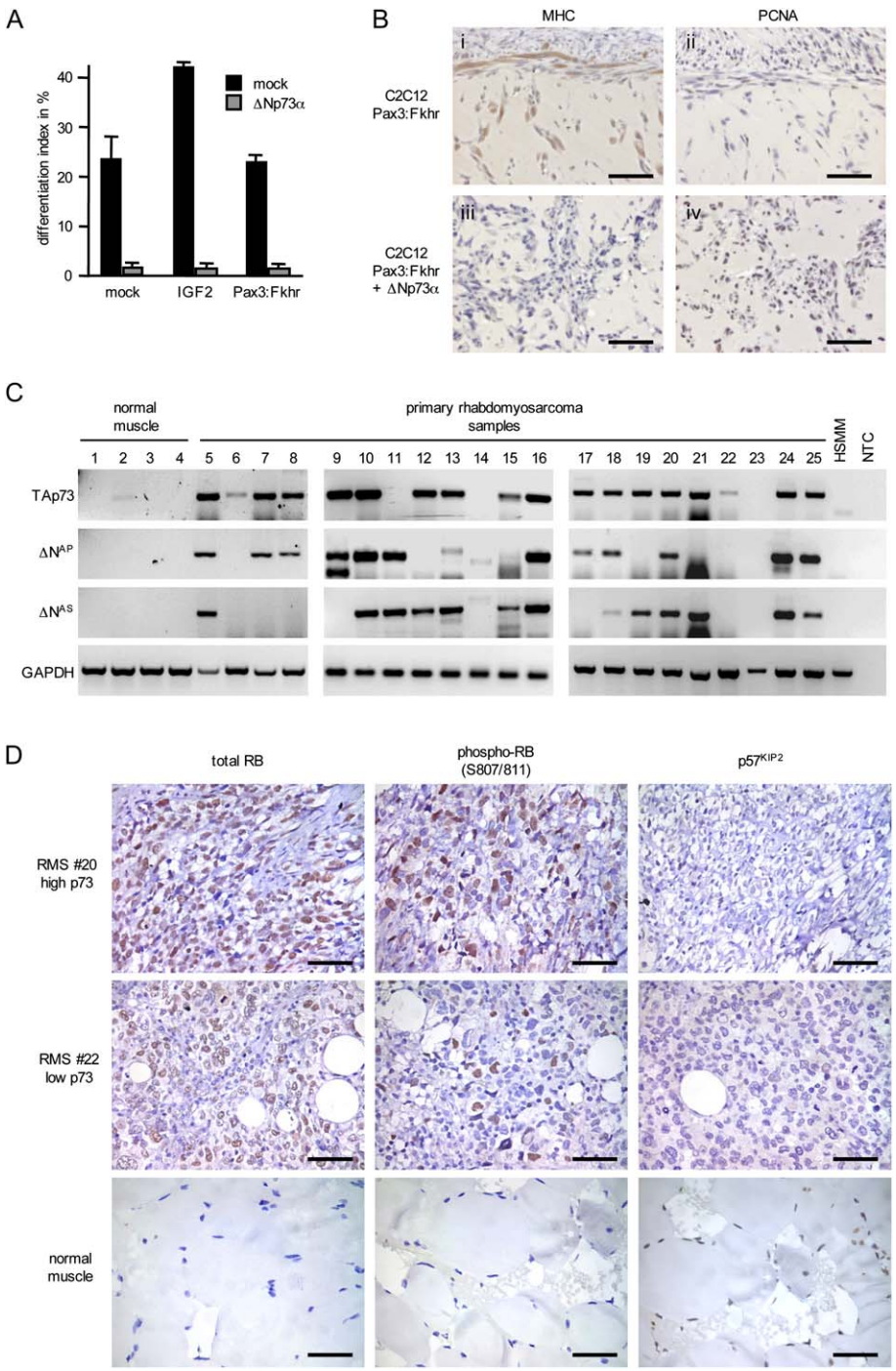

Figure 6. ΔNp73α in rhabdomyosarcoma development

A and B: ΔNp73α cooperates with IGF2 and Pax3:Fkhr in the malignant transformation of myoblasts. **A:** C2C12 myoblasts were transduced with retroviral constructs for IGF2, Pax3:Fkhr, and ΔNp73α. Empty vectors were used as controls. Following selection, the differentiation potential was analyzed by immunofluorescence staining for MHC. The calculated differentiation index is shown. **B:** Pax3:Fkhr (**Bi** and **Bii**) and Pax3:Fkhr+ΔNp73α (**Biii** and **Biv**) myoblasts were mixed with Matrigel Matrix HC and injected s.c. into nude mice. After 1 week, the Matrigel plugs were excised and analyzed by immunohistochemistry for MHC (**Bi** and **Biii**) or PCNA (**Bii** and **Biv**). Scale bar, 100 μm.

C: Expression of full-length (TAp73) and N-terminally truncated p73 isoforms generated by alternative splicing of exon 2 (ΔN^{AS}) or alternative promoter usage (ΔN^{AP}) was investigated in normal muscle and RMS tissues by semiquantitative RT-PCR. Human skeletal muscle myoblasts (HSMM) were used as a control.

D: Immunohistochemistry of two rhabdomyosarcoma samples, lanes 20 and 22 in **C**, and a normal muscle tissue for total RB, serine 807/811 phosphorylated RB, and p57. Scale bar, 50 μm.

Data are reported as the mean ± standard deviation.

formation assay demonstrating reduction in colony size but not colony number following p73 knockdown (Figure 7G). To understand why restoration of p57 expression by depletion of ΔNp73α is not sufficient to induce differentiation, we tried to rescue the differentiation defect directly by overexpression of p57. However, this approach also failed to rescue the differentiation defect, although p57 induced efficient dephosphorylation of the endogenous RB protein (Figure 7H). Simultaneously, however, RB expression was reduced to barely detectable levels, reminiscent of our observations in C2C12-ΔNp73α cells (Figure 3H). Restoration of RB levels by coexpression of RB together with p57 resulted in high levels of "active RB" that proved sufficient for differentiation (Figure 7H). Similarly, wild-type RB was efficiently activated in p73-depleted Rh30 cells again, resulting in morphological and biochemical signs of differentiation (Figure 7I). This indicates that differentiation of rhabdomyosarcoma cells requires the same two pathways shown to be important for the differentiation of nontransformed myoblasts: the p63/p73-p57 and the p53-RB pathways (Figure 7J). In Rh30 cells, induction of p57 by p63/p73 is blocked by ΔNp73α, whereas transactivation of the RB gene is prevented by a mutation in p53. Correction of these two common RMS-associated alterations by direct overexpression of RB and p57 as the two main downstream effectors of p53 and p63/p73, respectively, rescues the differentiation

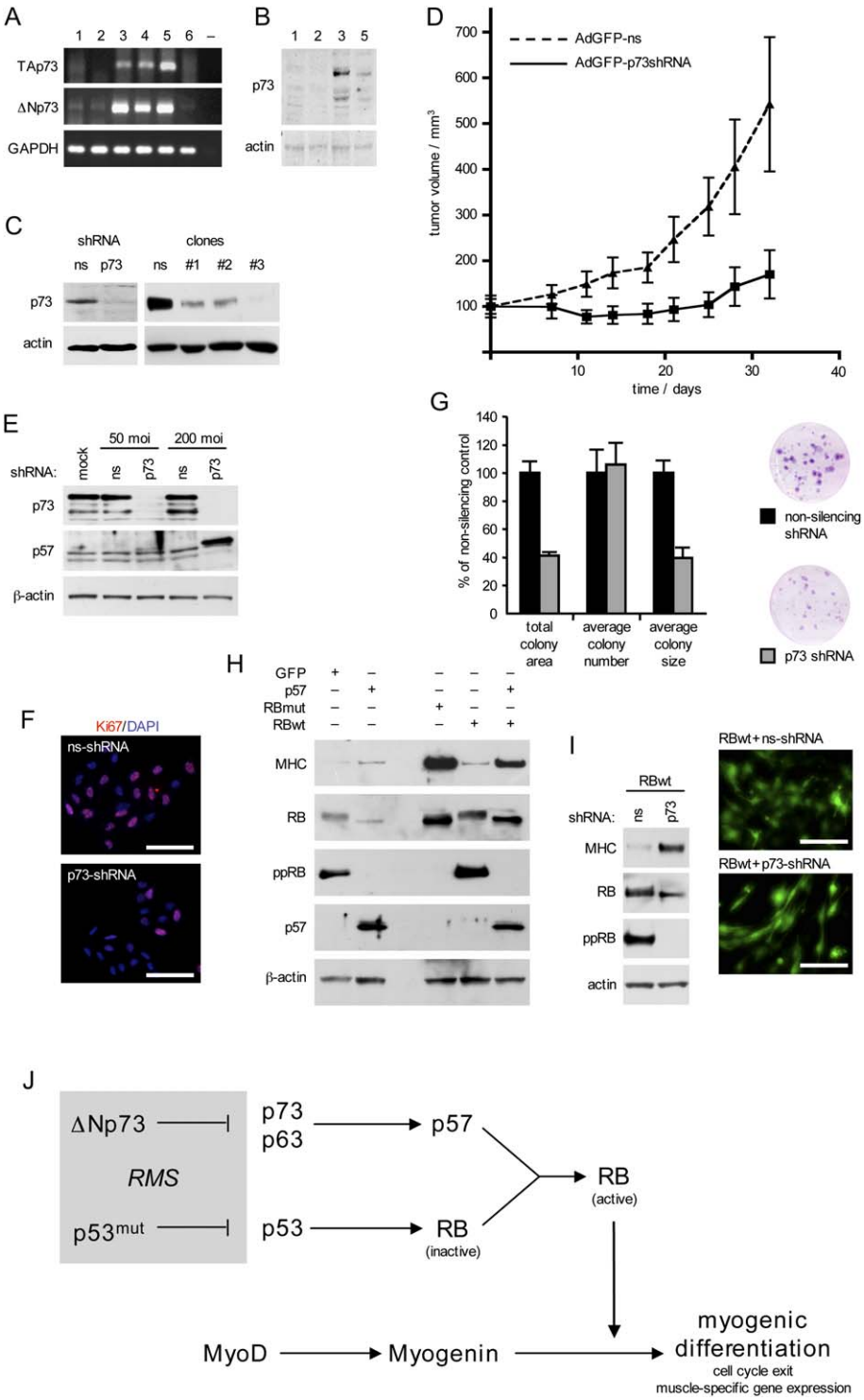

Figure 7. p73 functions as an oncogene in RMS

A and B: Human RMS cell lines (1, JR-1; 2, RD; 3, Rh30; 4, Rh18; 5, Rh41) and primary human fibroblasts (6, BJ) as a control were analyzed for p73 expression by isoform-specific RT-PCR (**A**) and immunoblotting (**B**).

C: Rh30 cells were infected with lentiviruses expressing a p73-directed shRNA or a nonsilencing shRNA as a control. Bulk cultures and individual clones were expanded, and endogenous p73 expression was analyzed by immunoblotting.

D: Rh30 cells (5×10^6) were injected subcutaneously into nude mice. After 4 weeks, 5×10^7 PFU of recombinant adenoviruses generating nonsilencing (AdGFP-ns) or p73-directed shRNAs (AdGFP-p73shRNA) were injected into established tumors of approximately 100 mm^3 volume (n = 5 for each treatment). Shown are the tumor volumes and the standard deviation (two-way ANOVA, $p < 0.002$).

E and F: Rh30 cells were infected for 2 days with a low (50 moi) or high (200 moi) dose of AdGFP-ns or AdGFP-p73shRNA. **E:** Immunoblot for p73, p57, and β-actin. **F:** Immunofluorescence staining for the proliferation marker Ki67. Scale bar, 100 μm.

G: Clonogenic formation assay. Rh30 cells were transfected with plasmids encoding nonsilencing or p73-directed shRNAs and selected with puromycin for 2 weeks. Shown is a quantitative analysis of the total colony area, the average colony number per dish, and the average colony size. A representative Giemsa staining is shown on the right.

H: Rescue experiment. Rh30 cells were infected with the indicated adenoviruses and shifted to differentiation medium for 3 days. Expression of MHC, RB, serine 807/811 phosphorylated RB, p57, and β-actin is shown by immunoblotting.

I: Rh30 cells were infected with a combination of AdGFP-RBwt and AdGFP-ns or AdGFP-p73shRNA, respectively. After 3 days in differentiation medium, differentiation was analyzed by fluorescence microscopy and immunoblotting for MHC, RB, and phospho-RB (S807/811). Scale bar, 100 μm.

J: Role of p53 family members during myogenic differentiation. Alterations found in rhabdomyosarcoma samples and their impact on the differentiation program are shaded in gray.

Data are reported as the mean ± standard deviation.

defect and therefore identifies dysfunction of p53 family-regulated differentiation pathways as the underlying cause of the failure of rhabdomyosarcoma cells to differentiate.

Discussion

In our study we show that all p53 family members cooperate to activate the differentiation function of RB, which is required for both the permanent cell cycle exit and the activation of muscle-specific genes. Whereas p53 regulates RB protein levels, p63 and p73 control the activity of RB by regulating its phosphorylation state via the CKI $p57^{KIP2}$ (Figure 7J). RMS cells maintain RB in the hyperphosphorylated state when exposed to mitogen-poor differentiation-inducing media, explaining in part their failure to arrest growth or differentiate under these conditions (Knudsen et al., 1998). Similarly, myoblasts transfected with ΔNp73α maintain hyperphosphorylated RB and fail to differentiate. Transfection with active forms of RB

(phosphorylation-insensitive RB or the combination of wild-type RB with p57) rescues the differentiation defect of both ΔNp73α-transfected myoblasts and RMS cells that express high endogenous levels of ΔNp73. This provides compelling evidence that RB dysfunction in RMS can be caused by genetic alterations that affect the function of the p53 family in differentiation control.

In addition to p57, p21 is another CKI involved in RB regulation, and only mice deficient for both p57 and p21 have severe muscle defects (Zhang et al., 1999). The $p21^{CIP1}$ promoter contains characteristic p53-binding elements and is activated by ectopic expression of all transactivating p53 family members (Figure 4D). However, during muscle differentiation p21 induction has been previously shown to be p53 independent, which has been explained by direct induction of p21 by MyoD (Parker et al., 1995). In our experiments ΔNp73α reduced but did not completely abrogate p21 induction (Figure 3C). The severe differentiation defect of ΔNp73α myoblasts, however, demonstrates that the low levels of p21 seen in the absence of p53 family function are inadequate to support differentiation. Furthermore, depletion of neither p57 nor p21 was alone sufficient to inhibit differentiation. Only the combined knockdown of both p57 and p21 significantly compromised the differentiation process (Figures 5H and 5I). Therefore, although p53 itself might be dispensable (Parker et al., 1995), the p53 family as a whole is certainly required for p21 to reach levels needed for completing the differentiation program.

Considering the profound impact of ΔNp73 on myogenic differentiation, it appears remarkable that none of the homozygous knockout mice of single p53 family members have an overt muscle phenotype. p53 null mice show normal muscle development, although p53-deficient myoblasts fail to induce RB, resulting in deficient MHC expression upon differentiation in vitro (Porrello et al., 2000; Figure 5G; Figure S4B). In our experiments the *Rb1* gene was transactivated not only by p53 but also by TAp63γ and TAp73β (Figure 4D), suggesting that p63/p73 might be able to compensate for p53 loss in the microenvironment of the myotome or the regenerating muscle tissue in vivo.

Although both p63 and p73 knockout mice present with developmental defects, severe muscle abnormalities have not been reported (Moll and Slade, 2004). However, in our experiments RNAi-mediated knockdown of either factor was sufficient to inhibit p57 induction and impaired the progression to late stages of differentiation. Furthermore, myoblasts from p73 knockout mice showed a significant reduction in p57 expression (Figures S4C and S4D). However, as p73 and p63 are both involved in regulation of p57 expression, developmental plasticity in signaling pathways might allow p63 to compensate for p73 loss and vice versa in vivo. Considering the similar function of the two proteins in the differentiation process, functional compensation appears very likely, so that a muscle phenotype in vivo might only become apparent in homozygous compound double or triple knockouts.

A role for p53 in suppression of RMS development has been previously suggested. $p53^{+/-}$ mice over a year old develop mostly sarcomas, 19% of which are RMS (Choi and Donehower, 1999). Furthermore, conditional Pax3:Fkhr knockin mice develop RMS frequently on a p53-compromised background but only very rarely in p53-proficient mice (Keller et al., 2004). Apart from mouse studies, the Li-Fraumeni cancer syndrome, which is associated with germline p53 mutations, was initially identified from a RMS patient as the index case and includes these tumors along with other soft-tissue sarcomas (Li and Fraumeni, 1969). Interestingly, in many tumors p53 is inactivated by missense mutations that endow p53 with new functions ("gain-of-function"), like the dominant-negative inhibition of the closely related family members p63 and p73 (Di Como et al., 1999; Marin et al., 2000). Therefore, many p53 mutations disable not only the tumor-suppressive function of p53 but rather the function of the complete family.

In addition, our study demonstrates that p73 is overexpressed in the majority of RMS samples. Just as high p53 protein levels in tumor tissues are an indicator for p53 mutations, high p73 levels correlate with compromised p73 activity. In many tumors, p16-RB-E2F pathway alterations result in elevated levels of the E2F-target gene TAp73 (Irwin et al., 2000; Stiewe and Pützer, 2000; Zaika et al., 2001). Increased TAp73 expression in tumors in turn is frequently associated with the generation of aberrantly spliced ΔNp73 encoding transcripts (Stiewe et al., 2002b, 2004; Pützer et al., 2003) and in transactivation of the ΔNp73 promoter (Grob et al., 2001; Kartasheva et al., 2002). Consistently, we observed simultaneous overexpression of both TAp73 and ΔNp73 in RMS samples and found that ΔNp73 expression in Rh30 cells is driven by TAp73 (Figures S5C–S5F). ΔNp73 forms transactivation-defective complexes with p73 (and also p63), resulting in the stabilization of p73 proteins on a high, but transcriptionally inactive level (Slade et al., 2004). In addition, ΔNp73 competes with p53 for binding to p53 target promoters (Grob et al., 2001; Stiewe et al., 2002a). However, higher concentrations of ΔNp73 are needed for inhibition of p53 than for inhibition of p63/p73 (data not shown). Likewise, most p53 missense mutations compromise p53 function, but only a few have a dominant-negative effect on p63/p73 (Marin et al., 2000). For example, although the p53 mutations 143A, 179Q, and 273H all abrogate p53 function, only the 143A and 179Q mutants inhibit transactivation by p73. ΔNp73 overexpression and p53 mutations can therefore cooperate to provide complete inhibition of the p53 family and are not necessarily mutually exclusive. An example is provided by the Rh30 cell line in our studies, which contains a DNA contact mutation in p53 (R273C). Although not formally proven for the R273C mutant, the similar R273H mutation prevents p53 from binding to target promoters but has no dominant-negative effect toward p63 and p73 (Marin et al., 2000). Although p63 and p73 are not inactivated by the p53 mutant, they are sequestered by high levels of ΔNp73. The importance of ΔNp73 overexpression for the tumorigenic phenotype even in the presence of a p53 mutation is illustrated by the dramatic reduction of tumor growth following RNAi-mediated knockdown of p73 in vivo. Consistent with ΔNp73 being a repressor of p57 expression (Figures 3A and 3C), depletion of p73 in Rh30 cells resulted in a strong increase in p57 levels associated with a cell cycle arrest (Figures 7E–7G). Ectopic expression of p57 alone induced a similar cell cycle arrest that was not associated with differentiation, presumably because of a simultaneous reduction in RB protein levels (Figure 7H; data not shown). However, differentiation was observed when RB was expressed together with p57 (Figure 7H) or expressed in the context of ΔNp73 depletion (Figure 7I), suggesting that the p53-RB pathway is required to maintain high levels of RB when p57 is induced by p63/p73. RB and p57 are therefore the major downstream targets of the p53 family in myogenic differentiation, and induction of both targets is typically impaired

during the development of RMS tumors by alteration of p53 family functions.

In summary, our experiments show that all p53 family members are involved in regulating the process of muscle differentiation. Whereas the early steps of differentiation, up to expression of Myogenin, can occur in the absence of the p53 family, the later stages involving permanent cell cycle exit and activation of muscle-specific gene transcription require active RB, which is dependent on p53 family functions. The functions of the individual members are distinct but complementary. p53 is required to induce transcription of the RB gene, whereas p63 and p73 control expression of p57, which maintains RB in an active hypophosphorylated state. Ablation of these p53 family functions by overexpression of ΔNp73 or mutations in p53 blocks myogenic differentiation and enables cooperating oncogenes to transform myoblasts to tumorigenicity. Induction of cellular differentiation therefore contributes to the tumor suppressor activities of the p53 family and provides an explanation for the high frequency of p53 pathway alterations in rhabdomyosarcoma.

Experimental procedures

In vivo experiments

For analysis of tumorigenicity, 5×10^6 cells were injected subcutaneously into the flank of 4- to 6-week-old nude mice. Tumor volumes were measured twice weekly using calipers. For later recovery, the cells were mixed with Matrigel Matrix HC (Becton Dickinson) before subcutaneous injection. After 1 week, the Matrigel plug was excised and processed for immunohistochemistry using standard procedures. For immunodetection of MHC and PCNA, the M.O.M. Peroxidase Kit (Vector Laboratories) was used. For in vivo gene delivery experiments, 5×10^6 Rh30 cells were subcutaneously injected into nude mice. After 4 weeks, tumors with approximately 100 mm^3 volume were injected with 5×10^7 PFU of CsCl-purified adenoviral vector. Tumor growth was measured twice weekly for a total of 6 weeks. All animal experiments were performed according to protocols approved by the Bezirksregierung Unterfranken.

Human rhabdomyosarcoma tissues

For analysis of human rhabdomyosarcoma tissues, 21 ex vivo RMS fresh-frozen specimen containing 12 embryonal and 9 t(2;13) Pax3:Fkhr-positive alveolar RMS were investigated by RT-PCR. This study was approved by the Ethics Committee of the University of Würzburg, Germany, and informed consent was obtained from all subjects.

Microarray experiments

Mock and ΔNp73α-expressing C2C12 myoblasts were analyzed under proliferating conditions as well as 6 and 24 hr after the shift to differentiation medium. Total RNA was extracted with TriPure Isolation Reagent (Roche). The samples were analyzed for gene expression using Affymetrix GeneChip Mouse Genome 430 2.0 Arrays and further analyzed with GeneSpring 7.0 (Silicon Genetics). Only genes flagged present or marginal in all samples were included in the analysis. A list of 646 genes that were changed >3-fold in mock myoblasts at 6 or 24 hr compared to the 0 hr time point was extracted (Table S1). The complete set of microarray data has been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE5447.

Statistical analysis

Statistical analysis of tumor growth curves was performed with GraphPad Prism software using two-way ANOVA analysis. All numerical data are reported as the mean ± standard deviation.

Other procedures are described in the Supplemental Data.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, five supplemental figures, and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/4/281/DC1/.

Acknowledgments

We thank Gerry Melino, Bert Vogelstein, Hans van Bokhoven, Antonio Giordano, Frederic Barr, Pumin Zhang, Eric Knudsen, William Kaelin, Frank McKeon, Didier Trono, Linda Harris, and Stefan Gaubatz for providing expression plasmids, cell lines, and mouse strains. We are grateful to Alea Mills and Martin Eilers for helpful comments. We further thank Nadja Karl, Andrea Staudigel, and Antje Barthelm for technical assistance. A.R. and P.F. are supported by the Interdisciplinary Center for Clinical Research (IZKF), University of Würzburg. This work was funded by grants from the Deutsche Krebshilfe-Dr. Mildred Scheel Stiftung (10-1884-St1, 10-2075-St2) to T.S. and the Deutsche Forschungsgemeinschaft (Transregio TR17, Forschungszentrum FZ82).

Received: February 22, 2006
Revised: June 5, 2006
Accepted: August 10, 2006
Published: October 16, 2006

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Accession numbers

The complete set of microarray data has been deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE5447.