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The role of PAX3-FOXO1 in the pathogenesis of alveolar rhabdomyosarcoma

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biomedical Sciences

by

Wendy Linette Roeb

Committee in charge:

Professor Webster Cavenee, Chair
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The dissertation of Wendy Linette Roeb is approved, and is acceptable in quality and form for publication on microfilm:

Co-Chair

Chair

University of California, San Diego

2007

DEDICATION

To my husband, Jim, for his unwavering love and support.

EPIGRAPH

It takes less time to do a thing right than explain why you did it wrong.

-Henry Wadsworth Longfellow

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AWARDS

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ABSTRACT OF THE DISSERTATION

The role of PAX3-FOXO1 in the pathogenesis of alveolar rhabdomyosarcoma

by

Wendy Linette Roeb

Doctor of Philosophy in Biomedical Sciences

University of California, San Diego, 2007

Professor Webster Cavenee, Chair
Professor Karen Arden, Co-chair

Rhabdomyosarcomas, malignant tumors of mesenchymal origin, are the most common soft tissue sarcomas in children. Of the two subtypes, alveolar tumors (ARMS) portend the worst prognosis. Most ARMS are characterized by a balanced reciprocal chromosomal translocation t(2;13) that fuses the *PAX3* to the *FOXO1* gene. Expression of the fusion gene is a negative prognostic factor independent of tumor subtype. Despite the overwhelming data implicating the PAX3-FOXO1 chimeric protein in the pathogenesis of ARMS, little is known about its function. To study its function in its

endogenous context, myogenic precursor cells were isolated from transgenic mice. These cells express *PAX3-FOXO1* under the control of the *PAX3* promoter. The absence of any additional genetic lesions enabled us to dissect the effect of PAX3-FOXO1 alone without the contribution of the additional genetic abnormalities in cells derived from tumors.

Chapter 1 introduces alvoleolar rhabdomyosarcomas and summarizes current knowledge of PAX3-FOXO1 function.

Chapter 2 describes the characterization of *PAX3-FOXO1* transgenic myoblasts and details the discovery of a novel mechanisms by which PAX3-FOXO1 regulates *p57Kip2* transcription through the degradation of EGR1.

Chapter 3 details the regulation of *Mdm2* transcription by PAX3-FOXO1 and discusses how this attenuation of TP53 function likely contributes to the relative resistance of ARMS to treatment.

Chapter 4 summarizes the progress made in this dissertation and examines future directions.

CHAPTER 1: OVERVIEW

1.1 ALVEOLAR RHABDOMYOSARCOMA

Rhabdomyosarcoma (RMS) is a pediatric cancer of mesechymal origin and is the most common soft tissue sarcoma in children. These tumors are generally divided into two major subgroups: embryonal (ERMS), representing the more favorable prognosis with an overall survival rate of 82%, and alveolar (ARMS), with a reduced overall survival rate of 65% This poorer responsiveness of ARMS is evident even for treatment regimens that combine radiation therapy, combination chemotherapies and surgery (38).

The most aggressive subtype of RMS, alveolar, is histologically composed of dense aggregates of poorly differentiated cells separated by a framework of dense, fibrous septa forming “alveolar” spaces (49). ARMS are distinguished from other soft tissue sarcomas by immunohistochemical reactivity for the striated muscle-specific markers desmin, MYOD1, and muscle-specific actin. Criteria for subtyping ARMS include: physical location of the tumor, alveolar appearance, and the expression of the *PAX3-FOXO1 (PAX3-FKHR)* chimera, resulting from the in frame fusion between chromosomes 2 and 13.

ARMS generally presents in adolescents (14), most commonly originating in the muscle tissue of the extremities (41). Solid variants of ARMS have been found to carry the same poor prognosis as do those ARMS with the more classical histology, suggesting that the aggressive behavior of these tumors may be a consequence of PAX3-FOXO1 expression (42).

1. 2 PAX3-FOXO1

The PAX3-FOXO1 fusion protein, created by the t(2;13) chromosomal translocation, is present in most cases of alveolar rhabdomyosarcoma (46). The translocation severs the transcriptional transactivation domain of *PAX3* but preserves both of its two DNA binding domains. The *FOXO1* gene is disrupted in a large intron that bisects its DNA binding domain. The chromosomal rearrangement creates a chimeric protein containing the transcriptional activation domain of FOXO1 and the DNA binding elements of PAX3, under the control of the *PAX3* promoter (23). It is notable that this translocation event also destroys one allele of both the *PAX3* and the *FOXO1* genes.

While some potential PAX3-FOXO1 downstream targets have been identified (19, 24, 36, 53) the mechanism by which PAX3-FOXO1 contributes to tumor pathogenesis is unknown. The FOXO1 transactivation domain has been shown to be more robust than that of PAX3, enabling PAX3-FOXO1 to more strongly activate PAX3 consensus sequence reporter constructs (6, 22, 37). The transactivation domain of PAX3-FOXO1 is required for tumor growth and maintenance (3, 21, 31) These reports have led to the hypothesis that PAX3-FOXO1 drives oncogenesis by overactivating PAX3 transcriptional targets (6, 7, 24, 36, 47). A recent study found that PAX3-FOXO1 bypasses cellular senescence by reducing CDKN2A ($p16^{INK4A}$) levels (12, 32), while others have suggested that expression of PAX3-FOXO1 results in aberrant regulation of genes involved in myogenic differentiation (51).

PAX3 contains two DNA binding domains and recruitment of both is required for gene activation (25). In contrast, PAX3-FOXO1 can activate transcription through the engagement of only one of its two intact DNA binding domains. The activation of *myogenin* transcription by PAX3-FOXO1 requires only its paired domain while activation of *PDGFα* transcription engages only the homeodomain (20). This relaxation of the requirement for coordination of binding between the paired and homeodomains is likely due to the substitution of the FOXO1 for the PAX3 transactivation domain. The PAX3 transactivation domain has been shown to prevent the homeodomain alone from interconnecting with DNA (11). Thus PAX3-FOXO1 is postulated to have a broader repertoire of target genes than PAX3, but few genes have been identified as PAX3-FOXO1 specific, suggesting that single DNA binding domain attachment may be an *in vitro* phenomenon.

Although a number of genome-wide approaches have been attempted in order to study the targets of PAX3-FOXO1 misregulation (5), the data from these studies is conflicting. In some instances PAX3-FOXO1 has been shown to induce myogenic characteristics in non-myogenic cells (29). In cells from the myogenic lineage, though, PAX3-FOXO1 represses the expression of myogenic markers (15). Whether the genes identified in these studies have a role in the pathogenesis of ARMS or are even direct targets of PAX3-FOXO1 is unclear.

ARMS have an immuno-inhibitory phenotype, with fewer neutrophils infilitrating PAX3-FOXO1-expressing tumors than ERMS. This effect was mediated by the direct interaction of PAX3-FOXO1 with STAT3, leading to downregulation of MHC and over-

production of IL-10 (40). These data suggest that PAX3-FOXO1 promotes tumor development by limiting immune surveillance.

The function of PAX3-FOXO1 is likely similar to the function of PAX3, since PAX3-FOXO1 contains the functional, intact DNA binding domains of PAX3 and is regulated by its promoter. Additionally, several genes have been identified as transcriptional targets of both PAX3 and PAX3-FOXO1 (19, 24, 36, 53).

1. 3 PAX3

The PAX family of transcription factors is defined by the presence of the paired DNA binding domain. In mammals, the *PAX* gene family consists of nine family members, divided into four subfamilies based on sequence similarity and function (35). PAX proteins play crucial roles in the development of a variety of organ systems.

In addition to the paired domain, the PAX3/7 subfamily also contains a second DNA binding domain, the homeodomain. These two DNA binding domains can function cooperatively or independently of each other adding complexity to PAX3/7's repertoire of target genes (48). Pax3 also interacts with the co-repressors DAXX and HIRA, enabling to repress as well as activate gene transcription (27, 34).

The phenotype of loss of PAX3 function reveals the variety of organ systems in which PAX3 orchestrates development. In humans, heterozygosity for *PAX3* leads to the development of Waardenburg Syndrome (types I and III). This autosomal dominant disorder is characterized primarily by deafness but is also associated with limb muscle hypoplasia, pigmentary defects, and dystopia canthorum (13). In mice, heterozygosity for

Pax3 yields only pigmentary disturbances. Homozygous loss of *Pax3* is lethal in mice and seemingly also so in humans. Analysis of homozygous null embryos reveals multiple defects including absence of limb musculature, skeletal dysmorphism, dysraphism, underdevelopment of PNS structures, and severe cardiac defects. (33).

Early in embryogenesis, *Pax3* expression originates in the neural plate and hold fold regions. A SHH signal from the notochord rapidly restricts *Pax3* expression to the neural folds. As the neural tube closes, SHH further limits *Pax3* expression to only neural crest cells in the neural tube. *Pax3* expression is a balance between the BMP4 induction signal from the epidermal ectoderm (39) and the SHH repression signal from the floorplate and notochord (1). The transcription factor SLUG, expressed in the neural folds, likely mediates the signaling between BMP 4 and PAX3 (10). *Pax3* is also expressed in the somites. Expression of *Pax3* coincides with the earliest stages of somitogenesis, expressed in the dorsal region of the new somite. After somites differentiate into sclerotome and dermomyotome, *Pax3* expression is restricted to the hypaxial dermamyotome that will form the limb muscles and body wall (16).

As evidenced by the lack of limb musculature in knockout mice, PAX3 plays an essential role in myogenesis. PAX3 induces *Met* expression in the hypaxial mesoderm. MET is critical for myogenic precursor pathfinding. The ligand for MET, HGF, is expressed in the limb buds, guiding migrating cells (9). *Met* null mice also completely lack limb musculature (18). *Pax3* null mice lack *Met*-expressing cells in their limbs, illustrating the involvement of these genes in the myoblasts migratory pathway (52).

Pax3 expression is at its peak when myoblasts are in a highly proliferative state. As cells *Pax3* expression abates, bHLH transcription factors are activated. Reduction in

PAX3 levels is a necessary step in myogenic determination (17). This downregulation is mediated, in part, by mono-ubiquitination of PAX3 leading to its proteasomal degradation (8).

PAX3 is crucial not only for early myogenic determination but also for terminal differentiation (26, 50). PAX3 induces expression of the myogenic regulator *Myf5* by upregulating the transcription of *Six1* and *Eya2*, promoting myogenic differentiation (45). *In vivo* studies have further validated this observation.(4). Introduction of dominant negative *Pax3* into adult muscle satellite cells interrupts differentiation through a lack of MyoD induction (43).

1.4 PAX3-FOXO1 MOUSE MODELS

Several studies have utilized transgenic or knock-in approaches to investigate the consequence of *PAX3-FOXO1* expression in the mouse (2, 28, 30, 44). The phenotype of mice expressing *PAX3-FOXO1* is similar to mice with reduced levels of *Pax3* and involved pigmentary abnormalities of the abdomen, hindpaws, and tail, with additional neurological related alterations. These phenotypic consequences of *PAX3-FOXO1* expression resulted from defects in hindlimb skeletal muscle and neural crest migration.

Analysis of the four *PAX3-FOXO1* mouse models described to date (2, 28, 30, 44) also suggests that *PAX3-FOXO1* expression alone is insufficient to produce a malignant phenotype. However, disruption of the *Ink4a/ARF* or *Tp53* pathways, targets of inactivation in human rhabdomyosarcoma, in *Pax3-Foxo1* mice substantially increases the frequency of tumor formation (28). These mouse models suggest that expression of

Pax3-Foxo1 is not a dominantly-acting transforming event. Rather, PAX3-FOXO1 is more likely to predispose cells to transformation by a secondary genetic event, perhaps by increasing the pool size of cells at a particular point in myogenic differentiation where they are most susceptible.

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CHAPTER 2: PAX3-FOXO1 CONTROLS EXPRESSION OF THE P57KIP2 CELL CYCLE REGULATOR THROUGH DEGRADATION OF EGR1

2.1 ABSTRACT

The PAX3-FOXO1 chimeric protein results from translocation between chromosomes 2 and 13, the most common genetic aberration in the alveolar subtype of the human skeletal muscle tumor, rhabdomyosarcoma. To understand how PAX3-FOXO1 contributes to tumor development, we isolated and characterized muscle cells from transgenic mice expressing *PAX3-FOXO1* under control of the *PAX3* promoter. We demonstrate that these myoblasts are unable to complete myogenic differentiation due to an inability to upregulate *p57Kip2* transcription. This defect is caused by reduced levels of the EGR1 transcriptional activator resulting from a direct, destabilizing interaction with PAX3-FOXO1 occurring through recognition of PAX3-FOXO1 by the quality control ubiquitin ligase, CHIP1. Neither PAX3 nor FOXO1 share the ability to regulate *p57Kip2* transcription, nor are they CHIP1 substrates. Thus, the breakage and fusion of the genes encoding these transcription factors creates a unique misfolded chimeric protein that controls a key cell cycle and differentiation regulator.

2.2 INTRODUCTION

Rhabdomyosarcoma constitutes a group of soft tissue sarcomas of childhood and adolescence that are thought to arise from undifferentiated mesenchyme resembling various stages of early embryonic skeletal muscle development. The most aggressive pediatric subtype, alveolar rhabdomyosarcoma (ARMS), is composed of dense aggregates of poorly differentiated cells separated by a framework of fibrous septa forming “alveolar” spaces. Typical features of ARMS include: physical location of the tumor, alveolar appearance, the presence of the characteristic translocation between chromosomes 2 and 13 and immunohistochemical reactivity for the myogenic markers desmin, MYOD, and myosin heavy chain (64). This latter feature might suggest that ARMS arise as a consequence of incomplete myogenic differentiation and abnormal proliferation coupled to transforming mutations.

The PAX3-FOXO1 fusion protein, created by the t(2;13) chromosomal translocation, is present in most cases of alveolar rhabdomyosarcoma (54). The translocation severs the transcriptional transactivation domain of *PAX3* but preserves both of its two DNA binding domains. The *FOXO1* gene is disrupted in a large intron that bisects its DNA binding domain. The chromosomal rearrangement creates a chimeric protein containing the transcriptional activation domain of FOXO1 and the DNA binding elements of PAX3, under the control of the *PAX3* promoter (22).

PAX3 is an essential myogenic regulator. Mice lacking *Pax3* have multiple skeletal muscle defects, most notably delays in muscle differentiation as well as an

overall decrease in muscle mass (17). In myogenesis, PAX3 functions to induce the expression of *SIX1* and *EYA2* (49), transcription factors that upregulate expression of *MYF5*, advancing the myogenic differentiation program. FOXO1 also plays a pivotal role in mediating myogenic differentiation (10, 28).

In myoblasts, terminal differentiation and proliferation are mutually exclusive processes. P57KIP2 (CDKN1C) promotes differentiation by stabilizing MYOD, inhibiting cyclin E-CDK2 activity and PCNA function while maintaining RB in an active hypophosphorylated state (12, 47, 48, 63). *P57KIP2*, located at 11p15, is a paternally imprinted gene whose decreased expression is a feature common to a variety of human tumors (5, 9, 11, 26, 35, 50). There are several mechanisms through which this occurs, commonly involving changes in genomic imprinting or loss of the active maternal allele (1, 27, 53). It is interesting to note that the embryonal subtype of rhabdomyosarcoma is characterized by loss of the maternal 11p15 chromosomal region (51). Loss of p57KIP2 function is also implicated in Beckwith-Wiedemann syndrome, a complex overgrowth condition associated with an increased risk for developing rhabdomyosarcomas (24).

While some potential PAX3-FOXO1 downstream targets have been identified, the mechanism by which PAX3-FOXO1 contributes to tumor pathogenesis is unknown. The FOXO1 transactivation domain has been shown to be more robust than that of PAX3, enabling PAX3-FOXO1 to more strongly activate PAX3 consensus sequence reporter constructs (7, 18, 40). These reports have led to the hypothesis that PAX3-FOXO1 drives oncogenesis by overactivating PAX3 transcriptional targets (7, 8, 23, 38, 58). A recent study found that PAX3-FOXO1 bypasses cellular senescence by reducing $p16^{INK4A}$ (CDKN2A) levels (13, 36), while others have suggested that expression of PAX3-

FOXO1 results in aberrant regulation of genes involved in myogenic differentiation (reviewed in (65)). The present studies were undertaken to identify PAX3-FOXO1 target genes in transgenic myoblasts, to test whether these affect myogenic differentiation and to determine how PAX3-FOXO1 expression leads to their misregulation.

2.3 MATERIALS AND METHODS

Plasmids. Mammalian expression vectors encoding EGR1, PAX3-FOXO1, PAX3, FOXO1, CHIP1, and PAX3-FOXO1 mutants, both wild-type and epitope-tagged, were derived from pcDNA3.1 (Invitrogen). SP1, SP3, and N-terminus Flag-tagged ubiquitin plasmids were derived from pCMV. Epitope-tags are as follows: the V5 tag (Invitrogen) was fused to the N-terminus of EGR1; the 6xHis tag was fused to the N-terminus of CHIP1 and PAX3-FOXO1; the 3xHA tag was fused the C-terminus of PAX3, FOXO1, PAX3-FOXO1, and all PAX3-FOXO1 deletion mutants.

Mouse *p57kip2* promoter deletions were constructed using the firefly luciferase vector, pGL3 basic (Promega). The Egr1 reporter, containing three tandem EGR1 consensus sites (GCGGGGGCG, (56)) separated by spacers, was derived from the pLucMCS vector (Stratagene).

Cell Culture. Except for Figures 7D and S2, all transfections were performed with human 293T cells, in DMEM with 10% FBS. Cells were transfected using 1 ug of DNA and 8 ul of Lipofectamine 2000 (Invitrogen) per $3x10^6$ cells. For the reporter assays, $1x10^6$ cells were transfected with 0.5 ug DNA and 1 ul Lipofectamine 2000.

For Figures 2-7 (D) and 2-9 (S2), COS7 and TS20 cells were maintained in DMEM with 10% FBS. 6 x10^5 cells were transfected using 2 ug of DNA and 5 ul Lipofectamine 2000. TS20 cells were transfected at the non-permissive temperature of 39°C. After six hrs, the permissive population was incubated at 35°C for 18 hrs while the non-permissive population remained at 39°C. All cells were harvested at 24 hrs after transfection for IP.

Western blots and antibodies. 10 ug of protein was harvested in RIPA buffer (150 mM NaCl, 1.0% TritonX100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and Complete protease inhibitor (Roche)), added to LDS sample buffer (Invitrogen) separated on either 4-12% or 3-8% Nupage gels (Invitrogen), transferred to nitrocellulose (Invitrogen), blocked in PBS containing 0.1% Tween 20 and 5% nonfat milk and incubated for one hr at RT with primary antibodies. The following antibodies were used: desmin (D3) and myosin heavy chain (MF20) from the Developmental Studies Hybridoma Bank; V5 (Invitrogen); HA (Roche); EGR1 (C19), p57KIP2 (C20), and FOXO1 (C20)(Santa Cruz); EGR1 and FOXO1 (for IP proteins - R&D Systems and EMD, respectively); Flag and β-Actin (Sigma); CHIP1 (Imgenex); and Strepavidin (GE Biosciences).

For Co-IP, 20% of the elutant was subjected to Western blotting. For the endogenous Co-IP from Rh28 cells, an HRP-linked anti-goat secondary antibody that preferentially detects non-reduced IgG was used (eBioscience).

Isolation of mouse myoblasts. Isolation and culture of mouse myoblasts was performed as described in (45). Briefly, hindlimb muscle tissue was dissected from 3

day-old mice, minced and digested with Liberase Blendzyme 3 (Roche) followed by trypsin. After washing, this mixture was plated and cultured in myoblast growth media (20% FBS in a 50/50 mix of DMEM/Ham's F10 (Invitrogen) and 25 ng/ml bFGF (Dako)). Fibroblasts were removed by selective plating at each subsequent passage. For expression analysis, RNA was harvested from early passage cells (≤ passage 7) from cultures grown in triplicate using the RNeasy Plus Kit (Qiagen).

For differentiation experiments, cells were cultured in DMEM with 5% Horse Serum (Invitrogen). After 24 hrs (48 hrs for p57KIP2) in differentiation medium, protein was harvested in RIPA buffer and RNA was extracted as above.

For viral transduction, viruses in the MSCV vector were obtained by co-transfecting 293T cells with an equal mass of MSCV plasmid and the envelope encoding vector, pCL-Eco (Imgenex). Viral supernatant was prepared 48 hrs post-transfection by passing the media through 0.45 u filters. Myoblasts ($3x10^6$ cells) were infected with fresh virus diluted 1:2 with myoblast growth media and 3.2 ug/ml fresh polybrene (Sigma) for 8 hrs. 48 hrs post infection transduction efficiency was measured by maintaining a separate pool of myoblasts infected with GFP. The entire procedure was repeated four times to obtain a cell population >90% GFP positive.

Quantitative real-time PCR. Total RNA was harvested using the RNeasy Plus Kit (Qiagen) and reverse transcribed with the Superscript III First Strand Kit (Invitrogen). Quantitative PCR was performed on the Icycler IQ (Biorad) using IQ Syber Green (Biorad) according to the manufacturer's instructions using the default machine settings using an annealing temperature of 60°C. Primers utilized were: *p57kip2* F, AAGAGAACTGCGCAGGAGAAAAG, *p57kip2* R,

GCCTCTAAACTAACTCATCTCAGAC, *Gapdh* F, GCCTGGAGAAACCTGCCAAGTATGAT, *Gapdh* R, TGGAAGAGTGGGAGTTGCTGTTGA. These primers were designed to gap introns and have a >90% amplification efficiency. Quantitative values were obtained by the $2^{-\Delta\Delta CT}$ method (37), using *Gapdh* as the reference gene.

EMSA. EGR1 was transcribed and translated *in vitro* (TNT T7 Quick, Promega) in a reaction with 10 uM $ZnSO_4$ (Sigma). The EMSA was performed with 0.2 pmol of ^{32}P- labeled *p57Kip2* promoter sequence (-400 bp to -350 bp) at 30°C for 30 min in a reaction buffer containing (20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl (pH 7.5), 0.25 mg/ml poly(dI-dC).poly(dI-dC), and 10 uM $ZnSO_4$). Reactions were separated on 6% DNA retardation gels (Invitrogen) that were dried and subjected to autoradiography.

Reporter Assays. 293T cells were co-transfected with the firefly luciferase reporter plasmid and the indicated combinations of expression plasmid. The *Renilla* luciferase plasmid pRL (Promega), driven by a minimal tk promoter, was included as an internal control. 48 hrs post-transfection, the Dual-Luciferase Reporter Assay (Promega) was performed according to the manufacturer's instructions and values were read on the GENios Pro (Tecan).

EGR1 Destruction Assay. 293T cells were transfected with a fixed mass of epitope-tagged EGR1 plasmid with increasing quantities of epitope-tagged PAX3-FOXO1 plasmid, total DNA mass was kept constant by the addition of pcDNA3.1 plasmid. 16 hrs prior to harvest, cells were treated with either 5 uM MG132 dissolved in DMSO or DMSO alone.

Co-IP. 293T cells were transfected with equal quantities of epitope-tagged PAX3-FOXO1 and EGR1 using Lipofectamine 2000 according the manufacturer's instructions. Cells were lysed in NP40 buffer and either 1 mg of lysate was incubated overnight with 2 ug of anti-V5 antibody or 2 mg of lysate was used with 2 ug of an anti-HA antibody in the presence of Dynabeads Protein G (Invitrogen). Immune complexes were washed (150 mM NaCl, 50 mM Tris-HCl (pH 8), 1% NP40, 0.5% sodium deoxycholate, 0.05% SDS, and Complete protease inhibitor (Roche)). Complexes were reduced, denatured, and eluted in LDS sample buffer.

For the Co-IP of endogenously expressed proteins, the ARMS-derived cell line Rh28 (25) was utilized. Cells were treated with 20 uM MG132 (EMD Biosciences) for 2 hrs prior to harvesting in NP40 buffer. For the EGR1 IP, 10 mg of protein was incubated overnight in the presence of 2 ug of the anti-EGR1 antibody (C19) with Dynabeads Protein A (Invitrogen). For the CHIP1 IP, 20 mg of protein was incubated overnight in the presence of 2 ug of the anti-CHIP1 antibody with Dynabeads Protein G. Immune complexes were processed as described above. For the IP of ubiquitinated proteins, 10 mM iodoacetamide (Sigma) was added to the lysis buffer.

***In Vitro* Ubiquitination Assay.** Ubch5b, Ubiquitinylation Buffer, Biotinylated ubiquitin, Mg-ATP, E1 were obtained from Biomol; DTT and Inorganic Pyrophosphatase (IPP) from USB. 293T cells were transfected with CHIP1-His tagged plasmid. 48 hrs after transfection, cells were lysed in NP40 buffer. For PAX3-FOXO1, His-tagged protein was transcribed/translated using the TNT T7 system (Promega). For both proteins, lysates were incubated with Dynabeads Talon (Invitrogen) for 1 hr and washed. Protein purity and yield were verified using the InVision His-tag In-gel Stain (Invitrogen)

as well as by immunoblot. Assay conditions were as follows: 1x Ubiquitinylation Buffer, 0.5U IPP, 1 mM DTT, 1.2 mM Mg-ATP, 0.1 uM E1, 2 uM UBCH5b (E2), 50 uM CHIP1 (E3), 20uM PAX3-FOXO1, 2.5 uM Biotinylated ubiquitin. Reactions were incubated at 30°C for two hrs and 10% of each reaction was reduced and diluted in LDS sample buffer. The remainder was diluted with NP40 buffer and incubated overnight with 1 ug FOXO1 antibody and 15ul Dynabeads Protein G. Immune complexes were processed as described above.

2.4 RESULTS

PAX3-FOXO1 Expression in Myoblasts Inhibits Differentiation and Decreases P57Kip2. In order to study the role of PAX3-FOXO1 on myogenic differentiation, we isolated myoblasts from mice harboring the *PAX3-FOXO1* fusion gene under the control of the *PAX3* promoter (2). *PAX3-FOXO1* transgenic myoblasts are phenotypically indistinguishable from their wild-type counterparts and express normal levels of the myoblast marker, desmin (30).

Rhabdomyosarcomas are characterized by deficiencies in myogenic differentiation and an inability to exit the cell cycle (4, 43, 44, 52, 57, 61). Cells derived from these tumors express MyoD and myogenin, but do not differentiate into myotubes (57). In order to determine if myoblasts derived from *PAX3-FOXO1* transgenic animals have similar defects, wild-type and transgenic myoblasts were cultured in media that induces myogenic differentiation. As shown in Figures 2-1 (A) and 2-1 (B), *PAX3-*

FOXO1 transgenic myoblasts differentiate poorly, they fail to form myotubes and they do not upregulate expression of the terminal differentiation marker, myosin heavy chain.

This led us to hypothesize that the *PAX3-FOXO1* fusion gene might play a role in suppressing differentiation and preventing cell cycle exit. In order to address the nature of the differentiation defect, expression profiling using Genechip microarrays was performed on two sets each of passage-matched primary myoblasts from transgenic and wild-type animals. For both data sets, among many genes with altered expression, the most profound effect was decreased expression of the CDK inhibitor, *p57Kip2*. This result was validated using quantitative PCR on independent primary myoblasts from *PAX3-FOXO1* transgenic animals, as shown in Figure 1C. Wild-type myoblasts showed approximately a 150-fold increase of *p57Kip2* expression upon differentiation induction, while the transgenic myoblasts had less than 15% of that response (Fig. 2-1 (E)) and this corresponded to the levels of p57KIP2 protein in the cells (Fig. 2-1 (D)). These results suggest that PAX3-FOXO1 might influence the balance between myogenic differentiation and proliferation by decreasing the levels of the proliferation inhibitor, p57KIP2.

To confirm that diminished quantities of p57KIP2 are sufficient to inhibit differentiation, we restored *p57Kip2* expression in *PAX3-FOXO1* transgenic myoblasts. Early passage transgenic myoblasts were infected with either empty vector or *p57Kip2* retroviruses. *P57Kip2* transcript levels were increased to slightly less than the levels seen in wild-type myoblasts, almost four fold. As shown in Figure 2-2, restoration of *p57Kip2* expression profoundly improves the ability of PAX3-FOXO1 transgenic myoblasts to

differentiate. This result demonstrates that p57KIP2 is a major effector of PAX3-FOXO1 in inhibiting myogenic differentiation.

The *P57Kip2* Promoter is Responsive to PAX3-FOXO1. To determine whether the *p57Kip2* promoter is PAX3-FOXO1 responsive and which of its elements are responsible for the transcriptional repression, a series of deletions of the full-length mouse promoter linked to a luciferase transcriptional reporter were constructed (Fig. 2-3 (A)). Sequence analysis showed that there are two putative FOXO1 binding sites and one putative PAX3 binding site in the full-length promoter, at positions -2130, -2650 and -2900, and all were contained in the PAX3-FOXO1-responsive full-length *p57Kip2* promoter construct. However, the deletion of a segment of the *p57Kip2* promoter containing all of these sites (-3000 to -1800 from the transcriptional start site) did not cause the *p57Kip2* promoter to become unresponsive to PAX3-FOXO1 inhibition. On the contrary, PAX3-FOXO1-responsive sites were scattered throughout the promoter, with the magnitude of the repression diminishing with decreasing promoter length. To further define the minimal sequence required for PAX3-FOXO1-dependent repression, additional deletion constructs were created. As shown in Figure 2-3 (B), deletion of 100 nucleotides from -400 to -300, with respect to the transcription start site of the minimal *p57Kip2* promoter, renders it PAX3-FOXO1 insensitive. These 100 nucleotides of sequence are also sufficient to mediate repression by PAX3-FOXO1 when placed upstream of a synthetic minimal promoter (Fig. 2-3 (B)). Moreover, this ability is specific to the PAX3-FOXO1 fusion protein and is not evidenced by wild-type PAX3 or FOXO1 alone (Fig. 2-3 (C)). These results indicate that PAX3-FOXO1 represses *p57Kip2* transcription through sequences in the -400 to -300 region of the promoter that are

distinct from PAX3 or FOXO1 binding sequences and that are unresponsive to PAX3 or FOXO1.

PAX3-FOXO1 Repression of the P57Kip2 Promoter is Mediated by EGR1 Binding Sequences. The minimum sequence of the *p57Kip2* promoter required for PAX3-FOXO1 repression, -400 to -300 bp, is highly GC rich unlike the PAX3 or FOXO1 binding sites that were the anticipated PAX3-FOXO1 targets. We performed chromatin immunoprecipitation several times using endogenous PAX3-FOXO1 from transgenic myoblasts but were unable to amplify p57 promoter sequences (data not shown). When combined with the absence of a PAX3 binding site in this region these data suggest that PAX3-FOXO1 controls *p57Kip2* expression indirectly. An alternative mechanism by which PAX3-FOXO1 might repress *p57Kip2* transcription without directly binding to the *p57Kip2* promoter could be by interfering with an activator of *p57Kip2* transcription. Since the region of the *p57Kip2* promoter from -400 to -300 bp is extremely GC rich, we assessed three of the most common GC box binding transcription factors, EGR1, SP1, and SP3, for their ability to activate *p57Kip2* transcription. As shown in Figure 2-4 (A), only EGR1 was able to activate *p57Kip2* transcription in a dose-dependent fashion. This result is consistent with microarray experiments that show that *p57Kip2* is upregulated by EGR1 (19, 55).

To establish whether EGR1 acts directly on the *p57Kip2* promoter, EMSA was performed. We used *in vitro*-translated EGR1 and a radiolabeled oligonucleotide representing -400 to -350 bp of the *p57Kip2* promoter sequence as this sequence contained the majority of the EGR1 binding sites in this region. While EGR1 could clearly shift the mobility of the oligonucleotide, disruption of the two EGR1 binding sites

in the unlabeled, competing DNA abrogates its ability to efficiently compete for EGR1 binding (Fig. 2-4 (B)). Thus EGR1 can specifically bind to and activate *p57Kip2* transcription through the minimal promoter sequence important for PAX3-FOXO1 repression.

To determine whether PAX3-FOXO1 could interfere with activation of the *p57Kip2* promoter by EGR1, luciferase reporter assays were performed using the full-length *p57Kip2* promoter in the presence of increasing quantities of *Egr1*. PAX3-FOXO1 represses EGR1 activation of the *p57Kip2* promoter approximately four-fold (Fig. 2-4 C). Since PAX3-FOXO1 has the ability to interfere with EGR1-dependent activation of the *p57Kip2* promoter, we tested whether it was able to more generally suppress EGR1-mediated transcription. Luciferase assays were performed with a synthetic reporter containing three tandem copies of the EGR1 consensus sequence. Consistent with the effects observed for the *p57Kip2* promoter, PAX3-FOXO1 was able to suppress EGR1 activity by about four-fold (Fig. 2-4 (D)) suggesting that this is one of its general activities.

PAX3-FOXO1 Destabilizes EGR1. In the course of performing these experiments, we observed that co-transfection of *PAX3-FOXO1* and *Egr1* resulted in significantly reduced levels of EGR1. One mechanism by which PAX3-FOXO1 might interfere with EGR1-dependent transcription is by destabilizing EGR1. Co-transfection of increasing quantities of *PAX3-FOXO1-HA* in the presence of a fixed amount of *Egr1-V5* showed that PAX3-FOXO1-HA reduced EGR1-V5 protein levels in a dose-dependent manner (Fig. 2-5 (A)). Inhibition of the proteasome by the addition of MG132 abolished this effect, suggesting that the observed reduction of EGR1 protein is due to proteasomal

degradation (Fig. 2-5 (A)). Consistent with this, levels of *Egr1-V5* transcript were unaffected by PAX3-FOXO1 (data not shown). To further demonstrate that destabilization of EGR1 is specific for PAX3-FOXO1, we also performed this assay using PAX3 and FOXO1. As shown in Figure 2-9 (S1), neither of these transcription factors alone has an effect on EGR1 stability.

Since proteasome inhibition prevents PAX3-FOXO1 from destabilizing EGR1, inhibition of proteasome function would be expected to prevent the repression of *p57Kip2* transcription by PAX3-FOXO1. To test this, reporter assays were performed in the presence of MG132 for 16 hours; such proteasome inhibition largely reversed the repressive effect of PAX3-FOXO1 on *p57Kip2* transcription (Fig. 2-5 (B)). The inclusion of MG132 for the entire course of the assay, 48 hours, might have completely restored *p57Kip2* transcription, however this experiment could not be carried out due to the toxicity of the drug (41, 67).

To establish whether the destabilization of EGR1 by PAX3-FOXO1 involves their direct interaction, we performed co-immunoprecipitations of transfected epitope-tagged proteins (Fig. 2-5 (C)). In the absence of proteasome inhibition, immunoprecipitation of PAX3-FOXO1-HA yields a faint smear when blots are probed for EGR1-V5. In the presence of MG132, EGR1-V5 co-immunoprecipitates with PAX3-FOXO1-HA, demonstrating that PAX3-FOXO1 and EGR1 directly associate in an unstable complex. We also performed the reciprocal experiment by immunoprecipitating EGR1-V5, resulting in a specific signal for PAX3-FOXO1 (data not shown). To demonstrate that the interaction between EGR1 and PAX3-FOXO1 was not simply a result of overexpression, we performed co-immunoprecipitation experiments of endogenous proteins from the

ARMS cell line, Rh28, which harbors the t(2;13) translocation (25). Treatment of these cells with MG132 increases the levels of EGR1 protein (data not shown) and PAX3-FOXO1 and EGR1 form a complex in ARMS cells (Fig. 2-5 (D)).

Lastly, we tested whether the observed decreases in *p57Kip2* mRNA were correlated with reduced EGR1 protein levels in our transgenic myoblasts. As shown in Fig. 2-5 (E), immoblot analysis revealed that PAX3-FOXO1 significantly reduces the levels of EGR1 protein in myoblasts.

We had expected that the observed proteasome-dependent destruction of EGR1 by PAX3-FOXO1 would involve the accumulation of ubiquitin on EGR1. To test this, we immunoprecipitated either PAX3-FOXO1-HA or EGR1-V5 from cells co-transfected with a fixed quantity of *Egr1-V5* in the presence of an increasing amount of *PAX3-FOXO1-HA* (Fig. 2-6 (A)). Surprisingly, although no ubiquitin was associated with EGR1, a large amount was detected on PAX3-FOXO1. Thus decreases in the stability of EGR1 are not accompanied by increases in its ubiquitination.

We next sought to determine which domains of PAX3-FOXO1 are required for suppression of *p57Kip2* transcription using a series of PAX3-FOXO1 deletions (Fig. 2-6 (B)). Interestingly, PAX3-FOXO1, despite the loss of its primary DNA binding domain (see construct 193-836, Fig. 2-6 (B)), still retains much of its activity. Additional deletions at either the N- or the C- terminus dramatically reduce most of the PAX3-FOXO1 transcriptional repression activity. This result suggests that the ability of PAX3-FOXO1 to suppress *p57Kip2* transcription is not due to a particular domain and implies that the overall protein conformation is responsible for suppression of *p57Kip2* transcription.

PAX3-FOXO1 is Recognized by the E3 Ubiquitin Ligase, CHIP1. Taken together, these results raise the intriguing possibility that in this context, PAX3-FOXO1 may be recognized as aberrantly folded by the cellular quality control machinery. The E3 ubiquitin ligase responsible for recognizing and targeting such proteins for degradation is CHIP1 (42). Aberrant proteins bound to the HSP70/90 chaperones are either encouraged to fold through an association with productive cofactors or targeted for degradation by CHIP1 (14). CHIP1 is broadly expressed with the highest levels found in skeletal muscle (6).

To investigate the possibility that PAX3-FOXO1 is a CHIP1 substrate, we sought to determine if these two proteins coexist in a complex by performing co-immunoprecipitation experiments. As shown in Figure 2-7 (A), immunoprecipitation of CHIP1 specifically co-immunoprecipitates PAX3-FOXO1-HA, but not PAX3-HA or FOXO1-HA. This association is not an artifact of overexpression since neither PAX3-HA nor FOXO1-HA co-immunoprecipitate with CHIP1. To further buttress this point, we were able to successfully co-immunoprecipitate endogenous PAX3-FOXO1 and CHIP1 from ARMS cells (Fig. 2-7 (B)). These results indicate that the fusion of PAX3 to FOXO1 creates a misfolded protein.

CHIP1 Ubiquitinates PAX3-FOXO1 and this Regulates EGR1 Degradation. The interaction between PAX3-FOXO1 and CHIP1 in a complex and the presence of ubiquitin on PAX3-FOXO1 suggests that CHIP1 is able to ubiquitinate PAX3-FOXO1. To test this, an *in vitro* ubiquitination assay was performed using purified PAX3-FOXO1 and CHIP1, as well as the ubiquitin activating enzyme, E1, and the E2 ubiquitin conjugating enzyme, UBCH5b. To determine the number of ubiquitinated lysines in

PAX3-FOXO1, biotinylated ubiquitin unable to be assembled into poly-ubiquitin chains by CHIP1 was used (29). As shown in Figure 2-7 (C), CHIP1 can ubiquitinate PAX3-FOXO1. Since the PAX3-FOXO1 and E1 proteins are similar in size, PAX3-FOXO1 was immunoprecipitated from the reactions. The presence of biotinylated ubiquitin at 220 kd on both the immunoprecipitation and the input immunoblots indicated that this signal results from ubiquitinated PAX3-FOXO1. This result also demonstrates that approximately fourteen lysines in PAX3-FOXO1 are modified by CHIP1.

Lastly, we sought to show that ubiquitination of PAX3-FOXO1 by CHIP1 is required for the destabilization of EGR1. For this experiment, COS7 cells were used because of their relatively low levels of CHIP1. Co-transfection of *PAX3-FOXO1-HA* and *Egr1-V5* left EGR1-V5 protein levels unchanged, presumably due to the small amount of CHIP1 in these cells. Co-transfection of *Egr1-V5* and *CHIP1* also did not affect EGR1-V5 levels. In addition, we were unable to successfully co-immunoprecipitate EGR1-V5 and CHIP1 (data not shown). Taken together, these results demonstrate that EGR1 itself is not a substrate of CHIP1. When *CHIP1*, *Egr1-V5*, and *PAX3-FOXO1-HA* were co-transfected, though, levels of both EGR1 and PAX3-FOXO1 proteins were reduced (Fig. 2-7 (D)). Thus, ubiqutination of PAX3-FOXO1 by CHIP1 is required for PAX3-FOXO1-mediated destabilization of EGR1 and, thereby, down-regulation of *p57Kip2* transcription and myogenic differentiation.

2.5 DISCUSSION

Here, we show that the accumulation of *p57Kip2* mRNA normally induced by differentiation signals is suppressed by PAX3-FOXO1, thus rendering primary *PAX3-FOXO1* myoblasts refractory to such stimuli. Taken together with other findings (39, 51, 67), our results suggest that loss of functional p57KIP2 is a common feature of both subtypes of rhabdomyosarcoma. *P57Kip2*-null mice display a variety of developmental defects resulting from an inability of cells to differentiate but they do not exhibit an increased susceptibility to tumors (66). Analysis of the four *PAX3-FOXO1* mouse models described to date (2, 31, 33, 46) also suggests that *PAX3-FOXO1* expression alone is insufficient to produce a malignant phenotype. However, disruption of the *Ink4a/ARF* or *Trp53* pathways, targets of inactivation in human rhabdomyosarcoma, in *PAX3-FOXO1* mice substantially increases the frequency of tumor formation (31). These mouse models and our present data suggest that loss of function of p57KIP2 is not a dominantly-acting transforming event. Rather, our data suggest that reduction of p57KIP2 levels by PAX3-FOXO1 is more likely to predispose cells to transformation by a secondary genetic event. PAX3-FOXO1 enables cells to bypass cellular senescence checkpoints through loss of $p16^{INK4a}$ (36). This function, in concert with the failure of PAX3-FOXO1-expressing cells to differentiate would create a large pool of proliferating cells primed for transformation. This mechanism of action is different from those described for any other cancer-related fusion gene (3).

It is interesting that in this context, PAX3-FOXO1 controls *p57Kip2* transcription not by acting as a transcription factor but, rather, by interfering with an intermediary

transcription factor, EGR1. In fact, the most significant transcriptional change was in a gene indirectly controlled by PAX3-FOXO1. It is reasonable to assume that since PAX3-FOXO1 itself, rather than EGR1 is ubiquitinated, any protein bound to PAX3-FOXO1 might also be co-degraded. Depending on the other proteins bound to PAX3-FOXO1, other cellular events, not simply transcription, could be perturbed by PAX3-FOXO1. Uncovering PAX3-FOXO1 binding partners will yield powerful insights into this enigmatic disease.

We had expected, based on the current model of PAX3-FOXO1 function, that previously identified PAX3 transcription targets, some of which also appear to be PAX3-FOXO1 targets, such as MITF (62), RET (34), TYRP1(21), MET (16), NCAM (32) and BCL-xL (38) would be profoundly upregulated in PAX3-FOXO1 transgenic myoblasts. However, transcription of these genes was unchanged in *PAX3-FOXO1* transgenic myoblasts. This might reflect the lower level of PAX3-FOXO1 expression in our transgenic myoblasts as compared to human ARMS tumor cell lines and which is also vastly less than in the overexpression studies. In our system, PAX3-FOXO1 expression is driven by the endogenous PAX3 promoter, as occurs *in vivo*. Therefore the expression levels in PAX3-FOXO1 transgenic myoblasts are likely similar to those that would be seen in affected myoblasts during the early stages of ARMS. Thus, this system should accurately model disease initiation. However, in transfection experiments using other cells that are wild-type for p53, we have routinely had difficultly in achieving even moderate levels of PAX3-FOXO1 expression. Our current findings provide a plausible explanation for these observations. If misfolded proteins accumulate to the extent that they overwhelm the chaperone system, then cells will undergo programmed cell death.

Apoptosis occurs through JNK, primarily through stabilization of p53 (20). Thus, overexpression of PAX3-FOXO1 would be expected to be selected against in otherwise wild-type cells.

The most likely scenario, then, may be that the chromosomal translocation occurs as the first genetic lesion. PAX3 is expressed during embryonic development, with expression ceasing shortly after birth (59). At this time the chromatin structure would be the most amenable to support a translocation event. Indeed, if p53 loss were the initiating event, then a mixture of genetic lesions would be expected in ARMS rather than the consistent t(2;13) translocation, due to the resultant genomic instability. When cells lose p53 function, then ARMS levels of PAX3-FOXO1 protein accumulation are tolerated. With this level of misfolded protein, it is likely that CHIP1 and other components of the misfolded protein response machinery are saturated. Non-ubiquitinated, transcriptionally-active PAX3-FOXO1 could then accumulate and increase the transcription of its previously identified, or other, target genes thereby promoting disease progression. In this model, PAX3-FOXO1 would play two roles in tumoriogenesis, first as a tumor-initiating factor then, after loss of functional p53, as a promoting factor.

The findings presented here raise the possibility that other chromosomal translocation-derived fusion proteins with poorly defined function, such as EWS-FLI, may also contribute to tumorigenesis by their malformation. EWS-FLI is a chimeric transcription factor characteristic of the Ewing Sarcoma Family of Tumors of bone and soft tissue (60). EWS-FLI has previously been shown to repress *p57Kip2* transcription through an unknown mechanism, mediated through multiple *Egr1* sites in the *p57Kip2*

promoter (15). Taken together with our results, this report suggests that EWS-FLI may also participate in the misfolding-mediated degradation of EGR1.

Our findings lead to the hypothesis that PAX3-FOXO1 contributes to rhabdomyosarcoma development by repressing the transcription of *p57Kip2.* Reduction of EGR1 protein levels by PAX3-FOXO1 results in inadequate quantities of p57KIP2 protein, preventing *PAX3-FOXO1* transgenic myoblasts from completing the differentiation program (Fig. 2-8). Thus, translocation-positive myoblasts that are unable to exit the cell cycle, could establish a pool of proliferating precursor cells susceptible to a secondary transforming event thus illustrating that tumor-specific genetic alterations could be central to the interplay between development and cancer. Finally, these results may have therapeutic applications because they suggest that ARMS is, at least in part, a protein degradation-driven disease. This implies that proteasome inhibitors, such as bortezomib, might be a new and effective approach for treating ARMS.

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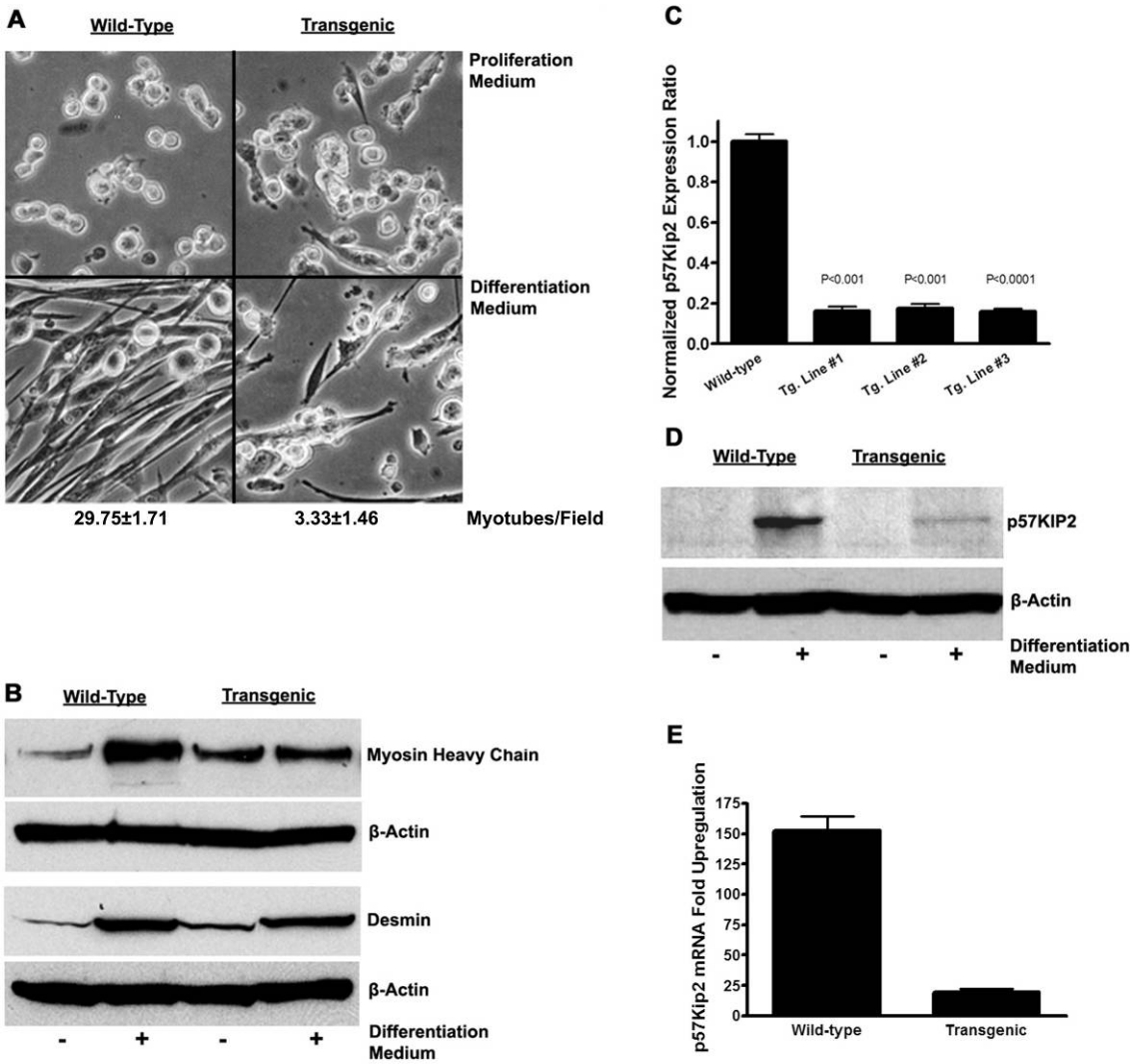


FIGURE 2-1. *PAX3-FOXO1* TRANSGENIC MYOBLASTS CONTAIN LOW LEVELS OF P57KIP2 AND ARE UNABLE TO DIFFERENTIATE.

(A) (upper) Proliferating myoblasts, seeded at constant density.
(lower) Myoblasts, plated at a constant density and maintained in differentiation medium for 24 hours.

(B) Immunoblot analysis of myogenic markers in wild type and transgenic myoblasts maintained in either proliferation or differentiation medium.

(C) Quantitative PCR analysis of *p57Kip2* mRNA from early-passage myoblasts. P values of the differences between wild-type and transgenic myoblasts were calculated using two-tailed Student's *T* test. Error bars represent standard deviations.

(D) Immunoblot analysis of p57KIP2 protein levels in wild-type or transgenic myoblasts maintained in either proliferation or differentiation medium.

(E) Quantitative PCR analysis of *p57Kip2* mRNA accumulation during differentiation. Values shown are the mean fold upregulation, ± SD.

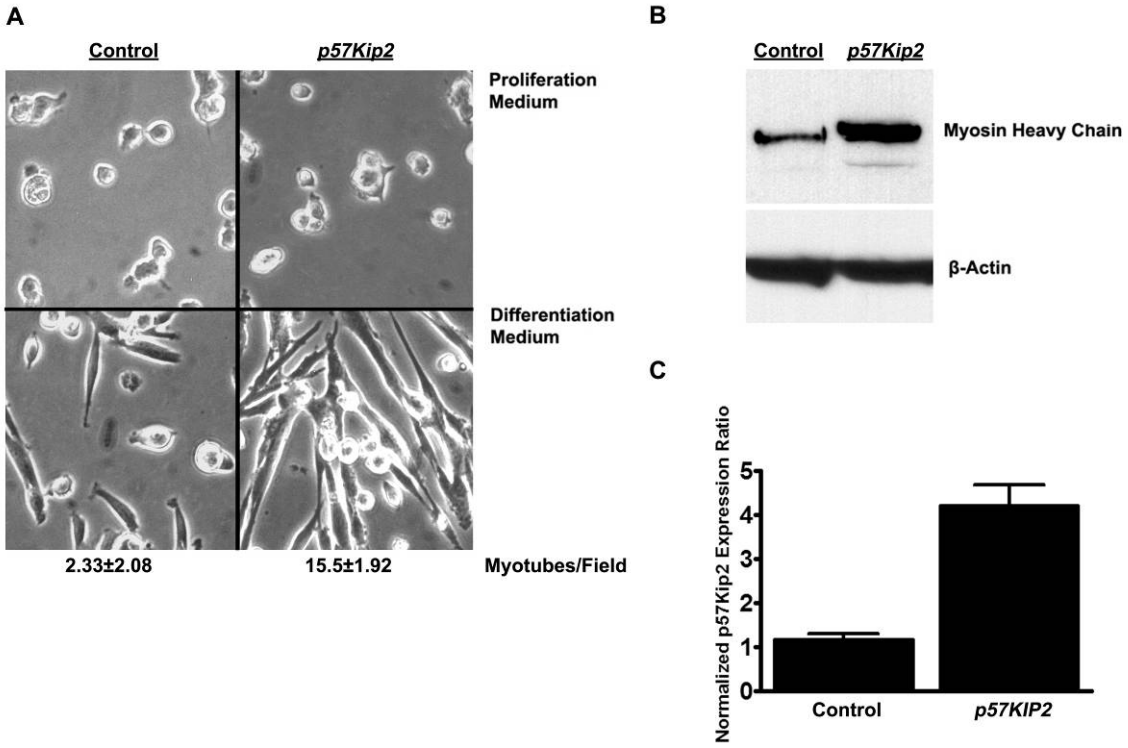


FIGURE 2-2. RESTORATION OF *P57KIP2* LEVELS IN PAX3-FOXO1 TRANSGENIC MYOBLASTS PROMOTES THEIR DIFFERENTIATION.

(A) (upper) Proliferating myoblasts, seeded at constant density.
(lower) Myoblasts, plated at a constant density and maintained in differentiation medium for 24 hours.

(B) Immunoblot analysis of myosin heavy chain in control and *p57Kip2* transduced transgenic myoblasts maintained in either proliferation or differentiation medium.

(C) Quantitative PCR analysis of *p57Kip2* mRNA from proliferating control and *p57Kip2* transduced transgenic myoblasts

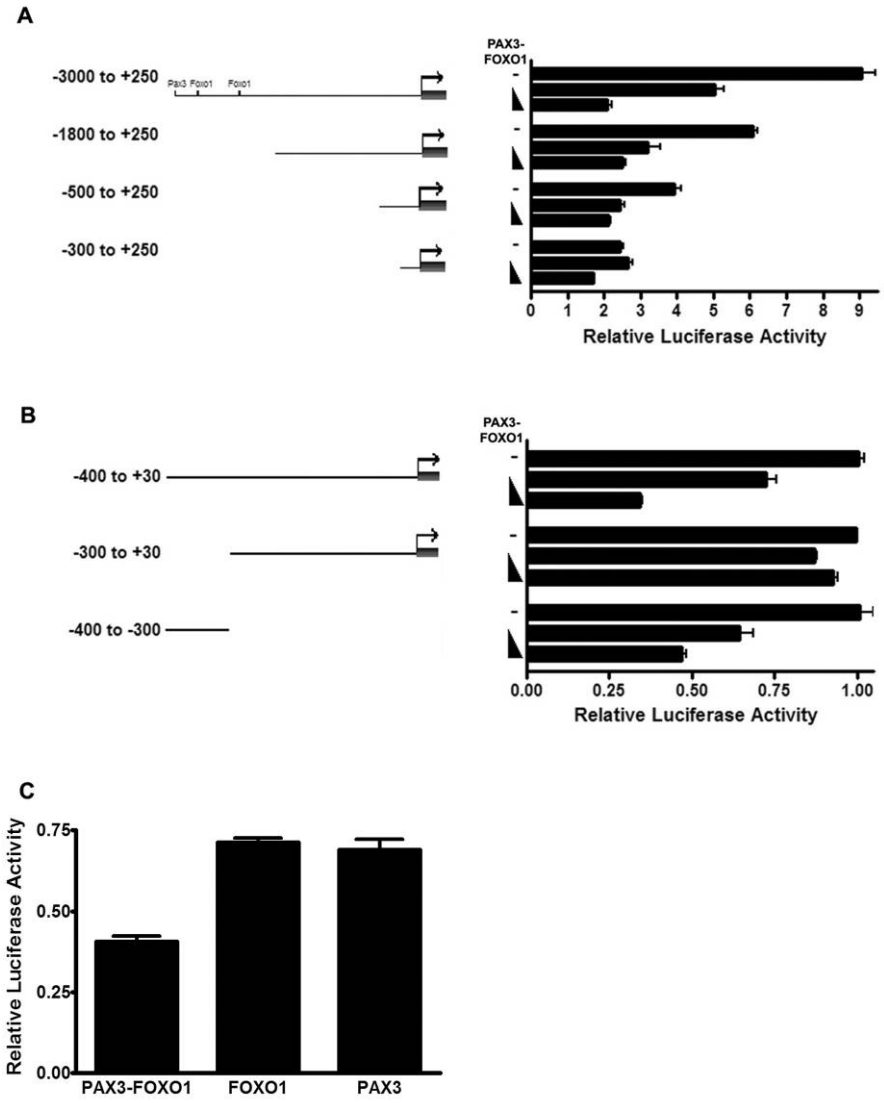


FIGURE 2-3. PAX3-FOXO1 REPRESSES *P57KIP2* TRANSCRIPTION THROUGH MULTIPLE GC-RICH SEQUENCE ELEMENTS.

(A) Luciferase reporter assays of sequential deletions of the *p57Kip2* promoter, co-transfected with increasing quantities of PAX3-FOXO1. Values are expressed as mean firefly luciferase activity normalized to control *Renilla* luciferase activity.

(B) Luciferase reporter assays of *p57Kip2* sequential deletions, co-transfected with increasing quantities of PAX3-FOXO1. The -400 to -300 bp *p57Kip2* promoter sequence is upstream of a minimal *tk* promoter. Values are expressed as mean fold activation relative to promoter co-transfected with empty vector.

(C) Luciferase reporter activity of PAX3, FOXO1, and PAX3-FOXO1 on the *p57Kip2* promoter, -400 to +30 bp, normalized to background activity on -300 to +30 bp. Values are expressed as mean fold activation relative to promoter co-transfected with empty vector.

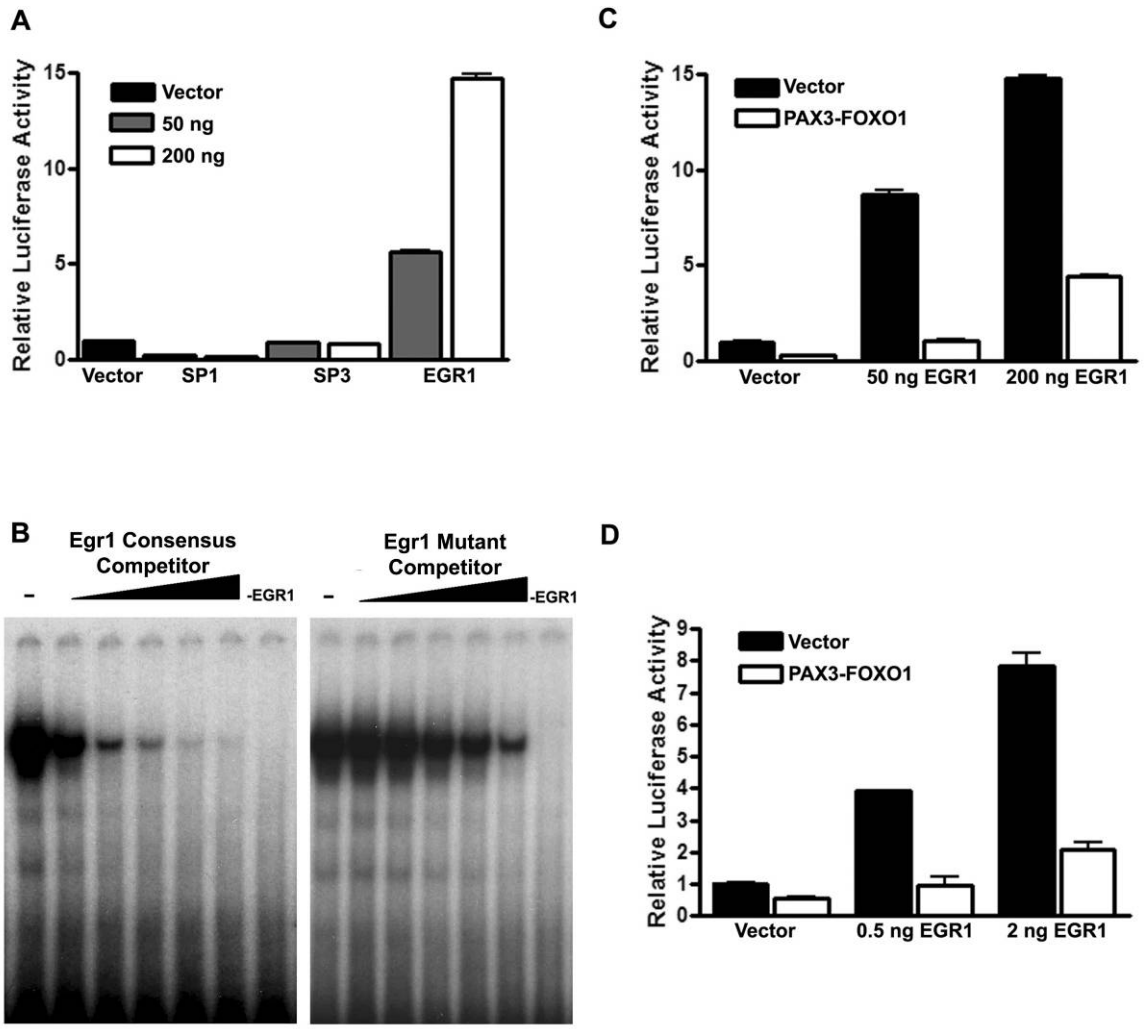


FIGURE 2-4. PAX3-FOXO1 INTERFERES WITH EGR1- DEPENDENT TRANSCRIPTION.

(A) Luciferase reporter assays of EGR1, SP1, and SP3 on the full-length *p57Kip2* promoter. Values are expressed as fold activation relative to promoter co-transfected with empty vector.

(B) EMSA of *in vitro* transcribed/ translated EGR1 on -400 to -350 bp *p57Kip2* promoter. Competing DNA was either wild-type sequence from -400 to -350 bp of the *p57Kip2* promoter (left), or mutated by disruption of EGR1-binding sites (right).

(C) Luciferase reporter assay of the full-length *p57Kip2* promoter co-transfected with an increasing quantity of EGR1, with or without PAX3-FOXO1.

(D) Luciferase reporter assay of an EGR1 synthetic reporter co-transfected with an increasing quantity of EGR1, with or without PAX3-FOXO1.

For all luciferase reporter assays, results are the mean values ± SD of triplicates. Results are representative of at least three independent experiments.

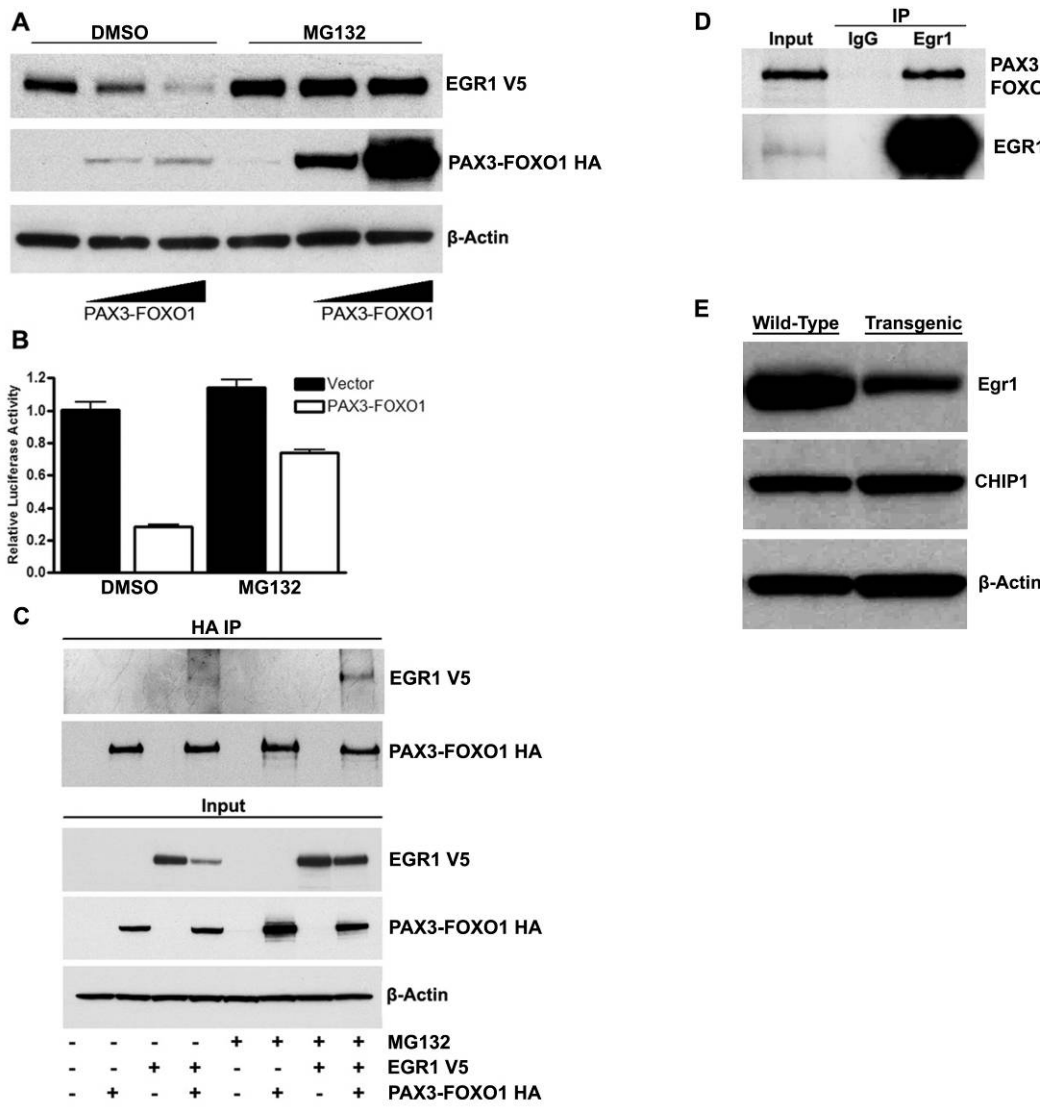


FIGURE 2-5. PAX3-FOXO1 INTERACTS WITH AND DESTABILIZES EGR1.

(A) Immunoblot analysis of EGR1 V5 protein in the presence of increasing quantities of PAX3-FOXO1 HA, with or without the proteasome inhibitor MG132.

(B) Luciferase reporter assay of the full-length *p57Kip2* promoter, co-transfected with PAX3-FOXO1, in the presence of MG132.

(C) Co-immunoprecipitation analysis of epitope-tagged proteins, with or without MG132.

(D) Co-immunoprecipitation of endogenous EGR1 and PAX3-FOXO1 from the cell line Rh28, in the presence of MG132.

(E) Immunoblot analysis of Egr1 in wild-type and *PAX3-FOXO1* transgenic myoblasts maintained in proliferation medium.

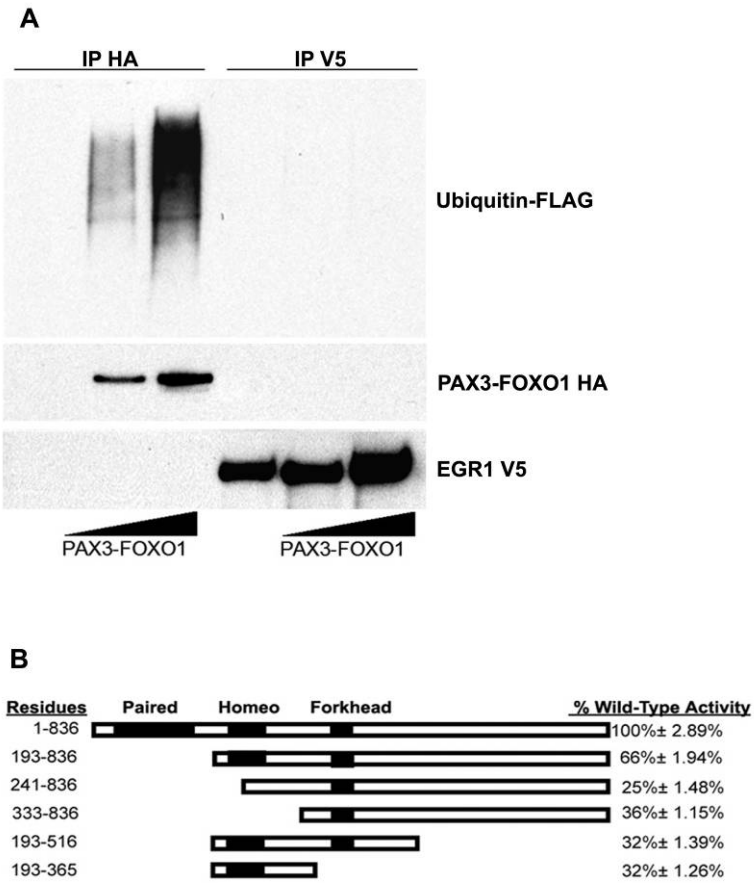


FIGURE 2-6. PAX3-FOXO1, BUT NOT EGR1, IS UBIQUITINATED.

(A) Immunoprecipitation of EGR1 V5 or PAX3-FOXO1 HA co-transfected with ubiquitin-FLAG, in the presence of MG132 and iodoacetamide.

(B) Deletion mutants of PAX3-FOXO1, shown at left, with their activity by luciferase reporter assay, as a mean percentage of wild-type, shown at right, ± SD.

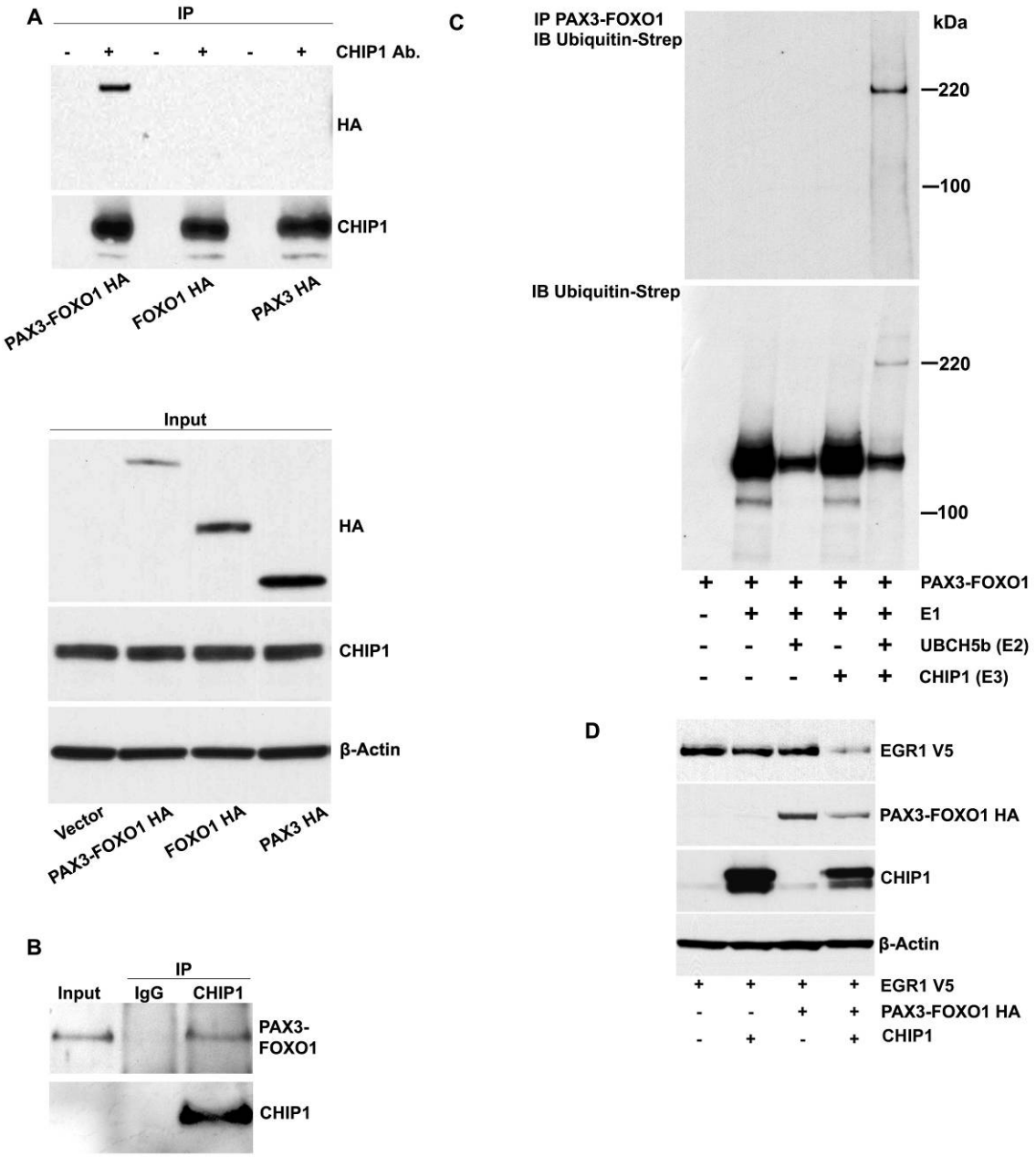


FIGURE 2-7. THE DIRECT UBIQUITINATION OF PAX3-FOXO1 BY CHIP1 IS REQUIRED FOR PAX3-FOXO1-DEPENDENT DEGRADATION OF EGR1.

(A) Co-immunoprecipitation analysis of epitope-tagged proteins.
(B) Co-immunoprecipitation of endogenous CHIP1 and PAX3-FOXO1 from the cell line Rh28, in the presence of MG132.
(C) (upper) Immunoprecipitation of PAX3-FOXO1 from *in vitro* ubiquitination reactions. (lower) Immunoblot analysis of *in vitro* ubiquitination reactions.
(D) Immunoblot analysis of EGR1 V5 and PAX3-FOXO1 HA proteins, co-transfected with or without CHIP1 in COS7 cells.

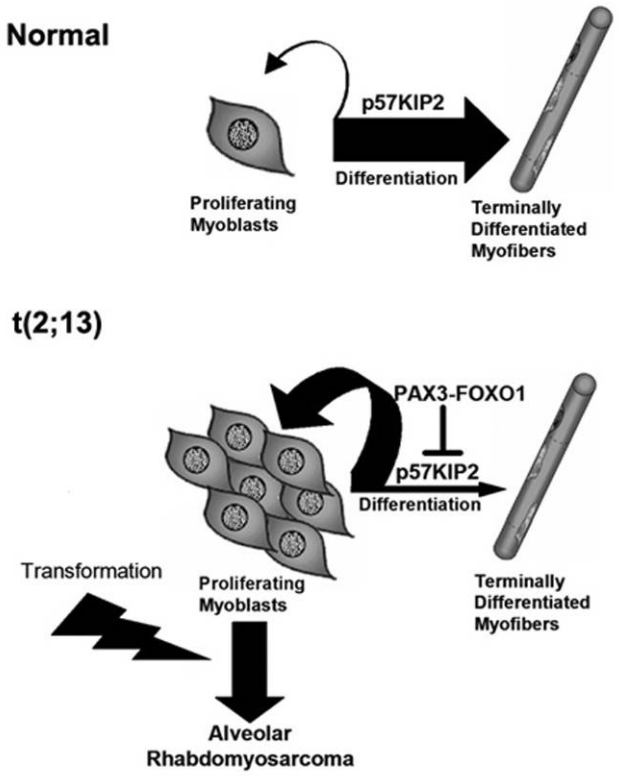
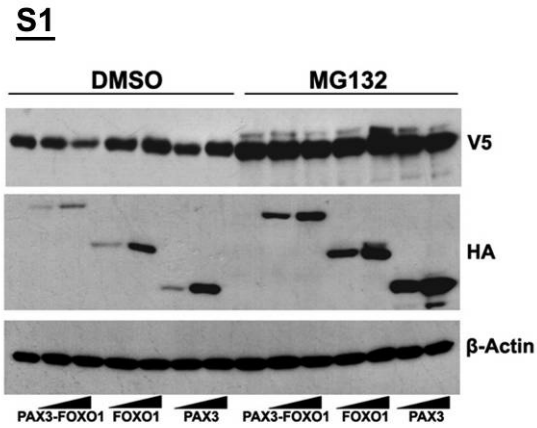


FIGURE 2-8. MODEL FOR PAX3-FOXO1-DRIVEN SUPPRESSION OF MYOGENIC DIFFERENTIATION.



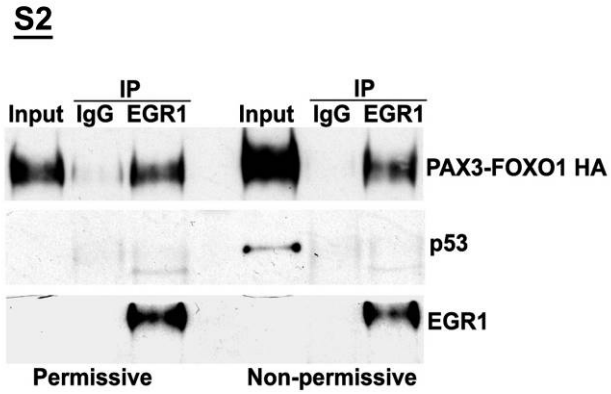


FIGURE 2-9. SUPPLEMENTAL DATA

(S1) Immunoblot analysis of EGR1 V5 protein in the presence of increasing quantities of PAX3-FOXO1 HA, PAX3 HA, or FOXO1 HA, incubated with MG132.

(S2) Co-immunoprecipitation of PAX3-FOXO1 and EGR1, from ubiquitination competent versus incompetent cells.

CHAPTER 3: PAX3-FOXO1 INTERFERES WITH TP53 FUNCTION BY UPREGULATING MDM2 EXPRESSION

3.1 ABSTRACT

Rhabdomyosarcomas (RMS), malignant tumors of striated muscle with a mesenchymal origin, are the most common soft tissue sarcomas in children. Tumors of the alveolar subtype (ARMS) are characterized by a balanced reciprocal chromosomal translocation t(2;13) that fuses the *PAX3* and *FOXO1* genes. In tumors harboring the translocation, expression of the *PAX3-FOXO1* fusion gene is independently prognostic of a poor response to therapy. While intensive multimodality therapies employing radiotherapies, chemotherapy, and surgery have increased the survival of patients with alveolar rhabdomyosarcoma, such approaches frequently result in devastating late-term effects. To understand how expression of *PAX3-FOXO1* renders mesenchymal cells resistant to treatment, we isolated muscle cells from *PAX3-FOXO1* transgenic mice. We found that these cells were resistant to radiation-induced cell death and that this was due to decreased TP53 activity. This down-regulation was mediated through a PAX3-FOXO1-depedent increase in *Mdm2* transcription that was effected through an element proximal to the second promoter of the *MDM2* gene. These results hold promise for the therapy of ARMS as treatment with an MDM2 antagonist in concert with standard therapies may improve long term patient outcome.

3.2 INTRODUCTION

Rhabdomyosarcoma is the most common soft tissue sarcoma of children and young adults (30). These tumors are generally divided into two major subgroups: embryonal, representing the more favorable prognosis with an overall survival rate of 82%, and alveolar, with a reduced overall survival rate of 65%. This poorer responsiveness of ARMS is evident even for treatment regimens that combine radiation therapy, combination chemotherapies, and surgery (21).

A balanced translocation between chromosomes 2 and 13 is the most common genetic aberration in ARMS (28) and results in a chimeric transcription factor, PAX3-FOXO1 (PAX3-FKHR), containing the DNA binding elements of PAX3 and the transcriptional activation domain of FOXO1 (11). Expression of *PAX3-FOXO1* is specific to ARMS and is independently prognostic of poor patient outcome (28).

Solid variants of ARMS have been found to carry the same poor prognosis as do those ARMS with the more classical histology, suggesting that the aggressive behavior of these tumors may be a consequence of *PAX3-FOXO1* expression (26). The present studies were undertaken to determine the role of PAX3-FOXO1 in response to radiotherapy.

3.3 **MATERIALS AND METHODS**

Plasmids. Mammalian expression vectors encoding *PAX3-FOXO1*, *PAX3*, and *TP53* were derived from pcDNA3.1 (Invitrogen).

Mouse *Mdm2* promoter deletions were constructed using the firefly luciferase vector, pGL3 basic (Promega). The Pax3 reporter, containing three tandem copies of the predicted PAX3 site (ATCGCCACTGAAC) as well as the mutated version (AAAACCACTGAAC) separated by spacers, were derived from the pLucMCS vector (Stratagene).

Cell Culture. All transfections were performed with mouse NIH3T3 cells, in DMEM with 10% FBS. Cells were transfected using 2.5 ug of DNA and 5 ul of Lipofectamine 2000 (Invitrogen) per $1x10^5$ cells.

Western blots. Protein was harvested in RIPA buffer (150 mM NaCl, 1.0% TritonX100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and Complete protease inhibitor (Roche)), 10ug of total protein was added to LDS sample buffer (Invitrogen) separated on either 4-12% or 3-8% Nupage gels (Invitrogen), transferred to nitrocellulose, blocked (PBS containing 0.1% Tween 20, 5% nonfat milk) and incubated for 1 hr at RT with primary antibodies.

Isolation of mouse myoblasts. p53 knockout mice were obtained from Jackson labs. Isolation and culture of mouse myoblasts was performed as described in (27). Briefly, hindlimb muscle tissue was dissected from 3 day-old mice, minced and digested with Liberase Blendzyme 3 (Roche) followed by trypsin. After washing, this mixture was plated and cultured in myoblast growth media (20% FBS in a 50/50 mix of DMEM/Ham's F10 (Invitrogen) and 25 ng/ml bFGF (Dako)). Fibroblasts were removed by selective plating at each subsequent passage. For expression analysis, RNA was harvested from cultures grown in triplicate using the RNeasy Plus Kit (Qiagen).

Quantitative real-time PCR. Total RNA was harvested as above and reverse transcribed (Superscript III First Strand Kit, Invitrogen). Quantitative PCR was performed on the Icycler IQ using IQ Syber Green (Biorad) according to the manufacturer's instructions and default machine settings using an annealing temperature of 60°C.

Reporter Assays. 3T3 cells were co-transfected with the firefly luciferase reporter plasmid and the indicated combinations of expression plasmid. The *Renilla* luciferase plasmid pRL (Promega), driven by a minimal tk promoter, was included as an internal control. 48 hrs post-transfection, the Dual-Luciferase Reporter Assay (Promega) was performed according to the manufacturer's instructions and values were read on the GENios Pro (Tecan).

3.4 RESULTS

Since ARMS apparently arises from largely undifferentiated cells in skeletal muscle [1], we isolated primary myoblastic cells from the muscles of transgenic mice that express *PAX3-FOXO1* under the control of the *PAX3* promoter (1). The use of primary cells enabled us to dissect the effect of PAX3-FOXO1 alone without the contribution of the additional genetic abnormalities found in cells derived from tumors.

To determine whether *PAX3-FOXO1* expression affects radiation responsiveness, cells were subjected to 10 Gy of ionizing radiation, a dose comparable to that given to patients undergoing intraoperative radiotherapy or brachytherapy (20). Cell survival was assessed at 28 hours after irradiation. As shown in Fig. 3-1, expression of PAX3-FOXO1

enables cells to resist radiation-induced cell death when compared to myoblastic cells isolated from non-transgenic mice.

Since radiosensitivity has been demonstrated in other instances to be mediated by TP53 (14, 32), we next tested the effect of *PAX3-FOXO1* expression on the sensitivity of TP53 target genes to TP53 dosage in myoblastic cells. Quantitative PCR was performed on transcripts from myoblasts isolated from either *Tp53* heterozygous or homozygous null animals. Levels of TP53 target genes from these cells were compared to identically isolated and cultured wild-type myoblasts. As might be predicted (9), the most sensitive gene was the Cdk inhibitor, $p21^{Waf1/Cip1}$(*Cdkn1c*) whose levels were decreased 10 fold in *Tp53* heterozygotes and an additional 10 fold in *Tp53* homozygous null myoblasts (Fig. 3-2).

Having established that $p21^{Waf1/Cip1}$ gene expression is a highly sensitive readout for p53 transcriptional activity in myoblasts, $p21^{Waf1/Cip1}$ transcript levels in the *PAX3-FOXO1* transgenic myoblasts were measured by quantitative PCR. The $p21^{Waf1/Cip1}$ transcript level was decreased approximately two fold in *PAX3-FOXO1* myoblasts (Fig. 3-3), suggesting that the transcriptional activity of TP53 is impaired by PAX3-FOXO1.

To determine whether PAX3-FOXO1 could suppress TP53-mediated transcription, luciferase reporter assays were performed using a synthetic reporter containing three tandem copies of the TP53 consensus sequence. PAX3-FOXO1 was able to reduce TP53's activation of its consensus sequence by 2-3 fold (Fig. 3-4). Thus PAX3-FOXO1 interferes with the transcriptional activity of TP53.

MDM2 is the primary regulator of TP53 activity, operating via three mechanisms. First, MDM2 binds to TP53, physically interfering with its transactivation of its

transcriptional targets (22). Second, MDM2 relocates TP53 from the nucleus to the cytoplasm (4, 12). Third, MDM2 acts as an E3 ubiquitin ligase targeting TP53 for proteasomal degradation (15). *Tp53/Mdm2* double null mice are phenotypically indistiquishable from *Tp53* null mice indicating that MDM2 primarily functions to regulate TP53 function (16, 23). Given the role that MDMD2 plays in orchestrating TP53 activity, we sought to determine if the levels of MDM2 were altered in *PAX3-FOXO1* myoblasts. Western blotting was performed on lysates harvested from cells plated at a constant density and harvested at similar passage numbers. Surprisingly, given that *Mdm2* is a TP53 target gene (3), levels of full-length MDM2 protein were found to be increased in *PAX3-FOXO1* transgenic myoblasts relative to wild-type myoblasts (Fig. 3-5). To determine if this increase was due to PAX3-FOXO1 upregulation of *Mdm2* transcription, RNA was harvested from wild-type and *PAX3-FOXO1* myoblasts and simultaneously subjected to quantitative PCR analysis. As shown in Figure 3-6, PAX3-FOXO1 increases the expression of *Mdm2 by* 2-3 fold, suggesting that PAX3-FOXO1 may impair TP53 activity by upregulating *Mdm2* transcription.

The transcription of *MDM2* is controlled from two promoters (31). The TP53-independent promoter is thought to be responsible for generating most of the *MDM2* transcript under normal conditions. The second promoter, containing two TP53 binding sites, modulates stress-induced increases in *MDM2* transcription (19). Transcripts derived from the second promoter are more efficiently translated and produce a greater percentage of full-length MDM2 protein (2, 5).

Since there is a putative PAX3 binding site in the second *MDM2* promoter, we speculated that PAX3-FOXO1 upregulates transcription through this promoter. To test

this hypothesis, three reporter plasmids were constructed. The first contained the entire promoter, 487 bp; the second construct lacked the GC rich sequence, beginning at 384; and the last construct was missing the p53 responsive elements, containing sequence slightly upstream of the core promoter. The reporter plasmids were tested for PAX3-FOXO1 responsiveness by luciferase reporter assays. Expression of all three constructs was increased by PAX3-FOXO1 (Fig. 3-7), indicating that PAX3-FOXO1 exerts it effects through elements proximal to the core promoter.

In the acute response to ionizing radiation, MDM2 is phosphorylated by ATM rendering it inactive (7). Even though the *PAX3-FOXO1* transgenic myoblasts have impaired TP53 function, they have a normal short-term response to ionizing radiation, likely due to the initial inactivation of MDM2 by ATM. Reflective of this, *PAX3-FOXO1* transgenic cells, in the acute response, upregulate *Tp53* transcription at a similar magnitude as wild-type cells (Fig. 3-8). If, as indicated in the reporter assays, PAX3-FOXO1 acts on the second promoter it might interfere with the stress-induced regulation of this promoter. To test this, wild-type and *PAX3-FOXO1* transgenic myoblasts were subjected to 10 Gy of gamma radiation. RNA was harvested two hours after treatment and QPCR was performed. As shown in Figure 3-9, these cells fail to upregulate the transcription of *Mdm2*. These results, taken together with the reporter assay data, indicate that PAX3-FOXO1 acts on the second, stress-responsive promoter of *MDM2* and it is this that regulates TP53 activity and thereby confers radiation resistance.

3.5 DISCUSSION

Here we show that primary myoblastic cell expressing *PAX3-FOXO1* are resistant to cell death induced by ionizing radiation. This property was conferred by PAX3-FOXO1 alone and did not require any cooperating genetic lesions. This finding has explanatory power for the clinical observation of a requirement for higher doses of radiation therapy to treat rhabdomyosarcomas of the alveolar subtype.

High levels of MDM2 are correlated with poor prognosis in a variety of tumors (reviewed in (8)). Nearly 50% of ARMS have amplified the chromosomal region enconding *MDM2*, as compared to the 10% seen in tumor of the embryonal subtype (29). Additionally, MDM2 positive tumors are significantly more resistant to both chemo- and radiotherapy. High levels of *MDM2* expression have been shown to convey multidrug resistance through upregulation of MDR1 in ARMS (6). Taken together, these data suggest that MDM2 is an important modulator of chemo- and radioresistance in ARMS.

Recent studies in mice have show that relatively small changes in *MDM2* expression profoundly affect TP53 function (18). For example, knock-in mice with a hypomorphic *Mdm2* allele had levels of *Mdm2* transcript that was roughly half of the wild-type dosage although levels of TP53 protein were unchanged. Of importance to our present results, this moderate reduction in MDM2 also resulted in a substantial increase in radiosensitivity. Taken together with our results, these data suggest that relatively modest changes in *Mdm2* mRNA levels are sufficient to mediate large changes in TP53 function.

Amplification or overexpression of *MDM2* are frequent events in ARMS (13, 17). Additionally, losses in TP53 function are the second most common genetic lesion in ARMS (10, 24). These observations suggest that maintaining the balance between MDM2 and TP53 is particularly critical in myogenic precursor cells in preventing oncogenic conversion.

These observations hold hope for the treatment of alveolar rhabdomyosarcomas. Only a small decrease in MDM2 function should be necessary to sensitize these tumors to radiation therapy. The extremely aggressive treatment regimen for ARMS yields therapy-related fatality rates of about 7-8% (25). Additionally, treatment of the ARMS occurring in the head can result in devastating late effects such as blindness or brain damage. Thus, it is possible that the administration of MDM2 inhibitors in conjunction with standard therapy may permit amelioration of the harsh treatment specific for the alveolar subtype of rhabdomyosarcoma.

3.6 ACKNOWLEDGEMENTS

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Chapter 3, in full, is in preparation for publication by Wendy Roeb, Antonia Boyer, Webster K. Cavenee, Karen C. Arden. The dissertation author was the primary investigator and author of this paper.

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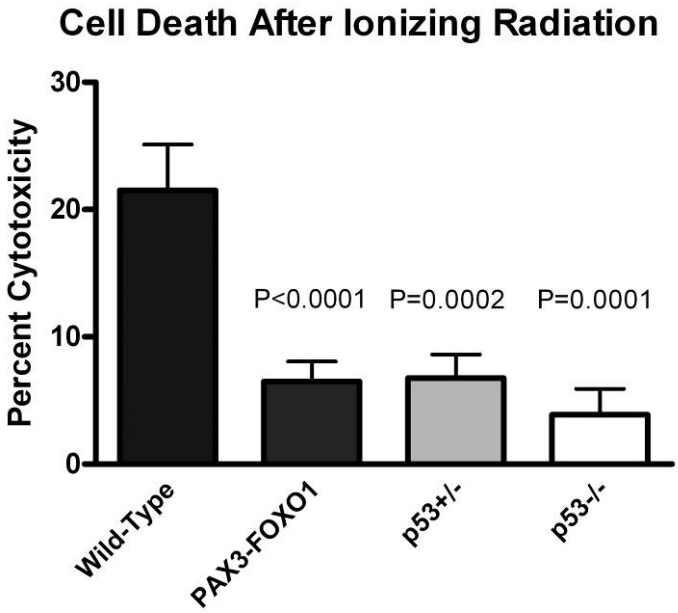

FIGURE 3-1. *PAX3-FOXO1* TRANSGENIC MYOBLASTS ARE RESISTANT TO RADIATION-INDUCED CELL DEATH. Proliferating mybolasts, plated at a constant density, were subjected to 10 Gy ionizing radiation. Cell death was quantitated 28 hours after treatment by measuring LDH activity in the media.

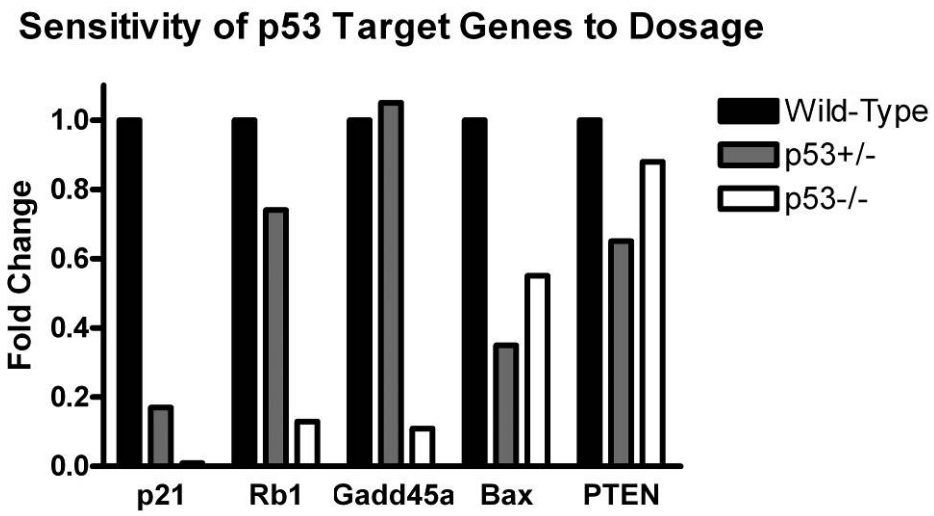

FIGURE 3-2. *P21*$^{WAF1/CIP1}$ GENE TRANSCRIPTION IS A SENSITIVE INDICATOR OF TP53 LEVELS. Quantitative PCR analysis of TP53 target genes in proliferating myoblasts.

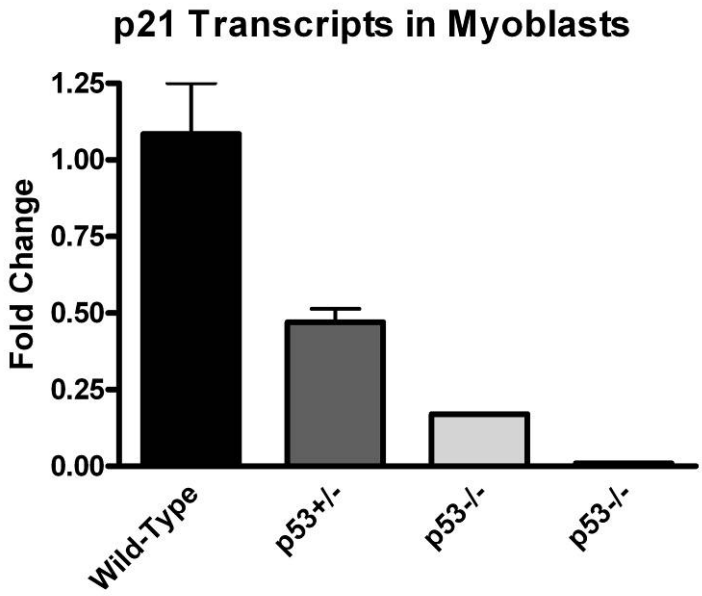

FIGURE 3-3. *PAX3-FOXO1* TRANSGENIC MYOBLASTS HAVE REDUCED LEVELS OF THE TP53 TARGET GENE *P21$^{WAF1/CIP1}$*. Quantitative PCR analysis of p21 mRNA in proliferating myoblasts.

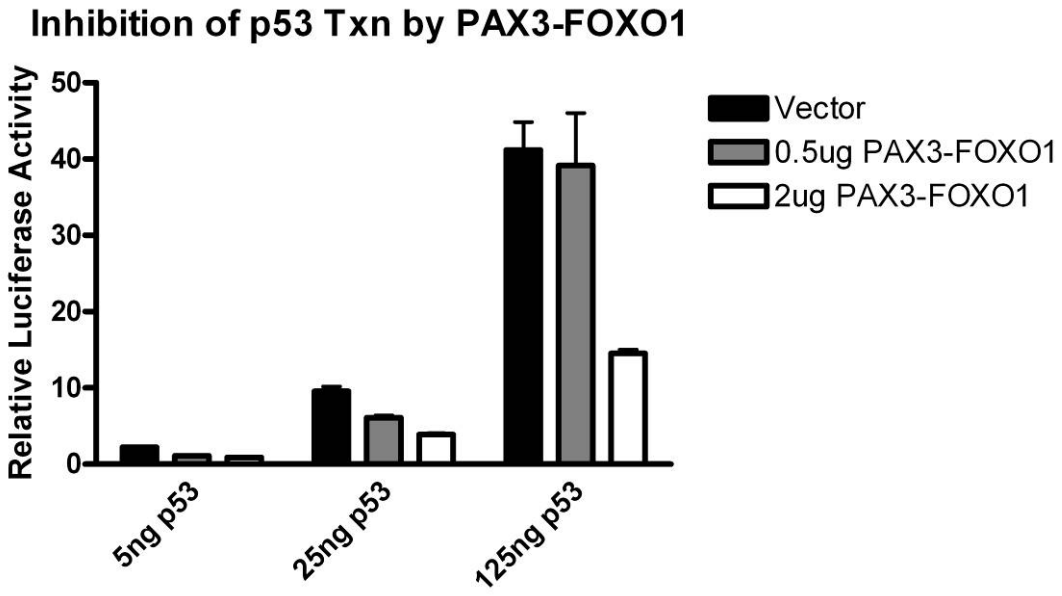

FIGURE 3-4. PAX3-FOXO1 INTERFERES WITH THE TRANSCRIPTIONAL ACTIVITY OF TP53. Luciferase reporter assays of a TP53 synthetic reporter co-transfected with an increasing quantity of *PAX3-FOXO1*.

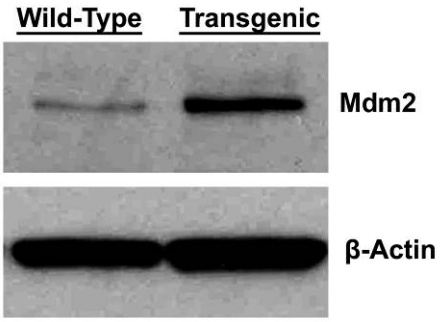

FIGURE 3-5. *PAX3-FOXO1* TRANSGENIC MYOBLASTS CONTAIN INCREASED QUANTITIES OF MDM2 PROTEIN. Immunoblot analysis of MDM2 protein in proliferating myoblasts.

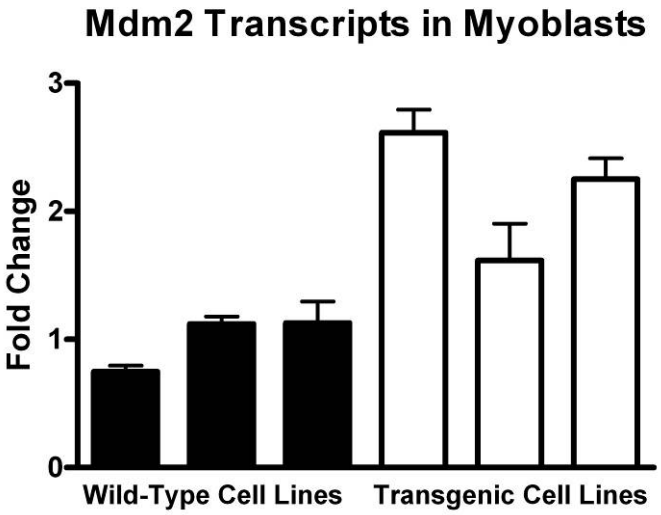

FIGURE 3-6. PAX3-FOXO1 TRANSGENIC MYOBLASTS CONTAIN INCREASED QUANTITIES OF *MDM2* MRNA. Quantitative PCR analysis of *Mdm2* mRNA in proliferating myoblasts.

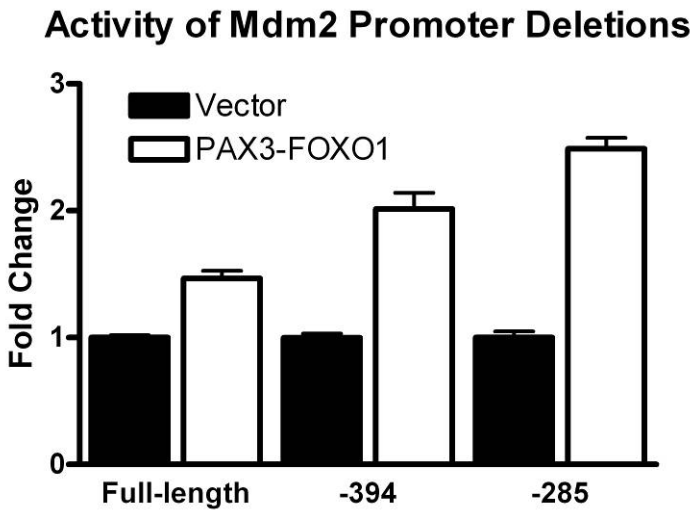

FIGURE 3-7. PAX3-FOXO1 EXERTS ITS EFFECTS THROUGH SEQUENCE PROXIMAL TO THE TRANSCRIPTION START SITE OF THE SECOND PROMOTER. Luciferase reporter assays of sequential deletions of the *MDM2* promoter, co-transfected with *PAX3-FOXO1*.

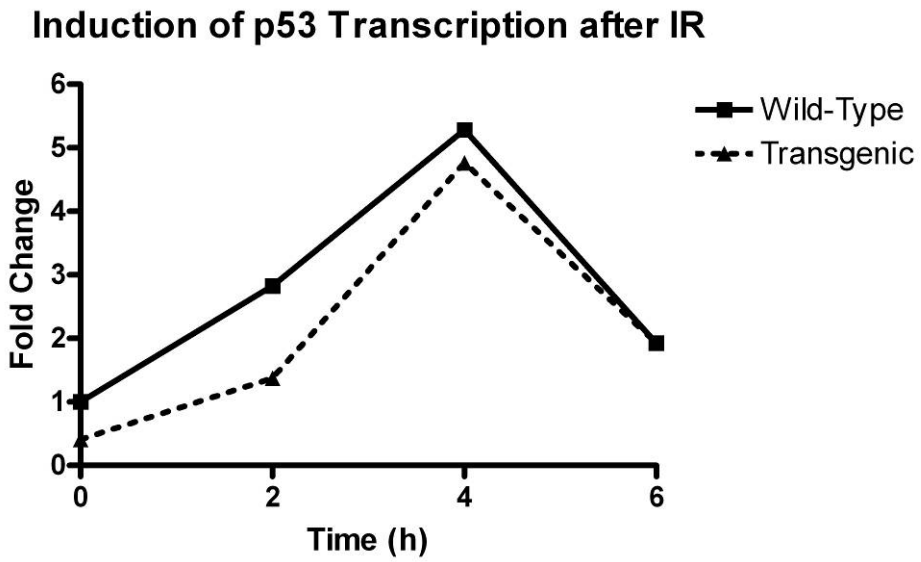

FIGURE 3-8. *PAX3-FOXO1* TRANSGENIC MYOBLASTS APPROPRIATELY UPREGULATE P53 EXPRESSION IN RESPONSE TO IONIZING RADIATION. Quantitative PCR analysis of upregulation of *Tp53* mRNA levels 2 hours after exposure to 10 Gy ionizing radiation.

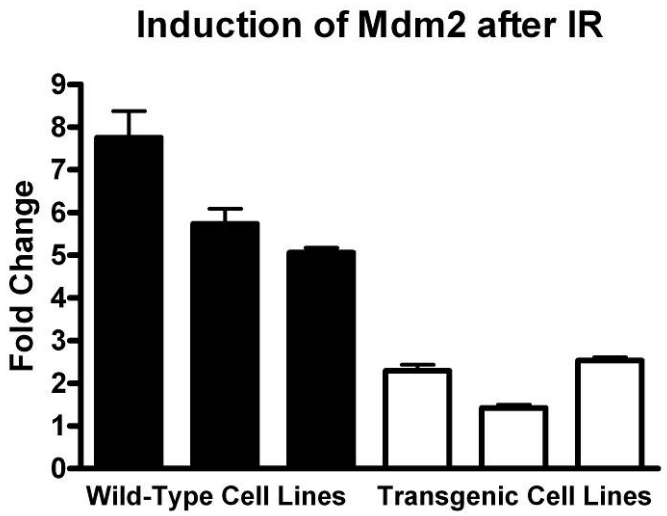

FIGURE 3-9. *PAX3-FOXO1* TRANSGENIC MYOBLASTS FAIL TO APPROPRIATELY UPREGULATE MDM2 EXPRESSION IN RESPONSE TO IONIZING RADIATION. Quantitative PCR analysis of upregulation of *Mdm2* mRNA levels 2 hours after exposure to 10 Gy ionizing radiation.

CHAPTER 4: FUTURE DIRECTIONS AND SUMMARY

4.1 FUTURE DIRECTIONS

It is interesting that in this context, PAX3-FOXO1 controls *p57Kip2* transcription not by acting as a transcription factor but, rather, by interfering with an intermediary transcription factor, EGR1. In fact, the most significant transcriptional change in transgenic myoblasts was in a gene indirectly controlled by PAX3-FOXO1. It is reasonable to assume that since PAX3-FOXO1 itself, rather than EGR1 is ubiquitinated, any protein bound to PAX3-FOXO1 might also be co-degraded. Depending on the other proteins bound to PAX3-FOXO1, other cellular events, not simply transcription, could be perturbed by PAX3-FOXO1. Immunoprecipitation of PAX3-FOXO1 followed by mass spectroscopy or, alternatively, yeast two hybrid screening will uncover novel PAX3-FOXO1 binding partners, yielding powerful insights into this enigmatic disease.

The findings presented here raise the possibility that other chromosomal translocation-derived fusion proteins with poorly defined function, such as EWS-FLI, may also contribute to tumorigenesis by their malformation. EWS-FLI is a chimeric transcription factor characteristic of the Ewing Sarcoma Family of Tumors of bone and soft tissue (2). EWS-FLI has previously been shown to repress *p57KIP2* transcription through an unknown mechanism, mediated through multiple *EGR1* sites in the *p57KIP2* promoter (1). Taken together with our results, this report suggests that EWS-FLI may also participate in the misfolding-mediated degradation of EGR1. Co-immunoprecipition and EGR1 degradation experiments will confirm this hypothesis.

4.2 SUMMARY

The research presented here show that PAX3-FOXO1 functions as a misfolded protein. This feature, in addition to PAX3-FOXO1's function as a transcription factor, demonstrates that PAX3-FOXO1 contributes to ARMS pathogenesis through multiple mechanisms. These findings describe the discovery of a new mechanism whereby tumor-associated genetic alterations increase the likelihood of cancer formation and may lead to new therapeutic approaches.

4.3 REFERENCES

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