

**The role of p53 family members
in myogenic differentiation and rhabdomyosarcoma
development**

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Summary

Disruption of differentiation pathways is one of the hallmarks of cancer. In rhabdomyosarcoma (RMS), a human tumor arising from myogenic precursors, the muscle differentiation program is disabled resulting in uncontrolled proliferation. Whether the differentiation block is due to overexpression of inhibitors, deficient function of activators, or both remained unknown. This study shows that RMS cells but not non-neoplastic muscle cells overexpress $\Delta Np73$, a pan-inhibitor of the p53 family of tumor suppressor genes. Experimental overexpression of $\Delta Np73$ in normal muscle precursor cells inhibited myogenic differentiation and promoted malignant transformation in cooperation with the RMS oncogenes IGF2 and PAX3/FKHR. *Vice versa*, RNAi knockdown of $\Delta Np73$ reduced the tumorigenicity of established RMS tumor cells. As $\Delta Np73$ is a dominant-negative inhibitor of the p53 family, inhibition of differentiation by $\Delta Np73$ suggests that the p53 family members (p53, p63 and p73) are critically involved in myogenic differentiation control. Indeed, this study demonstrates that all three p53 family members cooperate to activate the late stages of the differentiation process by regulating the activity of the retinoblastoma protein RB. The function of RB is known to be 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 activation state of RB by modifying its phosphorylation via the cyclin-dependent kinase inhibitor p57^{KIP2}. 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.

Zusammenfassung

Krebserkrankungen zeichnen sich häufig durch Störungen zellulärer Differenzierungsprozesse aus. So weisen Rhabdomyosarkome, die aus Muskelvorläuferzellen hervorgehen, Differenzierungsdefekte auf, die zur unkontrollierten Proliferation der Tumorzellen führen. Bislang ist ungeklärt, ob die Differenzierungsdefekte auf der verstärkten Expression von Inhibitoren, der defekten Funktion von Aktivatoren oder einer Kombination von beidem beruht. In dieser Arbeit wird gezeigt, dass im Unterschied zu normalen Muskelzellen RMS-Zellen verstärkt $\Delta Np73$, einen Pan-Inhibitor der p53-Tumorsuppressorfamilie, exprimieren. Die experimentelle Überexpression von $\Delta Np73$ in normalen Myoblasten blockierte die Muskeldifferenzierung und förderte in Kombination mit klassischen RMS-Onkogenen wie IGF2 oder PAX3/FKHR die maligne Transformation. Umgekehrt führte die Hemmung von $\Delta Np73$ durch RNAi zur Reduktion der Tumorigenität von RMS-Tumorzellen. Da $\Delta Np73$ als dominant-negativer Inhibitor der p53-Familie wirkt, lies die Hemmung von Differenzierungsprozessen durch $\Delta Np73$ vermuten, dass die p53-Familienmitglieder (p53, p63, und p73) an der Regulation der Muskeldifferenzierung beteiligt sind. Tatsächlich konnte in dieser Arbeit gezeigt werden, dass die drei p53-Familienmitglieder bei der Induktion später Differenzierungsstadien kooperieren, indem sie die Aktivität des Retinoblastoma-Proteins RB regulieren. Die Funktion von RB ist bekanntermassen sowohl für den permanenten Zellzyklusarrest als auch für die Aktivierung Muskel-spezifischer Gene notwendig. Während p53 die Proteinspiegel von RB reguliert, kontrollieren p63 und p73 den Aktivierungsgrad von RB, indem sie dessen Phosphorylierungszustand über den Zyklin-abhängigen Kinaseinhibitor p57^{KIP2} modifizieren. Eine Hemmung dieser Funktionen blockiert das Differenzierungsprogramm und fördert die Tumorentstehung. Die Aktivierung zellulärer Differenzierungsprozesse stellt somit einen entscheidenden Bestandteil der Tumorsuppressoraktivität der p53-Familie dar und liefert eine Erklärung für die Häufigkeit von Mutationen im p53-Signalweg bei Rhabdomyosarkom-Patienten.

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1. Introduction

1.1. The p53 Family Members

Introduction

The transcription factor and tumor suppressor p53 and its two homologues p63 and p73 form a protein family. Despite the structural and functional similarities between p53, p63 and p73 (Fig. 1), the knockout phenotypes and the expression patterns of p53 family members are quite different from each other. In the following sections, each p53 family members will be described in detail.

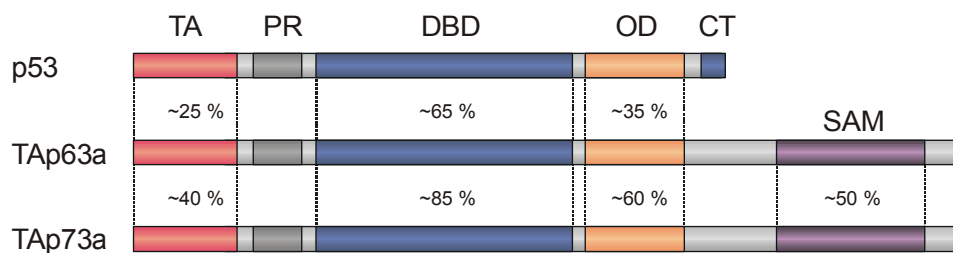
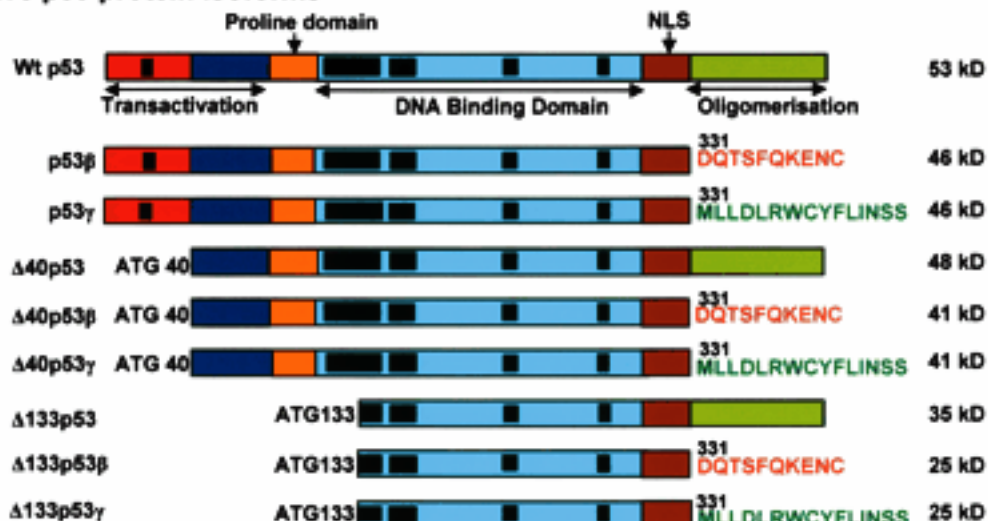


Figure 1. Gene structure of the p53 family members.

The percent identity at the amino acid level is indicated for the TA (transactivation domain), DBD (DNA binding domain) and OD (oligomerization domain).

1.1.1. The p53 Gene

p53 was discovered more than 25 years ago as a protein interacting with the oncogenic T antigen from SV40 virus (Linzer et al., 1979). The p53 gene contains an internal promoter and can transcribe twelve different mRNAs in normal human tissue, which can encode at least nine p53 protein isoforms (Fig. 2A and B). These p53 isoforms are expressed in several normal human tissues in a tissue dependent manner (Bourdon et al., 2005).

A Human p53 gene structure**B Putative p53 protein isoforms****Figure 2. The human p53 gene.**

A. The human p53 contains an alternative promoter and transcribes multiple splice variants. **B.** The human p53 gene generate nine different p53 protein isoforms : p53, p53 β p53 γ , Δ 133p53, Δ 133p53 β , and Δ 133p53 γ due to alternative promoters in intron 4 and alternative splicing of intron 9, but also Δ 40p53, Δ 40p53 β , and Δ 40p53 γ due to alternative splicing of intron 2 and alternative initiation of translation (Reviewed in Bourdon et al., 2005).

Molecular and biochemical assays revealed that p53 is a sequence-specific DNA-binding transcription factor. p53 is normally a short-lived protein that is maintained at low, often undetectable, levels in normal cells. Furthermore, tight regulation of p53 function is crucial for normal cell growth and development and one mechanism by which p53 function is controlled through the interaction with mouse double minute 2 protein (Mdm2) (HDM2, human homolog) (Kubbutat et al., 1997). Mdm2 possesses E3 ubiquitin ligase activity towards p53. Through its ability to ubiquitinate p53 and target it for proteasomal degradation, Mdm2 plays a key role in retaining p53 at very low concentration under non-stressed condition. At the same time, the *Mdm2* gene is a positive transcriptional target of p53, the expression of which is often elevated

subsequent to induction of p53 activity. This defines a negative feedback loop wherein p53 upregulates Mdm2, whereas Mdm2 downregulates p53. This loop can be viewed as a regulatory module, into which a plethora of incoming signals feed and thereby modulate p53 levels and activity in accordance with intracellular and extracellular cues (Daujat et al., 2001; Juven-Gershon and Oren, 1999; Momand et al., 2000).

1.1.2. p53 as a guardian of the genome

p53 is activated when cells are stressed or damaged. Recent research has confirmed the existence of several independent pathways by which p53 can be activated (Fig. 3). One pathway is triggered by DNA damage. It appears that different types of DNA damage activate different enzyme activities that modify the p53 protein at different amino-acid residues, and so the nature of the stress signal is transmitted to the protein, and presumably its activity, by a code inherent to the posttranslational modifications that reflect the different types of stress (Colman et al., 2000). For example, gamma radiation activates the ATM (Ataxia Telangiectasia Mutated) kinase and the Chk2 (checkpoint kinase 2), both of which can phosphorylate the p53 protein, while UV-radiation activates ATR (Ataxia telangiectasia and Rad3-related protein), Chk1 (checkpoint kinase 1) and casein kinase-2, which results in the modification of different amino-acid residues on the p53 protein. A second pathway is triggered by aberrant growth signals, such as those resulting from expression of the oncogenes Ras or Myc. In this case, activation of p53 depends on a protein called p14^{ARF} that physically interacts with Mdm2 and blocks Mdm2 induced degradation of p53 (Pomerantz et al., 1998). In addition, a wide range of chemotherapeutic drugs, hypoxia or both heat and cold shock conditions, which result in denatured proteins and RNA aggregation, induces p53. Furthermore, spindle poisons, which block chromosome segregation, or inflammation with its associated nitric oxide signalling can also trigger a p53 response.

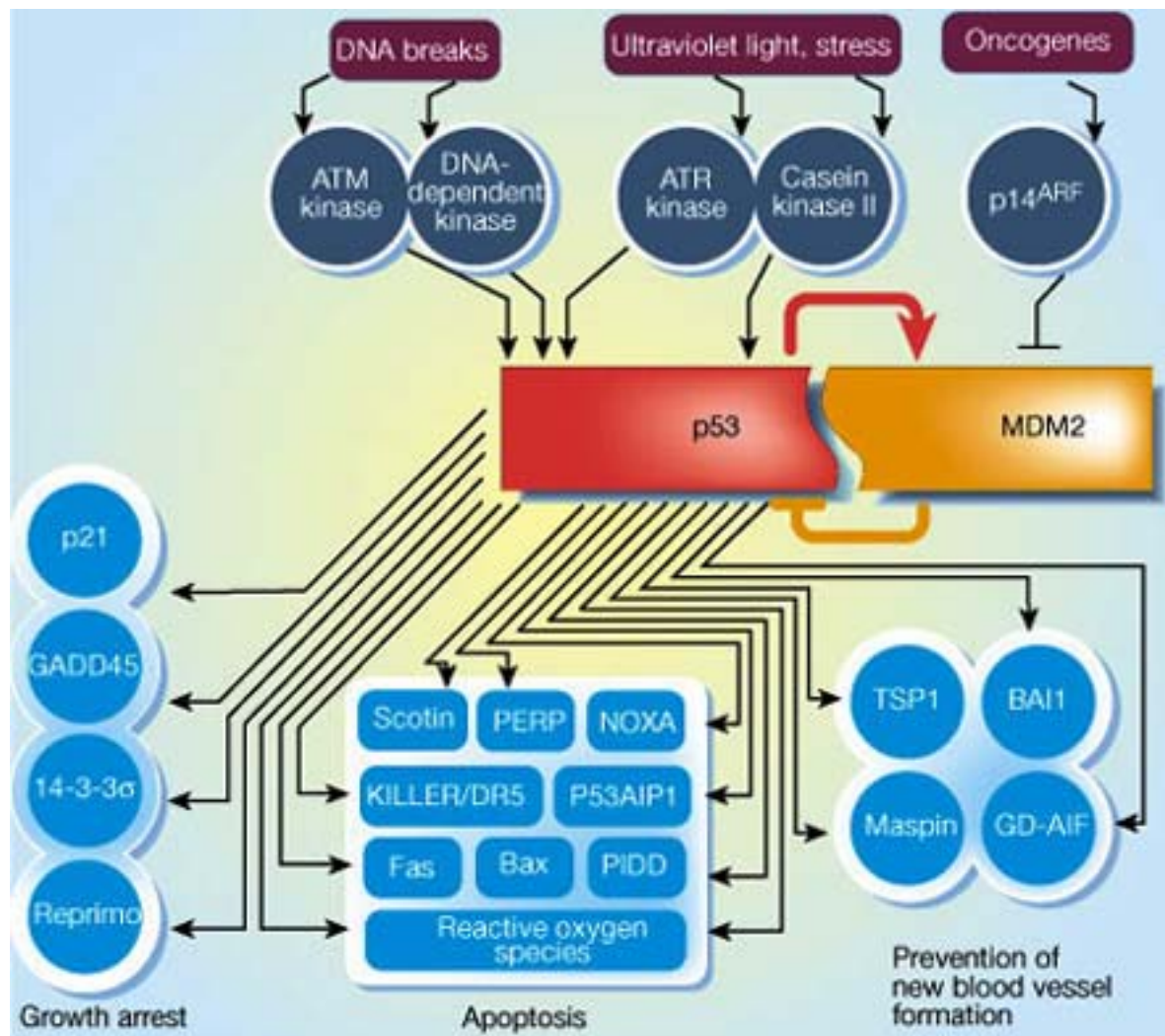


Figure 3. The diversity of cancer-related signals that activate p53 contributes to the central role the p53 protein as a tumor suppressor. See text for details (reviewed in Vogelstein et al., 2000).

When the cell is confronted with these various stress signals, activated p53 is a subject to a complex and diverse array of covalent post-translational modifications, which markedly influence the expression of p53 target genes. Post-translational modification of p53 involves the covalent addition of a functional group to the p53 protein after its translation. The most commonly reported post-translational modifications of p53 include phosphorylation of serines and/or threonines and acetylation, ubiquitylation and sumoylation of lysine residues (Buschmann et al., 2000; Gu and Roeder, 1997; Melchior and Hengst, 2002; Oda, 2002). The ensuing cellular response is dependent on the particular post-translational modifications, which are themselves dependent on the cell type and the nature of the external

stimuli. Post-translational phosphorylation and acetylation usually drive p53 activation because these modifications generally result in p53 stabilization, accumulation and activation in the nucleus.

Once the p53 protein is activated, it initiates a transcriptional program that reflects the nature of the stress signal. The activated p53 protein binds to a specific DNA sequence, termed the p53-responsive element (RE), and initiates one of three programs that result in cell cycle arrest, cellular senescence or programmed cell death (apoptosis) (Vogelstein et al., 2000). Major players in the p53-mediated cell cycle arrest are p21 and GADD45 (for G2 arrest). The identify of promyelocytic leukemia gene *PML* as a p53 target gene and *p53*-deficient cells are resistant to *PML*-induced cell cycle arrest indicate that *PML* plays a major role in p53 mediated cellular senescence (de Stanchina et al., 2004). A large number of genes (*bax*, *noxa*, *puma*) directly regulated by p53 are known that contribute to the apoptosis of cells. Several p53-regulated genes enhance the secretion of cytochrome c from mitochondria into the cytoplasm. Cytochrome c interacts with APAF-1 (another p53 regulated gene) to initiate a protease cascade, leading to the activation of caspase 9 and then caspase 3 followed by apoptosis. In addition, p53 regulate a series of genes (*Fas* ligand, *Killer/DR5*), resulting in the caspase 8 and 3 activation and apoptosis. Taken together, anti-proliferative response and senescence upon activation of p53 prevents the replication of damaged DNA and division of genetically altered cells. Therefore, p53 is thought to play an important role in maintaining the integrity of the genome (Lane, 1992).

1.1.3. The role of p53 in differentiation

Considerable experimental evidence has accumulated suggesting that a fine regulation of p53 activity is required for optimal development and differentiation. p53 expression and/or activity increases during differentiative processes such as hematopoiesis, spermatogenesis (Almon et al., 1993; Kastan et al., 1991) and myogenesis (Porrello et al., 2000; Soddu et al., 1996; Tamir and Bengal, 1998). Exogenous p53 expression can induce differentiation in a variety of tumor cells *in vitro* (Almog and Rotter, 1997) and *in vivo* (Bossi et al., 2000), while alterations of

the TP53 gene in vivo frequently correlate with undifferentiated phenotypes (Battista et al., 1995; Feinstein et al., 1991).

Moreover, it appears that maintaining of a fine balance of p53 protein levels within embryonic cells is important for optimal development. By *in situ* hybridization, it was observed that *Trp53* mRNA is present at high levels in all mouse embryonic cells from embryonic day (E) 8.5 to E 10.5. At later stages of development, *Trp53* expression becomes more pronounced during the differentiation of specific tissues and declines in mature tissues (Schmid et al., 1991). Furthermore, the complete absence of p53 can result in reduced fertility and exencephaly in some mouse strains (Armstrong et al., 1995; Sah et al., 1995) or in mice receiving low folic acid concentration in the diet (Choi and Donehower, 1999). Despite the viability of most p53- null mice, p53-depletion in *Xenopus* embryos results in gastrulation failure and defects in mesoderm formation (Cordenonsi et al., 2003). All these observations strongly support the involvement of the *Trp53* gene in cell differentiation and development. Nevertheless, the pathways modulated by p53 in these processes are still unknown, as are the effectors of these biological events.

1.1.4. The role of p53 in the tumor development

The function of the p53 tumor suppressor protein is directly or indirectly compromised in most sporadic human tumors (Harris et al., 1993; Nigro et al., 1989). Furthermore, germ-line p53 mutations cause hereditary cancer in both mice and humans (Levine et al., 1995). Patients with germ-line mutations in p53 develop the hereditary Li-Fraumeni cancer syndrome, characterized by an increased risk of developing a spectrum of tumors including breast cancer, sarcomas, and brain tumors (Malkin et al., 1990). Malignant progression is often associated with of p53 function either through mutations in the *TP53* gene itself or by defects in signaling pathways that are upstream or downstream of p53. Analysis of many tumors has shown that TP53 is mutated in about half of cancers (Fig. 4), resulting in a loss of its apoptotic function. These tumor-associated mutations in *TP53* are predominantly point mutations (93%).

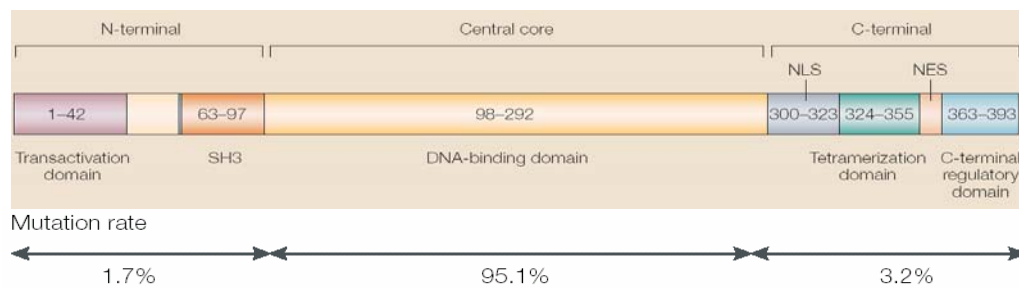


Figure 4. Location of tumor-associated mutation on p53 gene.

The most 95% of mutations occur within the DNA-binding domain, whereas the rest of the mutations occur in C-terminal and N-terminal region of p53.

The result of the mutational inactivation by single amino-acid substitution is that many tumor cells retain the ability to express a mutant p53 protein. These proteins are often more stable than wild type p53 and therefore present at very high levels in the tumor cells. One explanation for the selection of such mutations is that the mutant p53 proteins can act as dominant-negative inhibitors (a non-functional mutant protein that competes with the normal, non mutated protein, thereby blocking its activity) of wild-type p53 (de Vries et al., 2002; Ko et al., 1996). The observation that many tumors which harbor *TP53* mutations also show a loss of heterozygosity (effectively eliminating the wild-type allele) indicates that the efficiency of dominant-negative inhibition might not be complete and depends on the nature of the initial point mutation (Greenblatt et al., 1994).

In summary, the p53 tumor suppressor protein is known to regulate cell cycle checkpoints, apoptosis, differentiation and development. Nevertheless, whether apoptosis or developmental functions of p53 are equally important for the tumor suppressor activity is still unknown.

1.1.5. The p63 Gene

The p53 family member p63 gene was identified in 1998 (Yang A., 1998), and generates at least six protein isoforms which can be divided into two groups, those containing the transcription activation domain (TA isoforms) and those that do not (ΔN isoforms). In addition, alternative splicing of the C-terminus generates the α , β , or γ variants. The α -form of p63 contains a sterile alpha motif (SAM), which mediates protein-protein interactions (Fig. 5).

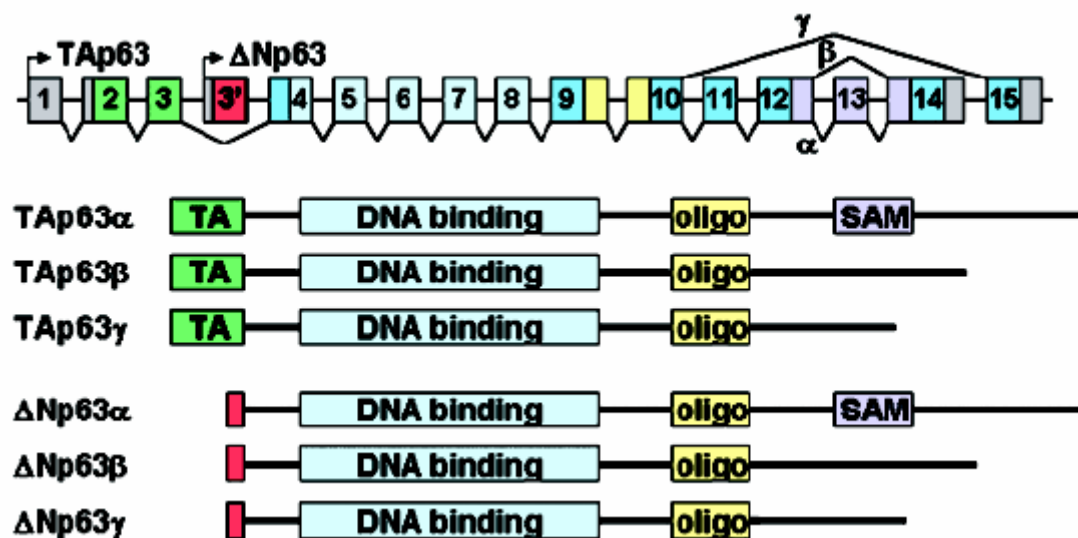


Figure 5. Scheme of the human p63 gene

The p63 gene is transcribed into six different transcripts that are generated by alternative promoter usage and alternative splicing. Exons are color coded to indicate the functional domains. (Adapted from Koster and Roop, 2004).

p63 is expressed in a confined manner, with the highest expression found in the basal cells of various epithelial tissues where ΔN p63 α transcripts are the most abundant. In contrast to p53, it is not yet clearly understood how the expression of p63 is regulated, nor what determines which transcripts are predominant, although DNA damage appears to upregulate the expression of TA forms (Kato et al., 2000) and downregulate the ΔN forms (Liefer et al., 2000).

Since the most significant degree of homology between p53 and p63 is in the DNA-binding domain, and the critical residues for the proper folding of the entire domain as well as for the binding to the target DNA sequences, are completely conserved (Celli et al., 1999; Yang et al., 1998), studies were performed to determine if p63 could regulate p53-responsive genes. Transient transfection assays showed that p63 activated or repressed transcription of a reporter gene downstream of an optimal p53 DNA-binding site (Yang et al., 1998). As predicted from their structures, the TA variants can transactivate p53 target genes, whereas the ΔN variants are believed to act in a dominant-negative manner (Yang et al., 1998). Furthermore, initial studies of p63 biochemical activities found that transient transfection TA-containing versions of p63 variants could induce both cell cycle arrest and apoptosis (Yang et al., 1998). Similar assays were performed with the $\Delta Np63$ variants and opposite effects on cell cycle regulation or apoptosis were observed as compared with those generated with TAp63 variants (Sasaki et al., 2001; Yang et al., 1998). However, further studies on TA- or $\Delta Np63$ variants are required to fully understand the role of p63 in cell cycle regulation and apoptosis.

1.1.6. The role of p63 in development and differentiation

In contrast to p53-null mice, which are highly tumor prone but lack a considerable developmental phenotype, the p63-null mice show several developmental defects. For instance, p63 expression is absolutely essential for limb formation and epidermal morphogenesis (integument and tongue) including the formation of adnexa (teeth, hair, mammary and prostate glands, and sweat and lacrimal glands) (Mills et al., 1999b). p63-null animals show severe limb truncations or absence of limbs and craniofacial malformations. They also fail to develop skin and most epithelial tissues (e.g., prostate and mammary glands). The animals do not survive beyond a few days postnatally (Mills et al., 1999b). Reminiscent of the knockout phenotype in mice, heterozygous germ line point mutations of p63 in humans cause six rare autosomal dominant developmental disorders with a strong but not absolute genotype-phenotype correlation. Ectrodactyly-ectodermal dysplasia-clefting (EEC Syndrome) or the related yet distinct ankyloblepharon-ectodermal dysplasia-clefting (Hay-Wells syndrome) were the first discovered (Celli et al., 1999; Hamada et al.,

2002). There are four additional related human developmental syndromes with p63 mutations (acro-dermato-ungual-lacrimal tooth (ADULT) syndrome, limb mammary syndrome (LMS), Rapp-Hodgkin syndrome, and split hand-split foot (SHFM) malformation) that extend the genotype-phenotype correlation (Brunner et al., 2002).

Together, these data clearly establish a fundamental role of p63 in epithelial stem cell biology and in the apical ectodermal ridge of the limb bud, where p63-expressing cells create a signalling center (Pellegrini et al., 2001). Whether this role is one in stem cell selfrenewal or in stem cell differentiation into stratified epithelium remains a matter of controversy (Mills et al., 1999a; Yang et al., 1999). In one model, p63 is required for the ectoderm to commit to epidermal lineages (Mills et al., 1999a; Yang et al., 1999), whereas, in the other model, p63 is not required to commit but to maintain the stem cell pool and prevent it from differentiation (Brunner et al., 2002).

1.1.7. The role of p63 in the tumor development

In contrast to the high incidence of tumors in p53-compromised mice, the tumor phenotype of mice with compromised p63 is much less clear. Whereas p63^{-/-} mice die at the birth, p63^{+/-} mice develop tumors, surmising that p63 plays a “broader role” in tumor suppression than was previously appreciated (Flores et al., 2005). Although p63^{+/-} mice did not display the highly penetrant tumor phenotype of p53^{+/-} mice, the wild type p63 allele was lost in the tumors that did develop. In addition, mice mutant for p63 in combination with p53 mutation lead to a more aggressive tumor phenotype (Flores et al., 2005).

Furthermore, recent studies have shown that p63 overexpression in human tumor. In addition to frequently amplification of the human *TP63* gene in squamous cell, cervical, and prostate carcinomas, some studies have shown tumor-suppressive activities of same p63 isoforms in human tumors (Hibi et al., 2000). In addition, certain tumor types (transitional cell carcinomas, mammary adenocarcinomas, squamous cell carcinomas, and osteosarcomas) exhibit loss or reduced expression of p63 (Di Como et al., 2002; Koga et al., 2003; Park et al., 2000; Rocco and Ellisen, 2006; Urist et al., 2002). More recent studies using antibodies or RT-PCR for specific isoforms of p63 have shown that $\Delta Np63\alpha$ are significantly overexpressed in

various human cancer cells. Such as, head, neck squamous cell carcinomas (HNSCC) (Sniezek et al., 2004) and in normal bronchus and squamous carcinomas (Massion et al., 2003). Many more studies using antibodies or RT-PCR for specific isoforms in additional human tumors are needed to determine the significance of the loss or gain of each isoform.

In summary, the role of p63 in tumorigenesis is complex. Unraveling the complex web of interactions between different p63 isoforms, in addition to determining how these proteins impact other members of the p53 protein family present important challenges that will help to better understand the role of p63 in cancer.

1.1.8. The p73 gene

During a search for novel interleukins, Caput and co-workers (Kaghad et al., 1997) identified a complementary DNA that was predicted to encode a p53-like protein. The corresponding gene, called p73, maps to chromosome 1p36, a region that is frequently deleted in a variety of human cancers. The p73 gene expresses at least six alternatively spliced C-terminal isoforms p73 α , β , γ , δ , ϵ , and ζ (Fig. 6A) and at least four alternatively spliced N-terminal isoforms (Fig. 6B), which contain different parts of the transactivation domain. Among the various isoforms, TAp73 α is the longest and contains a sterile alfa motif (SAM domain) involved in protein-protein interactions (Fig. 6C). These different mRNAs arise from both alternative splicing (AS) and the use of an alternative promoter (AP). The p73 isoforms that are regulated by the two promoters are named TAp73 and Δ Np73 (Stiewe et al., 2002a). Whereas TAp73 is capable of activating p53-responsive genes such as *CDKN1A* (which encodes p21^{WAF1/CIP1}), *p53R2*, *PUMA* and *BAX* (De Laurenzi et al., 1998; Jost et al., 1997; Kaghad et al., 1997), the Δ Np73 isoforms that lack the TA domain are incapable of inducing gene transcription and therefore do not induce growth arrest or cell death (Stiewe et al., 2002a).

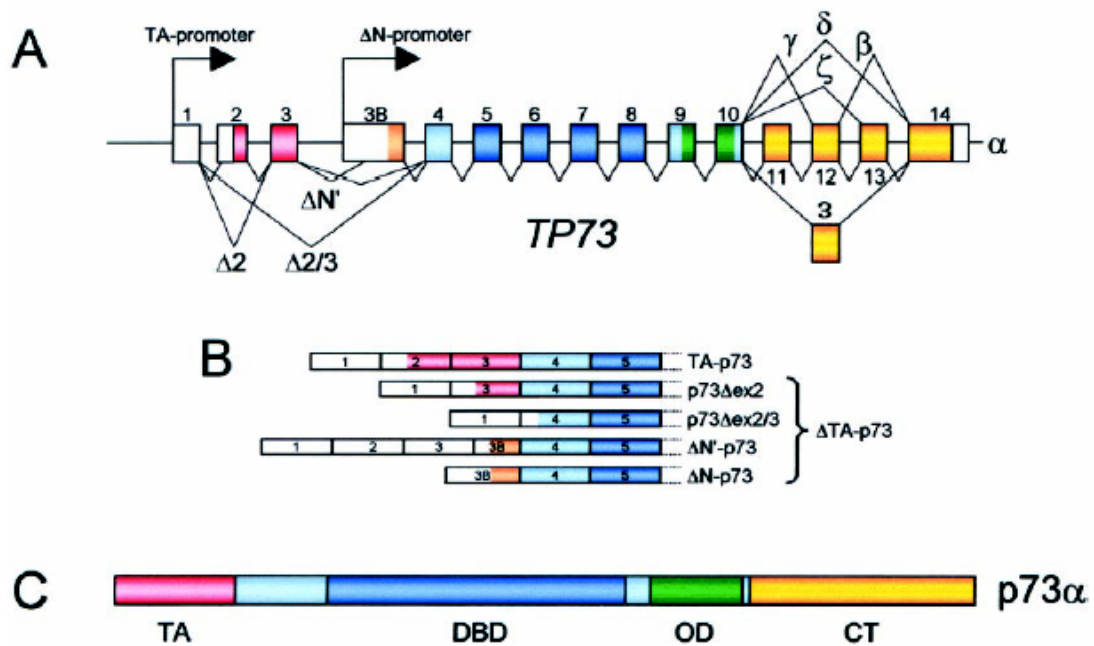


Figure 6. The human p73 gene structure and protein isoforms

A. The splicing patterns generating C-terminal isoforms p73 α , β , γ , δ , ϵ , ζ and the N-terminal isoforms p73 Δ ex2, p73 Δ ex2/3, Δ Np73, and Δ N'p73 are shown. The arrows indicate transcriptional start sites. The Δ Np73 isoform is generated from a cryptic promoter within intron 3. **B.** The exon structure of the N-terminal isoforms is shown in comparison to full-length TAp73 (exons 1–5 only). Noncoding sequences are depicted in white. **C.** Domain structure of full-length TAp73 α . TA, transactivation domain; DBD, DNA-binding domain; OD, oligomerization domain; CT, C terminus (Reviewed in Stiewe and Putzer, 2002)

However, the Δ Np73 isoforms have a very important regulatory role, as they exert a dominant-negative effect on p53, TAp63 and TAp73 by blocking their transactivation activity, and hence their ability to induce apoptosis (Grob et al., 2001; Kartasheva et al., 2002; Stiewe et al., 2002b). This inhibitory function is exerted either by competing for binding to the same DNA target sequence (for p53; Fig. 7A) or at the oligomerization level (for TAp73; Fig. 7B). Although the Δ Np73 forms can interact with TAp73, they seem to have lost the ability to interact with wild-type p53 (Grob et al., 2001; Kartasheva et al., 2002; Stiewe et al., 2002b).

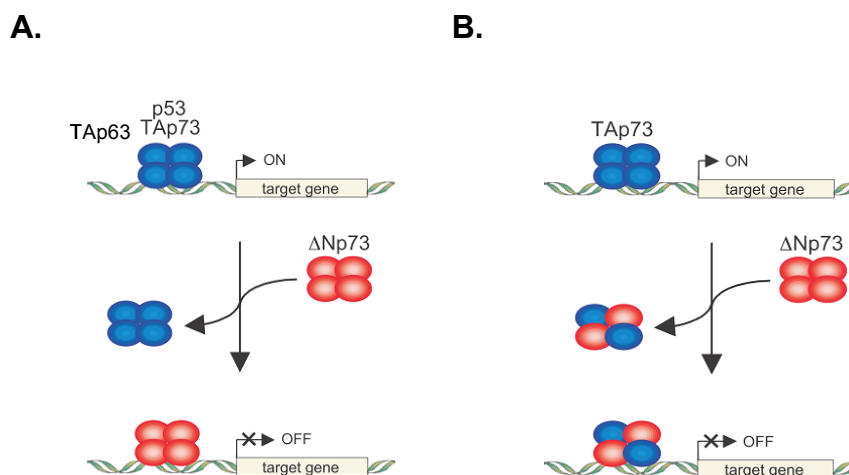


Figure 7. The dominant-negative effect of $\Delta Np73$ on p53, TAp73, and TAp63

A. Competition with p53, TAp63 and TAp73 for the same p53 promoter-targeting sequences. **B.** Formation of transcriptionally inactive TAp73- $\Delta Np73$ heterodimers.

p73 activity is regulated by some of the same mechanisms as p53 (Zaika et al., 2001). In addition, novel pathways have been described. A key player in the regulation of p73 is E2F1 which, apart from regulating cell proliferation, is also able to induce apoptosis (Reviewed in (Stiewe and Putzer, 2002). Whereas activation of p53 in response to E2F1 is indirect involving the tumour-suppressor ARF, E2F1 regulates p73 levels directly, through recognition and transactivation of the *TP73* promoter (Irwin et al., 2000; Stiewe and Putzer, 2000). E2F1-mediated transactivation of p73 results in the activation of p53-responsive target genes and apoptosis. On the other hand, disruption of p73 function by p73-mutants which exhibit a dominant-negative effect ($\Delta Np73$ or p73DD) inhibits E2F1-induced apoptosis in p53-defective tumour cells and p53^{-/-} mouse embryo fibroblasts (MEFs), indicating that endogenous p73 contributes to E2F1-induced apoptosis in the absence of p53 (Irwin et al., 2000; Stiewe and Putzer, 2000; Zaika et al., 2001). In the light of these findings, increased expression of p73 by E2F1 might possibly constitute a p53-independent anti-tumorigenic safeguard mechanism. Consistent with p73 being an important downstream target of E2F1-signalling overexpression of oncogenes, which increase E2F1 activity (such as SV40 T antigen, c-Myc and the adenoviral E1A protein), induces increased p73 expression, and results in induction of p73 target genes and p73-mediated apoptosis (Marin et al., 1998; Steegenga et al., 1999; Zaika et al., 2001).

Under normal physiological conditions, p73 protein levels are kept low by rapid degradation via the ubiquitin–proteasome pathway (Balint et al., 1999; Bernassola et al., 2004). Several mechanisms have been shown to stabilize p73, including phosphorylation by c-abl, p38 MAPK and c-Jun, acetylation by CBP/p300 and SUMOylation (reviewed in (Oberst et al., 2005)). For instance, DNA damage affects p73 stability and transcriptional activation through distinct mechanisms. The MLH-1/c-abl signaling cascade plays a central role in DNA damage-induced stabilization of p73 (Agami et al., 1999; Gong et al., 1999). In response to genotoxic stress, activated c-abl binds to the PXXP motif of p73 and phosphorylates it on Tyr residue 99 (Agami et al., 1999). In addition, c-abl indirectly promotes p73 phosphorylation on Ser/Thr-Pro residues through the activation of the p38 MAP kinase pathway (Sanchez-Prieto et al., 2000). p300-mediated acetylation of p73 upon DNA damage induced by chemotherapeutic drugs represents an alternative mechanism for p73 transcriptional activation (Costanzo et al., 2002). Furthermore, p300-mediated acetylation of p73 results in protein stabilization and is promoted by the tumor suppressor promyelocytic leukemia protein, PML, which, in turn, regulates p73 transcriptional activity in a PML-nuclear body (PML-NB)-dependent manner (Bernassola et al., 2004). Interestingly, p38 MAPK-mediated phosphorylation of p73 favors its binding to PML and recruitment in the PML-NB, hence assisting p73 stabilization. Concomitantly, DNA damage-induced phosphorylation of p73 on Ser/Thr-Pro residues allows the interaction of p73 with the peptidyl–prolyl *cis/trans* isomerase Pin1, which results in p73 prolyl isomerization, conformational changes, acetylation, stabilization, and functional activation (Mantovani et al., 2004). Moreover, the checkpoint kinases (Chk1 and Chk2) control p73 activity in response to DNA damage (Urist et al., 2004).

Furthermore, p53 and TAp73 can induce expression of the Δ Np73 isoform, which creates a dominant-negative feedback loop that regulates the function of both p53 and TAp73 (Grob et al., 2001; Kartasheva et al., 2002) (Fig. 8). Perturbations of these regulatory loops in cancer cells (Stiewe et al., 2002b) or in virally infected cells (Allart et al., 2002) resulting in excess or persistent expression of the Δ Np73 isoform might result in inhibition of p53 or TAp73. Consequently, loss of these regulatory pathways would be predicted to allow inappropriate p53 or TAp73 activity. Thus, it is

likely that a balance between the intracellular expression levels of pro-apoptotic TAp73 or p53 and antiapoptotic Δ Np73 plays an important role in regulating cell fate determination.

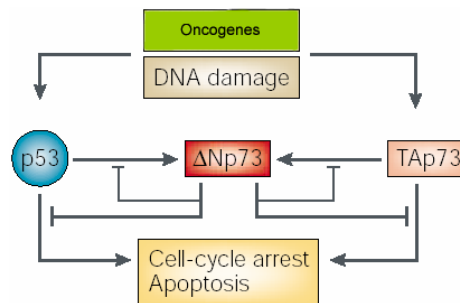


Figure 8. Δ Np73 creates a dominant-negative feedback loop that regulates the function of both p53 and TAp73.

Both p53 and TAp73 are activated in response to DNA damage or oncogenes and p53 and TAp73 transcriptionally transactivate Δ Np73; this in turn downregulates its own promoter to fine-control the steady-state protein levels. Δ Np73 is able to functionally inhibit cell-cycle arrest and apoptosis induced by either TAp73 or p53 (modified from Melino et al., 2002).

1.1.9. The role of p73 in development and differentiation

Unlike p63-deficient mice, p73^{-/-} mice survive postnatally and some live well into adulthood despite having multiple defects (Yang et al., 2000). p73^{-/-} mice show congenital hydrocephalus, hippocampal dysgenesis, due to disappearance of Cajal-Retzius neurons, and defects of pheromone detection that lead to lack of interest in sexually mature females. In addition to these severe neurological defects, p73^{-/-} mice show a generalized pan-mucositis with consequent microbiological infections, which are characterized by massive neutrophil infiltration at the affected sites. The massive inflammation, however, is not clear yet, since no major defects of the lymphoid and granulocyte populations are present in these mice. This raises the possibility that the inflammation and infections are related to epithelial barrier function (Yang et al., 2000).

In tissue culture models, p73 plays a role in the differentiation of several cell lineages. p73 accumulates during retinoic acid-mediated neuronal differentiation in neuroblastoma cell lines, whereas p53 levels remained unchanged in response to retinoic acid. Under experimental conditions, ectopic overexpression of p73 in undifferentiated neuroblastoma cell lines result in neurite extension as well as expression of neuronal differentiation markers (De Laurenzi et al., 2000a). Similar results were also observed during neuronal differentiation in P19 embryonal carcinoma cells exposed to retinoic acid (De Laurenzi et al., 2000a). In addition, the ectopic expression of p73 induces oligodendrocyte precursor cell (OPC) differentiation, which is inhibited by Δ Np73 (Billon et al., 2004). In addition, p73 gene expression is upregulated during muscle differentiation (Fontemaggi et al., 2001) and the p73 gene is an *in vivo* transcriptional target of the muscle regulatory factors MyoD, myogenin, Myf5 and Myf6 (Fontemaggi et al., 2005). Moreover, the expression of p73 is markedly enhanced during differentiation of myeloid leukemic cells (Tschan et al., 2000). Similar to p63, p73 involve in the terminal differentiation of human skin keratinocytes (De Laurenzi et al., 2000b).

In summary, these findings suggest a p73-specific role in differentiation that is not shared by p53 and, for the most part, not shared by p63 either. Moreover, some data provide evidence for the existence of a coordinated network of transcriptional activators and repressor controlling p73 expression during differentiation (Fontemaggi et al., 2005). Further experiments are necessary to characterize the downstream functions of p73 in the various differentiation processes.

1.1.10. The role of p73 in the tumor development

Similar to p63^{+/-} mice, p73^{+/-} mice develop malignant tumors at high frequency and their spectrum is quite different from that of p53-deficient mice. In addition, mice mutant for p73 in combination with p53 mutation present a more aggressive tumor phenotype (Flores et al., 2005). However, p73 does not conform to Knudson's two hit hypothesis because extensive studies have revealed only rare mutations in both cell lines and primary tumors (Irwin and Kaelin, 2001). Interestingly, p73 mRNA and protein levels tend to be higher, and not lower, in tumor tissue compared with the surrounding normal tissue. Ependymomas, breast, lung, prostate, ovarian,

colorectal, esophageal, and bladder cancers have all been reported to elevate p73 levels compared with their normal tissue counterparts. Most importantly, patients with high p73 protein expression had a worse survival than patients with undetectable levels (Sun, 2002; Tannapfel et al., 1999). There is an emerging sense that the dominant-negative Δ Np73 isoforms rather than TAp73 might be the physiologically relevant components of tumor-associated p73 overexpression, functionally overriding an often concomitant increase in TAp73 expression. Tumor-specific up-regulation of Δ Np73 has been found in ovarian cancer, breast and gynecological cancer, hepatocellular carcinoma, lung, gastric, and thyroid cancer and neuroblastoma (Casciano et al., 2002; Concin et al., 2004; Frasca et al., 2003; Stiewe et al., 2004; Uramoto et al., 2004; Zaika et al., 2002). Of note, Δ Np73 overexpression appears to have a clinical impact at least in some cancer types. Δ Np73 was found to be an independent prognostic marker for reduced progression-free and overall survival in lung (Uramoto et al., 2004) and neuroblastoma patients (Casciano et al., 2002).

Conclusion

Identification of the p53 homologues, p63, and p73 has opened a new chapter in developmental and cancer biology. While new p53 target genes and functions are published on a monthly basis, the regulation and function of p63 and p73 are still in the early stages of discovery. Differences in p53^{-/-}, p63^{-/-} and p73^{-/-} mouse phenotypes alone suggest that p63 and p73 regulate signaling pathways that differ from p53. Studies of human tumors and human genetic syndromes have shed some light on both p63 and p73 function, but a better biochemical understanding of p63 and p73 will undoubtedly be required to understand the role of these p53 family members in tumorigenesis and development.

1.2. Skeletal muscle differentiation (myogenesis)

Introduction

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 requires either of two members of the myogenic basic helix-loop-helix (bHLH) transcription-factor family, MyoD or Myf5, which are expressed in proliferating myoblasts prior to the onset of muscle differentiation. Further steps in myogenesis require another myogenic bHLH factor, myogenin, as well as the MEF2 transcription factor family, which cooperate with the myogenic bHLH proteins in the activation of many muscle structural genes. Studies on the differentiation of cultured myoblasts have revealed that muscle-differentiation-specific gene expression occurs in a stereotypic pattern. Within 24 hours of serum removal, proliferating myoblasts initiate the expression of myogenin. At the same time, the retinoblastoma protein (RB) is activated that is required for MEF2 dependent gene expression. Furthermore, these cells induce expression of both cyclin-dependent kinase (Cdk) inhibitors and RB to exit the cell cycle permanently. Once the cells have become post-mitotic, expression of myofibrillar proteins such as myosin heavy chain (myHC) and enzymatic genes such as muscle creatine kinase (MCK) begins, approximately 36-48 hours after the onset of differentiation, followed by fusion of cells into multinucleated myotubes (Fig. 9).

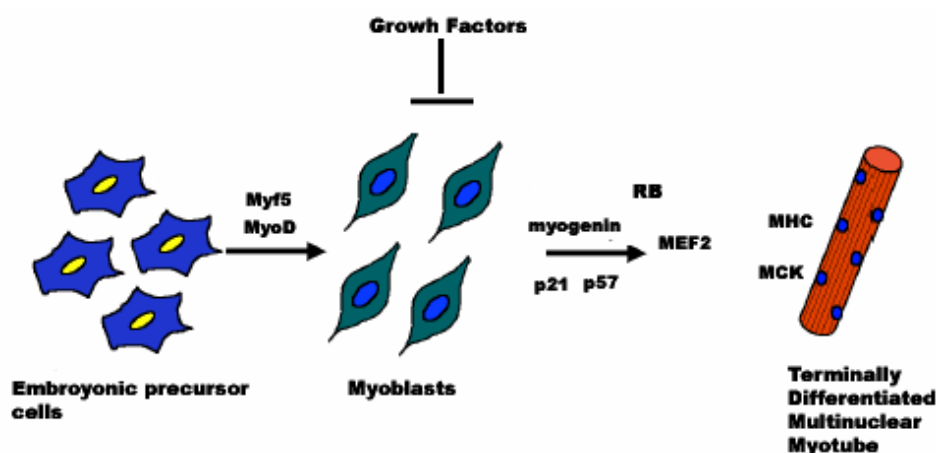


Figure 9. Regulation of myogenesis.

1.2.1. Regulation of myogenesis

1.2.1.1. The myogenic regulatory factors

The myogenic regulatory factors (MRFs) belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors. MRFs are necessary for the determination and terminal differentiation of skeletal muscle progenitor cells (Arnold and Winter, 1998; Cossu et al., 1996). The original cloning of MyoD and demonstration that it represents a master regulatory gene for the determination of skeletal muscle, ushered in a new era of research in skeletal myogenesis (Davis et al., 1987). This discovery led to the cloning of three other factors namely Myf5, myogenin and MRF4/Myf-6/Herculin (Braun et al., 1989; Ramirez et al., 2004; Taylor et al., 2000). In all cases, overexpression of these factors converts nonmuscle cells to the myogenic lineage, demonstrating their role in myogenic lineage determination and differentiation. Whereas MyoD and Myf5 act to determine the myoblast lineage, myogenin and MRF4 are important for differentiation and maintenance of the terminally differentiated state (Rawls et al., 1995; Rudnicki and Jaenisch, 1995). Furthermore, the ability of each factor to initiate the expression of one or more of the other three suggests they form a cross-regulatory loop.

1.2.1.2. Cell cycle and differentiation

In skeletal muscle cells, cell cycle regulation plays a fundamental role in the production and maintenance of the differentiated phenotype. Precursor cells first commit to a particular differentiation program, which is marked by expression of early differentiation markers. The committed precursors can immediately proceed to withdraw from the cell cycle. In contrast to entry into quiescence, this withdrawal is irreversible, and in most cases differentiated tissues of organs are unable to reenter the cell cycle even under ideal growth conditions. The early differentiation genes expressed in committed precursors are mostly tissue specific transcription factors required for activation of later genes essential for full differentiation. Typically these early transcriptional activators are present in the precursors before terminal cell cycle withdrawal, whereas the late differentiation markers are not expressed until the

cells become post-mitotic. Terminal cell cycle exit seems to be required in order to activate tissue specific gene expression. It follows that cellular mechanisms must exist to coordinate these two processes and prevent the onset of differentiation before the cells are fully arrested (reviewed in (Lassar et al., 1994).

The activity of the MRF is tightly coupled to the cell cycle (Lassar et al., 1994; Olson, 1992). In proliferating myoblasts, activated cyclin-dependent kinases (Cdk4) inhibit MyoD activity through direct interaction (Halevy et al., 1995; Skapek et al., 1996; Skapek et al., 1995; Zhang et al., 1999a). Expression of Id proteins precludes the formation of E protein-MRF heterodimers (Benezra et al., 1990). Upon differentiation, withdrawal from the cell cycle is initiated and maintained by a positive feedback loop in which high p21 and RB expression prevents re-entry into the cell cycle and the MRF-E protein complex is activated (Guo et al., 1995; Schneider et al., 1994). Furthermore, upregulation of p57^{KIP2} stabilizes MyoD by blocking cyclinE/cdk2 activity and by direct interaction with MyoD (Reynaud et al., 1999) (Fig. 10).

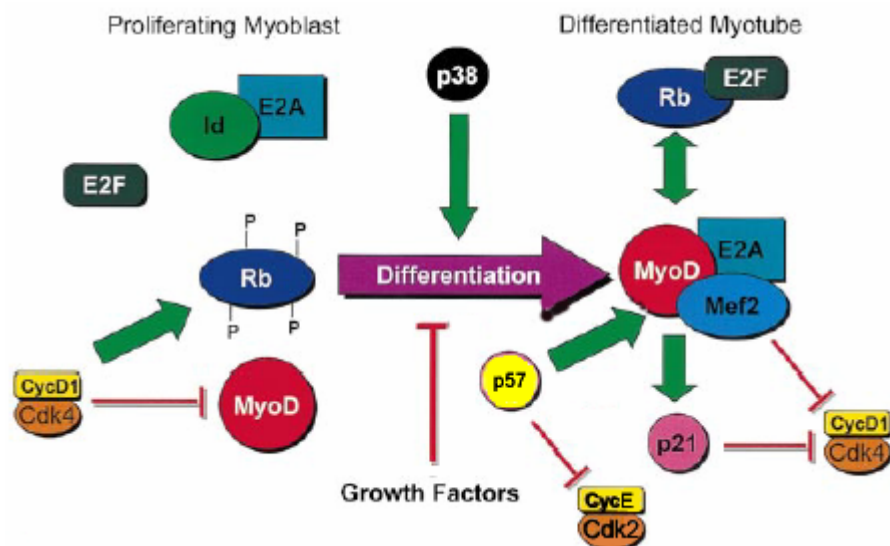


Figure 10. The activity of the *MyoD* family is coupled to cell-cycle control. Green arrows denote positive, whereas blunt red arrows denote negative regulatory relationships.

1.2.1.3. The retinoblastoma tumor suppressor protein RB in myogenesis

During myogenic differentiation, proliferating myoblasts need to terminally exit from the cell cycle before the G1 restriction point (Perry and Rudnick, 2000). RB plays a key role in controlling cell cycle progression through the G1 restriction point for entry into S-phase (Stevaux and Dyson, 2002). Therefore, it can be hypothesized that RB plays a critical role in myoblasts by regulating the switch from proliferation to differentiation.

The importance of RB has been demonstrated by the fact that $RB^{loxP/loxP}, Myf5-Cre$ mice, lacking RB in myoblasts, died immediately at birth and exhibited high numbers of apoptotic nuclei and an almost complete absence of myofibers (Huh et al., 2004). In contrast, $RB^{loxP/loxP}, MCK-Cre$ mice, lacking RB in differentiated muscle fibers, were viable and exhibited a normal muscle phenotype and ability to regenerate indicating that RB plays a crucial role in the switch from proliferation to differentiation rather than maintenance of the terminally differentiated state (Huh et al., 2004). Moreover, during muscle differentiation, RB becomes hypophosphorylated (activated) and mRNA and protein levels increase approximately 10-fold (Martelli et al., 1994). In addition, studies using MyoD-converted RB-deficient embryonic fibroblasts have suggested that RB is essential for both MyoD and MEF2 transcriptional activity as well as during the early stages of differentiation in order to properly control cell cycle exit and regulation of the progression of the differentiation program (Novitch et al., 1999). Although RB-deficient fibroblasts transfected with MyoD become myogenic and express early muscle markers such as myogenin, expression of late markers such as myosin heavy chain (MHC) is reduced (Novitch et al., 1996). In addition, serum restimulation of these partially differentiated RB-deficient myoblasts results in BrdU incorporation, S-phase entry, and DNA synthesis. However, these cells are unable to enter mitosis (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 (Korenjak and Brehm, 2005), the mechanisms that activate RB during myogenesis still remain unclear.

Conclusion

It is clear that a great deal of information has been obtained regarding many aspects of skeletal muscle development. However, several questions concerning coordination of cell cycle and terminal differentiation remain and we are only now beginning to understand the many factors involved in these processes.

1.3. Rhabdomyosarcoma (RMS)

Introduction

Rhabdomyosarcoma (RMS) is a tumor derived from the skeletal muscle lineage. RMS is the most common soft-tissue sarcoma of childhood, with an annual incidence of 4 to 7 cases per million children. Based on histopathologic features, two major subtypes, embryonal (ERMS) and alveolar (ARMS), were identified and associated with distinct clinical characteristics and genetic alterations. In RMS, the muscle program is only partially activated despite the presence of virtually all the MRF (Tapscott et al., 1993). RMS cells fail to both complete the myogenic program and irreversibly exit the cell cycle, resulting in uncontrolled proliferation and incomplete myogenesis (Merlino and Helman, 1999). Although the origin of RMS cells has not yet been precisely defined, it is known that these cells do not arise from differentiated myofibers. Nevertheless, the myogenic identity of these cells has been clearly established (Dias et al., 1990).

1.3.1. Epidemiology and Pathology of RMS

Soft tissue sarcomas are the sixth most common malignancy in childhood and rhabdomyosarcomas constitute 50% of soft tissue sarcomas. This tumor accounts for 10-15% of solid malignant tumors and 6% of all malignancies in children under 15 years of age. In the United States the male to female ratio is 1.5:1, the tumor is twice as common in Caucasians as in African-Americans and approximately 250 new cases are diagnosed every year (Adamson et al., 2005).

Rhabdomyosarcomas arise from undifferentiated mesenchymal cells. It manifests immunohistochemical expression of muscle specific proteins such as myosin, desmin, myoglobin and Z-band protein (Dagher and Helman, 1999). Histologically, there are four subtypes: embryonal, alveolar, botryoid and pleomorphic.

Embryonal: Fifty-four percent of rhabdomyosarcomas in the third Intergroup Rhabdomyosarcoma Study (IRS-III) (Crist et al., 1995) and approximately 70% of non-metastatic rhabdomyosarcomas in the fourth Intergroup Rhabdomyosarcoma Study (IRS-IV) were of the embryonal subtype (Crist et al., 2001). This subtype usually occurs before 8 years of age and accounts for 80% of genitourinary tumors, 60% of head and neck tumors and 50% of tumors at other sites. The tumor is poorly circumscribed, soft and whitish in color. Morphologically, it resembles developing skeletal muscle of the 7-10 week old fetus. (O'Neill et al., 2003). [See Fig. 11A for an example of an embryonal rhabdomyosarcoma.]

Botryoid: This variant of the embryonal subtype occurs in hollow cavities such as the vagina, biliary tract and nasopharynx. The tumor resembles a bunch of grapes. Microscopically, small round cells surround a loose myxoid stroma with a central zone of round and spindle cells. Botryoid tumors have the best prognosis and are typically found in children under five years of age (O'Neill et al., 2003). [See Fig. 11B for an example of a botryoid rhabdomyosarcoma.]

Alveolar: The tumor is named because of its lung-like architecture under the microscope. It is second in frequency to the embryonal subtype. Tumors are often more firm and less myxoid and occur more commonly on the limbs and trunk. Under the microscope, large round cells with a predominantly eosinophilic cytoplasm growing in thin strands of fibrovascular stroma with “free floating” tumor cells are seen. It generally occurs in 10-30 year old patients and 80% are associated with the t(2;13)(q37;q14) chromosomal translocation. Presence of an alveolar component in any rhabdomyosarcoma requires that it be labeled as alveolar. Only undifferentiated rhabdomyosarcoma has a worse prognosis than the alveolar variety (O'Neill et al., 2003). [See Fig. 11C for an example of an alveolar rhabdomyosarcoma.]

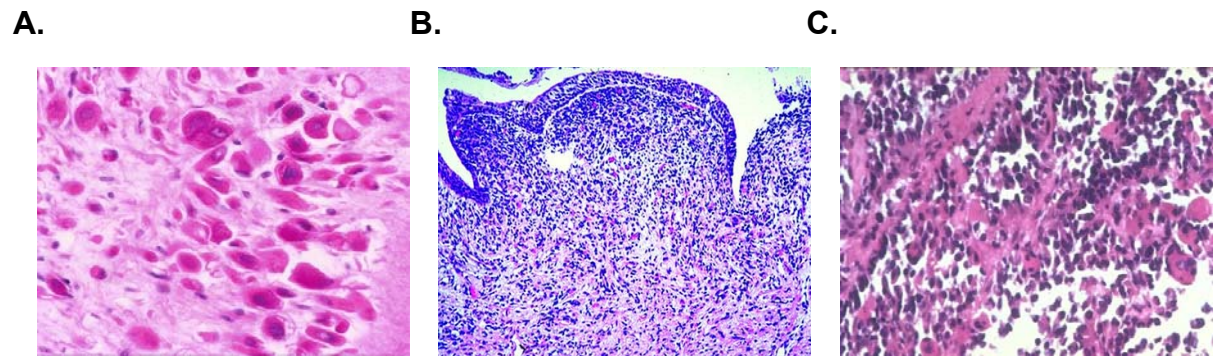


Figure 11. Pathologic Classification of Rhabdomyosarcomas

A. Embryonal rhabdomyosarcomas such as this case have abundant rhabdomyoblasts containing deeply eosinophilic cytoplasm. **B.** Botryoid rhabdomyosarcoma. A small-cell neoplasm abuts an epithelial surface, with condensation of tumor cells in the immediate subepithelial zone. **C.** The microscopic features of alveolar rhabdomyosarcoma are readily apparent in this micrograph. Undifferentiated tumor cells line fibrovascular septae, with the central cells "falling out" to give the alveolar appearance. (Adapted from *Pediatric Neoplasia book: Morphology and Biology* by David M. Parham)

Pleomorphic: This subtype usually occurs on the limbs and trunks of adults over 45 years old. It comprises only 1% of childhood rhabdomyosarcomas. Large pleomorphic cells with multinucleated giant cells are characteristic. Immunohistochemistry is usually required to distinguish it from liposarcoma or malignant fibrous histiocytoma. [See Fig. 12 for an example of Pleomorphic rhabdomyosarcoma.]

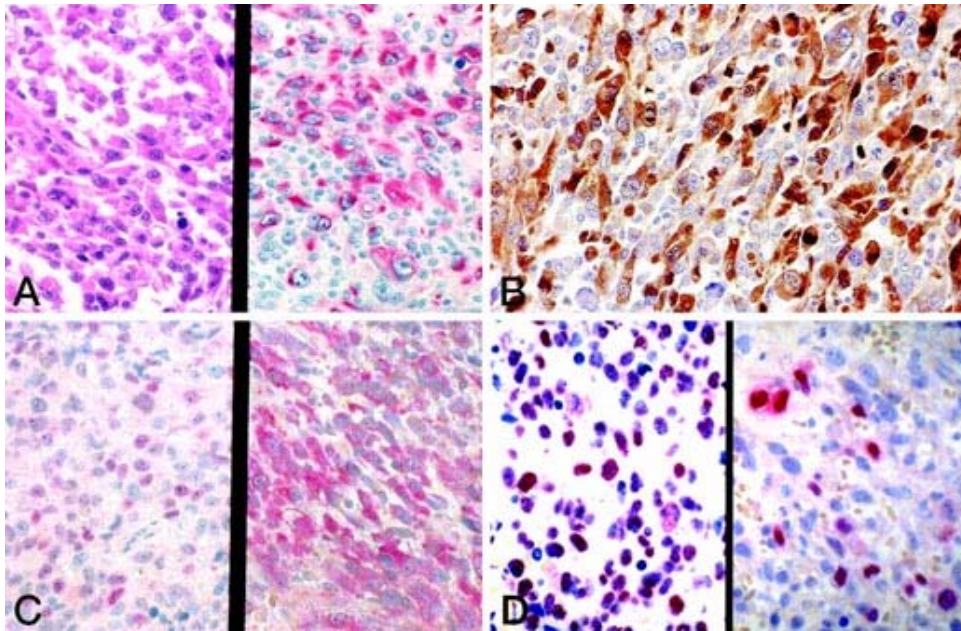


Figure 12. An example of a pleomorphic rhabdomyosarcoma (classic variant; *left*) and diffusely positive desmin reactivity (*right*; **A**); myoglobin positively (**B**); MyoD1 (nuclear, *left*) and fast myosin (cytoplasmic) positively (**C**); and myogenin, myf 3 (nuclear, *left*) and myf4 (nuclear) positively (**D**). (Adapted from *Pediatric Neoplasia book: Morphology and Biology* by David M. Parham)

1.3.2. Molecular pathogenesis of rhabdomyosarcoma (RMS)

1.3.2.1. Alveolar RMS

Chromosomal analyses of RMS cases demonstrated nonrandom translocations associated with the ARMS subtype (Barr et al., 1993). The most prevalent finding is a translocation involving chromosomes 2 and 13, $t(2;13)(q35;q14)$, that was detected in 70% of published ARMS cases. The chromosome 13 locus juxtaposed with either *PAX3* or *PAX7* is *FKHR* (*FOXO1A*), which encodes a member of the fork head transcription factor family (Davis et al., 1994; Galili et al., 1993). The encoded FKHR product is organized with an N-terminal fork head DNA binding domain and a C-terminal transcriptional activation domain. The translocations break within intron 7 of *PAX3* or *PAX7* and intron 1 of *FKHR* and thus create two chimeric genes on the derivative chromosomes (Barr et al., 1998; Davis et al., 1995; Fitzgerald et al., 2000). These gene fusion events result in alterations at the level of protein function, gene expression, and subcellular localization. For instance, the *PAX3/FKHR* and

PAX7-FKHR fusion proteins activate transcription from PAX binding sites but are 10-100-fold more potent as transcriptional activators than the wild-type PAX3 and PAX7 proteins (Bennicelli et al., 1996). In addition, when PAX3/FKHR was introduced into chicken embryo fibroblasts and NIH3T3 fibroblasts, the expression of this fusion protein leads to cellular transformation, including morphological changes, focus formation and anchorage independent growth (Lam et al., 1999; Scheidler et al., 1996). In these studies, wild-type PAX3 failed to produce these changes. Furthermore, downregulation of fusion gene expression in an ARMS cell line using antisense oligonucleotides resulted in cell death, presumably by promoting apoptosis (Fredericks et al., 2000). However, Keller et al. reported the first mouse model of alveolar rhabdomyosarcoma using a conditional PAX3/FKHR knock-in allele (Keller et al., 2004). In these mice, alveolar rhabdomyosarcomas occur but at very low frequency, and FKHR haploinsufficiency does not appear to accelerate tumorigenesis. However, PAX3/FKHR homozygosity with accompanying *Ink4a/ARF* or *p53* pathway disruption substantially increases the frequencies of tumor formation, indicating that PAX3/FKHR alone is not sufficient for tumorigenesis and additional cooperating genetic alteration are required to cause transformation.

1.3.2.2. Embryonal RMS (ERMS)

Although no consistent chromosomal rearrangements have been identified in ERMS, molecular analyses of polymorphic loci revealed frequent allelic loss on chromosome 11 (Koufos et al., 1985). Several results from allelic loss studies suggest that ERMS tumorigenesis frequently involves inactivation of an imprinted tumor suppressor by allelic loss of the active maternal allele and retention of the inactive paternal allele. For instance, studies of the human 11p15 chromosomal region and the corresponding mouse region demonstrated the imprinting of several genes within the region (Loh et al., 1992; Scrabble et al., 1989). For instance, *H19* is preferentially expressed from the maternally inherited alleles and *IGFII* is imprinted in the opposite direction so that the paternally inherited alleles are preferentially expressed (Tycko, 1994).

1.3.2.3. p53 and RB in rhabdomyosarcoma pathogenesis

Somatic mutations of *TP53* and dysregulation of its associated regulatory proteins have been implicated in the development of a variety of tumors, including RMS (Diller et al., 1995; Felix et al., 1992). Although lymphomas are the most prominent neoplasms in p53-null mice, p53 heterozygotes over a year old develop mostly sarcomas, 19% of which are RMS (Harvey et al., 1993). Furthermore, conditional PAX3/FKHR knock-in 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). In addition, further mutations affecting p53 function have been identified in RMS patients. For instance, RMS or other sarcomas demonstrate Mdm2 overexpression (Keleti et al., 1996) and homozygous deletions at the 9p21 region which contains both *CDKN2A* and *ARF* (Brookes et al., 2002). Both changes result in p53 dysfunction due to increased degradation via Mdm2.

Inherited alterations in the *RB1* gene, another tumor suppressor gene, are associated with the development of a variety of tumors, including retinoblastoma, osteosarcomas and other sarcomas including RMS (Cance et al., 1990). Though various sarcomas were found to have acquired RB mutations, no such mutations were identified in sporadic cases of RMS. However, genetic changes were detected in genes that regulate RB function. In particular, RMS cases have been identified with amplification of *CDK4* gene and deletions of the *CDKN2A* and *CDKN2B* (p15INK4B) (Iolascon et al., 1996). The CDK proteins inhibit RB function by phosphorylation and thereby promote cell-cycle progression, whereas the CDKN loci encode inhibitors of the cyclin-dependent kinases CDK4 and CDK6. Supporting a role of the CDK alteration for RMS development, transfection of *CDKN2A* into p16INK4A-deficient ERMS cell line RD, led to reduced CDK6 kinase activity, G1 growth arrest, and acquisition of a more differentiated morphology (Urashima et al., 1999).

Conclusion

Rhabdomyosarcoma (RMS) is an aggressive childhood muscle cancer for which outcomes are poor when the disease is advanced. RMS cells fail to both complete the myogenic program and irreversibly exit the cell cycle, resulting in uncontrolled proliferation and incomplete myogenesis (Merlino and Helman, 1999), which could contribute to an aggressive tumor phenotype. For a better understanding of the initiation and progression of RMS we need more information regarding the differentiation defect of RMS. Furthermore, this information needs to be integrated with studies of the clinical behavior of human tumors to optimize the diagnosis and to define directions in which possible therapeutic strategies can be designed to rescue these defective differentiation pathways.

1.4. Aim of the study

The p53 family is considered a family of proapoptotic transcription factors and the tumorsuppressor activity of p53 is typically attributed to its proapoptotic function (Schmitt et al., 2002). However, knockout mice of the p53 family members p63 and p73 present with a developmental phenotype indicative of essential functions in differentiation control. A link between the role of the p53 family in differentiation control and its tumor suppressor activity is appealing but experimental evidence has been missing. To identify the missing link, this study addresses the role of the p53 family in rhabdomyosarcomas, a tumor where loss of differentiation is intimately coupled to tumor formation. Rhabdomyosarcomas harbor a variety of genetic and molecular lesions that compromise the ability of the tumor cells to exit the cell cycle and complete the muscle differentiation program resulting in uncontrolled proliferation. Involvement of the tumor suppressor gene p53 in rhabdomyosarcoma is suggested by the presence of p53 mutations in RMS tumors and by the occurrence of rhabdomyosarcomas in human families and in knockout mice carrying a germ-line mutation in one p53 allele (Merlino and Helman, 1999). Interestingly, in many tumors p53 is inactivated by missense mutations that endow p53 with new functions („gain-of-function“) including the dominant-negative inhibition of the closely related family members p63 and p73 (Di Como et al., 1999; Marin et al., 2000). Therefore p53 mutations frequently disable not only the tumorsuppressive function of p53 but rather the function of the complete p53 family. Considering that myogenic differentiation is accompanied by changes in the expression level of all three p53 family members, we hypothesized that the p53 family has essential functions in coordinating differentiation. Ablation of these functions would compromise terminal cell cycle exit and differentiation and therefore contribute to tumor development in the muscle lineage. To test this hypothesis we investigated questions related to the role of the p53 family in both RMS development and myogenic differentiation.

1. *The p53 family in RMS development.* Do RMS tumors harbor functional alterations of the p53 family in addition to p53 mutations? Is p53 family dysfunction linked to the tumorigenic phenotype of RMS cells? Does experimental inhibition of p53 family function (via the pan-p53 family inhibitor Δ Np73) promote malignant transformation of muscle progenitor cells?

2. *The p53 family in myogenic differentiation.* What is the molecular function of the p53 family in myogenic differentiation control? How does a pan-p53 family inhibitor ($\Delta Np73$) affect differentiation? How do individual p53 family members contribute to differentiation?

2. Materials and Methods

2. 1. Materials

2.1.1. Buffers and media

All buffers were prepared and diluted in aqua_{bidest.}

1. Phosphate-buffered saline (PBS), pH 7.3

NaCl	137mM (0.9%)
KCl	2.7mM
KH ₂ PO ₄	1.5mM
Na ₂ HPO ₄ x 2 H ₂ O	8.0mM

2. Tris-buffered saline (TBS), pH 7.3

NaCl	137mM (0.9%)
Tris/HCl	2mM

3. PBS/T (PVDF membrane washing buffer)

Tween-20 in TBS	0.1%
-----------------	------

4. 10xStock Blotting buffer 1Liter.

Glycine	145.6g
Tris	30.28g

5. 5xStock SDS running buffer for 1 Liter.

Tris base	15.1g
Glycine	72g
SDS	5g

6. 50xTAE buffer for 1Liter.

Tris	242g
Acetic acid	27.1ml
0,5M EDTA	200ml

7. RIPA (radioimmunoprecipitation assay) buffer

Tris	50mM pH 7.2
NaCl	150mM
SDS	0.1%
Na-Doxyholat	1%
Triton X-100	1%

8. Annealing buffer

KAc	100mM
HEPES-KOH	30mM pH 7.4
MgAc	2mM

9. Blocking buffer for Immunofluorescence

Horse serum	10%
BSA (bovine serum albumin)	1%
PBS	1x

10. Naphthol AS-MX phosphatase stock for 1 Liter

Naphthol AS-MX stock	25mg
N:N Dimethylformamide	1.25ml
Tris/HCL buffer pH 8.3	0.2M

11. Incubating solution

Naphthol AS-MX stock	10ml
Fast blue BB	6mg
Filter, use immediately	

12. Cytomix (5ml)

FCS	10%
ATP	0.1M ATP
Reduced Glutathion	0.25M

13. RPMI medium

RPMI1640	500ml
Pen/Strep	5ml
Amphotercin B	2.25ml
FCS	50ml

14. DMEM medium

DMEM	500ml
Pen/Strep	5ml
FCS	50ml

15. Ham's F-10 Medium

Ham's F-10	500ml
FCS	15%
BSA	0.5mg/ml
Fetuin	0.5mg/ml
EGF	10ng/ml
Dexamethasone	0.39µg/ml
Insulin	0.18 mg/ml
Creatin	1mM
Pyruvate	100ng/ml
Uridine	50µg/ml

16. Differentiation Medium for C2C12 cells

DMEM	500ml
Pen/Strep	5ml
Horse Serum	10ml

17. Differentiation Medium for SH-SY5Y cells

DMEM	500ml
Pen/Strep	5ml
Retionic acid	10µM

18. Differentiation Medium for HSMM cells

DMEM	500ml
Pen/Strep	5ml
Amphotercin B	2.25ml
BSA	0.5mg/ml
EGF	10ng/ml

2.1.2. Primary antibodies

Mouse monoclonal Actin beta (ab6276), Klon AC-15	Abcam Ltd
Mouse monoclonal MHC (MF 20)	University of Iowa, Iowa city
Mouse monoclonal Myogenin (F5D)	University of Iowa, Iowa city
Mouse monoclonal anti-p57 ^{Kip2} (ab3223)	Abcam Ltd.
Mouse monoclonal PCNA (sc-56)	SantaCruzBiothech
Mouse monoclonal p73a/β (Ab-4) Coctail	Dunn Labortechnik
Mouse monoclonal anti-Human p73 (ER-15)	BD Pharminigen
Mouse monoclonal Rb (G3-245)	BD Pharminigen
Mouse monoclonal phospho-RB (S807/811)	Cell Signaling Technology
BrdU (Ab-3)	Oncogene Research Products
Mouse monoclonal p53 (ChIP) Ab-1	Oncogene Research Products
Mouse monoclonal p63 (ChIP) Ab-1	Oncogene Research Products
Mouse monoclonal TAp73 (ChIP) clone 5B429 (IMG-226)	Imgenex
Rabbit polyclonal Anti p21(C-19)	SantaCruzBiothech
Rabbit polyclonal MyoD (M-13)	SantaCruzBiothech
Mouse monoclonal p53 (DO1)	Dr. B. Vojtesek

2.1.3. Secondary antibodies

Alexa 680 anti rabbit IgG	MoBi Tec
Alexa 680 anti mice IgG	MoBi Tec
Alexa 546 anti mice IgG	Molecular Probes
Stabilized Goat Anti-Rabbit (HRP-Conjugated)	Pierce
Stabilized Goat Anti- Mice (HRP-Conjugated)	Pierce

2.1.4. Chemicals

Acrylamid Mix (29:1; 40%) Rotiphorese Gel 40 (29: 1)	Roth
Acetic acid	Roth
Agar-Agar	Roth
Agarose for DNA/RNA	Elektrophorese Roth
Albumin bovine, fraction V	Roth
Ammoniumpersulfate (APS)	Applichem

Ampicillin	Roth
Bradford-Solution: Protein Assay / Phosphoric Acid Methanol	Bio-Rad
Bone Morphogenic Protein-2 (BMP-2), kindly provided by Dr. Walter Sebald	
Cesiumchloride	Roth
Chloroform	Applichem
Chloroform: Isoamylalcohol 24 : 1	Applichem
DNA-Loadingbuffer 6x	Fermentas
Dimethylsulfoxid (DMSO)	Roth
Dithiotreitol (DTT)	Applichem
EDTA-Disodiumsalt-Dihydrat (Na ₂ EDTA)	Applichem
Ethanol	Roth
Ethidiumbromide-Solution 1% (10mg/ml)	Roth
α -D(+)-Glucose Monohydrat	Roth
Glycerole	Roth
Glycine	Roth
HEPES	Applichem
Hydrochloric acid (HCl)	Roth
Loadingbuffer DNA IV	Applichem
Milk powder	Roth
Magnesiumchloride (MgCl ₂)	Sigma
β -Mercaptoethanole	Sigma
Methanole	Roth
Fast blue BB	Sigma
Naphthol AS-MX phosphate	Sigma
Nonidet P-40 (NP40)	Fluka
2-Propanole (Isopropanole)	Roth
Potassiumacetate (KAc)	Roth
Potassiumchloride (KCl)	Applichem
Potassiumhydroxide (KOH)	Applichem
Propidiumiodine	Roth
Protease and phosphatase inhibitors Cocktail, EDTA-free	Roche
Roti-Load1,	4x konzentriert Roth
Roti-Phenol Phenol:Chloroform:Isoamylalcohol (25:24:1)	Roth
Sodiumchloride (NaCl)	Roth

Sodiumcitrate	Applichem
Sodiumdeoxycholate	Applichem
Sodiumhydroxide (NaOH)	Roth
Sodiumlaurylsulfate (SDS)	Roth
SDS-Solution 10%	Applichem
<i>N,N,N',N'</i> -Tetramethylethyldiamine (TEMED)	Applichem
Tris	Roth
Triton X100	Applichem
Tryptone	Roth
Tween 20	Applichem
Retionic acid	Sigma
Yeast extracts	Roth
X-Ray developer LX 24	Kodak
X-Ray fixer AL4	Kodak

2.1.5. Cell culture reagents

Dulbecco's modified Eagle-Medium (DMEM)	PAA Laboratories
Fetal Bovine Serum (FBS)	PAA Laboratories
Horse Serum	Biochrom AG
Hygromycine B-Lösung (50 mg/ml)	PAA Laboratories
jetPEI Q	Biogene
MEM Aminoacids (10x)	PAA Laboratories
Penicillin / Streptomycin (100x)	PAA Laboratories
Trypsin/EDTA 1:250 (10x)	PAA Laboratories

2.1.6. Enzymes and biomolecules

Adenosintriphosphate (ATP)	Applichem
γ - ³² P-Adenosintriphosphate ([γ - ³² P]-ATP)	Amersham
Alkaline Phosphatase CIAP	Fermentas
Desoxynucleotitriphosphate-Mix (dNTPs)	Fermentas
DNA-standard GeneRuler DNA Ladder Mix	Fermentas
DNA-standrad MassRuler DNA Ladder Mix	Fermentas
HotStarTaq DNA polymerase	QIAGEN

L-Glutathion, reduced	Roth
Klenow-Fragment	Fermentas
<i>Pfu</i> Ultra DNA polymerase	Stratagene
Poly (dIdC)	Sigma
PageRuler Prestained Protein Ladder	Fermentas
Random Hexamer Primer Mix	Roche
Restrictionsendonucleases (diverse)	Fermentas
Restrictionsendonucleases <i>PacI</i> and <i>NcoI</i>	NEB
Ribonuclease A, DNase-free (RNase)	Applichem
T4 DNA Ligase	Fermentas

2.1.7. Kits

Nucleobond PC 100 (Midi)	Macherey-Nagel
NucleoSpin Plasmid (250)	Macherey-Nagel
QIAshredder (250)	QIAGEN
Omniscript RT Kit 200	QIAGEN
RiboGreen RNA Quantitation Kit	Molecular probes
RNase-Free DNase Set (50)	QIAGEN
RNeasy Mini Kit (250)	QIAGEN
TriPure RNA Isolation Kit	Roche
Wizard SV Gel and PCR Clean-Up System	Promega

2.1.8. Laboratory equipments

Analytical balance CP225D	Sartorius
CCD-Camera CoolSNAP	Coherent
Centrifuge 5810R	Eppendorf
Centrifuge 5415R	Eppendorf
Centrifuge Avanti J-20 XP	Beckman coulter
Computer	Appel
Cuvette for Electroporation	Biorad
Dampfsterilisation HICLAVE HV-110	BPW
Deep freezer HERAfeeze	Heraeus

Electrophoresis-chamber HE33 mini	Amersham
Electrophoresis-chamber	Biometra
Electrophoresis-chamber HE 100 SuperSub	Amersham
Electrophoresis-chamber mighty small SE260	Amersham
Electroporation Gene Pulser II	Bio-Rad
Electroporation Micro Pulser	Bio-Rad
Extraction system for Cellcultur (BioChem-VacuuCenter BVC 21)	Vacuubrand
Fluorescence activated cell sorter (FACS)	Becton Dickinson
Fluorescence-Microscope	Axiovert 200 Zeiss
Freezer (-80)	Liebherr
Gel-Documentation E.A.S.Y. 440K	Herolab
Gel chamber PowerPac	Biorad
Hoods Class 2	BDK
Incubator HERAcell 240	Heraeus
Microscope DMIL	Leica
Refrigerator Profi line	Liebherr
Laser-Scanner Odyssey-Imager Li-Cor	Biosciences
Microwave 8022	Privileg
Nitrocellulose-Membran Hybond-ECL	Amersham
PCR-hood Captair bio	Erlab
pH-Meter BlueLine	Schott
Photometer BioPhotometer	Eppendorf
Precision balance 572-35	Kern
Refrigerator Profi line	Liebherr
Semy-dry Blot-Apparatur Trans-Blot SD cell	Bio-Rad
Thermocycler GeneAmp PCR System9700	Applied Biosystems
Thermocycler Mastercycler gradient	Eppendorf
Thermomixer comfort	Eppendorf
Water treatment plant Purelab ultra ELGA	LabWater
Ultracentrifuge L7-ultracentrifuge	Beckman coulter
UV-Transilluminator TFX-20.M Vilber	Lourmat
Vacuumpumpe N820AN.18	KNF
Vortex genie 2	Scientific industries
X-ray film	Biomax Kodak

X-ray box 13 x 18 cm

Dr. Goos-Suprema

2.1.9. Primers

Name	Sequence	used for
hp53for	5'-TTGCCGTCCCAAGCAATGGATG-3'	SQ-RT-PCR
hp53rev	5'-TCTGGGAAGGGACAGAAGATGAC-3'	SQ-RT-PCR
hp63-for	5'-CCCTCCAACACCGACTACCC-3'	SQ-RT-PCR
hp63-rev	5'-CACCGCTTCACCACCTCCGT-3'	SQ-RT-PCR
hTAp63-for	5'-GACCTGAGTGACCCCATGTG-3'	SQ-RT-PCR
hTAp63-rev	5'-CGGGTGATGGAGAGAGAGCA-3'	SQ-RT-PCR
hΔNp63 for	5'-TGCCCAGACTCAATTTAGTGAG-3'	SQ-RT-PCR
hΔNp63 for	5'-AGAGAGAGCATCGAAGGTGGAG-3'	SQ-RT-PCR
hp73-for	5'-GACGGAATTCACCACCATCCT-3'	SQ-RT-PCR
hp73-rev	5'-CCAGGCTCTCTTTCAGC-3'	SQ-RT-PCR
hTAp73-for	5'-GGCTGCGACGGCTGCAGAGC-3'	SQ-RT-PCR
hTAp73-rev	5'-GCTCAGCAGATTGAACTGGGCCAT-3'	SQ-RT-PCR
hDNp73-for	5'-CAAACGGCCCGCATGTTCCC-3'	SQ-RT-PCR
hDNp73-rev	5'-TGGTCCATGCTGCTGCTCAGC-3'	SQ-RT-PCR
hΔN2p73-for	5'-GGCTGCGACGGCTGCAGGGA-3'	SQ-RT-PCR
hΔN2p73-rev	5'-CAGGCGCCGGCGACATGG-3'	SQ-RT-PCR
mp53-F418	5'-GCAGTCTGGGACAGCCAAG-3'	SQ-RT-PCR
mp53-R418	5'-GGTAAGGATAGGTTCGGCGG-3'	SQ-RT-PCR
mp63-F388	5'-CCACAGGGCGCTGTTATC-3'	SQ-RT-PCR
mp63-R388	5'-CTGGGCAAGCACAGATCC-3'	SQ-RT-PCR
mTAp63-for	5'-CGACCCTTACATCCAGCGTTTCA-3'	SQ-RT-PCR
mTAp63-rev	5'-GAGAGAGGGCATCAAAGGTGGAG-3'	SQ-RT-PCR
mΔNp63-for	5'-CAATGCCAGACTCAATTTAGTGA-3'	SQ-RT-PCR
mΔNp63-rev	5'-GGCCCGGGTAATCTGTGTTGG-3'	SQ-RT-PCR
mp73-for617	5'-CCCACCACTTCGAGGTCACCTT-3'	SQ-RT-PCR
mp73-rev617	5'-ATGCACGTTTGCTGGCAGCTCC-3'	SQ-RT-PCR
mTAp73-for	5'-CCAAGAAAGGCGCTAAGC-3'	SQ-RT-PCR
mTAp73-rev	5'-TGGTGTCGAAGGTGGAGCT-3'	SQ-RT-PCR
mDNp73-for	5'-ACCTAGCCACCCAGACCCAT-3'	SQ-RT-PCR

mDNp73-rev	5'-GACTTGGCAGTGCTCGACTG-3'	SQ-RT-PCR
mActa-F	5'-CTGAGGAGCACCCGACTCTG-3'	SQ-RT-PCR
mActa-R	5'-AGGGAGGAGGAAGAGGCAGC-3'	SQ-RT-PCR
mALP-F386	5'-CCGATCGGGACTGGTACTC-3'	SQ-RT-PCR
mALP-R386	5'-GGAGAGCGAAGGGTCAGTC-3'	SQ-RT-PCR
mCcna2A-F	5'-CAGCCTGCCTTCACCATTG-3'	SQ-RT-PCR
mCcna2A-R	5'-TCACAGCCAAATGCAGGG-3'	SQ-RT-PCR
mCcnG2-F457	5'-TCTTGGCCCTTATGAAGGTG-3'	SQ-RT-PCR
mCcnG2-F457	5'-GTGTCGCTGAGCTTCAAATG-3'	SQ-RT-PCR
mCdc6-F372	5'-ACGGACCCCCTCGCTCACATAC-3'	SQ-RT-PCR
mCdc6-F372	5'-TCCCAGGAGCGCCAGAAAGG-3'	SQ-RT-PCR
hCKM-F355	5'-GGTCACCCCTTCATCATGAC-3'	SQ-RT-PCR
hCKM-F355	5'-CCTTCTCCGTCATGCTCTTC-3'	SQ-RT-PCR
mCKM-F280	5'-TCAAGGGTTACTCTGCCT-3'	SQ-RT-PCR
mCKM-R280	5'-TTCACCCACACAAGGAAGC-3'	SQ-RT-PCR
mGadd45a-F	5'-CGATAACGTGGTACTGTGCC-3'	SQ-RT-PCR
mGadd45a-R	5'-TTAATCACGGGCACCAC-3'	SQ-RT-PCR
mIGFII-F348	5'-CATCGTGGAAGAGTGCTG-3'	SQ-RT-PCR
mIGFII-F348	5'-CACTGATGGTTGCTGGAC-3'	SQ-RT-PCR
mIGFII Bam-for	5'-GACGGATCCATGGGGATCCCAGTGGGGAAG-3'	cloning
mIGFII Bam-rev	5'-GACGGATCCTCACTGATGGTTGCTGGACAT-3'	cloning
mMEF2C-F	5'-ATTCCTGCTGTTCCACCTCC-3'	SQ-RT-PCR
mMEF2C-R	5'-AACGCGGAGATCTGGCTTAC-3'	SQ-RT-PCR
mMHC-F384	5'-GAGATGGCCACCATGAAG-3'	SQ-RT-PCR
mMHC-R384	5'-CACCTTATTCTCCGTGGC-3'	SQ-RT-PCR
mMyf5-For301	5'-AGCTTGCAAGAGGAAGTC-3'	SQ-RT-PCR
mMyf5-Rev301	5'-AGGGCTGTTACATTCAGG-3'	SQ-RT-PCR
mMyogenin-F504	5'-GCGGACTGAGCTCAGCTTAAG-3'	SQ-RT-PCR
mMyogenin-R504	5'-GCTGTCCACGATGGACGTAAG-3'	SQ-RT-PCR
hMyogenin-F312	5'-CCGTGGGCGTGTAAGGTGTG-3'	SQ-RT-PCR
hMyogenin-R312	5'-ACTGCAGGAGGCGCTGTGAG-3'	SQ-RT-PCR
mMyoD-F416	5'-TGGCAGATGCACCACCAG-3'	SQ-RT-PCR
mMyoD-R416	5'-AGTGCAAGTGGCCTTCGC-3'	SQ-RT-PCR
mMyoD-for	5'-CGGGATCCATGGAGCTTCTATCGCC-3'	cloning

mMyoD-rev	5'-GCGGATCCTCAAAGCACCTGATAAATC-3'	cloning
myoDseq_1	5'-GACCTGGACCCGCGCCTG-3'	sequencing
myoDseq_2	5'-ATGGCGTTGCGCAGGATC-3'	sequencing
hMyoD-F311	5'-CCTACTGTGGGCCTGCAAG-3'	SQ-RT-PCR
hMyoD-F311	5'-AGTCGCCGCTGTAGTGCTC-3'	SQ-RT-PCR
hNEFH-F289	5'-GGAGATAACTGAGTACCGGCG-3'	SQ-RT-PCR
hNEFH-R289	5'-CCGACACTCTTCACCTTCCAG-3'	SQ-RT-PCR
mOsteocalcin-F258	5'-TTCTGCTCACTCTGCTGACC-3'	SQ-RT-PCR
mOsteocalcin-R258	5'-AGATGCGTTTGTAGGCGG-3'	SQ-RT-PCR
mp21-F358	5'-TGTCCAATCCTGGTGATGTCC-3'	SQ-RT-PCR
mp21-R358	5'-TCAGACACCAGAGTGCAAGAC-3'	SQ-RT-PCR
mp27-F274	5'-GTGTCCAGGGATGAGGAAG-3'	SQ-RT-PCR
mp27-R274	5'-TAATTCCTCCTGGCAGGC-3'	SQ-RT-PCR
mp57-F345	5'-CAGGACGAGAATCAAGAG-3'	SQ-RT-PCR
mp57-R345	5'-GGTTCCTGCTACATGAAC-3'	SQ-RT-PCR
hPAX3/FKHR-S	5'-GCACTGTACACCAAAGCACG-3'	SQ-RT-PCR
hPAX3/FKHR-AS	5'-CTGTGGATTGAGCATCCACC-3'	SQ-RT-PCR
hPAX3/FKHRNot-F	5'-CGAGCGGCCGCGCAGGATGACCACGCTGGCC-3'	cloning
hPAX3/FKHR-Pac-R	5'-CGCTTAATTAATCAGCCTGACACCCAGCT-3'	cloning
pSuperRetro	5'-CCCCTTGAACCTCCTCG-3'	sequencing
mRb-F450	5'-TGTGAGCATCGAATCATG-3'	SQ-RT-PCR
mRb-R450	5'-AATCTGGTCCAAATGTCTG-3'	SQ-RT-PCR
hRB-F371	5'-CCGAGAAGGACCAACTGATC-3'	SQ-RT-PCR
hRB-R371	5'-GGTCCAAATGCCTGTCTCTC-3'	SQ-RT-PCR
mRfc3-F	5'-GAGCATCAGACTATCACAACCC-3'	SQ-RT-PCR
mRfc3-R	5'-GAGCATCAGACTATCACAACCC-3'	SQ-RT-PCR
mTncc-F	5'-CAGAAACCCACACCTGAGGAG-3'	SQ-RT-PCR
mTncc-R	5'-AGGCAACCGTGCAAGACC-3'	SQ-RT-PCR
mp21-ChIP-S	5'-CGGAGACCAGCAGCAAATCG-3'	ChIP
mp21-ChIP-AS	5'-TGACACATACACACCCAGGCAC-3'	ChIP
mp57-ChIP-S	5'-TCTGTCAGGCCATGTCTGG-3'	ChIP
mp57-ChIP-AS	5'-AGTTGGGCCCATCCTAGC-3'	ChIP
mRB-ChIP-S	5'-GACGACGCGGGCGGAGACAGG-3'	ChIP
mRB-ChIP-AS	5'-AACGCTCCCCGAGGAAAACCGGACGC-3'	ChIP

2.1.10. siRNA oligos

pSUPER-NS-S

5'-GATCCCCTTCTCCGAACGTGTCACGTTTGGATCCAAACGTGACACGTTCCGGAGAATTTTAA-3'

pSUPER-NS-AS

5'-AGCTTAAAAATTCTCCGAACGTGTCACGTTTGGATCCAAACGTGACACGTTCCGGAGAAGGG-3'

pSUPER-mp53si1-S

5'-GATCCCCGCTGTTATGTGCACGTAATTGGATCCAAGTACGTGCACATAACAGACTTTTTTA-3'

pSUPER-mp53si1-AS

5'-AGCTTAAAAAGTCTGTTATGTGCACGTAATTGGATCCAAGTACGTGCACATAACAGACGGG-3'

pSUPER-mp63si1-S

5'-GATCCCCGAGACCGGAAGGCAGATGATTGGATCCAATCATCTGCCTTCCGGTCTCTTTTTTA-3'

pSUPER-mp63si1-AS

5'-AGCTTAAAAAGAGACCGGAAGGCAGATGATTGGATCCAATCATCTGCCTTCCGGTCTCGGG-3'

pSUPER-mp73si8-S

5'-GATCCCCGGCCATGCCTGTTTACAAGTTGGATCCAAGTGTAAACAGGCATGGCCTTTTTTA-3'

pSUPER-mp73si8-AS

5'-AGCTTAAAAAGGCCATGCCTGTTTACACGTTGGATCCAAGTGTAAACAGGCATGGCCGGG-3'

2.1.11. Adenoviral vectors

pAdEasy-1 vector (Stratagene)

pShuttle-CMV (Stratagene)

Ad-GFP (AG Stiewe)

Ad-mutRB (AG Stiewe)

Ad-RB (AG Stiewe)

Ad-p57 (AG Stiewe)

2.1.12. Retroviral vectors

pWPI (kindly provided by D. Trono)

pWPI- Δ Np73 α (AG Stiewe)

pRSV-Rev (kindly provided by D. Trono)

pCMV-dR8.74 (kindly provided by D. Trono)

pMD2G (kindly provided by D. Trono)

pSUPER siRNA Vector (Oligoengine)

pSUPER p53si (AG Stiewe)

pSUPER p63si	(AG Stiewe)
pSUPER p73si8	(AG Stiewe)
pQxIP	(BD Bioscience)
pQxIP-p53DD	(cloned in this study)
pQxIP- Δ Np73 α	(cloned in this study)
pQxIH	(BD Bioscience)
pQxIH-IGFII	(cloned in this study)
pQxIH-MyoD	(cloned in this study)
pQxIH-Pax3/FKHR3	(cloned in this study)
pQxIH-p73DD	(cloned in this study)

2.2. Methods

2.2.1. Cell culture

In this study, all cells were incubated in a scientific incubator at 37°C in a humid atmosphere of 95% air, 5% CO₂. Cells were grown in monolayer culture and harvested at appropriate confluence for further passage by standard culture techniques. Cells were routinely harvested by rapid exposure to 0.2% trypsin containing 0.001% EDTA. The cells were then plated in dishes or wells. C2C12 myoblasts were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS), 60 mg/l (100 U/ml) penicillin and 100 mg/l streptomycin. Human rhabdomyosarcoma cell lines (RD, JR1, CT-TC, Rh18, Rh30, Rh41), human neuroblastoma cells SH-SY5Y, murine NIH3T3, fibroblast and packaging cell lines (RP, 293T, Ad293) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 60 mg/l (100 U/ml) penicillin and 100 mg/l streptomycin. Human skeletal muscle myoblasts (HSMM) cell lines were cultured in growth medium consisting of Ham's F10 (Gibco, Grand Island, NY) supplemented with 15% fetal calf serum, bovine serum albumin (0.5 mg/ml), fetuin (0.5 mg/ml), epidermal growth factor (EGF; 10 ng/ml), dexamethasone (0.39 mg/ml), insulin (0.18 mg/ml), creatine (1 mM), pyruvate (100mg/ml), and uridine (50 mg/ml). Primary murine myoblasts were isolated as described (Watson et al., 1997).

2.2.2. Differentiations conditions

To prepare differentiated myotubes, myoblasts were grown to 70% confluence. After washing in PBS, the cells were exposed to differentiation medium (DMEM, 2% heat-inactivated horse serum). In our differentiations condition, we could detect multinucleated myotubes within 72h. The differentiation index was calculated by counting at least 200 nuclei per dish and applying the following formula: differentiation index = number of nuclei in differentiated cells / total number of nuclei. For osteoblast differentiation, C2C12 myoblasts were washed in PBS and growth medium was replaced by DMEM containing 5% FBS supplemented with 300 ng/ml BMP-2 for five days. NIH3T3–MyoD cells were prepared by infection with a retroviral vector encoding MyoD and the hygromycin resistance gene. Two days after infection, the cells were selected in medium containing Hygromycin. The MyoD-transformed cells formed multinucleated myotubes when exposed to differentiation medium. To induce neuronal differentiation, the SH-SY5Y cells were differentiated for seven days in the presence of 10 μ M retinoic acid (RA).

2.2.3. Construction of plasmids

Δ Np73 α and Δ Np73 α (R292H) were cloned into the retroviral vector pQCXIP through BamHI digestion. pQCXIH-p73DD and pMSCVpuro-p53DD were generated by inserting the expression cassette into the BamHI site of the retroviral vectors. A full-length IGFII cDNA was amplified from C2C12 cDNA by PCR using the following primers: 5'-GACGGATCCATGGGGATCCCAGTGGGGAAG-3' and 5'-GACGGATCCTCACTGATGGTTGCTGGACAT-3' and cloned into BamHI site of the retroviral vector pQCXIH. The sequence of the IGFII cDNA was confirmed by automated DNA sequencing. PAX3/FKHR cDNA was amplified from pcDNA3.1-PAX3/FKHR vector (kindly provided by Frederic G. Barr) by PCR using the following primers: 5'-CGAGCGGCCGCAGGATGACCACGCTGGCC-3' and 5'-CGCTTAATTAATCAGCCTGACACCCAGCT-3' and cloned into NotI/PacI sites of retrovirus vector pQCXIH. MyoD cDNA was amplified from a MyoD expression construct (kindly provided by Antonio Giordano) by PCR using the following primers: 5'-CGGGATCCATGGAGCTTCTATCGCC-3' and 5'-GCGGATCCTCAAAGCACCTGATAAATC-3' and cloned into BamHI site of the

retroviral vector pQCXIH. To generate the adenoviral vector AdGFP-p57, the p57 cDNA was isolated from pBSmp57 plasmid (kindly provided by P. Zhang) and cloned into pShuttle (kindly provided by B. Vogelstein). Insert was confirmed by restriction analysis. The plasmid was purified in sufficient quantity for the subsequent cotransformation steps. Linearized and dephosphorylated pShuttle-p57 DNA was electroporated into BJ5183 cells which have the cellular components that are necessary to carry out recombination between the pShuttle vector and the E1-deleted, pAdEasy-1 vector. Adenovirus vectors AdGFP-RB and AdGFP mutRB were described previously (Stiewe et al). All the plasmids were confirmed by DNA sequencing. Retroviral vectors pRSV-Rev, pCMV-dR8.74 (packaging plasmids) and pMD2G (envelop plasmid) were kindly provided by D. Trono.

2.2.4. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed by using a modified protocol from Upstate Biotechnology. Cells in a 20 cm dish were fixed at 37°C for 10 min with 1% formaldehyde. After crosslinking, cells were washed, harvested, and resuspended in 300 µl RIPA lysis buffer and sonicated under conditions yielding fragments ranging from 200 to 1000 bp. Samples were diluted ten times with ChIP dilution buffer and precleared for 2 hr at 4°C with recombinant protein A agarose beads coated with BSA and salmon-sperm DNA. Precleared lysates were used for an overnight immunoprecipitation using 5 µg of specific antibodies (IgG2, p53, p63 and p73) at 4°C. Complexes were collected for 1 hr using recombinant protein A agarose beads coated with BSA and salmon-sperm DNA. After washing, complexes were eluted for 1 hr at room temperature in elution buffer (1% SDS, 0.1 M NaHCO₃), and formaldehyde crosslinking was reversed overnight at 65°C. Eluted DNA was recovered through ethanol precipitation and used as a template for PCR. The PCR primer sets and antibodies used for ChIP are described in 2.1.2 and 2.1.9.

2.2.5. Muscle injury model and histology

To induce muscle regeneration *in vivo*, the tibialis anterior (TA) muscles of 6-week old C57BL/6 mice were injected with cardiotoxin. The muscle were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Paraffin sections were prepared

for hematoxylin/eosin staining by standard methods. Uninjected muscles were used as control. For gene expression analysis, total RNA from TA muscles was extracted with TriPure isolation reagent (Roche).

2.2.6. Flow cytometric cell cycle analyses

After 2 days in differentiation medium, cells were harvested in 500µl PBS. To fix the cells (1×10^7), cell pellets were resuspended dropwise in 5 ml 70 % cold ethanol. Then fixed cells were treated with RNaseA (100 µg/ml) and propidium iodide (10mg/ml), and examined with a fluorescence activated cell sorter (FACS, Becton Dickinson). Subsequently, data analysis was performed by using the CellQuest program (BD Biosciences, San Jose, CA).

2.2.7. Immunofluorescence assay

Cell lines were grown in 12-well tissue cultures plates containing sterile coverslips. Cells were washed once in Tris-buffered saline (TBS), fixed in 100% cold methanol (-20°C) for 10 min, washed three times for 5 minutes in 1xTBS and blocked with blocking buffer (10% horse serum, 1% BSA, 1XPBS) at room temperature for 30 min. Cells were then incubated with the diluted primary antibody in (1%BSA in TBS) for 60 min. Primary antibodies used were anti-myosin heavy chain (anti-MHC). After being washed three times in 1xTBS, the cells were incubated for 45 min secondary antibody. Secondary antibodies (see 2.1.3) were used at 1/2000 dilution. Cells were washed three times in 1xTBS. To visualize the nuclei, the final wash contained 1ng of 4',6-diamidino-2-phenylindole (DAPI) per ml. The immunochemically stained cells were viewed at 100X magnification under a fluorescence microscope (Axiovert 200, Zeiss).

2.2.8. BrdU staining

C2C12 mouse myoblasts were seeded at a density of 4 to 6×10^4 cells per 12-well on glass coverslips in differentiation medium and cultured for 5 days, followed by 24 h in DMEM supplemented with 10 % FBS and 10 µM bromodeoxyuracil (BrdU). Cells were then washed 3 times in ice-cold PBS, fixed with 70 % ethanol/ 50 mM glycine

(pH 2.0) and stained for myosin heavy chain (MHC; antibody: MF-20, University of Iowa, Iowa City; dilution 1:1000) and BrdU antibody: (Ab-3, Oncogene; dilution 1:100) for one hour at room temperature. Secondary antibodies were isotype-specific anti-mouse IgGs (Molecular Probes; dilution 1:1000) conjugated with Alexa Fluor 488 (BrdU) or Alexa Fluor 546 (MHC). Nuclei were counter-stained with 1 μ M DAPI. Dried coverslips were mounted using the ProLong Antifade Kit (Molecular Probes) according to the manufacturer's protocol.

2.2.9. Whole-cell extract preparation

Cells were washed once with phosphate-buffered saline (PBS) and lysed in 200 μ l of RIPA buffer containing protease and phosphatase inhibitors cocktails (Roth) Cellular debris was removed by centrifugation at 14,000 rpm for 10 min. Cell extracts were stored at -80°C until use. Total protein concentration was quantitated by Bradford assay (Bio-Rad).

2.2.10. Western Blot analysis

Whole-cell lysates (100 or 200 μ g of protein) were separated in Tris acetat acrylamid gels and blotted onto PVDF membrane (Amersham Bioscience). After 1 h blocking with 10% milk powder in TBS, the membrane was incubated overnight at 4°C with primary antibodies (see 2.1.2). After being washed three times in 1xTBST, the membrane was incubated with HRP-conjugated secondary antibody (see 2.1.3). Proteins were visualized by ECL and membranes were exposed to film (Kodak).

2.2.11. Generation of retroviruses

2.2.11.1. Transfection of packaging cells and generation of retrovirus containing supernatant

The amphotropic packaging cells were transfected with retroviral vector plasmids by using Jet Pei Transfection Kits (Biogene). Briefly, packaging cells (5×10^5) were plated into 60-mm tissue culture dish (Greiner) and 24h later transfected with 5 μ g of vector DNA. Three hours later, cells were washed once with 1xPBS and replaced

with fresh medium. To transduce the target cells, virus vector particle-containing supernatant was harvested after approximately 2 days.

2.2.11.2. Transduction of cell lines with retroviruses

5×10^4 target cells were transduced once with virus supernatant for 36h in 6-well tissue culture plates in the presence of 4 $\mu\text{g/ml}$ Polybrene. After approximately 5 days, when nontransduced control cells died in the presence of appropriate selection, bulk cultures were expanded and used for experiments.

2.2.12. Generation of adenoviruses

2.2.12.1. Cloning and generating AdEasy™ Recombinants

To produce adenoviruses, we used the AdEasy™ Adenoviral Vector System from stratagene. First, our gene of interest was cloned into pShuttle vector by using an appropriate site(s) in the MCS. Insert was confirmed by restriction analysis and DNA (shuttle vector plus gene of interest) was purified in sufficient quantity for the subsequent cotransformation steps. Linearized shuttle plasmid DNA was electroporated into BJ5183 cells which have the cellular components that are necessary to carry out recombination between pShuttle vector and E1-deleted adenovirus vectors such as the pAdEasy-1 vector.

2.2.12.2. Transfection of 293 cells and preparation of primary viral stocks

To produce adenovirus, the purified recombinant adenovirus plasmid was transfected into 293 cells by using Jet Pei Transfection Kits.. After approximately seven days adenovirus containing supernatant was harvested. To prepare high titer virus stock, 293 cells were seeded on eight 150mm cell culture dishes in DMEM 2% with FCS and infected with 10 μl adenovirus containing supernatant. After two to three days, cells were harvested in 5 ml 1xPBS + 10% glycerol and frozen at -80°C .

2.2.12.3. Transduction of cell lines with adenoviruses

After washing with PBS, cells were infected once with high titer adenovirus for one hour in the presence of DMEM 2% FCS. To control transgenic protein expression, cells were harvested and analyzed by western blot.

2.2.13. shRNAs construction

In this study, we used the pSUPER.retro RNAi System as a vector system for stable expression of short interfering RNA. This system provides a mammalian retroiral expression vector that directs intracellular synthesis of siRNA-like transcripts. To induce silencing of our target genes, the pSUPER.retro vector was used in concert with a pair of 64-nt oligonucleotides, each containing a unique 19-nt sequence (see 2.1.10) derived from the target transcript. These oligos were annealed and ligated into the vector between the BglIII/HindIII sites. After transformation into competent cells (Top10F'), the presence of the insert was confirmed by restriction analysis.

2.2.14. RNA isolation, cDNA Synthesis, semi quantitative RT-PCR and microarray analysis

In this study, for total RNA isolation from primary muscle cells we used TriPure RNA Isolation Reagent (Roche). For all other cells, the RNeasy Mini Kit (Qiagen) was used. The RNA extraction and reverse transcription were performed as described in the manufacturer's instruction. RNA quantitation was performed with the RiboGreen RNA Quantitation Reagent Kit (Molecular Probes). RNA (2 µg) from each cell line was reverse-transcribed into cDNA using the Omniscript cDNA Synthesis Kit (Qiagen). cDNA (2 µl) was amplified by PCR. For the microarray analysis, total RNA from cells was extracted TriPure isolation reagent (Roche). These RNA samples were labeled as described in the manufacturer's instruction (Qiagen) and hybridized to Affymetrix high-density oligonucleotide arrays, the murine genome 430 2.0 Array. Raw expression data obtained with Affymetrix's software were analyzed with the software GeneSpring QXVersion 7.1 (Silicon Genetics).

2.2.15. Alkaline phosphatase activity (ALP) analysis

To examine alkaline phosphatase activity histochemically, cells were fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS, the cells were incubated for 20 min with a mixture of 0.1 mg/ml of naphthol AS-MX phosphate (Sigma), 0,5% N, N-dimethylformamide, 2 mM MgCl₂, and 0.6 mg/ml of fast blue BB salt (Sigma) in 0.1 M Tris-HCl, pH 8.5, at room temperature.

2.2.16. The 3D collagen matrix model and confocal microscop.

C2C12 myblasts were added to collagen solution (100µl MEM, 50µl Bicarbonat and 750 µl Vitrogene) and mixed (1:2) with DMEM supplemented with 20% FBS. After polymerization of the lattice, 3D collagen matrix cultures (collagen concentration: 1.67 mg/mL) of C2C12 myoblasts were allowed to differentiate for 5 days. Samples were fixed by PFA (4%) and stained for myosin heavy chain and beta actin. 3D confocal backscatter microscopy of fixed samples was carried out on a Leica TCS 4D system (Leica, Bensheim, Germany).

2.2.17. Mice

Cells (5×10^6 cells/100 µl) were injected subcutaneously into flank of 7-9 week old CD-1 athymic nude mice (*nu/nu*). The animals were monitored twice weekly until the tumor size reached 10 mm in diameter.

3. Results

3.1. Increased expression of p73 in rhabdomyosarcoma (RMS)

Δ Np73 is frequently overexpressed in a variety of human tumors, but not in normal tissues (Casciano et al., 2002; Concin et al., 2004; Frasca et al., 2003; Stiewe et al., 2004; Uramoto et al., 2004; Zaika et al., 2002). Deregulated expression of Δ Np73 can bestow oncogenic activity upon the *TP73* gene by functionally inactivating the tumor suppressor function of p53 and TAp73 (Grob et al., 2001; Slade et al., 2004; Stiewe et al., 2002a). Δ Np73 expression might therefore be selected for in human cancer. Thus, we analyzed Δ Np73 expression in various human cancer types by semiquantitative RT-PCR. Unique primers (see 2.1.9) were designed for isoform-specific amplification of TAp73 and the two different Δ Np73 variants Δ N^{AS} (generated by alternative splicing) and Δ N^{AP} (generated by the alternative promoter). Compared to normal skeletal muscle tissues and human skeletal muscle myoblasts, we detected increased expression of TAp73 (15 of 20 samples), Δ N^{AP} (12 of 20 samples) and Δ N^{AS} (12 of 20 samples) in primary human rhabdomyosarcoma (Fig. 13). Interestingly, almost all RMS tumors show overexpression of at least one of the oncogenic Δ Np73 isoforms (16 of 20 samples). Importantly, there is no tumor with high levels of the tumor-suppressive TAp73 isoform in the absence of concomitant Δ Np73 expression.

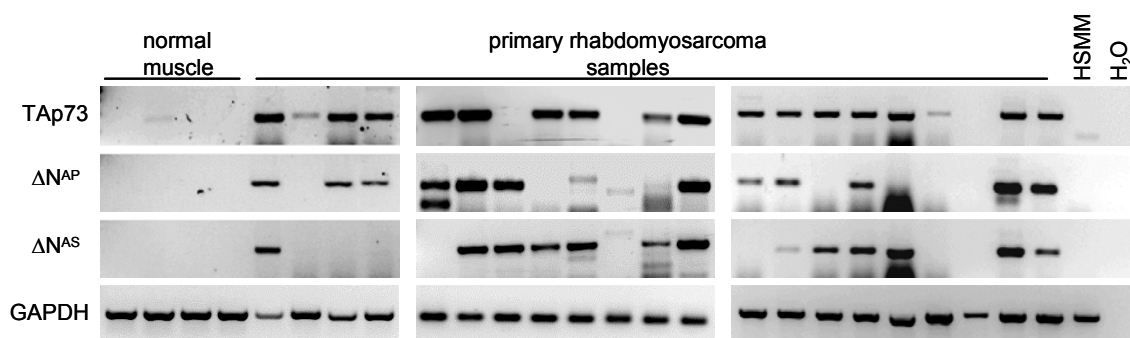


Figure 13. Tumor-specific up-regulation of p73 isoforms in primary rhabdomyosarcoma.

Expression of NH₂-terminal p73 isoforms was analyzed by semiquantitative RT-PCR in 20 primary RMS tumors, compared with four normal skeletal muscle tissues or human skeletal muscle myoblasts (HSM). Amplification of GAPDH demonstrates use of equal amounts of total RNA. H₂O, no template control.

In addition to primary rhabdomyosarcoma samples, we analyzed the p73 expression level in various rhabdomyosarcoma cell lines. Similarly, both TAp73 and Δ Np73 isoforms were simultaneously up regulated in 3 of the 5 cell lines on the RNA (Fig. 14A) and protein level (Fig. 14B). Although these results suggested an association of increased p73 expression with rhabdomyosarcoma of the alveolar subtype, no such correlation was observed with primary patient samples (Fig. 13).

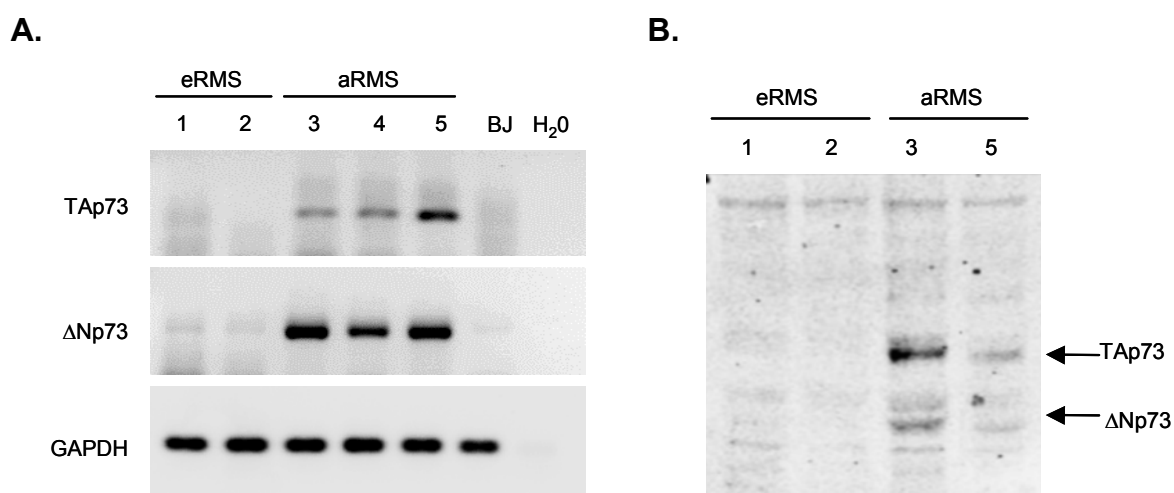


Figure 14. Increased expression of TAp73 and Δ Np73 in rhabdomyosarcoma cell lines.

A. Semiquantitative RT-PCR analysis of p73 isoforms (TAp73, Δ Np73) in rhabdomyosarcoma cell lines. p73 transcripts were amplified with isoform-specific primer pairs. Amplification of GAPDH demonstrates use of equal amounts of total RNA. 1, RD; 2, JR1; 3, Rh30; 4, Rh18; 5, Rh41; 6, BJ fibroblasts, negative control; H₂O, no template control. eRMS: embryonal subtype, aRMS : alveolar subtype). **B.** Cell extracts were subjected to immunoblot analysis for p73 (anti-p73, ER15). Arrows indicate p73 protein isoforms.

Taken together, our results show that high levels of TAp73 and oncogenic Δ Np73 isoforms (Δ N^{AS} and Δ N^{AP}) are seen in both primary rhabdomyosarcoma samples and established cell lines. High levels of the E2F target gene TAp73 are typically a result of p16-RB-E2F pathway alterations which are commonly observed in multiple tumor types (Irwin et al., 2000; Stiewe and Putzer, 2000; Zaika et al., 2001). Because TAp73 transcripts are frequently aberrantly spliced in tumor cells (Δ N^{AS}) and TAp73 is a transactivator of the Δ Np73 promoter (Δ N^{AP}) (Grob et al., 2001; Kartasheva et al., 2002), we hypothesized that high TAp73 levels in RMS cells drive

expression of oncogenic Δ Np73 isoforms. To evaluate this, we specifically silenced TAp73 by RNA interference. Small interfering RNAs (siRNAs) are 21-23 bp double-stranded RNA molecules that elicit gene-specific silencing in mammalian cells. When the RNA polymerase III is used to transcribe siRNA strands that are linked *in cis*, so forming a hairpin structure, these small RNAs were called short hairpin RNAs (shRNAs). Here, the Rh30 RMS cell line was transduced with retroviruses expressing short-hairpin RNAs (shRNAs) directed against p73 sequences specific for TAp73 but not Δ Np73. A non-silencing control shRNA was used as a control for unspecific silencing effects. After puromycin selection, successful downregulation of TAp73 was verified by western blot analysis (Fig. 15A). As shown in Figure 15B, efficient knockdown of TAp73 induces downregulation of Δ Np73 confirming that TAp73 is indeed the major cause for high levels of the oncogenic Δ Np73 isoform. As Δ Np73 in turn sequesters TAp73 (and also TAp63) in transcriptionally inactive complexes, high levels of p73 in RMS tumors are indicative of p63/p73 dysfunction.

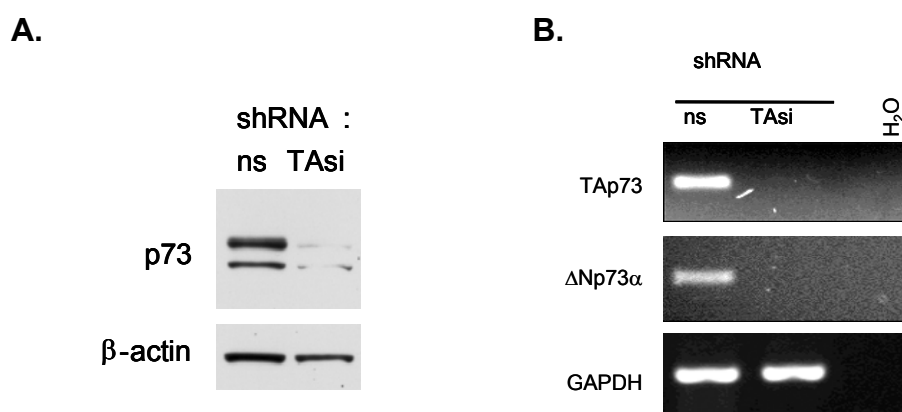


Figure 15. Knockdown of TAp73 induces downregulation of Δ Np73.

A. Whole cell extracts of Rh30 cells, expressing TAp73-shRNAs (TAsi) or non-silencing shRNAs (ns) as a control, were immunoblotted with anti-p73 (ER15) and anti-actin (ab6276) antibodies. **B.** Expression of TAp73 and Δ Np73 mRNA in shRNA-expressing cell lines. The cDNAs were amplified with TAp73- and Δ Np73-specific primers. Amplification of GAPDH demonstrates use of equal amounts of total RNA. H₂O, no template control.

3.2. RNAi-mediated downregulation of p73 inhibits RMS development in nude mice

To determine whether tumor-specific up-regulation of p73 isoforms plays a critical role in the development and progression of rhabdomyosarcoma, we generated RMS cell lines, in which p73 levels are reduced by RNA interference. To silence p73 expression in the human rhabdomyosarcoma Rh30 cell line, the cells were transduced with retroviral vectors expressing a p73-directed or a non-silencing shRNA as a control. After puromycin selection, successful downregulation of p73 was verified by western blot analysis (Fig. 16). Compared to non-silenced control cells, endogenous p73 protein was nearly undetectable in three different p73-shRNA expressing clones, while the level of actin remained unchanged.

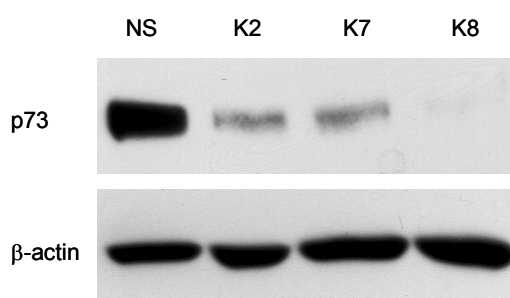


Figure 16. RNAi ablation of endogenous p73 expression in Rh30 cells.

Whole cell extracts from three different Rh30-p73 shRNA clones (K2, K7, K8) or a Rh30-non-silencing shRNA control clone (NS) were immunoblotted with anti-p73 (ER15) and anti-β-actin (ab6276) antibodies.

We injected three selected Rh30-p73 shRNA cell clones subcutaneously into nude mice. The injected animals were monitored until the tumor size in control mice reached 10 mm in diameter. 23 of 25 mice injected with the control Rh30 clone developed tumors. In contrast, only 6 of 35 mice, which were injected with the three selected Rh30-p73 shRNA cell clones developed tumors (Table 1). Taken together, Rh30 cells stably transfected with p73-directed shRNA expressing retroviral vectors displayed highly reduced tumorigenicity *in vivo*. To exclude clonal artifacts, we also injected polyclonal Rh30-p73 shRNA cells into nude mice. Similarly, RNAi ablation of

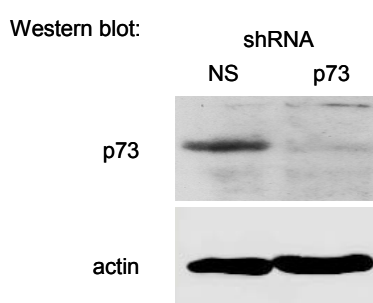
p73 in polyclonal Rh30 cells resulted in inhibition of tumorigenicity (Fig. 17) indicating that high p73 expression is critical for RMS tumor development *in vivo*.

tumorigenicity in nude mice (s.c. injection of 5×10^6 cells)	
Rh30-non-silencing shRNA	23/25
Rh30-p73 shRNA-K2	2/10
Rh30-p73 shRNA-K7	1/10
Rh30-p73 shRNA-K8	3/15

Table 1. RNAi ablation of endogenous p73 expression inhibits tumorigenicity of RMS cell lines in nude mice.

Cells (5×10^6 cells in a volume of 100 μ l) from transduced cell lines were injected s.c. into the right or left flank of 7-9 week old CD-1 athymic nude mice (*nu/nu*). The animals were observed until the tumor size in controls reached 10 mm in diameter. *Numbers* represent the number of mice with tumor/total number of mice injected.

A.



B.

tumorigenicity in nude mice (s.c. injection of 5×10^6 cells)	
Rh30-non-silencing shRNA	9/10
Rh30-p73 shRNA	3/10

Figure 17. RNAi ablation of endogenous p73 expression in Rh30 cells.

A. Whole cell extract from bulk cultures of Rh30-p73 shRNA (p73) cells or control Rh30 non-silencing shRNA (NS) cells were immunoblotted with anti-p73 (ER15) and anti-actin (ab6276) antibodies. **B.** Cells (5×10^6 cells in a volume of 100 μ l) from transfected cell lines were injected s.c. into the right or left flank of 7-9 week old CD-1 athymic nude mice (*nu/nu*). The animals were observed until the tumor size in controls reached 10 mm in diameter. *Numbers* represent the number of mice with tumor/total number of mice injected.

3.3. Δ Np73 cooperates with IGFII and PAX3/FKHR to transform C2C12 myoblasts to tumorigenicity

To evaluate further the oncogenic function of Δ Np73 α *in vivo*, the murine myoblast cell line C2C12 was transduced with Δ Np73 α or empty vector (mock) expressing retroviruses. The expression of Δ Np73 α was verified by western blot analyses (Fig. 18A). C2C12-mock and Δ Np73 α myoblasts were injected subcutaneously (s.c) into nude mice. As shown in Figure 18B, no tumor formation was detected in the control group and only 1 of 20 mice, which were injected with C2C12- Δ Np73 α myoblasts, developed a tumor. Δ Np73 expression alone is therefore not sufficient to transform C2C12 myoblasts to tumorigenicity.

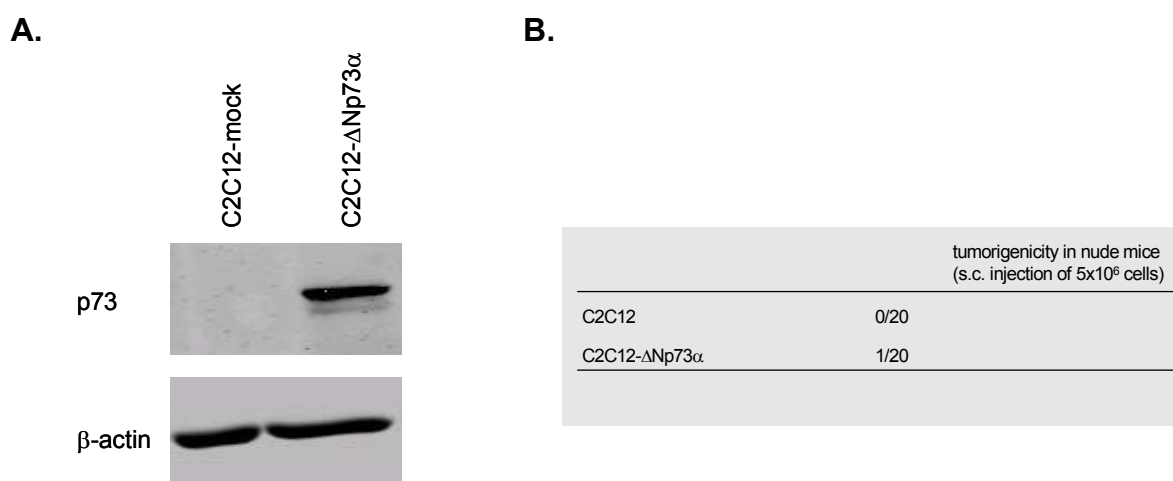


Figure 18. C212- Δ Np73 α myoblasts failed to form tumors in nude mice.

A. Stable expression of Δ Np73 α in C2C12 cells. After appropriate selection, cell extracts were subjected to immunoblot analysis for p73 (anti-p73, ER15). **B.** Cells (5×10^6 cells/100 μ l) from transfected cell lines were injected s.c. into right or left flank of 7-9 week old CD-1 athymic nude mice (*nu/nu*). The animals were observed until the tumor size reached 10 mm in diameter. *Numbers* represent the number of mice with tumor/total number of mice injected.

Since single oncogenes are rarely sufficient to induce transformation, we tested cooperation of Δ Np73 α with the known RMS oncogenes IGFII and PAX3/FKHR (Merlino and Helman, 1999). Thus, C2C12-mock and C2C12- Δ Np73 α myoblasts were transduced with retroviral vectors expressing PAX3/FKHR or IGFII. After

hygromycin selection, successful transduction was verified by semiquantitative RT-PCR (Fig. 19A). When the various myoblasts populations were injected subcutaneously into nude mice only IGFII+ Δ Np73 α and PAX3/FKHR+ Δ Np73 α cells formed tumors at a high rate (Fig. 19B). These results strongly support that Δ Np73 α interacts with PAX3/FKHR and IGFII to play a critical role in the development of rhabdomyosarcoma.

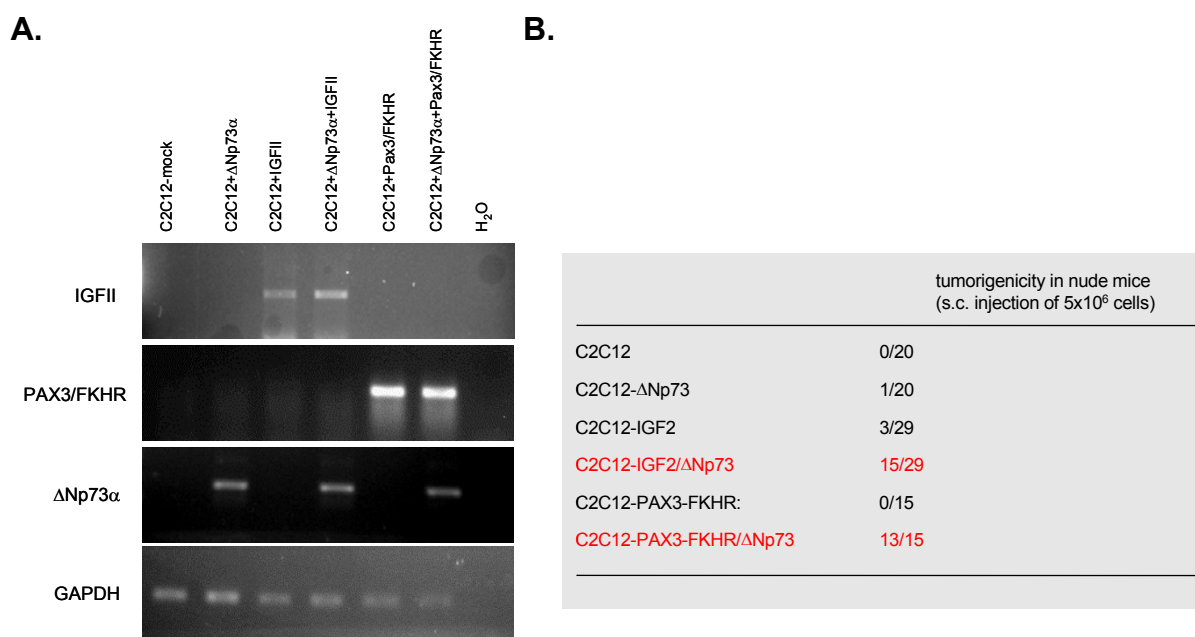


Figure 19. Cooperative oncogenic effect of Δ Np73 α *in vivo*.

A. The expression of Δ Np73, PAX3/FKHR and IGFII mRNA in different transduced cell lines. The cDNAs were amplified with Δ Np73, PAX3/FKHR and IGFII primers. Amplification of GAPDH demonstrates use of equal amounts of total RNA. H₂O, no template control. **B.** Cells (5×10^6 cells/100 μ l) from transfected cell lines were injected s.c. into right or left flank of 7-9 week old CD-1 athymic nude mice (*nu/nu*). The animals were observed until the tumor size reached 10 mm in diameter. *Numbers* represent the number of mice with tumor/total number of mice injected.

A characteristic feature of rhabdomyosarcoma is an inherent block of the myogenic differentiation program. Interestingly, Δ Np73 α inhibited differentiation of parental and PAX3/FKHR myoblasts and also the accelerated and enhanced differentiation of IGFII myoblasts (Fig. 20A, B) (Florini et al., 1996). These findings suggest that by interfering with differentiation Δ Np73 α unleashes the oncogenic activities of PAX3/FKHR (and IGFII) and enables tumor development.

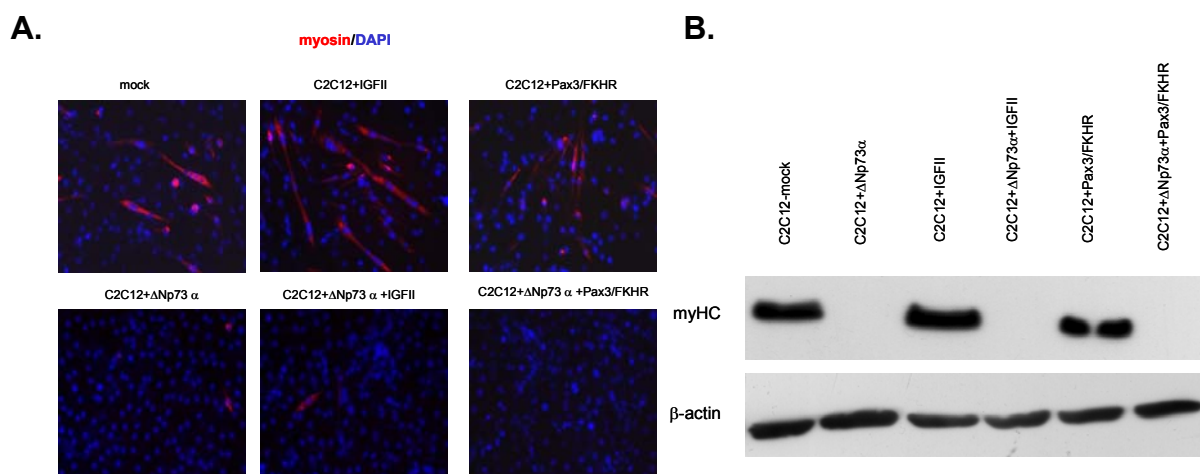


Figure 20. Δ Np73 blocks differentiation of parental and oncogene-expressing myoblasts.

A. Immunofluorescence staining for myosin (myHC) of C2C12 cell lines expressing the indicated transgenes (IGFII, PAX3/FKHR, Δ Np73). The cells were fixed and stained for myHC (anti-myHC, MF20) before and 5 days after the start of differentiation. The cells were counterstained with DAPI to visualize nuclei. **B.** The indicated C2C12 cell lines were allowed to differentiate for five days. Proteins were extracted from cells and myosin (myHC) expression was determined by western blot analysis. The amount of protein in each sample was normalized by β -actin expression.

3.4. Ectopic expression of Δ Np73 α blocks myogenic differentiation

To explore more precisely the underlying molecular mechanism of the Δ Np73 α induced differentiation block, we took advantage of the C2C12 differentiation model *in vitro*. C2C12 myoblasts can be induced to differentiate by culturing them in mitogen poor medium (DM). Upon serum starvation, these cells terminally exit from the cell cycle. After induction of intermediate muscle specific genes such as myogenin, C2C12 cells elongate and fuse to form multinuclear myotubes, which express late muscle specific markers such as myosin (myHC). To examine the effect of Δ Np73 α on myogenic differentiation, C2C12-mock and C2C12- Δ Np73 α myoblasts were shifted into differentiation media (DM) and allowed to differentiate for 5 days. On day 3 of differentiation, C2C12 control cells formed multinuclear myotubes. In contrast, the differentiation of C2C12- Δ Np73 myoblasts into multinuclear fibers was absent (Fig. 21). Interestingly, we observed that Δ Np73 expressing C2C12 myoblasts continued to proliferate under differentiation conditions.

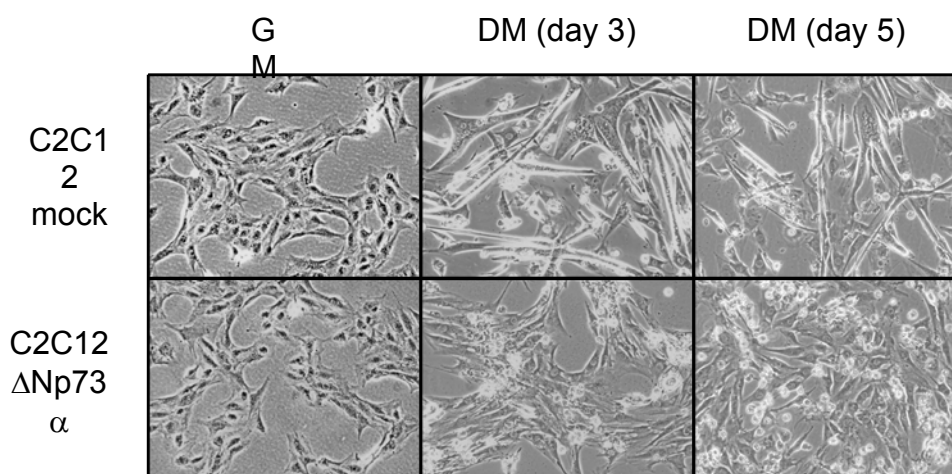


Figure 21. Ectopic expression of Δ Np73 α blocks myogenic differentiation.

C2C12-mock and $-\Delta$ Np73 myoblasts were allowed to differentiate for five days and monitored by phase-contrast microscopy. On day 3 and 5, control C2C12 cells formed long and large multinucleated cells or fibers. In contrast, C2C12- Δ Np73 myoblasts continued to grow and were unable to form myotubes.

The absence of myotubes in the Δ Np73 α population was further confirmed by immunofluorescence staining and immunoblotting for myosin heavy chain (myHC) as a marker of mature muscle fibers (Fig. 22A and B).

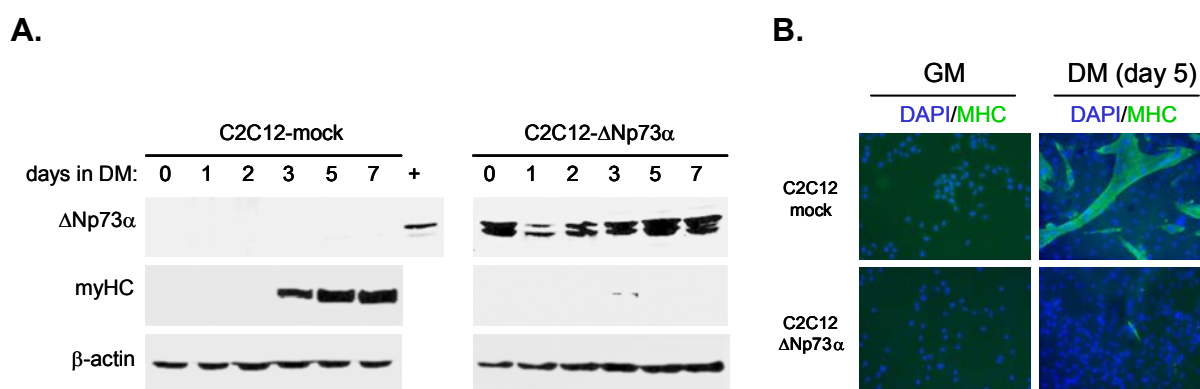


Figure 22. Ectopic expression of Δ Np73 α blocks myogenic differentiation.

A. C2C12-mock and $-\Delta$ Np73 myoblasts were cultured in differentiation media. At the indicated time points of differentiation, cells were harvested for western blotting with antibodies against p73 (ER15) and myHC (MF20). Cell lysates were normalized for expression of β -actin (ab6276). **B.** Immunofluorescence staining for myHC. Cells were placed into differentiation media (DM). At days 0 and 5, the cells were fixed and stained for myHC (green). To visualize nuclei, the cells were counterstained with DAPI (blue).

Furthermore, we investigated the effect of Δ Np73 on myogenesis in both human skeletal muscle myoblasts (HSMM) and primary murine muscle satellite cells. HSMM cells were transduced with viral vectors expressing Δ Np73 α or an empty vector control (mock). Transduced HSMM cells were differentiated for 3 days with 2% horse serum and observed by phase-contrast microscopy. Under differentiation condition, HSMM-mock cells formed multinuclear myotubes expressing muscle differentiation markers such as muscle creatin kinase (MCK). In contrast, HSMM- Δ Np73 myoblasts failed to form differentiated myotubes and to induce muscle differentiation markers (Fig 23A and B).

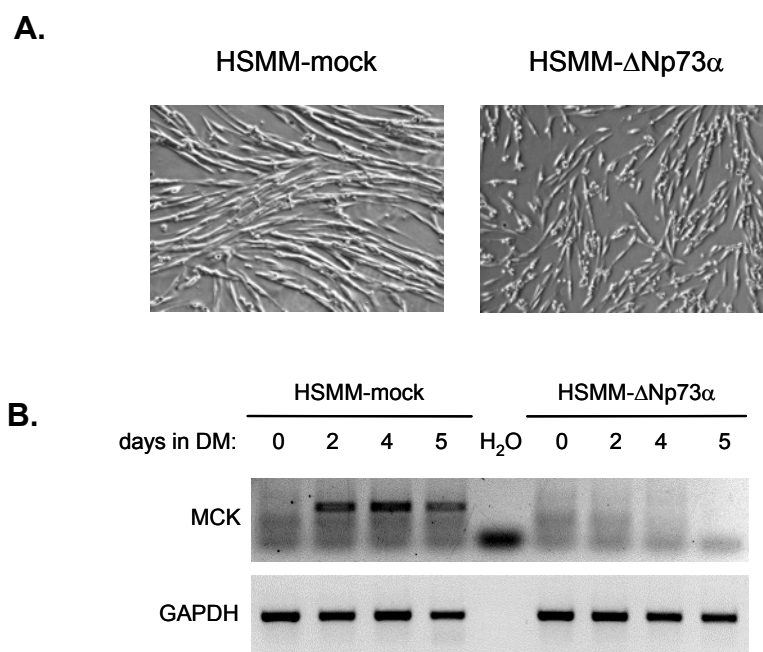


Figure 23. Ectopic expression of Δ Np73 α blocks myogenic differentiation of human skeletal muscle myoblasts (HSMM).

A. Transduced HSMM cells were differentiated for 3 days with 2% horse serum and observed by phase-contrast microscopy. **B.** Empty vector (mock) or the Δ Np73 α expressing HSMM cells were cultured in differentiation media. At the indicated time points, cells were harvested for semi-quantitative RT-PCR for muscle creatin kinase (MCK), a late muscle differentiation marker. Amplification of GAPDH demonstrates use of equal amounts of total RNA. H₂O, no template control.

To analyze the impact of Δ Np73 on myogenic differentiation in primary murine muscle satellite cells, myoblasts were isolated from Δ Np73 α ^{fl^{ox}} transgenic mice as

described in materials and methods. In these mice expression of $\Delta\text{Np73}\alpha$ is prevented by a transcriptional stop cassette that is flanked by loxP sites. To induce $\Delta\text{Np73}\alpha$ expression in the isolated myoblasts, recombination was induced by infection with 100 moi (multiplicity of infection) of Adeno-Cre. Recombination efficiency was analyzed by RT-PCR using primers specific for $\Delta\text{Np73}\alpha$ (Fig. 24A). With Adeno-Cre or Adeno-GFP (control) infected cells were incubated either in growth medium (GM) or induced to differentiate for 30 hours in differentiation medium (DM). Subsequently, cells were analyzed for the expression of the muscle differentiation marker myosin (myHC) by semi-quantitative RT-PCR analysis and observed by phase-contrast microscopy (Fig. 24A and B). Similar to the results obtained in the C2C12 myoblast cell line, $\Delta\text{Np73}\alpha$ induced a complete differentiation block in primary murine muscle satellite cells.

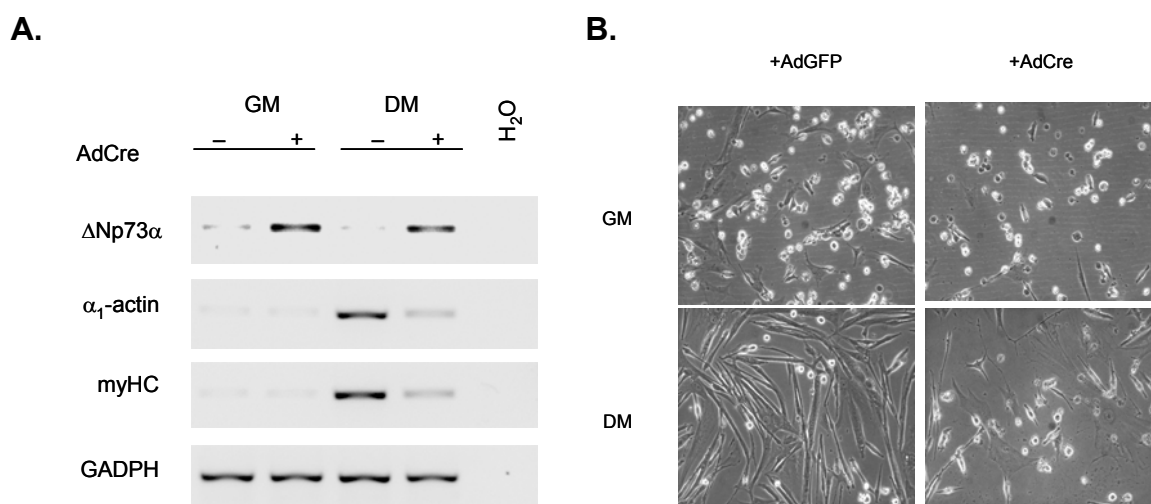


Figure 24. Inducible expression of the $\Delta\text{Np73}\alpha$ transgene interferes with myogenic differentiation in isolated primary murine muscle satellite cells.

A. Cells were analyzed for the expression of $\Delta\text{Np73}\alpha$, α_1 -actin and the muscle differentiation marker gene myosin (myHC) by semi-quantitative RT-PCR. Amplification of GAPDH demonstrates use of equal amounts of total RNA. H₂O, no template control **B.** Adeno-Cre or Adeno-GFP infected cells were allowed to differentiate for 30 hours and observed by light microscopy. Whereas control cells (+AdGFP) undergo terminal differentiation into multinuclear myotubes, induction of $\Delta\text{Np73}\alpha$ by Adeno-Cre inhibits execution of the myogenic differentiation program.

To test whether $\Delta Np73\alpha$ 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 myHC and differentiated into multicellular muscle fiber-like structures, $\Delta Np73\alpha$ prevented myHC upregulation and maintained a dispersed single-cell state (Figure. 25). Thus, $\Delta Np73\alpha$ not only inhibits differentiation induced by serum withdrawal but also overrides synergistic growth factor and ECM-induced differentiation signals.

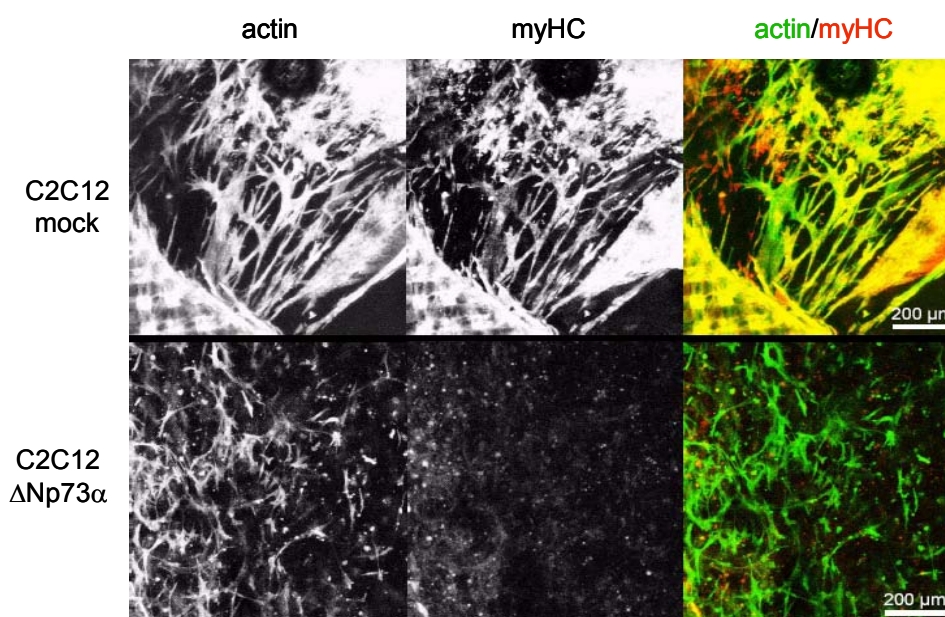


Figure 25. $\Delta Np73\alpha$ overrides growth factor and ECM-induced differentiation signals in a 3D collagen lattice.

Mock and $\Delta Np73\alpha$ expressing myoblasts were embedded in a 3D collagen lattice for 5 days and analyzed for expression of actin (green) and myHC (red) by confocal fluorescence microscopy.

3.5. Δ Np73 expressing myoblasts fail to induce cell cycle arrest in differentiation medium

Differentiation conditions cause a cell cycle arrest in G_0/G_1 and subsequently the timely ordered activation of muscle specific gene expression. Interestingly, under differentiation conditions, Δ Np73 expressing C2C12 myoblasts continued to proliferate (Fig. 21). Thus, we examined the effect of Δ Np73 on cell cycle progression in more detail during myogenesis. In growth factor rich medium mock and Δ Np73 α myoblasts showed no significant difference in their proliferation rate (Fig. 26A). Under conditions of mitogen deprivation mock myoblasts completely withdrew from the cell cycle within one day. In contrast, Δ Np73 α myoblasts slowed down markedly but continued to proliferate resulting in a progressive increase in cell number (Fig. 26A). Similarly, expression of the proliferating cell nuclear antigen (PCNA) was completely repressed in mock but not in Δ Np73 α cells (Fig. 26B).

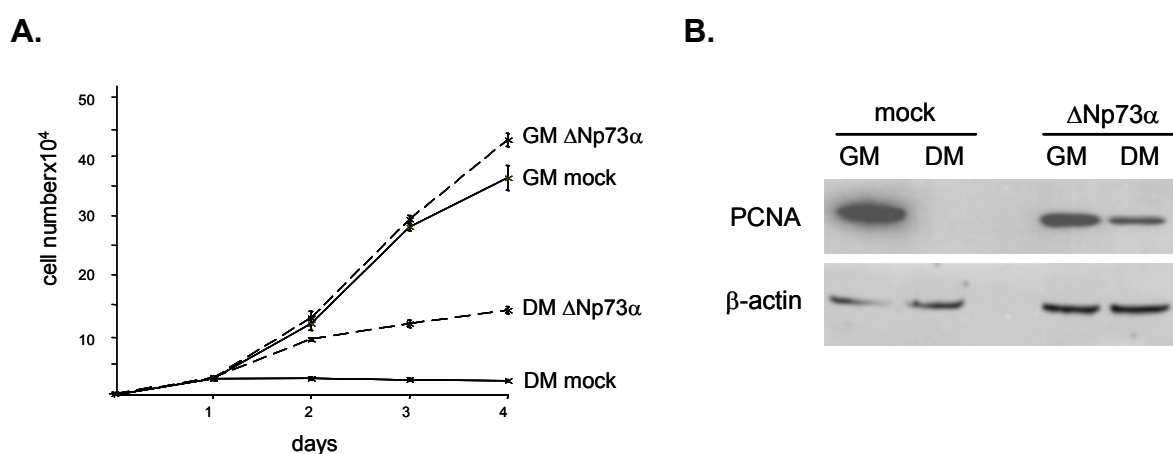


Figure 26. C2C12- Δ Np73 myoblasts fail to arrest in differentiation medium.

A. C2C12-mock and C2C12- Δ Np73 cells were allowed to differentiate for four days. The cells were counted at the indicated time points. Results are the mean \pm standard deviation of three independent experiments.

B. C2C12- Δ Np73 and C2C12-mock cells were incubated either in growth medium (GM) or induced to differentiate for 48 hours in differentiation medium (DM) and harvested for western blot analysis with antibodies against PCNA (sc-56). The cell lysates were normalized for expression of β -actin (ab6276).

Cell cycle profiles demonstrate that only very few mock cells (6%) remain in S-phase 2 days after differentiation induction compared to more than 22% of the $\Delta Np73\alpha$ cells (Fig. 27).

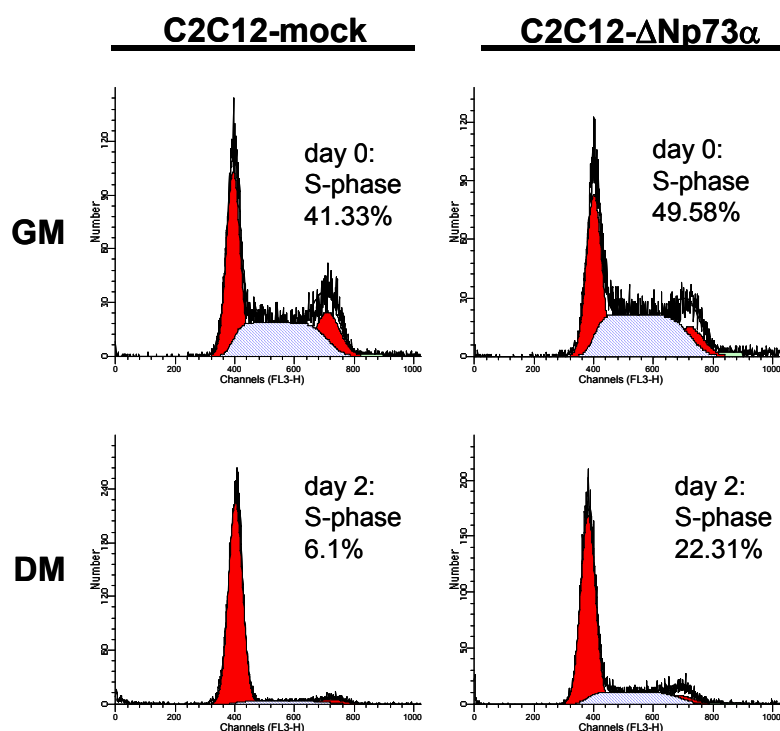


Figure 27. C2C12- $\Delta Np73$ myoblasts fail to arrest in differentiation medium.

Cell cycle analysis was performed on C2C12-mock and C2C12- $\Delta Np73\alpha$ myoblasts (see methods). Cells were incubated either in growth medium (GM) or induced to differentiate for 48 hours in differentiation medium, stained with propidium iodide for DNA content and examined with a flow cytometer.

Importantly, cell cycle withdrawal during myogenic differentiation is known to be permanent and restimulation with growth factors does not cause cell cycle reentry (Fig. 28A). All nuclei within multinuclear, myHC expressing myotubes remained permanently arrested and failed to incorporate BrdU whereas $\Delta Np73\alpha$ cells rapidly increased their proliferation rate and showed a BrdU incorporation index of more than 90% (Fig. 28A and B). These data indicate, that $\Delta Np73\alpha$ myoblasts are sensitive to growth factor depletion and slow down their proliferation rate but fail to permanently withdraw from cell cycle progression.

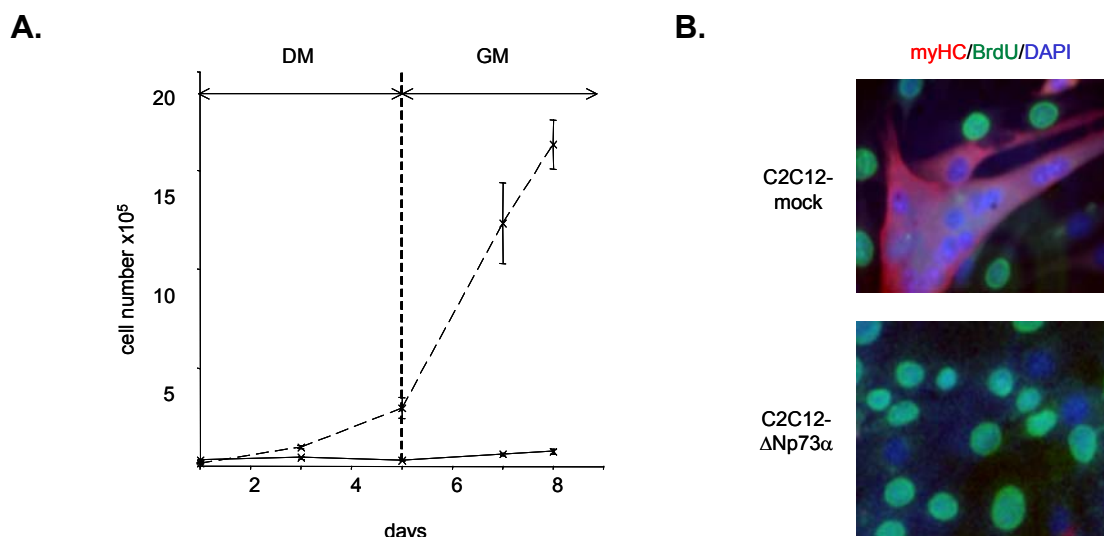


Figure 28. C2C12- Δ Np73 α myoblasts fail to permanently withdraw from cell cycle progression.

A. Proliferation curve of mock (solid line) and Δ Np73 α (dashed line) myoblasts during differentiation (DM) and subsequent restimulation with 10% FCS (DM). **B.** Immunofluorescence staining for myHC (red), BrdU (green) and DAPI (blue) of differentiated myocytes 24 hours after restimulation with FCS in the presence of BrdU.

3.6. Ectopic expression of Δ Np73 blocks progression but not initiation of the myogenic differentiation program

The myogenic differentiation program is initiated by a limited set of transcription factors that progressively unfold a complex program of gene expression (Jahson et al., 1995; Melendez et al., 2003). Δ Np73 α is a protein with sequence-specific DNA-binding properties suggesting that it interferes with the expression of critical genes during differentiation (Grob et al., 2001; Kartasheva et al., 2002; Stiewe et al., 2002a). With the advent of microarray technology, it has become possible to investigate changes in gene expression profiles in a biological process on an unprecedented scale.

To identify the critical genes affected by Δ Np73 expression, the gene expression profiles of C2C12-mock with C2C12- Δ Np73 α myoblasts were compared by microarray analysis. C2C12-mock and - Δ Np73 α myoblasts were induced to differentiate by culturing in differentiation medium. At different time-points (0, 6, and

24h after induction of differentiation), total RNA was extracted from the cells. These RNA samples were labeled and hybridized to high-density oligonucleotide arrays (Affymetrix GeneChip® Mouse Genome 430 2.0 Array). Raw expression data obtained with Affymetrix's software were further analyzed with the GeneSpring software (Silicon Genetics). A total of 646 genes were induced or repressed by more than 3-fold during the differentiation of mock myoblasts. The expression levels of these 646 genes correlate between the two cell types both in proliferation medium and 6 hours after induction of differentiation. Only 23/646 genes (3.6%) were expressed differentially in the two cell types at the 0 hour (Fig. 29A) and 19/646 (2.9%) at the 6-hour time point (Fig. 29B). In contrast, 24 hours after induction of differentiation 261/646 genes (40.4%) are expressed differently (> 3-fold) between the two cell types (Fig. 30). Of 73 genes that show expression changes during the first 6 hours of differentiation in mock cells, 53 genes (72.6%) were also altered in the $\Delta Np73\alpha$ myoblasts. Of 188 genes that are changed more than 3-fold between 6 hours and 24 hours of differentiation in the mock cells, only 5 (2.6%) were changed in $\Delta Np73\alpha$ cells (Fig. 30).

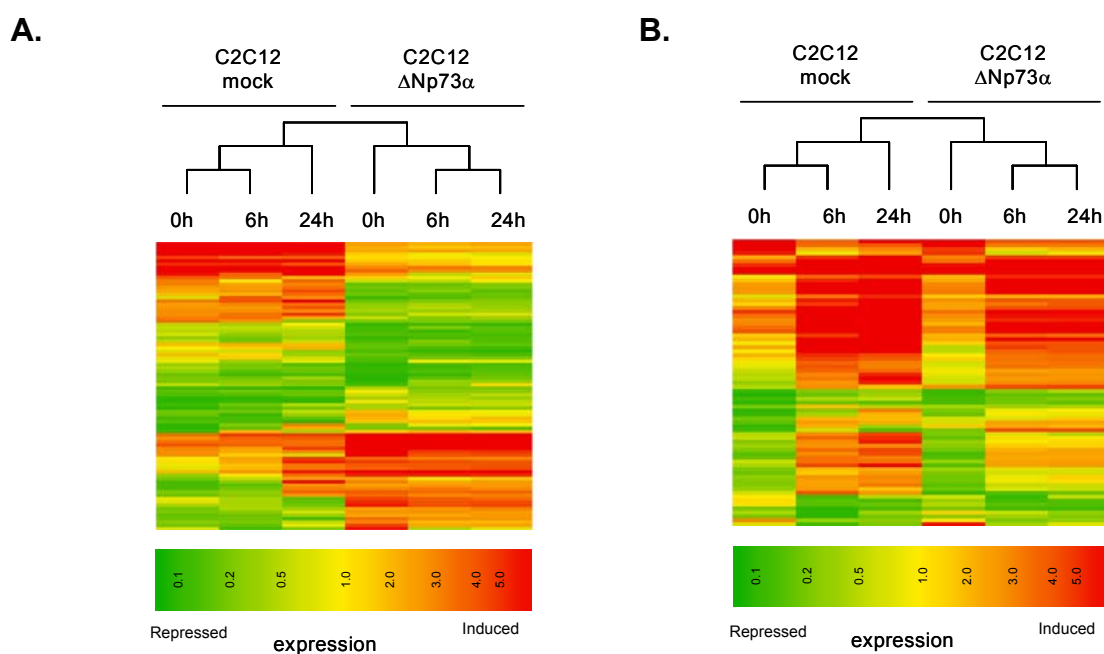


Figure 29. Expression profiles of genes that change their expression levels 3 folds.

A. In comparison to C2C12- $\Delta Np73\alpha$ myoblasts, 23 genes in C2C12-mock showed 3 fold change or higher in their relative expression levels at time 0h in differentiation.
B. At time from 0h to 6h in differentiation, 19 genes in both cell lines are similarly 3 fold up or down regulated.

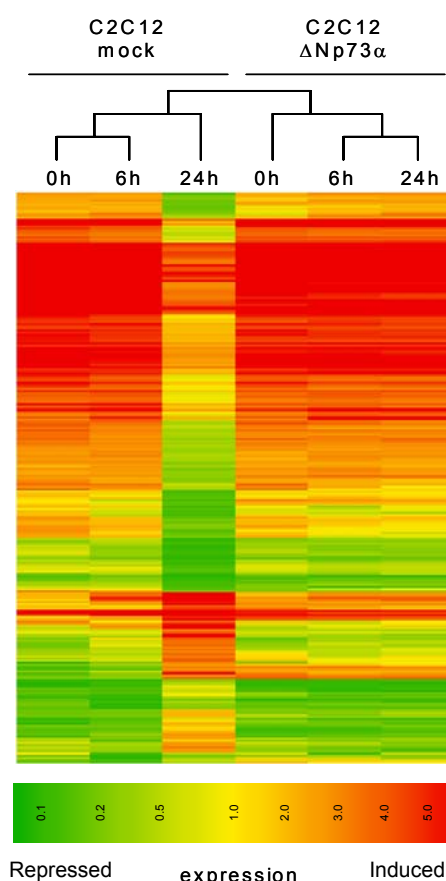


Figure 30. Expression profiles of genes that change their expression levels 3 folds from 6h to 24h in myogenic differentiation.

261 genes in C2C12-mock myoblasts showed 3 fold change whereas only 5 genes change their expression pattern in C2C12- Δ Np73 α myoblasts

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 (Fig. 31). In mock cells the muscle-related genes were induced or repressed most prominently at 24 hours of differentiation (Fig. 31A). Consistent with the observed differentiation defect, only few changes were observed in Δ Np73 α myoblasts (Fig. 31A). Furthermore, as expected from the function of Δ Np73 as a dominant-negative inhibitor of p53 family members, most of the p53 target genes that showed expression changes in the mock cells were unchanged in the Δ Np73 α cells (Fig. 31B). Finally, consistent with the defects in permanent cell cycle withdrawal, a number of cell proliferation related genes failed to be silenced in the Δ Np73 α myoblasts (Fig. 31C).

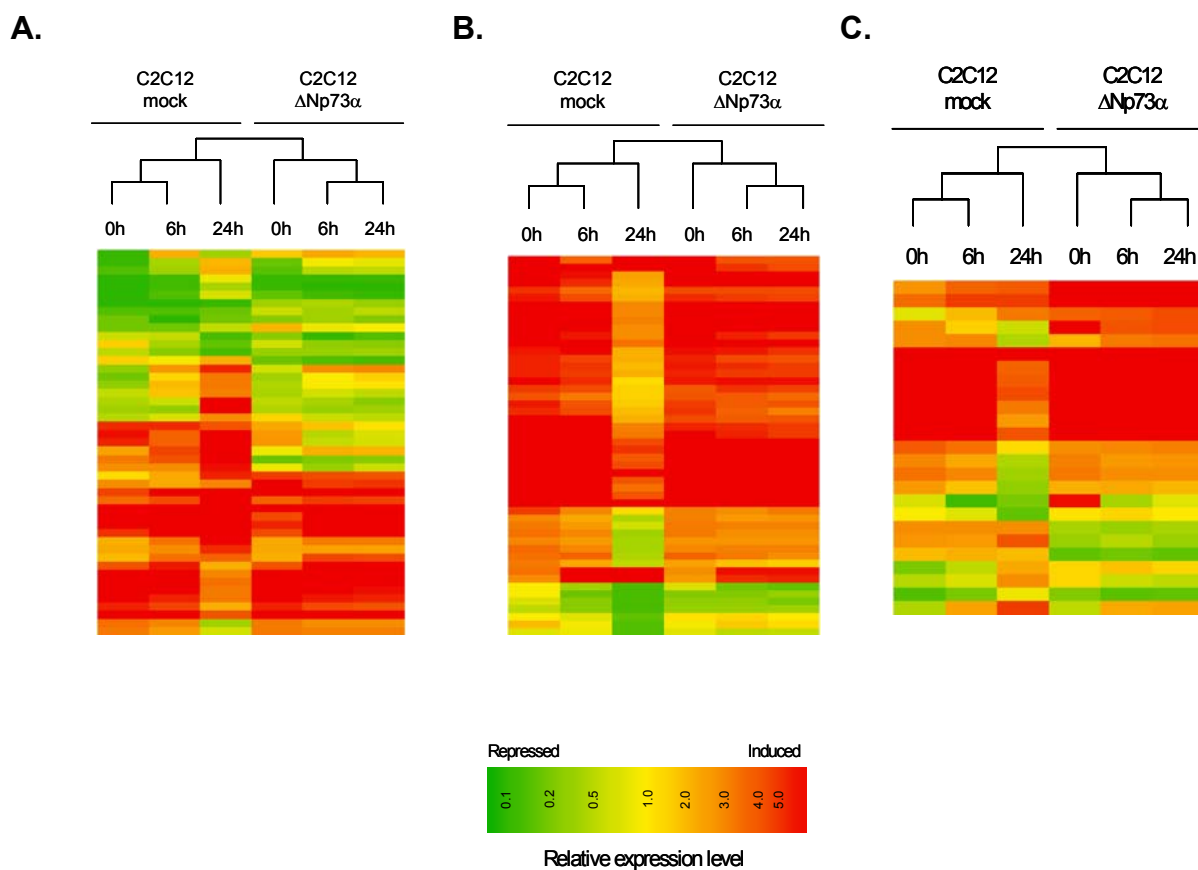


Figure 31. Expression profiles of muscle specific (A), cell cycle regulating (B) and p53 related target genes (C) that change their expression levels 3 fold during myogenic differentiation.

Although a number of genes known to be involved in myogenesis were identified, we took a further step to verify our microarray results. Thus, the expression of representative genes from muscle specific, cell cycle regulating and p53 related target genes were analyzed with semiquantitative RT-PCR. As shown in Figure 32, these genes show an expression pattern very similar to those detected by the microarray analysis.

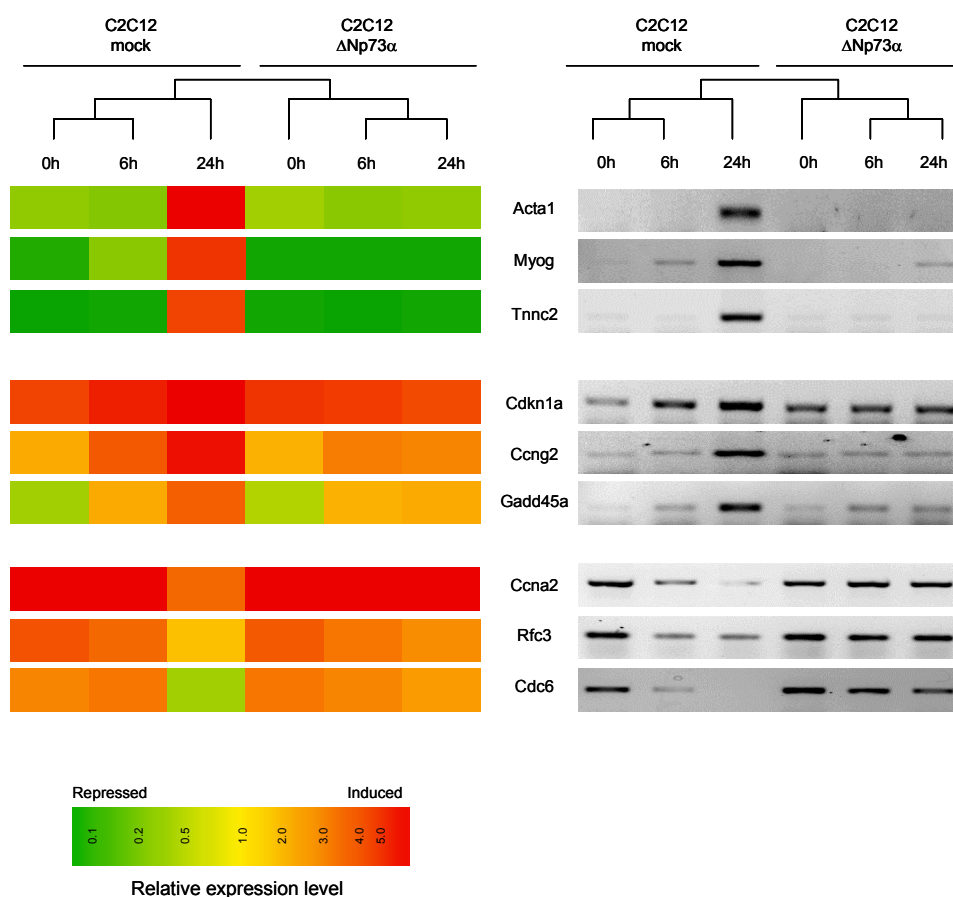


Figure 32. Semiquantitative RT-PCR analysis for the expression of muscle specific genes (Acta1, myogenin and Tnnc2), p53 target genes (Cdkn1a, Ccng2 and Gadd45a) and cell cycle related genes (Ccna2, Rfc3 and Cdc6).

Together, these data show on the basis of genome-wide expression profiles that Δ Np73 α does not interfere with the early, initiating steps but rather has profound impact on the progression to later stages of differentiation that require terminal cell cycle exit.

3.7. Δ Np73 inhibits late stages of myogenic differentiation

To locate the differentiation block more precisely, C2C12-mock and C2C12- Δ Np73 α myoblasts were allowed to differentiate for 0, 1, 2, 3, 5, and 7 days and harvested for semi-quantitative RT-PCR analysis of muscle specific differentiation markers. During *in vitro* differentiation, similar to mock cells, the expression of muscle specific transcription factors MyoD and Myf5 remained unchanged in the C2C12- Δ Np73 α myoblasts (Fig. 33). Whereas in C2C12-mock myoblasts the expression of myogenin, the major driving force for myogenic differentiation, was highly induced on day 1, its expression was present but delayed in C2C12- Δ Np73 α myoblasts (Fig. 33). Interestingly, downstream targets of myogenin, MEF2C, myosin (myHC) and muscle creatine kinases (CKM), were absolutely absent in C2C12- Δ Np73 α myoblasts (Fig. 33). The block to differentiation therefore occurs primarily after the myogenin expression step.

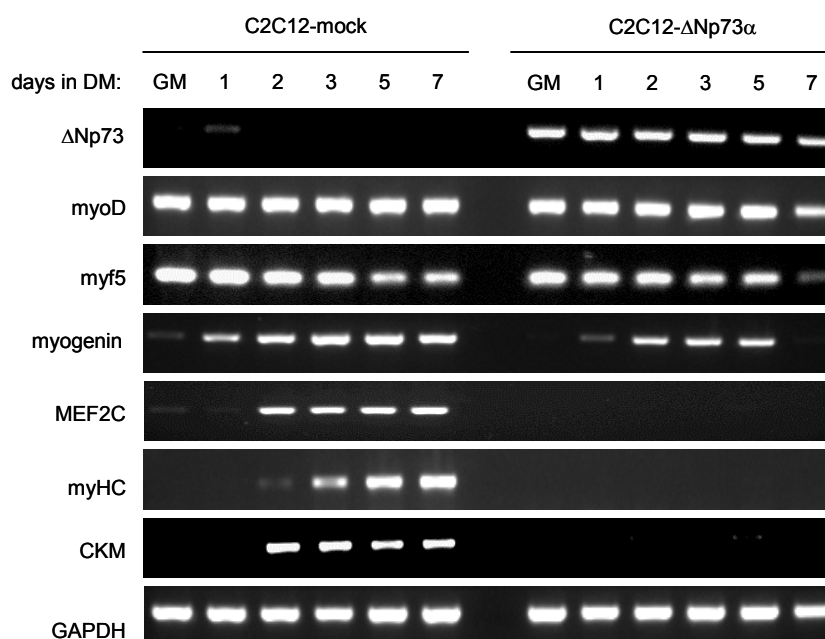


Figure 33. Δ Np73 inhibits late stages of myogenic differentiation.

C2C12-mock and - Δ Np73 α myoblasts were differentiated by culturing them in medium containing 2 % horse serum (DM). At the indicated days in DM, cells were harvested. The expression of muscle specific genes was analyzed by semiquantitative RT-PCR. Amplification of GAPDH demonstrates use of equal amounts of total RNA. H₂O, no template control.

It has been previously demonstrated that the retinoblastoma protein (RB) promotes myoblast cell cycle arrest and is required for high-level expression of late markers of skeletal muscle differentiation. However, expression of intermediate muscle differentiation markers, such as myogenin, is not effected by RB-loss (Shimizu-Yoshida et al., 2001). Thus, we analyzed RB activity in C2C12- Δ Np73 α myoblasts during myogenesis. Initially, we compared the differentiation potential of Δ Np73 α - expressing with RB-/- 3T3-fibroblasts in a myogenic conversion assay. *In vitro*, 3T3 fibroblasts can be induced to differentiate by transfection with MyoD, a myogenic basic helix-loop-helix (bHLH) transcription factor. Firstly, we generated Δ Np73 α expressing 3T3 cell lines by retroviral gene transfer. The expression of Δ Np73 was verified by western blot (Fig. 34A). Then, we compared the differentiation ability of 3T3-wt, 3T3- Δ Np73 α and 3T3-RB-/- fibroblasts following transduction with MyoD retrovirus containing supernatant. After hygromycin selection, cells were incubated for 3 days in differentiation medium and harvested for semiquantitative RT-PCR analysis. As shown in Figure 34B, MyoD induced myogenic differentiation only in wild-type 3T3 fibroblasts. In contrast, MyoD did not induce differentiation of 3T3- Δ Np73 α and 3T3-RB-/- fibroblasts, although both cell types expressed the intermediate muscle differentiation marker myogenin (Fig 34B).

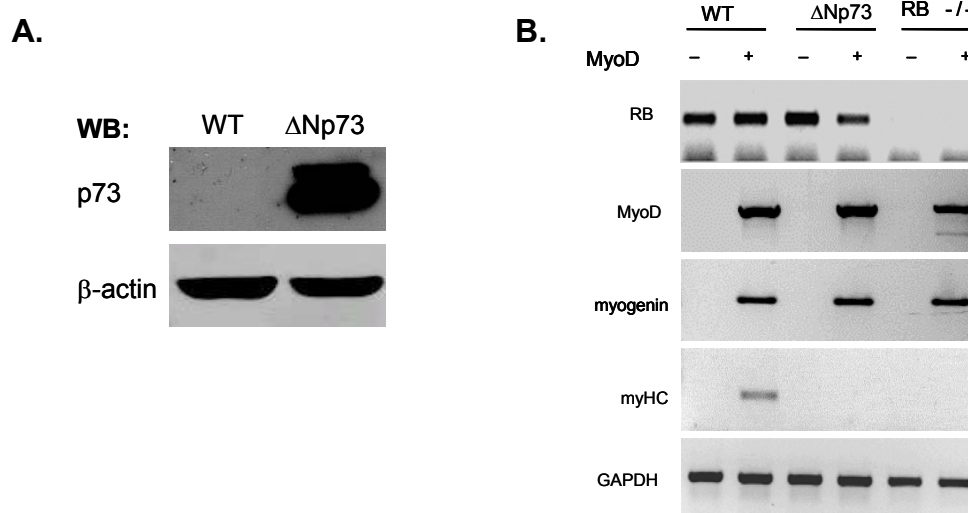


Figure 34. RB-loss and Δ Np73 α -expression block MyoD-induced differentiation at the myogenin step.

A. Stable expression of Δ Np73 α in 3T3 fibroblasts. After puromycin selection, cell extracts were subjected to immunoblot analysis for p73 (anti-p73, ER15). **B.** 3T3-wt, 3T3- Δ Np73 α and 3T3-RB-/- fibroblasts were transduced with MyoD(+) or empty (-) vector containing retroviral supernatant. After selection, cells were allowed to differentiate for 3 days and harvested for semiquantitative RT-PCR analysis.

Taken together, $\Delta Np73\alpha$ and RB-loss both block myogenic differentiations at the transition from the myogenin- to the myosin-positive state. The similar phenotype of RB-loss and $\Delta Np73\alpha$ expression suggests that RB function, which is required for the high level expression of late markers of differentiation, might be compromised by $\Delta Np73\alpha$.

3.8. C2C12- $\Delta Np73\alpha$ myoblasts show a defect in RB activation during myogenesis

Because the similar phenotype of RB-loss and $\Delta Np73\alpha$ -expression suggests that RB function might be compromised by $\Delta Np73\alpha$, we further analyzed RB expression in C2C12- $\Delta Np73\alpha$ myoblasts during myogenesis. C2C12-mock and C2C12- $\Delta Np73\alpha$ myoblasts were differentiated and harvested either for semi-quantitative RT-PCR or for western blot analysis. The induction of RB gene transcription is a key event in the process of skeletal muscle differentiation. As expected, the expression of the retinoblastoma gene *Rb1* was strongly induced in C2C12-mock cells at day 2 (Fig. 35A). In contrast, the upregulation of RB expression was abolished in C2C12- $\Delta Np73\alpha$ myoblasts (Fig. 35A). Furthermore, in differentiating wild-type myoblasts we observed an increase in protein amount and in electrophoretic mobility indicating a shift from inactive, hyperphosphorylated to active, hypophosphorylated RB species (Fig. 35B). This activation of RB was absent in $\Delta Np73\alpha$ myoblasts. Whereas the reduced levels of RB protein can be explained by transactivation defects of the *Rb1* gene (Fig. 35B), the failure to dephosphorylate RB has other reasons.

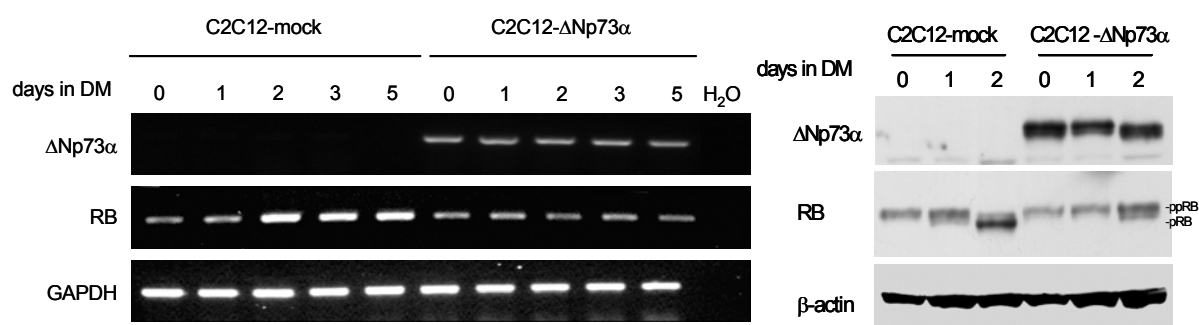


Figure 35. C2C12-ΔNp73 α myoblasts fail to activate RB during myogenesis.

C2C12-mock and -ΔNp73 α myoblasts were differentiated and harvested at the indicated time points. The expression of the retinoblastoma gene (*Rb1*) was analyzed by semiquantitative RT-PCR. Amplification of GAPDH demonstrates the use of equal amounts of total RNA. H₂O, no template control. **B.** At days 0, 1, and 2 of differentiation, cells were harvested. Cell extracts were subjected to immunoblot analysis for p73 (anti-p73, ER15) and RB (anti-RB, G3-245). ppRB: hyperphosphorylated RB ; pRB: underphosphorylated RB. The cell lysates were normalized for β -actin expression (anti-actin, ab6276).

In summary, C2C12-ΔNp73 α myoblasts fail to activate RB during myogenesis. Physiologically various cyclin-dependent kinases (CDKs), which are regulated by cyclin-dependent kinase inhibitors (CKI), control RB activity (Stevaux and Dyson, 2002). CKIs induce cell cycle arrest in response to anti-proliferative signals, including myogenic (Skapek et al., 1995), myeloid (Yaroslavskiy et al., 1999) and neural differentiation (Sasaki et al., 2000). Furthermore, it has been shown that mice deficient for the CKIs p21^{Cip1} and p57^{Kip2} display severe defects in skeletal muscle development (Zhang et al., 1999b). Thus, we analyzed these two CKIs in C2C12-mock and ΔNp73 α myoblasts during differentiation. Whereas mock myoblasts induce expression of both p21 and p57, expression of these CKIs is almost absent in ΔNp73 α myoblasts both on the mRNA and protein level (Fig. 36A, B). These findings provide a simple explanation for the inability to activate RB and the failure of ΔNp73 α myoblasts to exit the cell cycle and induce muscle-specific gene expression.

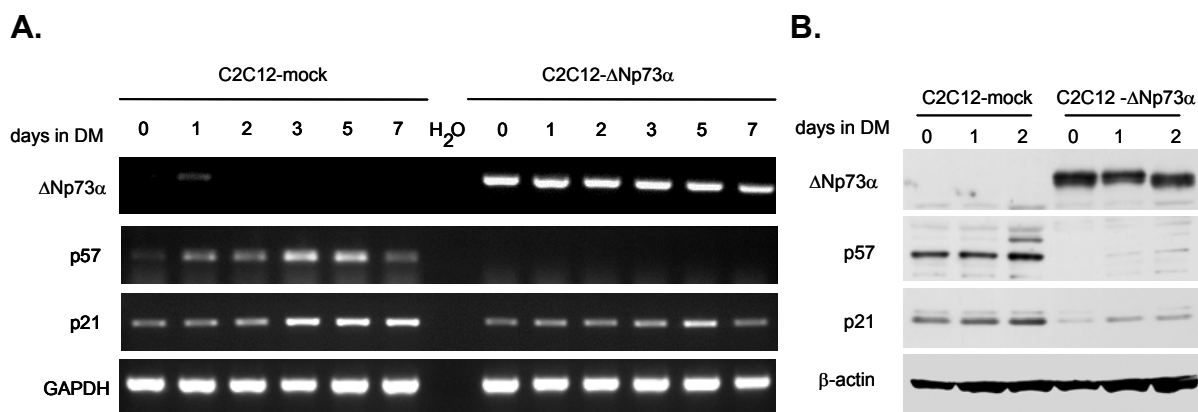


Figure 36. C2C12-ΔNp73α myoblasts show defects in the expression of the CDK inhibitors p21^{CIP1} and p57^{KIP2}.

A. C2C12-mock and C2C12-ΔNp73α cells were harvested for RNA isolation at the indicated time points. The expression of p21^{CIP1} and p57^{KIP2} genes was analyzed by semiquantitative RT-PCR. Amplification of GAPDH demonstrates the use of equal amounts of total RNA. H₂O, no template control **B.** Myoblasts were analyzed by western blot with antibodies against p73 (anti-p73, ER15), p21^{CIP1} (anti-p21, C-19) p57^{KIP2} (anti-p57, ab3223) and PCNA (anti-PCNA, sc56). The cell lysates were normalized for β-actin expression (anti-actin, ab6276).

3.9. Active RB rescues the differentiation defect of C2C12-ΔNp73α myoblasts

To investigate if the observed defect in RB inactivation is causally related to the block in differentiation, we tried to rescue the differentiation defect with ectopic expression of RB. 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). After infection, cells were allowed to differentiate for 3 days. Subsequently, cells were photographed under a fluorescence microscope and harvested for western blot analysis. As shown in Figure 37A, C2C12-ΔNp73α cells infected with AdGFP or AdGFP-RBwt did not show any signs of differentiation. In contrast, C2C12-ΔNp73α cells infected with AdGFP-RBmut(PS) formed differentiated myotubes (Fig. 37A). Consistent with this observation, only C2C12-ΔNp73α myoblasts expressing constitutively active RB expressed the differentiation marker

myosin. Importantly, wild-type RB was inactivated by hyperphosphorylation and therefore unable to induce differentiation (Fig. 37B).

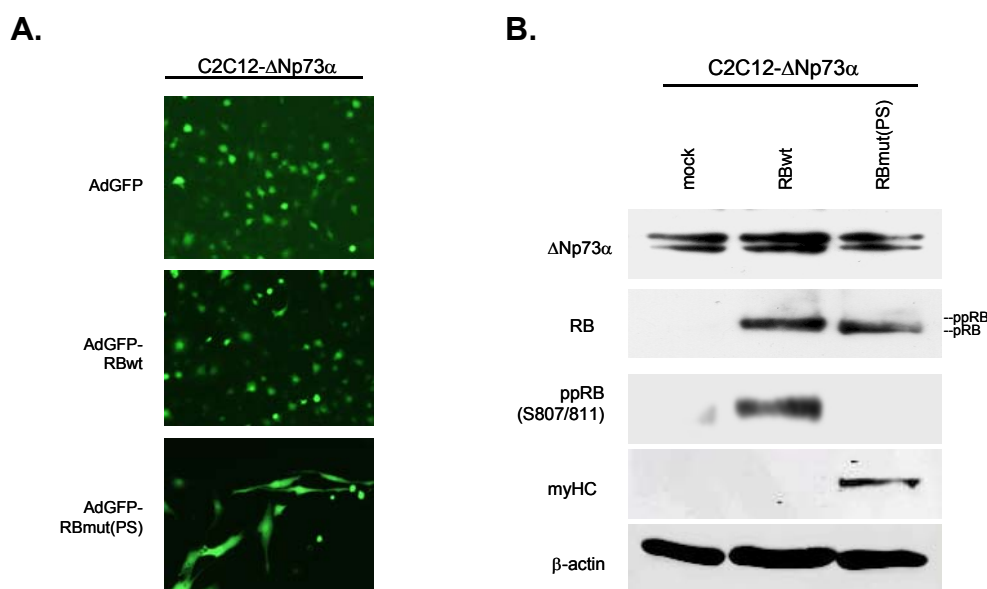


Figure 37. The introduction of constitutively active RB rescues the differentiation defect of C2C12- Δ Np73 α myoblasts.

A. C2C12- Δ Np73 α myoblasts were infected with the adenoviral vectors Ad-GFP, AdGFP-RBwt and AdGFP-RBmut(PS). After infection, cells were allowed to differentiate for 3 days and observed under the fluorescence microscope. **B.** At day 3 after differentiation, cells were harvested for western blotting with antibodies against p73 (ER15), RB (G3-245), ppRB (anti phospho-RB, s807/811), myHC (MF20). The cell lysates were normalized for β -actin expression (anti-actin, ab6276).

However, in combination with p57 (AdGFP-p57), hyperphosphorylation of wild-type RB was prevented resulting in expression of MHC (Fig. 38). Thus, active RB, either in form of a constitutively active mutant or as a combination of wild-type RB with p57, rescues the Δ Np73 α -induced differentiation block proving that defects in activating RB are indeed the underlying cause.

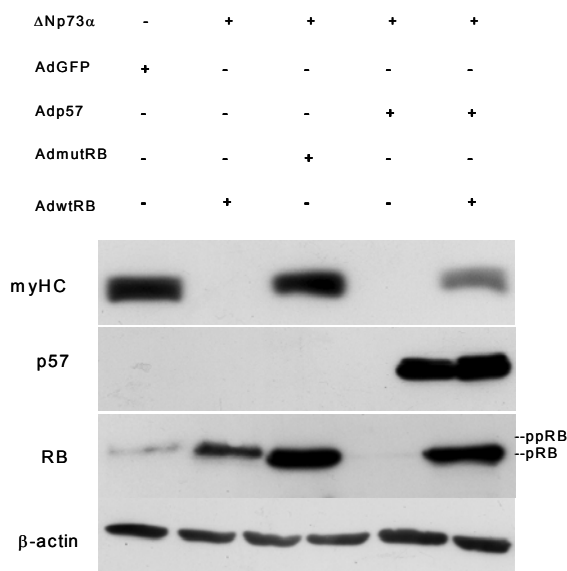


Figure 38. Combination of p57 with wtRB rescues differentiation defect of C2C12- Δ Np73 α myoblasts.

C2C12- Δ Np73 α myoblasts were infected with the adenoviral vectors AdGFP, AdGFP-p57, AdGFP-RBwt, and AdGFP-Rbmut(PS) alone or in the indicated combinations. After adenovirus infection, cells were allowed to differentiate for 3 days. At day 3 of differentiation, cells were harvested for western blotting with antibodies against myHC (MF20), p57 (ab3223), and RB (G3-245). The cell lysates were normalized for β -actin expression (anti-actin, ab6276).

To test, whether rhabdomyosarcoma cells and C2C12- Δ Np73 α myoblasts have a similar defect in myogenesis. Rh30 cells were infected with AdGFP, AdGFP-RBwt, AdGFP-Rbmut(PS), AdGFP-p57 or a combination of AdGFP-RBwt and AdGFP-p57. After infection, cells were allowed to differentiate for 3 days. Subsequently, Rh30 cells were observed under fluorescence microscope and harvested for western blot analysis. As shown in Figure 39A, Rh30 cells infected with Ad-GFP, AdGFP-RBwt, AdGFP-p57 did not show any signs of differentiation. In contrast, Rh30 cells expressing the constitutively active form of RB or a combination of AdGFP-RBwt and AdGFP-p57 exhibit morphological and biochemical signs of differentiation (Fig. 39A and B). Similar to Δ Np73 α myoblasts, these results provide a simple explanation for the inability of Rh30 cells to differentiate due to inactivation of RB.

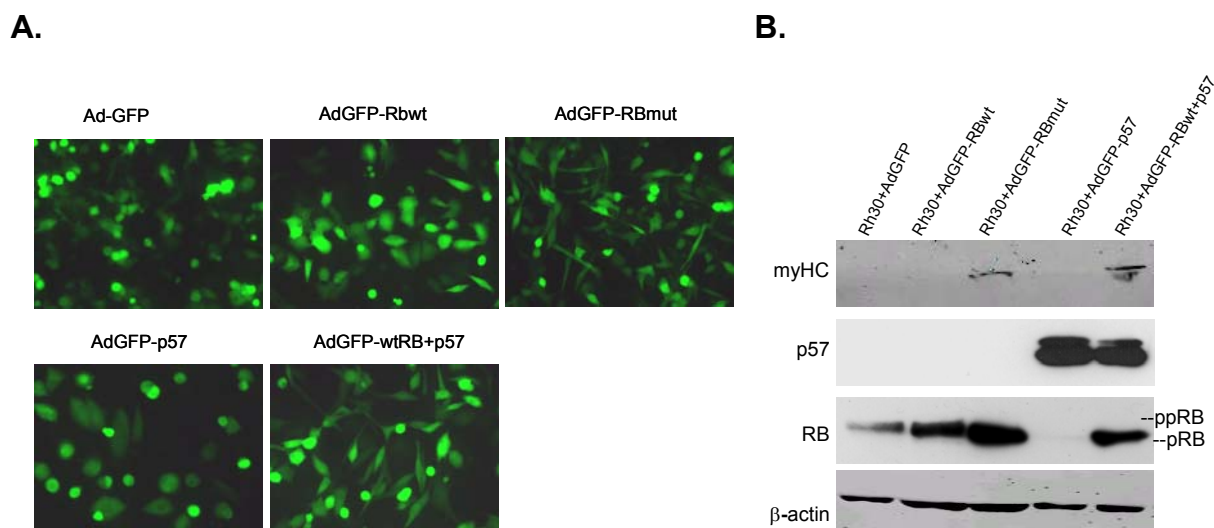


Figure 39. Combination of p57 with wtRB rescues differentiation defect of Rh30 rhabdomyosarcoma cells.

A. The Rhabdomyosarcoma cell line Rh30 was infected with AdGFP as a control, AdGFP-RBwt, AdGFP-Rbmut(PS), AdGFP-p57 or a combination of AdGFP-p57 and AdGFP-RBwt. After adenovirus infection, cells were allowed to differentiate for 3 days and observed under the fluorescence microscope. **B.** At day 3 of differentiation, cells were harvested for western blotting with antibodies against, myHC (MF20), p57 (ab3223), RB (G3-245). The cell lysates were normalized for β -actin expression (anti-actin, ab6276).

3.10. p53 family members are activated during muscle differentiation *in vitro* and muscle regeneration *in vivo*

Since $\Delta Np73\alpha$ functions as a dominant-negative inhibitor of the transactivation-competent p53 family members, $\Delta Np73\alpha$ might inhibit the activation of RB by interfering with essential functions of the p53 family in this process. In murine C2C12 myoblasts myogenic differentiation induced by growth factor withdrawal is associated with increasing expression of all three p53 family members (Fig. 40). Whereas p53 transcription progressively increases during the first 36 hours, expression of p63 and p73 peaks between 6 and 12 hours of differentiation (Fig. 40).

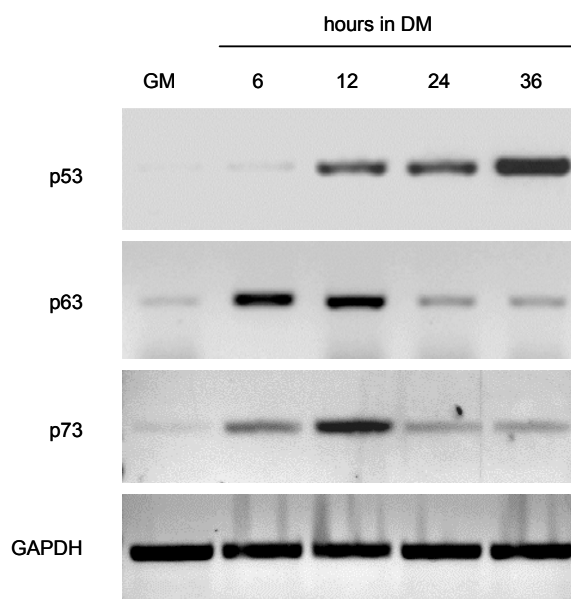


Figure 40. p53 family members are activated during muscle differentiation.

C2C12 cells were induced to differentiate into myotubes by incubation in medium containing 2% horse serum. RNA was isolated from the cells at the indicated time points (hours) and expression of p53, p63 and p73 was measured by semiquantitative RT-PCR. Expression of the housekeeping gene GAPDH is shown as a control.

Furthermore, we analyzed the expression of all three p53 family members in primary murine muscle satellite cells. As shown in Figure 41A, similar to C2C12 myoblasts, p53 transcription progressively increases during the first 48 hours. In addition, both p63 and p73 expression peaks early during the differentiation process. By using isoform-specific primer pairs, we detected that expression of TAp73 peaked between 6 and 12 hours of differentiation, whereas the Δ Np73 isoform was expressed at low but constant levels (Fig. 41C). Interestingly, Δ Np63 expression was increased at 24h during differentiation of primary myoblasts, whereas expression of TAp63 remained unchanged over the time course of differentiation (Fig. 41B). Isoform-specific upregulation of individual p53 family members during the myogenic differentiation process suggests possible functions in differentiation control.

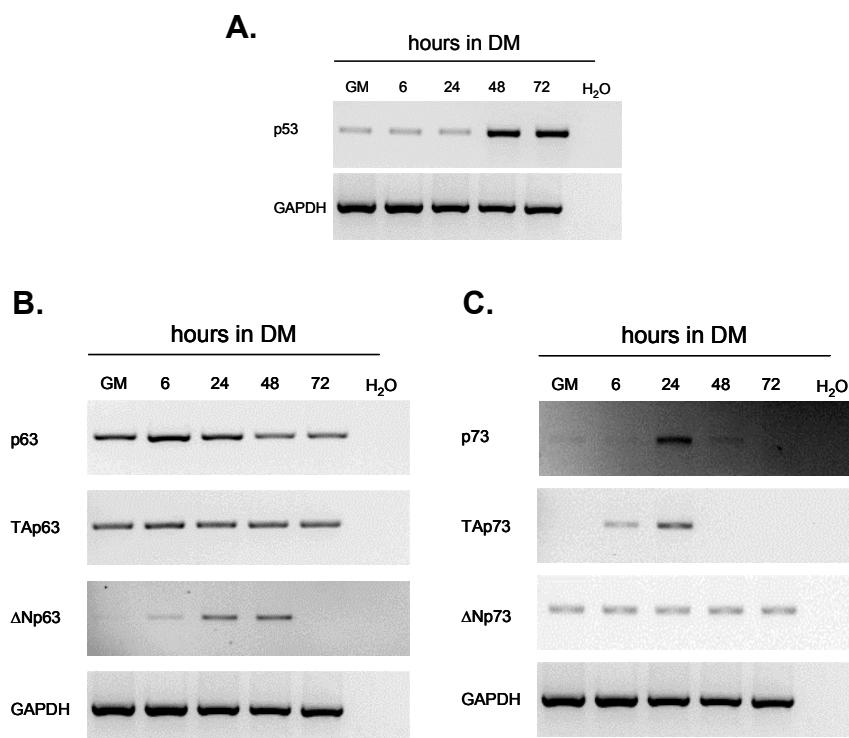


Figure 41. p53 family members are activated during muscle differentiation.

Primary murine muscle satellite cells were either incubated in growth medium (GM) or induced to differentiate for 6, 12, 24, 48 and 72 hours in differentiation medium (DM). At the indicated time points, cells were analyzed for the expression of total p53, p63, p73 and the TAp63, TAp73, ΔNp63 and ΔNp73 isoforms by semi-quantitative RT-PCR analysis. Amplification of GAPDH demonstrates the use of equal amounts of total RNA. H₂O, no template control.

Furthermore, we analysed the expression of p53 and p63/p73 during muscle regeneration in an *in vivo* muscle injury model. Upon injury, muscle satellite cells reenter the cell cycle, proliferate, and then exit the cell cycle either to renew the quiescent satellite cell pool or to differentiate into mature myofibers. To induce muscle regeneration, we injected cardiotoxin into tibialis anterior (TA) muscles of 6 week-old C57BL/6 mice. As shown in Figure 42, histological analysis demonstrated global myofiber fragmentation and edema at days 1 and 2 after injury. The number of mononucleated cells/cross-section area increased significantly after cardiotoxin injection with a peak around day 3. This increase in cell number is attributable to both inflammatory cell infiltration and proliferation of satellite cells. Myotubes started to appear at day 5 and became more evident at days 7 and 10 postinjection. The morphology at day 14 was characterized by the presence of central nuclei, a known

hallmark of recent muscle regeneration in nearly all myofibers. The regeneration process shifted morphologically from a phase of proliferation to differentiation at around day 3 after injury.

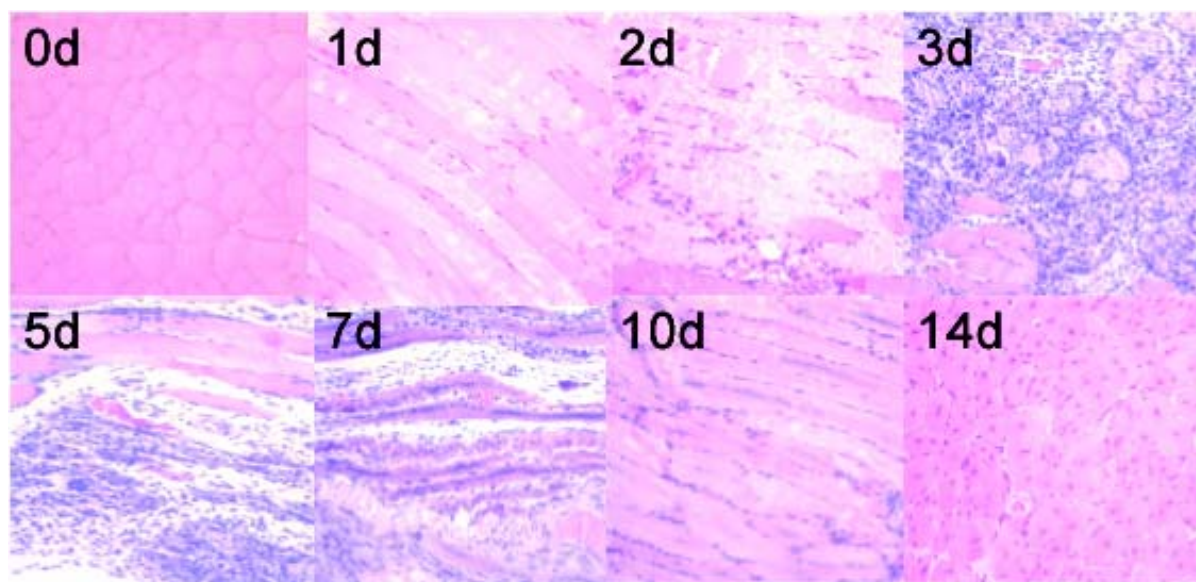


Figure 42. Histological analysis of muscle regeneration *in vivo*.

Tibialis anterior muscles of 6 week old C57/BL6 mice were injected with 100 μ l of 10 μ M cardiotoxin, which induces necrolysis of myofibers without damaging satellite cells. At the indicated days, the muscles were harvested, fixed in 4% paraformaldehyde, and embedded in paraffin. Histological sections were prepared for hematoxylin/eosin staining by standard methods.

To investigate the gene expression of p53 family members during muscle regeneration, RNAs were isolated from cardiotoxin injected muscles. As shown in Figure 43, muscle regeneration induced by cardiotoxin is associated with changes in the expression of all three p53 family members. After an initial increase in p53 transcription (most likely due to toxic effect of cardiotoxin), p53 expression peaked at the days 3 to 10 when new myotubes began to develop. In contrast to p53, p73 expression strongly increased before RB, p21, and p57, which are all essential for cell proliferation or myogenesis, were upregulated (on day 3). In contrast, p63 expression was induced between days 3 and 5. Taken together the results show that p53 family members were activated during both muscle differentiation *in vitro* and muscle regeneration *in vivo*.

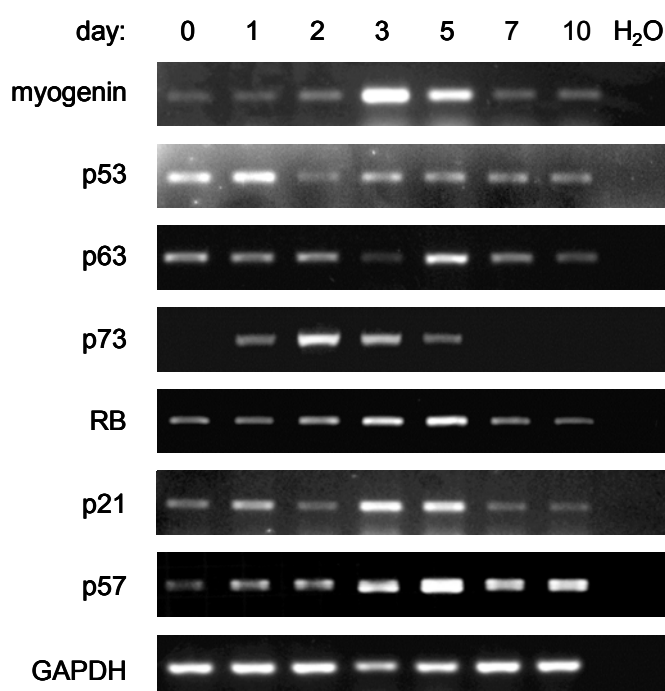


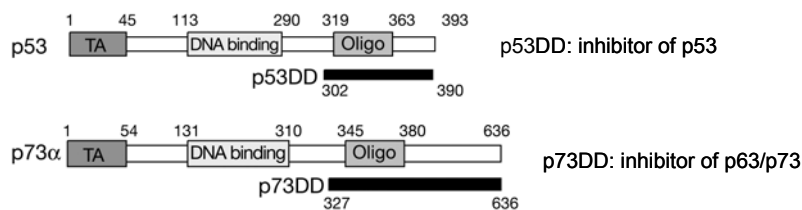
Figure 43. p53 family members are activated during muscle regeneration.

Muscle regeneration was induced by intramuscular injection of cardiotoxin in 6 week-old C57BL/6 mice. Total RNA was extracted at the indicated days and analyzed for the expression of p53, p63, p73, RB, p21, and p57 by semi-quantitative RT-PCR analysis. Amplification of GAPDH demonstrates the use of equal amounts of total RNA. H₂O, no template control.

3.11. Interference with p53 family members compromises myogenic differentiation

To evaluate, whether the p53 family members transcriptional activity is required for the induction of muscle differentiation, we used dominant-negative mini-proteins of p53 (p53DD) or p63/73 (p73DD), which allowed us to characterize the specific function of either p53 or p73 during myogenesis. These dominant-negative proteins are short C-terminal fragment of p53 or p63/73 that can complex with wild-type p53 or p63/73 and inhibit their activities (Fig. 44A). Thus, C2C12 myoblasts were transduced with p53DD, p73DD or a combination of p53DD and p73DD retrovirus containing supernatant. The expression of dominant-negative proteins in C2C12 myoblasts was verified by western blot (Fig. 44B).

A.



B.

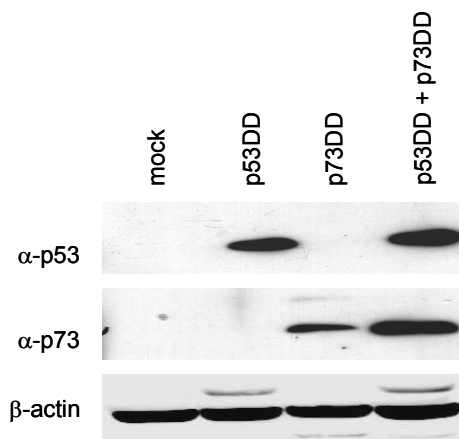


Figure 44. Dominant-negative inhibitors of p53 and p63/p73.

A. p53DD (inhibitor of p53) and p73DD (inhibitor of both p63 and p73) are short C-terminal fragments of p53 or p63/p73 that can complex with wild-type p53 or p63/p73 and inhibit their activities (Irwin et al., 2000). **B.** Expression of dominant-negative proteins in C2C12 myoblasts. After retroviral infection and subsequent selection, cell extracts were subjected to immunoblot analysis for p53 (anti-p53, DO1), p73 (anti-p73, ER15) and β -actin (anti-actin, ab6276).

To analyze, whether the inhibition of p53 family members by dominant negative proteins interferes with myogenic differentiation, the cell lines were allowed to differentiate for 5 days. The cultures were examined by immunofluorescence staining (Fig. 45A) and western blot (Fig. 45B) for the differentiation marker myHC (myosin heavy chain). As shown in Figure 45B, in comparison to mock cells, a reduction of ~65% in the differentiation indices was observed in p53DD-expressing myoblasts, whereas p73DD-myoblasts showed a reduction of ~60%. Interestingly, expression of both dominant-negative proteins (p53DD + p73DD) mimicked the effect of Δ Np73 α and blocked the differentiation process almost completely.

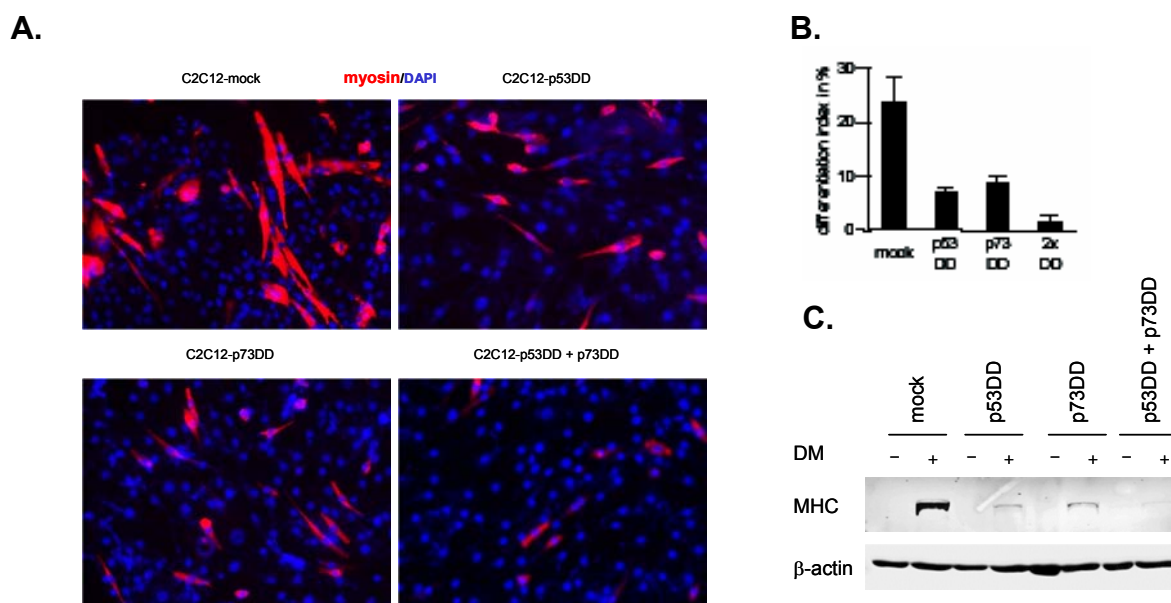


Figure 45. Expression of dominant-negative inhibitors of the p53 family interferes with myogenic differentiation.

A. Immunofluorescence staining for MHC of C2C12 cell lines expressing the indicated dominant-negative inhibitors of p53 family members. The cells were fixed on day 5 of differentiation and stained for MHC (red). The cells were counterstained with DAPI to visualize nuclei. **B.** The differentiation index was calculated as described in Materials and Methods. **C.** Immunoblot for myHC (anti-myHC, MF20). The amount of protein in each sample was normalized for β -actin (anti-actin ab6276) expression.

These results indicate that the two inhibitors have additive functions supporting the hypothesis that p53 and p63/p73 have different but complementary functions in differentiation control. To delineate these different functions of p53 and p63/73 at the molecular level, dominant-negative inhibitor expressing myoblasts were analyzed for expression of several differentiation-regulated genes. Total RNA was extracted from control and dominant-negative protein expressing C2C12 myoblasts incubated in the presence of differentiation medium. As shown in Figure 46, similar to Δ Np73 α expressing myoblasts, the two inhibitors (alone or in combination) have no significant effect on myogenin or MyoD expression, but interfere with expression of myHC or muscle creatin kinase (MCK) (Fig. 46).

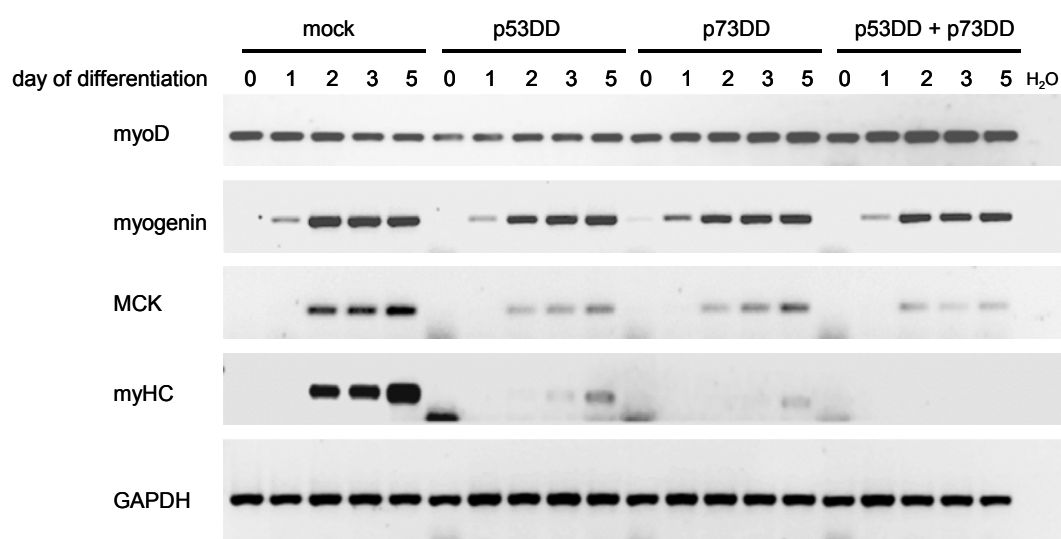


Figure 46. Dominant-negative inhibitors of p53 family members inhibit late muscle specific gene expression.

C2C12-mock, C2C12-p53DD, C2C12-p73DD and C2C12-p53DD+p73DD myoblasts were differentiated in medium containing 2 % horse serum. At the time indicated days, cells were harvested and expression of muscle specific genes was analyzed by semiquantitative RT-PCR. Amplification of GAPDH demonstrates the use of equal amounts of total RNA. H₂O, no template control

Furthermore, we investigated the expression of RB and p57. As expected, RB and p57 mRNA levels increase (Fig. 47A) and RB becomes hypophosphorylated (activated) during muscle differentiation in mock cells (Fig. 47B). Whereas p73DD myoblasts only induced the expression of RB, p53DD myoblasts induced the expression of p57 (Fig. 47A). Importantly, expression of both RB and p57 in p53DD+p73DD myoblasts is almost absent both on the mRNA and protein level (Fig 47A and B). Taken together, RB induction is primarily compromised by p53DD, whereas p57 induction is impaired by p73DD. This confirms that the p53 family members coordinate progression to later stages of differentiation by regulating RB.

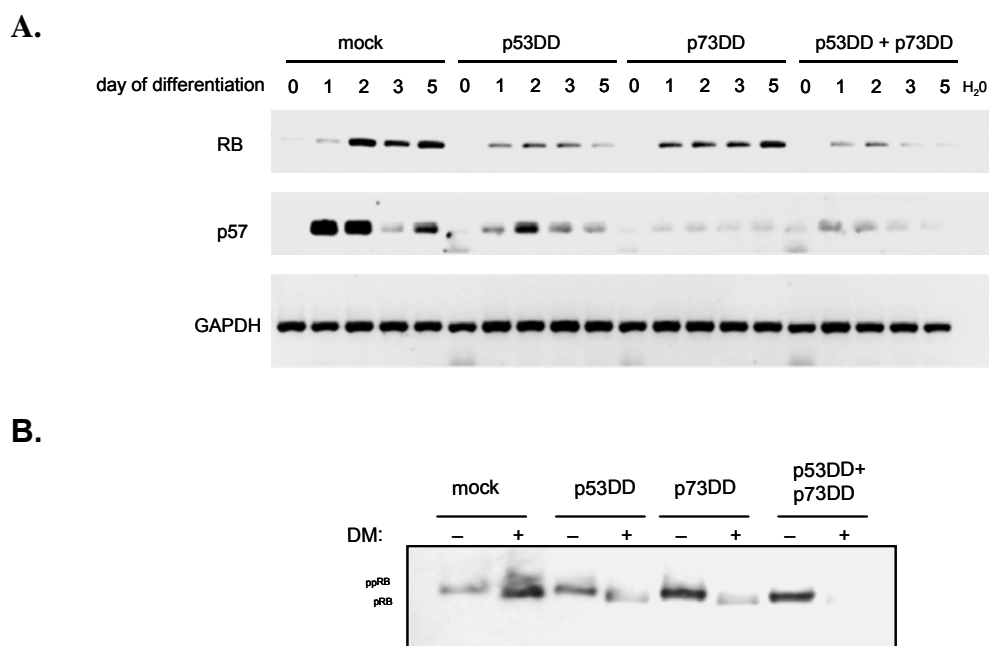


Figure 47. p53 family members coordinate progression to later stages of differentiation by regulating RB activity

C2C12-mock, C2C12-p53DD, C2C12-p73DD and C2C12-p53DD+p73DD myoblasts were differentiated in medium containing 2 % horse serum. At the indicated days, cells were harvested and expression of RB and p57 was analyzed by semiquantitative RT-PCR. Amplification of GAPDH demonstrates the use of equal amounts of total RNA. H₂O, no template control **B**. Proteins were extracted from the indicated cells and RB (anti-RB, G3-245) protein was analyzed in a western blot.

To rule out effects of p53DD and p73DD unrelated to their dominant-negative function, we also used RNA interference as an independent method. C2C12 myoblasts were transfected with shRNA constructs directed against p53, p63, and p73. A non-silencing shRNA was used as a control. Efficient knock-down was confirmed by RT-PCR (Figure 48).

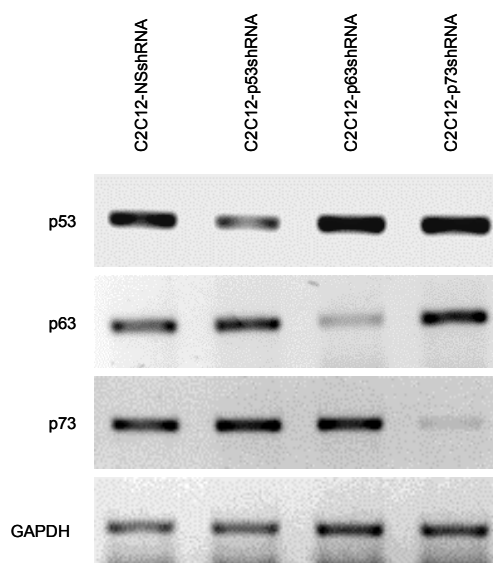


Figure 48. RNAi ablation of endogenous p53 family members in C2C12 cells

C2C12 myoblasts were transfected with shRNA constructs directed against p53, p63, and p73. A non-silencing shRNA was used as a control. After puromycin selection, successful downregulation of the targeted genes was verified by RT-PCR. Amplification of GAPDH demonstrates the use of equal amounts of total RNA.

To analyze, whether specific knock-down of single p53 family members impaired differentiation similar to expression of dominant-negative inhibitors, cells were induced to differentiate and harvested for semi-quantitative RT-PCR. As shown in Figure 49, specific knock-down of any single p53 family member impaired the expression of the muscle differentiation marker myHC. Consistent to our dominant-negative experiments, inhibition of p53 primarily compromised induction of RB, whereas shRNAs directed against either p63 or p73 interfered with p57 induction (Fig. 49).

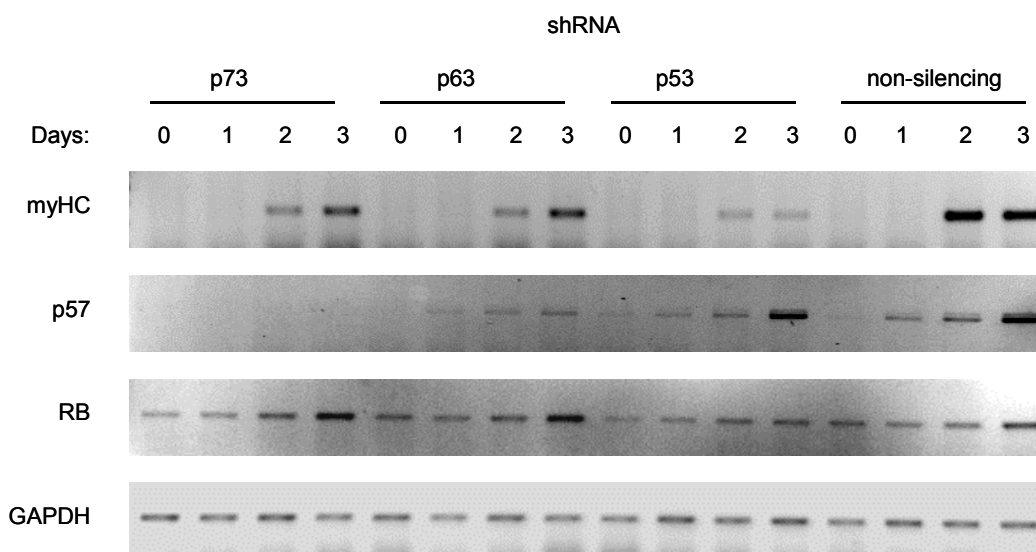


Figure 49. Specific knock-down of single p53 family members impaired differentiation

Myoblasts expressing the indicated shRNAs were differentiated in medium containing 2 % horse serum. At the indicated time points, cells were harvested and analyzed for expression of myosin (myHC), p57 and RB by semiquantitative RT-PCR. Amplification of GAPDH demonstrates the use of equal amounts of total RNA.

To investigate further p53- and p73-specific roles in myogenesis, primary myoblasts were explanted from both p53^{-/-} and p73^{-/-} knockout mice and compared to wild-type myoblasts. The isolated myoblasts were incubated either in growth medium or induced to differentiate for 6, 24, 48 or 72 hours. Subsequently, the cells were analyzed for the expression of the late muscle differentiation marker gene myosin (myHC), p57 and RB by semi-quantitative RT-PCR. As shown in Figure 50, consistent with dominant-negative or RNAi-mediated inhibition of p53 in C2C12 cells, p53^{-/-} primary myoblasts showed reduced expression of myosin (myHC) in comparison to wild-type (p53^{+/+}) myoblasts. Furthermore, our semi-quantitative RT-PCR analysis showed that p53^{-/-} primary myoblasts did not upregulate RB, whereas p57 upregulation was similar to wild-type myoblasts. These results demonstrate that p53 is essential for activating RB transcription.

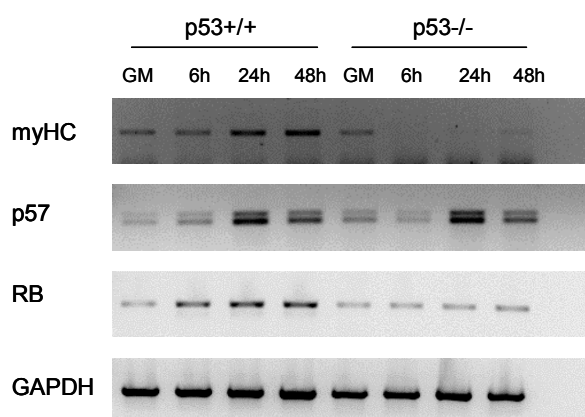


Figure 50. p53^{-/-} myoblasts have reduced differentiation capacity

Primary p53^{-/-} and wild-type (p53^{+/+}) myoblasts were incubated either in growth medium (GM) or induced to differentiate for 6, 24 and 48 hours in differentiation medium (DM). At the indicated hours, cells were harvested to analyze expression of the late muscle differentiation marker gene myosin (myHC), p57 and RB by semi-quantitative RT-PCR.

Interestingly, we observed no differentiation defect in p73^{-/-} myoblasts. As shown in Figure 51, primary p73^{-/-} myoblasts expressed muscle structural genes, like myosin (myHC), and the cell cycle related genes p57 and RB at similar levels as wild-type myoblasts. Since p63 and p73 have similar activities in regulating p57 expression in C2C12 cells, it can be suspected that p63 compensates for p73-loss.

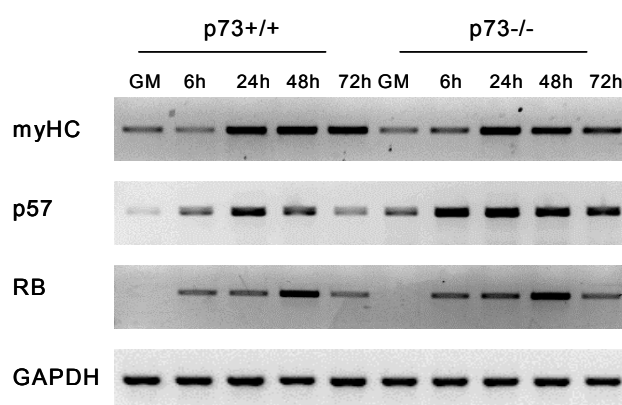


Figure 51. Absence of differentiation defect in p73^{-/-} myoblasts

Primary p73^{-/-} and wild-type (p73^{+/+}) myoblasts were incubated either in growth medium (GM) or induced to differentiate for 6, 24, 48 and 72 hours in differentiation medium (DM). Expression of the late muscle differentiation marker gene myosin (myHC), p57 and RB was analyzed by semi-quantitative RT-PCR.

3.12. Regulation of Rb and p57 expression by p53 family members

We further investigated regulation of RB and p57^{KIP2} expression by the p53 family. C2C12 myoblasts were electroporated the expression plasmids encoding p53, TAp73 α , TAp73 β , Δ Np73 α , Δ Np63 α and TAp63 γ . As shown in Figure 52, the major transactivating p53 family members (p53, TAp73 β , TAp63 γ) induced transcription of the *Rb1* gene, which has a typical p53 binding site in the promoter region. The difference between TAp73 α and TAp73 β can most likely be attributed to transactivation inhibitory functions located in the C-terminus of the α -isoform (Liu and Chen, 2005). In contrast, consistent with recent reports, p57^{KIP2} is transactivated only by TAp73 β and Δ Np63 α but not by p53 (Beretta 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 TAp73 β and Δ Np63 α . Our experiment further revealed, that although both TAp73 α and Δ Np73 α fail to transactivate RB and p57^{KIP2}, only Δ Np73 α has dominant-negative activity when co-expressed with p53 or TAp73 β (Fig. 52B).

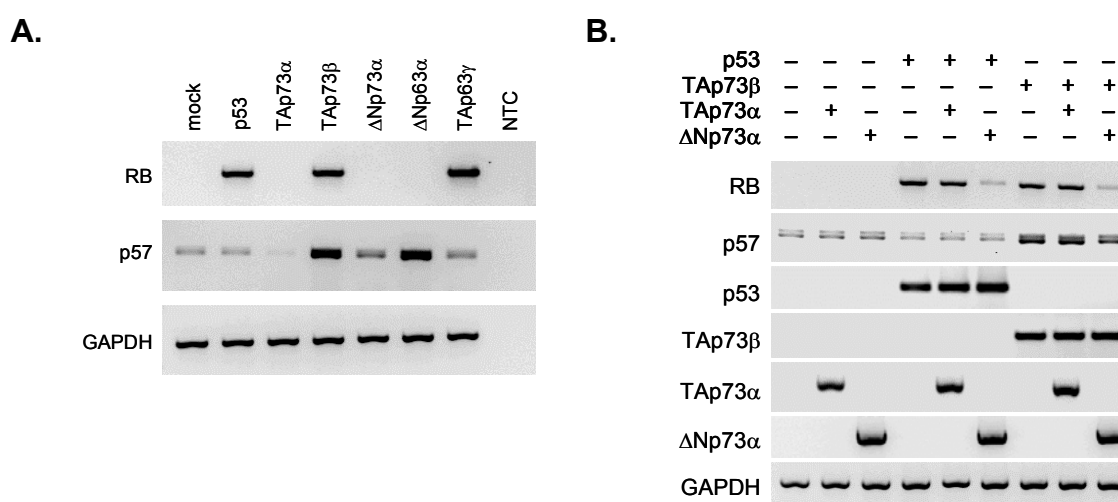


Figure 52. Regulation of RB and p57 expression by p53 family members.

C2C12 myoblasts were electroporated with the indicated expression plasmids. Subsequently, cells were harvested for semi-quantitative RT-PCR.

To analyze if the p53 family members contribute to RB and p57 regulation during myogenic differentiation, we investigated recruitment of p53, p63 and TAp73 to the respective promoter regions by chromatin immunoprecipitation. As shown in Figure 53, upon differentiation p53 associated with the RB promoter and p63 and TAp73 were recruited to the p57^{KIP2} promoter, whereas none of the investigated proteins was bound to these promoters in proliferating myoblasts.

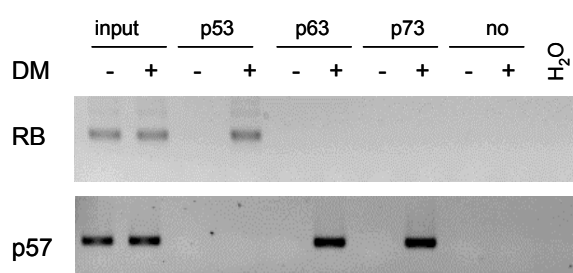


Figure 53. Recruitment of p53 family members to the RB and p57 promoter regions *in vivo*.

C2C12 cells were maintained in the presence of GM or DM for 2 days. Proteins were cross-linked to DNA by direct incubation of living cell with formaldehyde. Genomic DNA was extracted, immunoprecipitated with anti-p53, p63, p73 or without antibody and the bound promoter sequences were amplified from the immunoprecipitate by PCR.

Taken together these data correlate with the dominant-negative and RNAi 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/p73 on the other side are in control of p57. The two p53 family functions are complementary and cooperate to fully activate RB which is essential for efficient myogenic differentiation.

3.13. Δ Np73 interferes with osteoblastic and neuronal differentiation

To evaluate whether the inhibition of differentiation by Δ Np73 α is limited to myogenesis or represents a more general phenomenon, we analyzed the effect of

Δ Np73 α overexpression on osteoblastic and neuronal differentiation processes. It is known that bone morphogenetic protein-2 (BMP2) converts the myogenic differentiation pathway of C2C12 myoblasts into that of osteoblast lineage. To induce osteoblasts differentiation, C2C12-mock and C2C12- Δ Np73 α myoblasts were differentiated for 5 days in medium containing 5% FCS in the absence or presence of 300 ng/ml BMP2 and harvested for semi-quantitative RT-PCR analysis or stained for the osteoblast marker gene alkaline phosphatase. As shown in Figure 54A, differentiation of mock myoblasts in the presence of BMP2 almost completely inhibited the formation of multinucleated, myosin heavy chain expressing myotubes, and induced the appearance of numerous alkaline phosphatase (ALP) expressing, blue-stained cells. By contrast, no ALP-positive cells were seen in C2C12- Δ Np73 α myoblasts. Furthermore, semi-quantitative RT-PCR showed that BMP2 induced expression of osteoblastic differentiation markers such as alkaline phosphatase (ALP) or osteocalcin only in mock cells but not in C2C12- Δ Np73 α myoblasts (Fig. 54B).

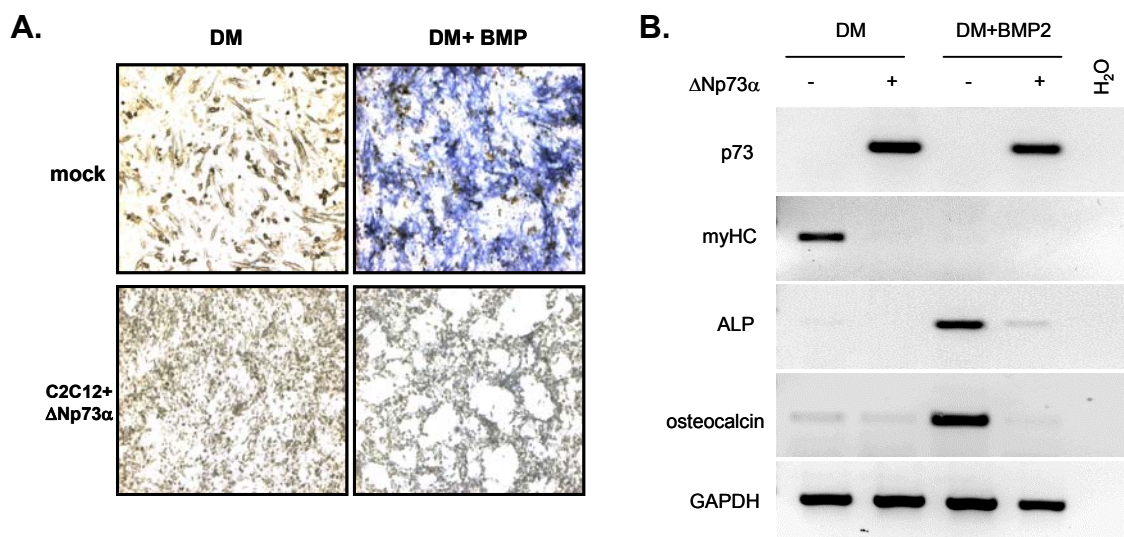


Figure 54. Δ Np73 α interfered not only with the myogenic differentiation program but also effectively inhibited BMP2 induced conversion into the osteoblast lineage.

A. C2C12-mock and - Δ Np73 α myoblasts were maintained in 5% FCS. To induce osteoblastic differentiation, 300 ng/ml BMP2 was added. Subsequently, cultures were allowed to differentiate for 5 days. After five days, cells were stained for the osteoblast marker gene alkaline phosphatase and photographed at 10x magnification under the phase-contrast microscope. **B.** Cells were harvested and analyzed by semi-quantitative RT-PCR for the expression of muscle (myHC) or osteoblast (alkaline phosphatase, ALP ; osteocalcin) marker genes.

To evaluate the effect of overexpressed $\Delta\text{Np73}\alpha$ on neuronal differentiation, we used human SH-SY5Y neuroblastoma cells. These cells have a basal neuroblast-like morphology with rounded cell bodies and occasional short processes, but they differentiate into a neuronal-like phenotype on contact with retinoic acid (RA) or neurotrophic factors. SH-SY5Y neuroblastoma cells were transfected with pQCXIP (mock) or pQCXIP- $\Delta\text{Np73}\alpha$ plasmids. After puromycin selection, the expression of $\Delta\text{Np73}\alpha$ in C2C12 was verified by western blot (Fig. 55A). To induce neuronal differentiation, the cells were differentiated for seven days in the presence of 10 μM retinoic acid (RA). In the presence of retinoic acid (RA), SH-SY5Y-mock cells showed neurite extension as a morphological marker of neuronal differentiation, whereas SH-SY5Y- $\Delta\text{Np73}\alpha$ cells remained in a basal neuroblast-like morphology with rounded cell bodies (Fig. 55B). Consistent with this observation, RA treatment strongly induced expression of neurofilament as a biochemical marker of neuronal differentiation in SH-SY5Y-mock cells, whereas its expression was significantly reduced in $\Delta\text{Np73}\alpha$ transfectants. (Fig. 55C). In summary, these data clearly show that the p53 family inhibitor $\Delta\text{Np73}\alpha$ is a potent repressor of differentiation in multiple experimental settings including myogenic, osteoblastic and neuronal differentiation.

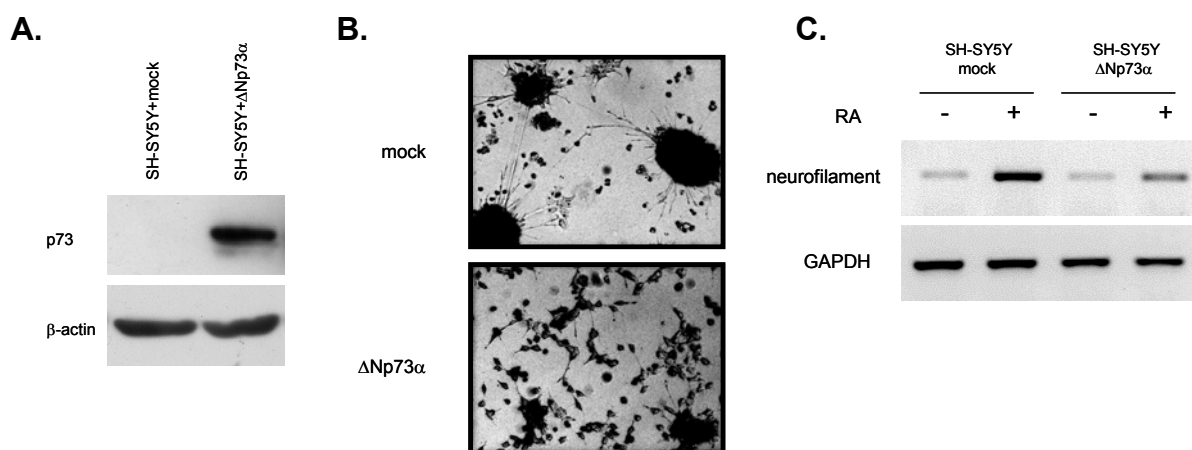


Figure 55. Ectopic expression of $\Delta\text{Np73}\alpha$ blocks retinoic acid (RA) induced neuronal differentiation of SH-SY5Y neuroblastoma. **A.** Expression of $\Delta\text{Np73}\alpha$ in SH-SY5Y cells. After puromycin selection, cell extracts were subjected to immunoblot analysis for p73 (anti-p73, ER15). **B.** SH-SY5Y morphology after 7 days in differentiation medium containing 10 μM retinoic acid. Cells were photographed using an inverted phase-contrast microscope at 100x magnification. **C.** Cells were analyzed by semi-quantitative RT-PCR for the expression of neurofilament as a biochemical marker of neuronal differentiation.

4. Discussion

4.1 Oncogenic function of p53 family members in human rhabdomyosarcoma (RMS)

A role for p53 in suppression of RMS development has been previously suggested. Although lymphomas are the most prominent neoplasms in p53-null mice, p53 heterozygotes over a year old develop mostly sarcomas, 19% of which are RMS (Choi and Donehower, 1999). Furthermore, conditional PAX3/FKHR knock-in 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 and Kaelin, 2000). Therefore p53 mutations frequently disable not only the tumorsuppressive function of p53 but rather the function of the complete family.

A detailed analysis of p73 in tumor cells indicated that Δ Np73 is frequently upregulated in a variety of primary cancers. In some cases, overexpression of p73 could even be correlated with an advanced tumor stage or poor prognostic parameters (Casciano et al., 2002; Concin et al., 2004; Frasca et al., 2003; Stiewe et al., 2004; Uramoto et al., 2004; Zaika et al., 2002). In this study we provide first evidence that Δ Np73 is also upregulated in human rhabdomyosarcoma. Interestingly, almost all RMS tumors showed overexpression of at least one of the oncogenic Δ Np73 isoforms (~75%). Furthermore, there was no tumor with high levels of the tumor-suppressive TAp73 isoform in the absence of concomitant Δ Np73 expression. Similarly, both TAp73 and Δ Np73 isoforms were simultaneously up-regulated in RMS cell lines. Concomitant upregulation of both p73 isoforms can be explained by the regulatory feedback loop connecting TAp73 and Δ Np73. p16-RB-E2F pathway alterations result in elevated levels of the E2F-target gene TAp73

in many tumors (Irwin et al., 2000; Stiewe and Putzer, 2000; Zaika et al., 2001). Increased TAp73 expression in turn is associated with the generation of aberrantly spliced Δ Np73 encoding transcripts and in transactivation of the Δ Np73-promoter (Grob et al., 2001; Kartasheva et al., 2002; Stiewe et al., 2002b). Consistently, we observed overexpression of both TAp73 and Δ Np73 in RMS samples and showed that knockdown of TAp73 induces downregulation of Δ Np73 confirming that TAp73 is indeed the major cause for high levels of the oncogenic Δ Np73 isoform.

Steady-state expression levels of endogenous p73 are kept extremely low under physiological conditions (Balint et al., 1999; Bernassola et al., 2004). It has been demonstrated that the induced Δ Np73 forms transactivation-defective complexes with p73 (and also p63) resulting in the stabilization of p73 proteins on a high, but transcriptionally inactive level through inhibition of its degradation (Fig 56) (Moll and Slade, 2004).

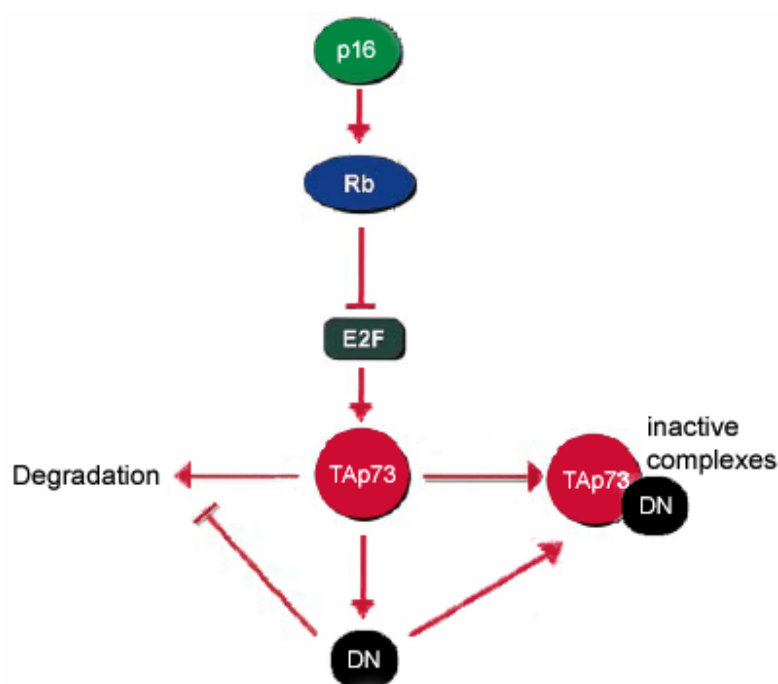


Figure 56. Δ Np73 forms transactivation-defective complexes with p73.

p16-RB-E2F pathway alterations result in elevated levels of the E2F-target gene TAp73 in many tumors and increased TAp73 expression in turn is associated with the generation of aberrantly spliced Δ Np73 and in transactivation of the Δ Np73-promoter. Subsequently, the induced Δ Np73 forms transactivation-defective complexes with p73 (and also p63) and inhibits its degradation, which results the stabilization of p73 proteins. Arrows represent activation, whereas bars represent inhibition.

In addition, $\Delta Np73$ competes with p53 for binding to p53 target promoters (Grob et al., 2001; Stiewe et al., 2002a). However, higher concentrations of $\Delta Np73$ are needed for inhibition of p53 than for inhibition of p63/p73 (data not shown). Likewise, p53 missense mutations inactivate p53 better than p63/p73. p73 ($\Delta Np73$) 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). The importance of p73 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*. Because our shRNA targets not only the oncogenic $\Delta Np73$ but also all other p73 isoforms, it can be assumed that this reduction of tumor growth in the presence of a mutant p53 is a result of the activated function of p63 following RNAi-mediated knockdown of $\Delta Np73$. Although these experiments need to be confirmed in other rhabdomyosarcoma types as well as in different cell lines, our experiments with RNA interference based knockdown of p73 (eventually in combination with p53 knockdown) establish on experimental basis for new p73-targeted therapies in RMS treatment.

Since single oncogenes are rarely sufficient to induce transformation (Merlino and Helman, 1999) and C2C12- $\Delta Np73\alpha$ myoblasts failed to form tumors in nude mice, we tested cooperation of $\Delta Np73\alpha$ with the known RMS oncogenes IGFII and PAX3/FKHR. When the various myoblasts populations were injected subcutaneously into nude mice only IGFII+ $\Delta Np73\alpha$ and PAX3/FKHR+ $\Delta Np73\alpha$ cells formed tumors at a high rate. These results strongly support that $\Delta Np73\alpha$ interacts with PAX3/FKHR and IGFII to play a critical role in the development of rhabdomyosarcoma. Importantly, this finding provides evidence for collaborative interactions between primary and secondary alterations in rhabdomyosarcoma. To understand why single oncogenes do not result in tumor formation whereas combinations together with $\Delta Np73\alpha$ do requires further experiments.

4.2. Molecular mechanisms of myogenic inhibition by Δ Np73 α

Disruption of differentiation is hallmark of cancer (Alema and Tato, 1994). In rhabdomyosarcoma (RMS), a human tumor arising from myogenic precursors (Merlino and Helman, 1999), the muscle program is only partially activated despite the presence of virtually all the MRFs (Tapscott et al., 1993). Although the origin of RMS cells has not yet been precisely defined, it is known that these cells do not arise from differentiated myofibers. Nevertheless the myogenic identity of these cells has been clearly established (Dias et al., 1992). RMS cells fail to both complete the myogenic program and irreversibly exit the cell cycle, resulting in uncontrolled proliferation and incomplete myogenesis (Merlino and Helman, 1999). Whether these differentiation inactivation in RMS is achieved by overexpression of inhibitors, deficient function of activators, or both remains until now unknown. In our study, we show that ectopic expression of Δ Np73 α inhibits myogenic differentiation. Thus, we speculate that Δ Np73 α unleashes the oncogenic activities of PAX3/FKHR (and IGFII) and enables tumor development by interfering with differentiation.

To understand the mechanistic basis, we investigated the impact of Δ Np73 on myogenic differentiation in C2C12 myoblasts, which faithfully mimic skeletal muscle differentiation *in vitro* (Blau et al., 1985). 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 (Blau et al., 1985). 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. In our experiments, C2C12 myoblasts transduced with an empty retroviral vector (mock) arrest, elongate, align and fuse to form multinuclear myotubes that stain positive for myosin heavy chain as a marker for differentiated muscle cells within three days, whereas Δ Np73 α expressing C2C12 cells fail to differentiate. Similar results were obtained in primary human and murine myoblasts indicating that Δ Np73 α induces a complete differentiation block in both established

murine and primary human myoblasts. Moreover, $\Delta Np73\alpha$ not only inhibits differentiation induced by serum withdrawal but also overrides synergistic growth factor and ECM-induced differentiation signals in a 3D tissue environment.

In skeletal muscle cells, cell cycle regulation plays a fundamental role in the production and maintenance of the differentiated phenotype. In growth factor rich medium mock $\Delta Np73\alpha$ myoblasts showed no significant difference in their proliferation rate. Under conditions of mitogen deprivation mock myoblasts completely withdrew from the cell cycle within one day whereas $\Delta Np73\alpha$ myoblasts slowed down markedly but continued to proliferate resulting in a progressive increase in cell number. Terminal cell cycle exit is required in order to activate tissue specific gene expression. Once the muscle cells exit from the cell cycle, they are unable to reenter the cell cycle even under ideal growth conditions. As expected after restimulation with growth factors all nuclei within multinuclear, myHC-expressing control myotubes remained permanently arrested and failed to incorporate BrdU. Interestingly, $\Delta Np73\alpha$ cells rapidly increased their proliferation rate and showed a BrdU incorporation index of more than 90%. Our data clearly demonstrate that $\Delta Np73\alpha$ myoblasts are sensitive to growth factor depletion and slow down their proliferation rate but fail to permanently withdraw from cell cycle progression. In addition, microarray analyses demonstrated that consistent with the defects in permanent cell cycle withdrawal a number of cell proliferation related genes failed to be silenced in the $\Delta Np73\alpha$ myoblasts. Furthermore, as expected from the function of $\Delta Np73\alpha$ as a dominant-negative inhibitor of p53 family members, most of the p53 target genes that showed expression changes in the mock cells were unchanged in the $\Delta Np73\alpha$ cells. Importantly, consistent with the observed differentiation defect, only few changes were observed in C2C12- $\Delta Np73\alpha$ myoblasts whereas in mock cells the muscle-related genes were induced or repressed most prominently at 24 hours of differentiation. In summary, we show on the basis of genome-wide expression profiles that $\Delta Np73\alpha$ does not interfere with the early, initiating steps but rather has profound impact on the progression to later stages of differentiation that require terminal cell cycle exit.

The retinoblastoma tumorsuppressor 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). Our analysis on the expression of typical muscle differentiation markers during myogenesis demonstrated that MyoD and Myf5, which play roles in specifying muscle lineage, are 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 $\Delta Np73\alpha$ myoblasts. However, downstream targets of Myogenin (MEF2C, MHC, CKM) were absent in $\Delta Np73\alpha$ expressing cells. These data demonstrate that the block to differentiation occurs primarily after the Myogenin expression step. In addition, in a direct comparison both $RB^{-/-}$ and $\Delta Np73\alpha$ -expressing fibroblasts stopped the MyoD-induced differentiation program at the Myogenin step. The similar phenotype of RB-loss and $\Delta Np73\alpha$ -expression demonstrates that RB function might be compromised by $\Delta Np73\alpha$. As expected, the expression of the retinoblastoma (RB) gene was strongly induced in C2C12-mock cells during myogenesis. In contrast, the upregulation of RB expression was abolished in C2C12- $\Delta Np73\alpha$ myoblasts. Furthermore, in differentiating wild-type myoblasts we observed an increase in protein amount and in electrophoretic mobility indicating a shift from inactive, hyperphosphorylated to active, hypophosphorylated RB species. This activation of RB was absent in $\Delta Np73\alpha$ myoblasts. Whereas the reduced levels of RB protein can be explained by transactivation defects of the *Rb1* gene, the failure to dephosphorylate RB has other reasons.

The activity of RB is regulated by cyclin-dependent kinases (CDKs), which phosphorylate and inactivate RB. During myogenic differentiation CDKs are redundantly inhibited by the two CDK inhibitors (CKIs) $p21^{CIP1/CDKN1A}$ and $p57^{KIP2/CDKN1C}$ resulting in the activation of RB. Furthermore, mice lacking both p21 and p57 fail to form myotubes, display increased proliferation and apoptotic rates of myoblasts, and display endoreplication in residual myotubes (Zhang et al., 1999b). Thus, we analyzed these two CKIs in C2C12-mock and $\Delta Np73\alpha$ myoblasts during differentiation. Whereas mock myoblasts induced expression of p57, expression of this CKI was absent in $\Delta Np73\alpha$ myoblasts both on the mRNA and protein level. In addition to p57, p21 is another CKI involved in RB regulation. The $p21^{CIP1}$ promoter

contains characteristic p53 binding elements and was activated by ectopic expression of all transactivating p53 family members (el-Deiry et al., 1993). 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 $\Delta Np73\alpha$ reduced but not completely abrogated p21 induction. The severe differentiation defect of $\Delta Np73\alpha$ myoblasts, however, demonstrates that the low levels of p21 in the absence of p53 family function are inadequate to support differentiation. 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.

To analyze 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. In our study, we infected $\Delta Np73\alpha$ myoblasts 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 of differentiation. Furthermore, 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. Thus, active RB, either in form of a constitutively active mutant or as a combination of wild-type RB with p57, rescued the $\Delta Np73\alpha$ -induced differentiation block proving that defects in activating RB were indeed the underlying cause.

Similar to myoblasts transfected with $\Delta Np73\alpha$, RMS cells maintain RB in the hyperphosphorylated state when exposed to mitogen-poor differentiation-inducing media, explaining in part their failure to growth arrest or differentiate under these conditions (Knudsen et al., 1998). Transfection with active forms of RB (phosphorylation-insensitive RB or the combination of wild-type RB with p57) rescues the differentiation defect of RMS cells that express high endogenous levels of $\Delta Np73$. This provides compelling evidence that RB dysfunction in RMS can be caused by inhibition of essential functions of the p53 family in differentiation.

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 (Korenjak and Brehm, 2005), the mechanisms that activate RB during myogenesis and other cellular differentiation processes via the expression of CKIs still remained unclear. Here, we demonstrate that the oncogenic $\Delta Np73\alpha$ inhibits essential functions in the activation process of RB and that these functions are commonly disabled in rhabdomyosarcomas (RMS). In addition, our results show that the p53 family inhibitor $\Delta Np73\alpha$ is a potent repressor of differentiation in multiple experimental settings including osteoblastic and neuronal differentiation, although further experiments are needed to understand the molecular basis for these differentiation blocks.

4.3 The role of p53 family members in myogenesis

p63, p73 and p53 compose a family of transcription factors involved in the cell response to stress and development. They encode for multiple p63, p73 or p53 proteins containing different protein domains (isoforms) due to alternative splicing, promoter usage and initiation of translation. p53 is the most frequently mutated gene in cancer (50%) and loss of p53 activity is considered to be ubiquitous to all cancers. 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 (Macleod et al., 1995). The importance of p53 in preventing genotoxic stress is undisputed. However, the physiological role of p53 in unstressed cells is still a matter of debate. p63 and p73 are likely candidates to compensate for p53 functions in various processes since all p53 family members share >60% amino acid identity within the DNA binding domain allowing them to regulate an overlapping set of target genes (Kaghad et al., 1997; Yang et al., 1998). In this study, we describe that in primary murine myoblasts all p53 family members are expressed. Whereas p53 mRNA levels progressively increase during differentiation, both $\Delta Np63$ and TAp73 expression peak around the first day during differentiation. TAp63 and $\Delta Np73$ mRNAs are detectable but unchanged over the

time course of differentiation. Similar expression changes were observed in regenerating muscles *in vivo*.

The retinoblastoma gene has a typical p53 binding site in the promoter region (Porrello et al., 2000). Since $\Delta\text{Np73}\alpha$ functions as a dominant-negative inhibitor of the transactivation-competent p53 family members, $\Delta\text{Np73}\alpha$ might inhibit the activation of RB by interfering with essential functions of the p53 family in this process. When ectopically expressed all the major transactivating proteins of the p53 family (p53, TAp73 β , TAp63 γ) induce RB. The observed difference between TAp73 α and TAp73 β can most likely be attributed to transactivation inhibitory functions located in the C-terminus of the α -isoform (Liu et al., 2005). The activity of RB is regulated by cyclin-dependent kinases (CDKs), which phosphorylate and inactivate RB. Thus, the regulated expression of cyclin-dependent-kinase inhibitors (CKIs) plays a key role in controlling the cell cycle during differentiation of many cell types. However, despite the clearly recognized abilities of p21 and p57 to promote G1 arrest, the precise regulation of these two CKIs during myogenesis is not yet clear. We demonstrate that $p57^{\text{KIP2}}$ is transactivated only by TAp73 β and $\Delta\text{Np63}\alpha$ but not by p53. The analysis of the sequences upstream of the $p57^{\text{KIP2}}$ transcriptional start or in the introns of $p57^{\text{KIP2}}$ failed to reveal any p53-binding sites arranged in tandem, as usually is seen in p53-responsive promoters (Beretta, 2005; Blint et al., 2002; Vaccarello et al., 2005). 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 TAp73 β and $\Delta\text{Np63}\alpha$. Moreover we demonstrate that upon differentiation p53 associated with the RB promoter and p63 and TAp73 were recruited to the $p57^{\text{KIP2}}$ promoter, whereas none of the investigated proteins was bound to these promoters in proliferating myoblasts. Regarding the mechanism of p73 up-regulation, it has been recently suggested by others that MyoD is a transcriptional regulator of these genes which displaces the negative regulator $\delta\text{EF1/ZEB}$ from E-boxes present in its regulatory region (Fontemaggi et al., 2005; Strano et al., 2001). Our observation in wild-type muscle cells that TAp63 γ and TAp73 β were recruited to the $p57^{\text{KIP2}}$ promoter, indicates a possible MyoD-p63/73-p57 pathway that might play a physiological role in myogenesis.

Considering the profound impact of $\Delta Np73$ on myogenic differentiation, it appears remarkable that none of the homozygous knockout mice of single p53 family members have an overt muscle phenotype *in vivo*. However, similar to myoblasts expressing p53DD or a p53-directed shRNA, myoblasts isolated from p53-null mice fail to induce RB resulting in deficient MHC expression upon differentiation *in vitro* (Porrello et al., 2000). Despite this clear requirement for p53 *in vitro*, additional factors in the microenvironment of the myotome or the regenerating muscle tissue might compensate for the loss of p53 *in vivo*. In fact, our studies demonstrate that TAp63 γ and TAp73 β can both activate RB suggesting that p63/p73 might compensate for p53-loss *in vivo*.

Although both p63 and p73 knockout mice present with developmental defects, severe muscle abnormalities have not been reported (Slade et al., 2004). However, our experiments with RNAi-mediated knockdown of p63 or p73 have revealed essential functions for both p63 and p73. Knockdown of either factor was sufficient to inhibit p57 induction and impaired the progression to late stages of differentiation. In contrast to the acute depletion of p63/p73 in our experiments, knockout mice have developed in the absence of p63 or p73. We therefore assume that during myogenesis developmental plasticity in signalling pathways might allow p63 to compensate for p73-loss and *vice versa*. Considering the similar function of the two proteins in the differentiation process functional compensation appears very likely, so that a muscle phenotype might only become apparent in homozygous compound double or triple knockouts or upon acute ablation of a single factor *in vivo*.

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/p73 control expression of p57 which maintains RB in an active hypophosphorylated state (Fig. 57). In tumors of muscle origin, p53 family function is frequently disabled by p53 mutations and $\Delta Np73$ overexpression. Inhibition of $\Delta Np73$ by RNA interference

reduces tumor growth *in vivo* providing evidence for $\Delta Np73$ as an attractive new target for RMS treatment. Recent publications may have a profound impact on our understanding of p53 family member's tumour suppressor activity. But in this study, we demonstrate that differentiation control contributes to the tumor suppressor activity of the p53 family.

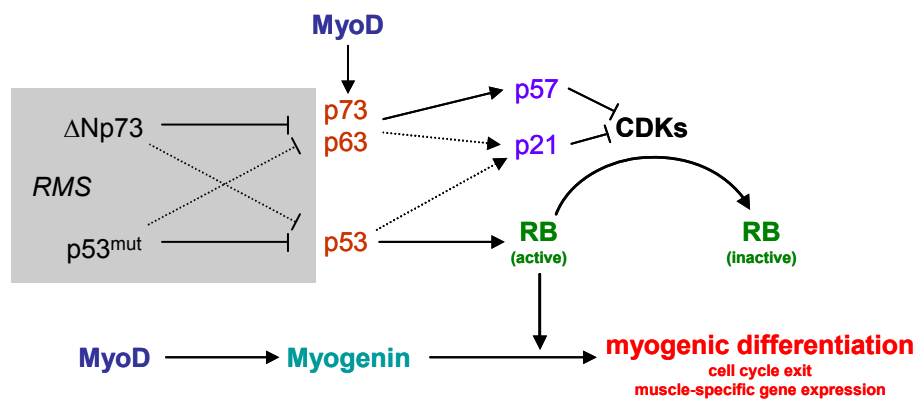


Figure 57. p53 family members are involved in regulating the process of muscle differentiation.

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6. Appendix

6.1. Curriculum vitae

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School education

1981-1991 primary, secondary and high school, Turkey
1991 Turkish high school diploma

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- Professor Peter Friedl for his great help 3D collagen matrix model and confocal microscope
- Dr. Stefan Gattenlöhner for kindly providing primary RMS samples
- All other members of the lab for the nice atmosphere, the nice chats and laughs that we had together.
- Last but not least for my parents and Maren Syta for their continuous help and support.

6.3. Publications

Articles

p53 family members in myogenic differentiation and rhabdomyosarcoma development

Hakan Cam, Heidi Griesmann, Lars Hofmann, Nicole Hüttinger-Kirchhof, Claudia Oswald, Peter Friedl, Stefan Gattenlöhner, Christof Burek, Andreas Rosenwald, Thorsten Stiewe

In revision (Cancer Cell.)

The p53 family inhibitor DeltaNp73 interferes with multiple developmental programs.

Hüttinger-Kirchhof N*, **Cam H***, Griesmann H, Hofmann L, Beitzinger M, Stiewe T.
Cell Death Differ, 2006Jan;13(1):174-7.

*contributed equally to the work

Retroviral vectors for high-level transgene expression in T lymphocytes.

Engels B*, **Cam H***, Schuler T, Indraccolo S, Gladow M, Baum C, Blankenstein T, Uckert W.

Hum Gene Ther. 2003 Aug 10;14(12):1155-68

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Oral presentations

The p53 family inhibitor Δ Np73 interferes with multiple differentiation programs

Congress :

Cell fate and decision

1st International Graduate College of University Würzburg,

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Posters

ΔNp73 – An Inhibitor of Myogenic Differentiation in Rhabdomyosarcomas

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p53 Family Members in Muscle Differentiation and Rhabdomyosarcoma Development

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