Tumorigenesis and Neoplastic Progression

Impaired Wnt Signaling in Embryonal Rhabdomyosarcoma Cells from *p53/c-fos* Double Mutant Mice

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Rhabdomyosarcoma is a primitive neoplasm with a poorly understood etiology that exhibits features of fetal skeletal muscle. It represents the most frequent malignant soft tissue sarcoma affecting the pediatric population and is often treated very aggressively. Embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma constitute the two major subtypes and exhibit different molecular features. We investigated one potential molecular basis for ERMS by using cells derived from tumors produced in *p53*^{-/-}/*c-fos*^{-/-} mice. This model closely recapitulates the timing, location, molecular markers, and histology seen in human ERMS. A combined chromatin immunoprecipitation/promoter microarray approach was used to identify promoters bound by the c-Jun-containing AP-1 complex in the tumor-derived cells that lacked c-Fos. Identification of the *Wnt2* gene and its overexpression in ERMS cells was confirmed in human rhabdomyosarcoma cell lines and prompted further analysis of the Wnt signaling pathway. Contrary to our expectations, the canonical Wnt/ β -catenin signaling pathway was down-regulated in ERMS cells compared with normal myoblasts, and activating this pathway promoted myogenic differentiation. Furthermore, the identification of both *survivin* and *sfrp2* through promoter and expression analyses suggested that increased resistance to apoptosis was associated with the inhibition of the Wnt signaling pathway. These results suggest that altered AP-1 activity that leads to the down-regulation of the Wnt pathway

may contribute to the inhibition of myogenic differentiation and resistance to apoptosis in ERMS cases. (Am J Pathol 2010; 177:2055–2066; DOI: 10.2353/ajpath.2010.091195)

Efforts to unravel the molecular events underlying the origin of different types of cancer have contributed to finding treatments for these diseases. However, largely left behind in this effort are tumors with poorly understood etiologies like rhabdomyosarcoma (RMS). RMS describes a heterogeneous group of poorly differentiated pediatric sarcomas that display features of developing muscle.¹ Representing 60% of all pediatric sarcomas and accounting for 5% to 10% of all childhood malignancies, treatment is often very aggressive, involving local irradiation, lengthy rounds of combination chemotherapy, and tumor resection.² RMS is broadly categorized into two subtypes, embryonal (ERMS) and alveolar (ARMS), that possess distinctive clinical, pathological, and biological properties.³

ARMS portends a poor prognosis and predominantly occurs in the extremities.¹ Cytogenetically, most ARMS harbor one or both of two distinct chromosomal translocations: t(2;13)(q35;q14) or t(1;13)(p36;q14), resulting in the formation of the fusion genes *PAX3-FOXO1* or *PAX7-FOXO1* that contribute to pathogenesis.⁴ Conversely, ERMS represents 75% of all cases of RMS, most frequently occurs in the orbit, head and neck, and genitourinary tract,³ and lacks any of the signature chromosomal rearrangements identified in ARMS.⁵ However,

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ERMS often exhibits a characteristic loss of heterozygosity or loss of imprinting on the short arm of chromosome 11 (11p15.5).⁶

Genetically engineered mouse models that recapitulate ARMS have been reported.^{7,8} ERMS models are more complex requiring multiple genetic perturbations to generate⁹ and most demonstrate low tumor penetrance and/or exhibit long latency periods that are not typical of human RMS.^{10–13} While investigating the interactions of p53 and c-Fos in the context of bone physiology, Fleischmann et al¹⁴ crossed two knockout strains of mice to generate *p53*^{-/-}/*c-fos*^{-/-} double mutants. These mice developed highly proliferative and invasive RMS in the facial and orbital regions, similar to that seen in human ERMS patients. Tumors from the *p53*^{-/-}/*c-fos*^{-/-} double mutant mice resemble human ERMS morphologically and express characteristic markers such as desmin, α -Actin, Pax7, and MyoD. Cell lines established from the primary tumors expressed muscle-specific markers such as MyoD and myosin Heavy Chain (MyHC), and displayed several cytochemical features of human ERMS tumor cells. Analogous to most *in vivo* tumors, the cells in culture are highly proliferative but fail to fuse to form myotubes and progress through terminal myogenic differentiation. Thus, the *p53/c-fos* deficient mouse represents a straightforward and predictable animal model of ERMS.

That *p53* inactivation plays a role in the development of a variety of tumors, including RMS, is unequivocal.^{7,15,16} Additional genetic lesions are required for tumor development,¹⁷ and it was unexpected that deletion of the *c-fos* proto-oncogene, a major component of the ubiquitously expressed AP-1 family of transcription factors^{18,19} in *p53/c-fos* double mutant mice, would lead to development of ERMS. AP-1 transcription factors are composed of basic leucine-zipper proteins that require dimerization to transactivate gene expression, thereby regulating a wide range of cellular processes.^{19,20} Their versatility has been explained by the heterogeneity of dimerization partners²¹ that alter DNA binding affinity and specificity so that depending on the composition of the AP-1 complex, genes involved in cell proliferation, differentiation, apoptosis, and oncogenesis are differentially affected.²² During myogenesis, differentiation of myoblasts in culture is triggered by withdrawal of mitogens and is associated with down-regulation of *c-fos*.²³ That c-Fos is a negative regulator of *MyoD* expression and myoblast differentiation is well-established.^{24–26} Apparently, c-Fos also possesses tumor-suppressive activity within the context of mutant *p53*.¹⁴ However, re-expression of *c-fos* in the *p53*^{-/-}/*c-fos*^{-/-} ERM cells did not promote differentiation,¹⁴ consistent with c-Fos overexpression inhibiting myogenic differentiation.²⁷

In the current study we used a cell line derived from *p53*^{-/-}/*c-fos*^{-/-} mouse tumors to investigate the molecular basis underlying the generation of ERMS. We hypothesized that since c-Fos:c-Jun represents the most predominant AP-1 heterodimer in myoblasts, study of the *c-fos*- mutant would lead to identification of genes regulated by the altered AP-1 complex that may contribute to ERMS tumorigenesis. Our studies revealed that in the absence of c-Fos, AP-1 activity is associated with mis-

regulation of the Wnt pathway, which may contribute to blocked myogenic differentiation and resistance to apoptosis in ERMS.

Materials and Methods

Mouse Cell Culture

JW41 cells, derived from embryonal rhabdomyosarcoma tumors of the facial/orbital region in *p53*^{-/-}/*c-fos*^{-/-} mice,¹⁴ were maintained in growth medium composed of Dulbecco's modified Eagle's medium, 4 mmol/L L-glutamine, 4.5 g/L glucose (Invitrogen, Carlsbad, CA) supplemented with 10% to 20% fetal bovine serum (Hyclone, Logan, UT), and 0.5% penicillin-streptomycin (Invitrogen) at 37°C in a humidified 5% CO₂–95% air atmosphere. To induce differentiation, cells were grown in differentiation medium composed of Dulbecco's modified Eagle's medium containing 2% horse serum (Hyclone). Primary normal myoblasts (NMs) were derived from the hindlimbs of C57BL/6 mice. To avoid loss of competence, cells were passaged only four to five times.

Human Cell Culture

The human embryonal rhabdomyosarcoma cell line RD was obtained from the American Type Culture Collection (Rockville, MD).²⁸ All other human ERMS cell lines (RH6, RH18, and RH36) were generously provided by Dr. Peter J. Houghton (St. Jude's Children's Research Hospital, Memphis, TN).²⁹ Cells were maintained in RPMI 1640 containing 10% fetal bovine serum at 37°C in a humidified 5% CO₂–95% air atmosphere. Human primary normal myoblasts (HNMs) were isolated from the vastus lateralis of a 9-year-old patient and grown in growth medium. For differentiation, cells were grown in α -minimal essential medium supplemented with 2% fetal bovine serum, 0.5% penicillin-streptomycin, and 2 mmol/L glutamine.

Chromatin Immunoprecipitation: ChIP-Chip Assay

Chromatin immunoprecipitation (ChIP) was performed by using EZ ChIP reagents (Upstate Biotechnology, Lake Placid, NY) in the presence of phosphatase and protease inhibitors (Roche, Indianapolis, IN) according to the manufacturer's instructions. Proliferating cells ($\geq 1 \times 10^7$) were treated with 1% formaldehyde to crosslink protein to DNA and Nuclei were lysed after cross-linking was stopped with glycine. DNA was sonicated to 600-bp to 800-bp fragments by using a Branson 450 sonifier (Branson Sonic Power Co., Danbury, CT) in an ice bath with 8×30 seconds bursts at 25% power, each separated by 30-second periods. The lysate (90 μ l) was diluted 1:20 times in IP dilution buffer (Upstate Biotechnology), pre-cleared with bovine serum albumin and yeast tRNA-saturated protein A/G agarose mix (Invitrogen). Part of the lysate was saved as the control (C) "input" DNA and frozen until the crosslink reversal step. Remaining lysate

was immunoprecipitated sequentially with antibodies against phospho-c-Jun and c-Jun (sc-7981 and sc-1694; Santa Cruz Biotechnology, Santa Cruz, CA) as per the manufacturer's protocol. ChIP analysis with nonspecific IgG was used as a negative control to assess the specificity of immunoprecipitation. Captured immunocomplexes were assessed by Western blot analysis with the c-Jun antibody to assess enrichment in c-Jun bound DNA. Antibody-bound chromatin was precipitated with protein G conjugated agarose beads, washed with gradient stringent buffers, and eluted with elution buffer to generate enriched (E) DNA. Both C and E samples were incubated at 65°C overnight to reverse cross-links and purified by treatment with RNase, proteinase K, and phenol extraction. Equivalent amounts of C and E DNA were amplified in parallel, using the Round A/B/C protocol.³⁰ The PCR products were purified by using MiniElute Reaction Columns (Qiagen, Germantown, MD) and visualized by electrophoresis through 1% agarose gels to evaluate their size. Two micrograms of C and E DNAs were labeled in parallel with Cy3 or Cy5 mono-reactive dyes (Amersham, Piscataway, NJ).

Promoter Design and Printing

The mouse promoter arrays consisted of regions from 2200 gene promoters (1000 bp upstream and 500 bp downstream from transcription initiation sites, extracted from the Transcription Start Site Database, Japan) containing AP1, CREB, and C/EBP recognition sites as identified by custom sequence analysis using PERL scripts (<http://dbtss.hgc.jp/>, last accessed August 6, 2010). Sixty-five base pair flanking either side of the binding site were computationally selected by using two criteria: low level of homology to any other sequence in the genome and melting temperature between 63°C and 67°C. The 4400 oligonucleotides were synthesized by Sigma-Genosys (Houston, TX) and each printed twice onto epoxy-coated microarray slides (TeleChem International, Inc., Sunnyvale, CA), using a GeneMachines OmniGrid Printer (Genomics Solutions, Ann Arbor, MI) at the Microarray Core Facility (University of Arkansas for Medical Sciences, Little Rock, AR). The printed and preprocessed microarrays were tested, and conditions were optimized for hybridization and washing by using custom test probes at the facility.

Hybridization and Scanning

Three independent chromatin isolates were processed through the ChIP, amplification, and hybridization steps, in parallel. Two arrays were used for each sample to allow the dyes to be reversed when labeling E and C DNAs (dye swapping). Each labeled E DNA was competitively hybridized with labeled C DNA to the promoter array slides. Labeled DNAs were ethanol precipitated, and 2 µg of Cy5-labeled E DNA were mixed with an equal mass of Cy3-labeled C DNA (or the reverse labeling scheme). To this was added yeast tRNA, COT1 DNA, polydATP (10 µg each), and 20 µl of 2 × Agilent hybridization buffer.

The solution was heated to 99°C for 10 minutes before applying it to the array. A 25 mm × 60 mm coverslip was then gently placed on top of the sample and hybridization carried out in a hybridization chamber at 42°C overnight in a water bath. After hybridization, slides were washed once with wash buffer 1 (2 × standard saline citrate, 0.1% SDS) followed by two washes with buffer 2 (0.2 × standard saline citrate, 0.1% SDS) for 10 minutes at RT and three times with buffer 3 (0.2 × standard saline citrate), 1 minute each, at room temperature. The slides were then dried by a brief spin at 1000 × *g* in a table-top centrifuge and immersed in DyeSaver (Genisphere, Hatfield, PA) and scanned with a Perkin Elmer Scan Array 5000 (Perkin Elmer, Boston, MA). Image analysis was performed by using the adaptive circle method in ScanArray Express (Perkin Elmer).

Statistical Analysis of ChIP-Chip Arrays

A rank-based technique was used to identify enriched genes.³¹ Preprocessing was performed to minimize contribution of experimental artifacts³² and included background subtraction, Lowess normalization. Subsequently, a channel sum filter (Cy3 + Cy5) > 1000 was used to eliminate lowly represented genes. Transcripts that passed the above criteria across all three arrays and the dye-swap replicates were subsequently log-transformed. Fold differences between genes in the immunoprecipitated enriched versus control samples on each of the replicate arrays were subsequently ranked in ascending order (ie, highest fold change gets lowest rank) following the rank product approach.³¹

SYBR Green Real-Time RT-PCR

Total RNA was isolated from cells by using the Ambion RNA Aqueous kit (Austin, TX) following the manufacturer's instructions. The quantity and quality of the isolated RNA was determined by using the Agilent 2100 Bioanalyzer with RNA 6000 Nano Chips (Agilent Technologies, Santa Clara, CA). Primer sets for each gene were designed by using Primer Express Software (Applied Biosystems, Foster City, CA) and synthesized (IDT, Coralville, IA). Sequences of the primers are listed in Table 1. Two-step real-time RT-PCR was performed on the ABI PRISM 7700 Sequence Detection System by using random hexamers and the TaqMan Reverse Transcription Reagents, and the SYBR Green PCR Master Mix for the PCR step (Applied Biosystems) as described in Hidestrand et al.³³

RT² Profiler PCR Arrays

RNA (1 µg) isolated and quantified as described above was used to generate cDNA with the Reaction Ready First Strand cDNA Synthesis kit (SuperArray Bioscience, Frederick, MD). PCR array analysis was performed with the RT² Profiler PCR array targeted for the mouse Wnt Signaling Pathway (SuperArray Bioscience Corp.), according to the manufacturer's instructions. The array contains a panel of

Table 1 Primer Sequences, GeneBank Accession Numbers, and Annealing Temperatures for Genes Whose Transcripts Were Measured by Real-Time RT-PCR

Gene	Accession no.	Primer sequences
<i>Wnt 2</i>	NM.009520	5'-CCGCGTCTGCAGCAAGA-3' 5'-CTCGGCCACAACACATGATT-3'
Forward primer		
Reverse primer		
<i>birc5</i>	NM.001012273	5'-AATCCTGCGTTTGAGTCGTCTT-3' 5'-GCCGGAGCTCCCATGAT-3'
Forward primer		
Reverse primer		
<i>mafG</i>	NM.010756	5'-AGACCGAATCGCCGGTTAT-3' 5'-ACCCATCGGACACACAGACA-3'
Forward primer		
Reverse primer		
<i>sh2d2A</i>	NM.021309	5'-TTCACGTGGACTCAGCTTTGTC-3' 5'-GAGTGGTTGAAGGAAACCTGAAGT-3'
Forward primer		
Reverse primer		
<i>aldh2</i>	NM.009656	5'-GCCATCGCATCCCATGA-3' 5'-CCTCCGTGGAGCCTGTGA-3'
Forward primer		
Reverse primer		
<i>sFRP2</i>	NM.009144	5'-CGGCGAGCTGGTGATCA-3' 5'-TGAACCTCTCTGGCCCTTCTG-3'
Forward primer		
Reverse primer		
<i>Wnt5a</i>	NM.009524	5'-GCAGACCGAACGCTGTCTATT-3' 5'-CCACAATCTCCGTGCACCTTCT-3'
Forward primer		
Reverse primer		
<i>Wnt7b</i>	NM.009258	5'-TGCCCGTGAGATCAAAAAGAA-3' 5'-CCCGCCTCATTGTGTGAA-3'
Forward primer		
Reverse primer		
<i>MyoD</i>	NM.010866	5'-CGCTCTTCTCTTCTCATAGCA-3' 5'-AGGGCTCCAGAAAGTGACAAAC-3'
Forward primer		
Reverse primer		
<i>18S rRNA</i>	NR.003278	5'-TTCGAACGTCTGCCCTATCAA-3' 5'-ATGGTAGGCACGCGACTA-3'
Forward primer		
Reverse primer		
<i>Twist1</i>	NM.011658	5'-GGACAAGCTGAGCAAGATTCA-3' 5'-CGGAGAAGGCGTAGCTGAG-3'
Forward primer		
Reverse primer		
<i>Fibronectin</i>	NM.010233	5'-GCAGTGACCACCATTCCTG-3' 5'-GGTAGCCAGTGAGCTGAACAC-3'
Forward primer		
Reverse primer		
<i>CTGF</i>	NM.010217	5'-GGGCCTCTTCTGCGATTTC-3' 5'-ATCCAGGCAAGTGCAATTGGTA-3'
Forward primer		
Reverse primer		
<i>Human c-fos</i>	NM.005252	5'-AAAAGGAGAATCCGAAGGGAAA-3' 5'-GTCTGTCTCCGCTTGGAGTGAT-3'
Forward primer		
Reverse primer		
<i>Human birc5</i>	NM.001168	5'-GCCAAGAACAAAATTGCAAAGG-3' 5'-TTTCTCCGAGTTTCTCAAA-3'
Forward primer		
Reverse primer		
<i>Human mafG</i>	NM.002359	5'-CCCAACTCCTCTTTTATCCTAACATG-3' 5'-CTGTCCCTGCTGGGTATTCC-3'
Forward primer		
Reverse primer		
<i>Human Wnt 2</i>	BC029854	5'-CCTGGTGTCTGTCAGTCATCTC-3' 5'-AGCCTGTCATGCTATTTCCAAAG-3'
Forward primer		
Reverse primer		
<i>Human sh2d2A</i>	NM.003975	5'-CAAGCCCTCCAATCCTATCTACA-3' 5'-GGCCCATGGCATAGAAAGC-3'
Forward primer		
Reverse primer		
<i>Human aldh2</i>	NM.000690	5'-AGCTACACACGCCATGAACCT-3' 5'-CAGGAGCGGGAATTCCA-3'
Forward primer		
Reverse primer		

89 primer sets for a thoroughly researched set of 84 relevant, pathway-focused genes, plus five housekeeping genes and three RNA and PCR quality controls. The list of the genes is available at the company's website (SABiosciences, Frederick, MD). Gene expression was quantified by using the comparative $\Delta\Delta C_t$ (cycle threshold) method by using the manufacturer's instructions. The fold change of gene expression in JW41 cells compared with NMs was

calculated with Microsoft Excel software (Redmond, WA). Candidate genes with fold changes >3.0 were selected for confirmation by real time RT-PCR.

Induction and Detection of Apoptosis

To induce apoptosis, cells were seeded at 5×10^4 cells per 35-mm dish, cultured overnight, and treated with

varying concentrations of staurosporine (Sigma, St. Louis, MO). Dose response (0.5 to 5.0 $\mu\text{mol/L}$ in growth medium for 2 hours at 37°C) showed that 0.5 $\mu\text{mol/L}$ of staurosporine resulted in 50% of the normal myoblasts undergoing apoptosis and was used in all experiments. After staurosporine, cells were fixed with 4% paraformaldehyde and apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN). Positive nuclei were detected with TSA-Fluorescein (Perkin Elmer) and are expressed as percent TUNEL positive nuclei per plate.

Transfection and Luciferase Reporter Assays

To quantify β -catenin activity, the TOPflash/FOPflash reporter plasmid system (gift from Dr. Randall T. Moon, University of Washington, Seattle, WA) was used, with or without LiCl treatment as a positive control. The TOPflash reporter construct contains multiple optimal TCF/LEF binding sites that, when bound by TCF/LEF/ β -catenin, induce transcription of the firefly luciferase reporter gene. FOPflash contains mutated TCF/LEF-binding sites that cannot be activated by β -catenin and was used as a negative control for measuring nonspecific activation of the reporter gene. One day before transfection, 1×10^5 cells were seeded per well into 24-well plates. All transfections were performed with 0.75 μg of TOPflash or FOPflash reporter gene plasmids by using Lipofectamine LTX and Plus reagents (Invitrogen) according to the manufacturer's specifications. To normalize for differences in transfection efficiency, cells were cotransfected with 15 ng of a control reporter plasmid, *Renilla reniformis* luciferase, driven by the TK promoter (pRL-TK; Promega, Madison, WI). Lithium Chloride (L9650; Sigma) was added 24 hours posttransfection at a concentration of 12.5 mmol/L. Cells were harvested 24 hours later, lysates prepared, and luciferase activity was determined by using the Dual Luciferase Reporter Assay System in accordance with the manufacturer's protocol (Promega). Reporter luciferase activity (Firefly) was expressed relative to control luciferase activity (*Renilla*) for each sample. To directly activate the β -catenin pathway, constitutively active β -catenin S37A³⁴ plasmid or vector pcDNA3 (1 μg each) were transfected as above into JW41 cells. All transfections were performed in triplicate.

Protein Extraction and Western Blot Analysis

The NE-PER system (Pierce, Rockford, IL) was used for isolation of cytosolic and nuclear fractions in accordance with the manufacturer's instructions. Equal amounts of protein (30 μg per lane), as determined by Bradford assay, was separated on 10% Tris/HCl Criterion gels (Bio-Rad Laboratories, Hercules, CA) and transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA) for Western blot analysis as described.³⁵ Subsequently, membranes were incubated in Ponceau S solution (Sigma) to evaluate the transfer of

protein between lanes. The stained images were scanned and saved for normalization of total protein after Western blot analysis. Proteins of interest were detected by using target specific primary antibodies for β -catenin (Transduction Laboratories, San Jose, CA) and secreted Frizzled Related Protein 2 (sFRP2; Santa Cruz Biotechnology), both at 1:200 dilutions. Detection was performed with species-specific horseradish peroxidase-conjugated secondary antibodies (Pierce and Santa Cruz Biotechnology) and SuperSignal (Pierce), followed by visualization with a Chemilmager 5500 (AlphaInnotech, San Leandro, CA), and quantification by using Chemilmager software.

Immunocytochemistry

Cells in culture were fixed with 4% paraformaldehyde for 15 minutes. For the nuclear antigen MyoD, JW41, and NMs cells were permeabilized following fixation for 10 minutes with 0.05% Igepal (Sigma), blocked with 2.5% normal horse blocking serum provided in the ImmPRESS Anti-mouse Ig staining kit (Vector Laboratories, Burlingame, CA), and incubated overnight at 4°C, with anti-MyoD antibody (BD Biosciences Pharmingen, San Diego, CA) at a concentration of 2.5 $\mu\text{g/ml}$ in ImmPress blocking buffer + 0.1% Igepal. Cells were washed twice with PBS + 0.1% Igepal for 15 minutes each at RT on a shaker. Cells were then incubated for 1 hour with the horseradish peroxidase coupled anti-mouse Ig ImmPress reagent diluted 1:2 in PBS + 0.1% Igepal and washed as described above. TSA-Fluorescein (Perkin Elmer), diluted 1:200 in amplification buffer (provided in the kit), was added to cells for 20 to 30 minutes in the dark and cells were given a final wash with PBS for 5 minutes before visualization.

For β -catenin detection, cells were fixed as above and incubated overnight at 4°C with anti- β -catenin antibody at a dilution of 1:100 (250 $\mu\text{g/ml}$; Transduction Laboratories). Washing was repeated three times with PBS + 0.1% Igepal, followed by incubation with horseradish peroxidase conjugated anti-mouse secondary antibody (Zymed, San Francisco, CA) for 1 hour at RT. TSA-Fluorescein was used to detect antibody binding as described above. Nuclear DNA was detected by Hoechst staining (H3570; 16.2 mmol/L; Invitrogen) that was diluted to 2 $\mu\text{mol/L}$ in PBS. Incubation was done for 30 minutes after which the cells were rinsed three to four times in PBS.

For MyHC staining, cells were postfixed in absolute methanol for 20 minutes and rinsed three times for 5 minutes each at RT with PBS. A4.1025 hybridoma supernatant (Helen Blau, Stanford Medical School, Stanford, CA), recognizing an epitope common to all MyHCs, was applied to the cells enough to cover them completely and incubated for 1 hour at RT. Washes were repeated as described above, and Texas Red conjugated goat anti-mouse secondary antibody (Rockland, Gilbertsville, PA) was added to cells at a concentration of 1:200 in PBS. Three washes for 5 minutes each were done at RT, and a final wash with water was done before visualization. Color

assigned was changed to blue for MyHC detection for achieving a better contrast of images.

All Images were acquired by using a digital Nikon Eclipse E600 microscope, with a Nikon Plan Fluor 20 $\times$ /0.50 or 40 $\times$ /0.8 objectives. For nonfluorescence chromogens, a Photometrics CoolSnapES color camera was used, whereas for fluorescence, a Photometrics CoolSnap black and white camera was used, both with MetaVue software (Molecular Devices Corp., Sunnyvale, CA). Images were optimized globally for contrast and brightness and assembled into figures by using Adobe Photoshop 6.0 (San Jose, CA).

Statistical Analysis

All data were expressed as the mean $\pm$ SD or SE as appropriate. The Student's *t*-test was used for comparing two groups, and the one-way analysis of variance and Holm-Sidak posthoc test was used for multiple comparisons, with the level of statistical significance set at *P* < 0.05 unless otherwise specified.

Results

Tumor-Derived JW41 Cells Mirror Embryonal Rhabdomyosarcoma

Myogenic potential of JW41 tumor-derived cells was tested at both mRNA and protein levels and compared with NMs isolated from mouse hind limb muscles. Although readily detectable, MyoD mRNA was three- to fourfold less abundant in JW41 cells (Figure 1A), which extended to the protein level with less than half of JW41 nuclei accumulating immunocytochemically detectable MyoD, compared with nearly 100% of NMs (Figure 1B). Myogenic differentiation potential of JW41 was determined by immunostaining for MyHC and MyoD after mitogen withdrawal. NMs fused to form multinucleated MyHC-positive myotubes with more than 90% of the myotubes containing MyoD positive nuclei (Figure 1C). This number was considerably lower for the JW41 cells with only 8% to 10% of cells expressing both cytoplasmic MyHC and nuclear MyoD (Figure 1C). Of the MyHC positive JW41 cells, approximately 2% were multinucleated. Thus, as in human ERMS, JW41 cells display heterogeneity with only a small percentage of cells able to undergo terminal myogenic differentiation.³⁶

Identification of AP-1 Transcriptional Targets in JW41 Cells

To identify putative target gene promoters preferentially bound by the c-Jun-containing AP-1 complex in JW41 cells, we used a chromatin immunoprecipitation coupled to microarray technique (ChIP-chip). The array was comprised of oligonucleotides corresponding to unique DNA sequences immediately surrounding AP-1, CREB, and C/EBP recognition sites located -1000 bp to $+500$ bp from the transcription start sites of Pol II transcribed

Figure 1. Myogenic potential of JW41 cells derived from an embryonal rhabdomyosarcoma in *p53*^{-/-}/*c-fos*^{-/-} mice. **A:** Gene expression analysis of *MyoD* in JW41 cells and NMs maintained in growth medium. Results were obtained from three independent RNA isolates analyzed by real-time RT-PCR. Data are presented as the mean $\pm$ SD normalized to 18S rRNA. Results were compared by Student's *t*-test (**P* < 0.005). **B:** Immunocytochemical visualization of MyoD protein (green) in JW41 cells and normal myoblasts maintained in growth medium. Scale bar = 50 μ m. **C:** Percentage means of cells that were MyHC- and MyoD-positive in JW41 and NMs cultures in differentiation media. Three different areas in four separate cultures were used to quantify the expression of MyHC- and MyoD-positive cells. Results were compared by Student's *t*-test (***P* < 0.001).

genes. Immunoprecipitated chromatin array binding patterns were compared with control chromatin from JW41 cells subjected to the same procedure but omitting the c-Jun antibodies. The two samples were differentially labeled and hybridized to a single array as a two-color experiment. The experiment was performed three times with different biological JW41 chromatin isolates. Data obtained from the ChIP-chip experiments were analyzed by Breitling's test³¹ to identify sequences showing increased signal (ie, promoter DNA fragment enrichment) relative to the control. A sequence was assumed to be enriched if its rank-product estimated from the given

replicate arrays was different from those estimated from the random shuffled counterparts. As each sequence surrounding the AP-1 binding site was spotted four times on the array, hybridization results from three independent experiments generated a total of 12 data points for each gene (see Supplemental Table S1 at <http://ajp.amjpathol.org>). The five gene promoters that showed consistent enrichment were wingless-type MMTV integration site family member 2 (Wnt2), Baculoviral IAP repeat-containing 5 (survivin), v-Maf musculoaponeurotic fibrosarcoma oncogene family protein G (mafG), aldehyde dehydrogenase-2 (aldh2), and a T-cell specific adapter protein (sh2d2A). Among these, Wnt2, birc5/survivin, and mafG are well documented to play a role in tumorigenesis.^{37–39} To test whether the genes identified by ChIP-chip are exclusively regulated by the altered AP-1 complex in JW41 cells, we hybridized the arrays with c-Jun immunoprecipitated chromatin from NMs. None of the five probes identified in JW41 cells showed enrichment in NMs (data not shown), suggesting that a different set of genes is regulated by the AP-1 complex in the two cell types. Real time RT-PCR demonstrated overexpression of the associated genes by at least twofold in JW41 cells compared with NMs, with Wnt2 showing the largest differential expression (Figure 2A). These results suggest that preferential binding of c-Jun-containing AP-1 to the promoters of these target genes leads to preferential transactivation in the ERMS-derived JW41 cells.

Wnt Pathway Gene Expression Analysis

As Wnts have not been previously implicated in these tumors, we used Wnt Signaling Pathway RT² Profiler PCR Array (Superarray) to explore the pathway in more detail and to identify candidate genes for further analysis. Detectable PCR products were obtained for 82 Wnt pathway genes (defined as requiring <35 cycles), of which 32 genes (39%) showed differential expression by at least threefold in JW41 cells compared with NMs (Table 2). Wnt2, Wnt10a, and Wnt8b showed higher expression in JW41 cells. Further, RT-PCR analysis of putative downstream targets of Wnt2, twist, connective tissue growth factor, and fibronectin showed higher expression in JW41 cells (Figure 2B). However, the genes that were most highly overexpressed in the ERMS cell line encoded the Wnt binding antagonists, sFRP2 and 4, and negative regulators of Wnt signaling Dickkopf homolog 1 (Dkk1) and Naked cuticle homolog 1 (Nkd1). Additionally, the majority of well-characterized downstream target genes of canonical Wnt/ β -catenin signaling, such as *c-myc* and cyclins, did not demonstrate differential expression (Table 2). Genes that showed down-regulation were Wnt receptors (Fzd1, 3 and 5), the signaling mediator Dishevelled (Dvl1), factors involved in recruiting and forming the activation complex with β -catenin in the nucleus (LEF-1 and Pygo). These results suggest that although overexpression of specific Wnt ligands may lead to alterations in downstream gene expression in JW41 cells, the canonical Wnt/ β -catenin signaling pathway is inhibited relative

to NMs. Interestingly, components of Wnt signaling that have been implicated in myogenesis such as Wnt7b that promotes myogenic differentiation³⁵ and sFRP2 that inhibits myotube formation⁴⁰ demonstrated differential expression. Real time RT-PCR analyses of the genes most relevant to myogenic differentiation confirmed the array results with statistical significance (see Supplemental Figure S1 at <http://ajp.amjpathol.org>).

Wnt/ β -catenin Pathway Analysis

As stabilization and nuclear accumulation of β -catenin are hallmarks of activated Wnt signaling,⁴¹ Western analysis of cytosolic and nuclear fractions (Figure 3A) and immunocytochemistry (Figure 3B) were performed to determine whether the canonical pathway was activated. Not only was β -catenin less abundant in JW41 cells, but the ratio of nuclear to cytosolic β -catenin was also lower than NMs (Figure 3A). Further, β -catenin primarily localized to the nucleus (visualized by counterstaining with Hoechst dye) and peri-nuclear space in NMs, but remained cytoplasmic in JW41 cells (Figure 3B). Transactivation of gene expression by β -catenin requires binding

Figure 2. Identification of altered AP-1 target genes using DNA arrays. **A:** Expression analysis by real-time RT-PCR of genes identified by ChIP-chip in JW41 cells and NMs. Bars represent the mean $\pm$ SD from three independent RNA isolates normalized to 18S rRNA. Data were analyzed by Student's *t*-test and expressed as different from NM (* $P \leq 0.05$; ** $P \leq 0.005$). **B:** Expression of Wnt2 target genes. Results for three independent RNA isolates normalized to 18S rRNA. Bars represent the mean $\pm$ SD. Results were analyzed by Student's *t*-test, and the difference between the expression analysis of all genes in JW41 cells and NMs was found to be statistically significant. * $P < 0.005$.

Table 2 List of Genes That Were Up-Regulated/Down-Regulated More Than Threefold in JW41 Cells Relative to Normal Myoblasts in Wnt Superarray Analysis

Genes	Fold down-regulated	Function	Genes	Fold up-regulated	Function
<i>Wnt7b</i>	420.22	Signaling	<i>sFRP2</i>	3915.55	Inhibitor
<i>Wnt9a</i>	32.56	Signaling	<i>Nkd1</i>	50.39	Inhibitor
<i>Wnt5a</i>	23.83	Signaling	<i>Wnt10a</i>	20.61	Signaling
<i>LEF1</i>	20.18	Regulation of transcription, Activator	<i>sFRP4</i>	15.73	Inhibitor
<i>Frzb</i>	15.30	Inhibitor	<i>Wnt8b</i>	13.78	Signaling
<i>Wif1</i>	9.16	Inhibitor	<i>Dkk1</i>	9.68	Inhibitor
<i>Pygo1</i>	7.70	Regulatory, transcription factor	<i>Dixdc1</i>	9.48	Signaling
<i>Wnt11</i>	6.08	Signaling	<i>Wnt7a</i>	5.48	Signaling
<i>Wnt4</i>	5.88	Signaling	<i>Wnt5b</i>	5.22	Signaling
<i>Fzd1</i>	5.05	Receptor for Wnt proteins	<i>Wnt2b</i>	5.05	Signaling
<i>Slc9a3r1</i>	5.01	Scaffold protein, enhance Wnt signaling	<i>Wnt2</i>	3.75	Signaling
<i>Dvl1</i>	4.68	Signaling	<i>Sox17</i>	3.69	Regulatory
<i>Porcn</i>	4.64	Signaling			
<i>Foxn1</i>	3.64	Regulatory, transcription factor			
<i>Fzd5</i>	3.62	Signaling			
<i>c-jun</i>	3.17	Regulatory, transcription factor			
<i>Kremen1</i>	3.15	Receptor for Dickkopf protein			
<i>Wnt6</i>	3.13	Signaling			
<i>Fzd3</i>	3.07	Signaling			
<i>Frat1</i>	3.05	Signaling			

to the TCF/LEF DNA binding site, which was quantified by using the TOPflash/Fopflash luciferase-based reporter plasmid system. Consistent with the immunocytochemical data, TCF/LEF/ β -catenin reporter gene activity was higher in NMs than JW41 cells (Figure 3C).

We next used LiCl, a prototypical inducer of Wnt pathway that inhibits GSK-3 β activity and stabilizes β -catenin,⁴² to attempt to activate the pathway in JW41 cells. JW41 cells showed up-regulation in luciferase activity (fivefold) albeit not to the same level as in NMs (Figure 3D), presumably due to less abundant β -catenin in JW41. Nonetheless, the results indicate that the Wnt/ β -catenin pathway can be activated, which provides the impetus to determine whether its activation leads to the differentiation of JW41 cells.

Induction of the Wnt/ β -catenin Pathway Promotes Myogenic Differentiation

JW41 cells and NMs were cultured in differentiation medium, and LiCl (12.5 mmol/L) was added every day for 4 days. In both JW41 cells and NMs, a significant increase in the number of MyHC positive myotubes was observed with LiCl treatment (Figure 4, A and B, quantified in C). JW41 cells showed a 3.5-fold increase in the frequency of MyoD positive nuclei compared with cells without LiCl treatment. This increase in the number of MyoD positive cells is associated with augmentation of fusion and MyHC expression (Figure 4A), suggesting that activation of the Wnt/ β -catenin pathway partially rescues the ERMS phenotype of JW41 cells by promoting myogenic differentiation. Overexpression of constitutively active β -catenin by transient transfection resulted in greater than fivefold induction of MyoD gene expression 24 hours after transfection (Figure 4D), confirming that β -catenin signaling directly promotes myogenic gene expression in the JW41 ERM cell line.

Susceptibility to Apoptosis

sFRP2, the Wnt antagonist that displayed high level gene expression in JW41 cells (Table 2), contributes to oncogenesis by promoting survival of tumor cells.⁴³ Analysis was extended to the protein level by Western blot, which showed a high level of sFRP2 protein accumulation in JW41 cells compared with NMs (Figure 5A). To determine whether JW41 cells were resistance to apoptosis, cells were treated with varying concentrations of staurosporin, a known initiator of apoptosis. At a concentration of 0.5 μ mol/L, approximately 50% of the NMs were TUNEL positive, whereas JW41 were completely resistant (Figure 5, B and C). A concentration dependent response was seen thereafter with JW41 cells showing more resistance even at higher concentrations of staurosporin at which all NMs underwent apoptosis (data not shown), suggesting that JW41 cells have a survival advantage that may contribute to the ERMS phenotype.

Analysis of Human ERMS Cell Lines

The human ERMS cell lines RD, RH36, RH18, and RH6 were analyzed to validate the results obtained in JW41 cells. All showed significantly lower *c-fos* expression compared with HNMs (Figure 6A). Genes identified as targets of AP-1 in JW41 cells were also examined, and the results obtained mirrored the findings in the JW41 cells (Figure 6B). Key players of the Wnt pathway misregulated in JW41 cells were likewise affected in human ERMS cell lines. Western blot analysis showed less overall β -catenin and relatively less nuclear β -catenin (Figure 6C). TCF/LEF/ β -catenin-dependent transcriptional activity was fivefold higher in HNMs compared with RD cells (Figure 6D). LiCl induced reporter gene activity 10-fold in HNMs, whereas only a fourfold increase occurred in RD cells. Further, analysis in the RD cell line showed an

Figure 3. Wnt/ β -catenin pathway analysis. **A:** Western blot analysis of β -catenin protein in cytosolic (Cyto) and nuclear (Nuc) extracts from JW41 cells and NMs. **B:** Immunofluorescence analysis of the subcellular localization of β -catenin (green) in NM and JW41 cells. Nuclear staining was performed with Hoechst Dye 33342 (blue). Scale bar = 20 μ m. **C:** LiCl induces β -catenin-dependent transcription. The TCF/LEF/ β -catenin-dependent TOPflash/FOPflash reporter plasmids were used to quantify β -catenin-dependent transcriptional activity following transient transfection of NMs and JW41 cells. Luciferase activities were determined and corrected for transfection efficiency by using *Renilla* pRL-TK activity. Data from three independent experiments, performed in triplicate, are expressed as the mean $\pm$ SE. As a positive control, cells were treated with LiCl. * P < 0.05 as determined by one-way analysis of variance and the Holm-Sidak posthoc test. **D:** Ratio of Luciferase activity $\pm$ LiCl in JW41 cells and NMs obtained after subtracting FOPflash $\pm$ LiCl luciferase activity, expressed as fold induction.

elevated expression of *sFRP2* and reduced abundance of *Wnt7b* transcripts (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). These results are consistent with results obtained in JW41 cells and suggest that defects in the Wnt/ β -catenin pathway may contribute to ERMS development through blocked myogenic differentiation and resistance to apoptosis.

Discussion

To delineate the etiology of ERMS development, gene targets of the AP-1 complex in tumor cells from *p53*^{-/-}/*c-fos*^{-/-} mice were identified. These mice developed ERMS in the facial and orbital regions at 10 weeks of age. Unlike normal myoblasts, tumor-derived JW41 cells differentiate poorly, recapitulating the block in terminal differentiation in ERMS. Thus, it is possible that tumors arise in the *p53*^{-/-}/*c-fos*^{-/-} mice as a consequence of regulatory disruption of muscle progenitor cells that has been proposed for human ERMS.^{44,45} We hypothesized that the absence of c-Fos results in a change in the stoichiometry of the available AP-1 components, altering dimer composition and AP-1 target gene preferences that contribute to the generation of ERMS. Using comprehensive

Figure 4. LiCl induces myogenic differentiation. JW41 cells and NMs were cultured in differentiation medium for 96 hours without (**A**) or with (**B**) 12.5 mmol/L LiCl and quantified in **C**. Double immunofluorescence was used to visualize myosin heavy chain (blue) and MyoD (green). Representative images show increased myogenic differentiation in response to LiCl. Scale bar = 50 μ m. **C:** Number of MyoD-positive nuclei in MyHC-expressing myotubes $\pm$ LiCl at the end of 96 hours was counted. Bars represent the means $\pm$ SE, n = 4. * P < 0.05 as determined by one-way analysis of variance and the Holm-Sidak posthoc test. **D:** *MyoD* gene expression analysis in JW41 cells transfected with constitutively active β -catenin (β -cat) plasmid or empty vector (EV). Results were obtained from three independent RNA isolates analyzed by real-time RT-PCR. Data are presented as the mean $\pm$ SD normalized to 18S rRNA. Results were compared by Student's *t*-test (* P < 0.05).

data analysis of our ChIP-chip results followed by evaluation of mRNA expression levels, we identified five gene promoters, *mafG*, *aldh2*, *sh2d2A*, *survivin*, and *Wnt2*, that all show preferential binding to c-Jun-containing AP-1 complexes and overexpression in JW41 cells. Epithelial tumor cells deficient in c-Fos also show binding of c-Jun to Wnt gene promoters,⁴⁶ suggesting Wnt2 may play a role in the tumor phenotype.

We investigated the Wnt signaling pathway in detail because of its importance in regulating cell proliferation and differentiation and because of extensive evidence linking it to cancer-related deregulation of cellular homeostasis.⁴⁷ However, Wnt/ β -catenin activity has not been reported to be associated with RMS in contrast to other pediatric and soft tissue tumors.^{48,49} To date, 19 different human and mouse Wnt proteins and 13 Fzd receptors have been shown to signal through β -catenin-dependent canonical and noncanonical pathways.⁵⁰ Our use of a Wnt superarray allowed a comprehensive analysis of many of the key molecules in these pathways. Unexpectedly, JW41 cells showed higher expression of extracellular antagonists of the canonical pathway, including sFRPs that bind Wnt ligands directly⁵¹ and Dkk

Figure 5. Staurosporin treatment demonstrates that JW41 cells are more resistant to apoptosis than NMs. **A:** Western blot analysis of sFRP2 protein accumulation in JW41 cells and NMs. **B:** Representative images showing TUNEL-positive nuclei (green) after a 2-hour staurosporin treatment (0.5 μ mol/L) of NMs and JW41 cells. **C:** Quantification of TUNEL-positive nuclei obtained in three independent experiments.

family proteins that bind to the Wnt co-receptor LRP5/6. These Wnt antagonists have been implicated in tumorigenesis.^{52,53} In addition, several Wnt family ligands including Wnt7b, Wnt5a, Wnt4, and Wnt11 showed down-regulation relative to normal myoblasts. JW41 cells also showed underexpression of many critical mediators, ef-

factors, and receptors of the signaling cascade. Down-regulation or no change in down-stream targets of Wnt signaling, reduced β -catenin abundance and cytoplasmic localization, as well as decreased TCF/LEF/ β -catenin-dependent reporter gene activity all indicated that the canonical Wnt signaling pathway was inhibited, supporting observations that β -catenin activating mutation does not contribute to RMS tumorigenesis.⁴⁹ Consistent with our results, other cancer studies have revealed down-regulation of the tumor suppressor *Wnt5a*. *Wnt5a* deletion or its reduced expression can occur in sarcomas,⁵⁴ *Wnt5a* methylation frequently takes place in colorectal cancer, and loss of Wnt5a happens with early recurrence in invasive ductal breast carcinomas.^{55,56}

Although overall the canonical β -catenin pathway appeared inhibited in the JW41 ERMS cells, the Wnt ligand Wnt2, and the putative Wnt2 downstream target, *twist*,⁵⁷ were overexpressed. Twist inhibits myogenic differentiation⁵⁸ and may affect a similar blockage of terminal differentiation in JW41 cells. Other downstream target genes of Wnt2, such as *fibronectin* and *ctgf*, were also overexpressed, which may contribute to the stroma-rich appearance of some ERMS.⁵⁹ Expression of Wnt2 and other markers of fibrosis increases in aging normal myoblasts, which show impaired myogenic differentiation³³ potentially mediated through a noncanonical pathway.

Numerous studies have shown that nuclear localization of β -catenin and stimulation of transcription of TCF/LEF/ β -catenin-dependent genes are essential for myogenic differentiation.⁶⁰ Evidence of inhibition of MyoD-mediated transactivation in muscle cells deficient in β -catenin and a direct β -catenin-MyoD interaction via E box elements suggest that MyoD may be a primary target of β -catenin and an effector in the Wnt canonical.^{61–63} Exogenous expression of β -catenin leading to enhanced transcription of *MyoD* in JW41 cells implies that β -catenin is necessary for MyoD activity in ERMS and consequently for myogenic differentiation. Thus, underexpression of Wnt

Figure 6. Evaluation of human ERMS cell lines. **A:** Real-time RT-PCR analysis shows decreased *c-fos* mRNA in human ERMS cell lines (RH6, RH18, RH36, and RD) compared with HNMs. **B:** ERMS cell lines and HNM were profiled for expression of genes identified through the mouse promoter array. Bars represent the mean $\pm$ SD from three independent RNA isolates normalized to 18S rRNA. One-way analysis of variance analysis and the Holm-Sidak posthoc test were performed and expressed as different from HNM (* $P < 0.05$). **C:** Western blot analysis of β -catenin protein in cytosolic (Cyto) and nuclear (Nuc) extracts from human ERMS cell lines RD, RH18, and RH36 and HNM. **D:** TCF/LEF-dependent TOPflash/FOPflash reporter plasmids were used to quantify β -catenin-dependent transcriptional activity following transfection of RD cells and HNM. Luciferase activities were determined and corrected for transfection efficiency by using *Renilla* pRL-TK activity. Data from three independent experiments, performed in triplicate, are expressed as the mean $\pm$ SE. As a positive control, cells were treated with LiCl. Bars with the same letter designation are significantly different with $P < 0.05$ as determined by one-way analysis of variance and the Holm-Sidak posthoc test.

ligands and overexpression of Wnt antagonists in JW41 cells inhibits Wnt/ β -catenin signaling that apparently suppresses myogenesis. *Wnt7b* promotes myogenic differentiation^{35,40} but shows dramatic down-regulation in JW41 cells compared with myoblasts. High levels of sFRP2 in JW41 cells may also block myogenic differentiation, as overexpression of sFRP2 in embryonic limbs reduces their number of terminally differentiated muscle cells.⁶⁴ Several other studies document the myosuppressive role of sFRP2, suggesting that it antagonizes Wnt-activation of myogenesis.⁴⁰

Treatment of JW41 cells with LiCl to stimulate the canonical Wnt pathway resulted in a significant increase in TCF/LEF/ β -catenin-dependent reporter gene transcription, associated with increased myogenic differentiation. Although the increase in expression of myogenic markers was apparent, LiCl did not completely rescue the myogenic block in JW41 cells, likely due to lower total β -catenin present. Additionally, as Wnt/ β -catenin and AP-1 pathways synergistically regulate various TCF/LEF-dependent genes, absence of c-Fos in JW41 cells may limit β -catenin function. Direct interaction of β -catenin and AP-1 and the presence of binding sites for both AP-1 and TCF/LEF in the promoters of the *c-jun*, *matrilysin*, *cyclin D1*, and *c-myc* genes suggest a vital synergism that is necessary for expression of certain genes,⁶⁵ which was not recapitulated by LiCl to promote normal myogenic differentiation. A vital characteristic common to all tumors is their intrinsic ability to resist apoptosis. A myriad of factors acting in concert through common pathways can result in resistance to apoptosis, which in turn contributes to tumor maintenance and progression. In JW41 cells, absence of c-Fos and overexpression of *birc5/survivin* may contribute to this phenotype. *Birc5/Survivin*, a member of the inhibitor of apoptosis (IAP) family, identified in the ChIP-chip analysis and shown to be overexpressed in JW41 cells, promotes tumor cell survival.³⁹ sFRP2 may also promote survival, as shown in human malignant glioma cells.⁵²

To validate the results obtained in the JW41 mouse model, we subsequently tested the expression of AP-1/Wnt pathway genes in well-established human ERMS cell lines. All human ERMS cell lines showed significantly less *c-fos* mRNA than normal human myoblasts. Furthermore, the human ERMS cell lines showed up-regulation of the five genes that were up-regulated in the JW41 cells. Most importantly, analysis of the Wnt pathway in human ERMS cells revealed results consistent with those from the JW41 model. Overexpression of Wnt signaling antagonists and underexpression of β -catenin were associated with low inherent activity of the LiCl-inducible, β -catenin-dependent reporter gene. Data both at protein and mRNA levels support the hypothesis that compromised Wnt signaling and altered AP-1 activity may synergistically contribute to cellular transformation, myogenic suppression, and apoptotic resistance of ERMS. Although activation of canonical Wnt signaling is not associated with ERMS,^{48,49} our results suggest for the first time a critical role for suppression of the canonical Wnt pathway in ERM tumorigenesis mediated by AP-1. Future experiments must verify these results *in vivo* and identify mechanisms underlying *c-fos* dependent rhabdomyosarcoma de-

velopment. Targeted activation of the Wnt pathway could thus affect a rational therapeutic approach to treating embryonal rhabdomyosarcoma.

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