Supplementary data:

Supplementary Materials and Methods

Cells

Mouse U20497T and U20325 ARMS cell lines were established from (*PAX3-FOXO1*$^{+/+}$, *INK4a/ARF*$^{-/-}$) and (*PAX3-FOXO1*$^{+/+}$, p53$^{-/-}$) conditional mouse models of ARMS (1) respectively. Rh30-PRSLuc and RD-PRSLuc polyclones were derived via transduction of lentivirus expressing the PAX3-FOXO1 responsive 6XPRS-Luc reporter into Rh30 (ARMS) and RD (ERMS) cells respectively, followed by selection against puromycin. A similar strategy was used to generate U20325-CMVLuc polyclones expressing the CMV-driven luciferase reporter into U20325 ARMS cells under selection of puromycin. All cells used in this study were cultured at 37^0C, 5% CO_2 and 95% humidity.

Reagents

A stock solution of AKT inhibitor MK-2206 (Active Biochem), Proteasome inhibitor MG132 (EMD Millipore), Thapsigargin (Santa Cruz) were prepared in DMSO at 10 mM concentration. Molecular biological grade $CaCl_2$ solution (2M) was purchased from Quality Biological, Inc. Dithiothreitol (DTT) (Sigma Aldrich) was reconstituted in tissue culture grade water at 100 mM concentration. All reagents except $CaCl_2$ (kept at 4^0C) were stored at -20^0C.

Plasmids

Lentiviral pLA-6XPRSLuc (referred herein PRSLuc) reporter luciferase construct was described previously (2). CMV-driven luciferase (CMVLuc) reporter gene in such viral vector was received from Andrei V. Gudkov (Roswell Park Cancer Institute). pBabe-myrAKT-HA construct was generated by sub-cloning myrAKT-HA insert from pCMV5-myrAKT-HA (Addgene) into SnaB1/EcoR1 sites of retroviral pBabe vector. Lentiviral pLV-PAX3-FOXO1-HA construct was

generated by cloning PCR amplified fragment of PAX3-FOXO1 from pCDNA3.1(+) Zeo-PAX3-FOXO1-3XHA (Dr. Karen Arden, University of California, San Diego) using primers, forward (5'-CCGCTCGAGCGGATGACCACGCTGGCCGGCGCT-3') and reverse (5'CTAGCTAGCTAGTCAAGCGTAGTCTGGCACGTCGTATGGGTAGCCTGACACCCT

ATGTGTCGTTG-3'), into Xho1/Nhe1 sites of pLV vector (3). Lentiviral constructs expressing shRNA oligonucleotide sequence targeting *PAX3-FOXO1*, and the scramble control were described in our previous study (2). PCR derived sequence was verified by sequencing.

Antibodies

Antibodies used were as follows: rabbit monoclonal antibodies specific for FOXO1 (C29H4) to detect PAX3-FOXO1, AKT (pan) (C67E7), Phospho-AKT (Ser473) (D9E) and rabbit polyclonal cleaved caspase-3 (ASP175) antibody were obtained from Cell signaling. Rabbit polyclonal antibodies for MyoD (C20) and PARP-1/2 (H-250) antibodies were purchased from Santa Cruz Biotechnology. Mouse monoclonal antibodies for PAX3 (MAB2457) and HA.11 (MMS-H101P) were obtained from R&D System and Covance respectively. Antibody against Ki-67 was obtained from Thermo Scientific. While peroxidase-conjugated β-actin (A3854) and anti-mouse IgG (A3682) antibodies were purchased from Sigma-Aldrich, peroxidase-conjugated anti-rabbit IgG (170-6515) antibody was obtained from Bio-Rad. Biotinylated goat anti-rabbit IgG (BA-1000) were purchased from Vector laboratories.

Retro- and lenti- viral production and transduction

Retro- and lenti- viruses were produced in Phoenix-Ampho and 293FT cells respectively as previously described (4). Briefly, Phoenix-Ampho cells were transfected with retroviral construct using PureFection nanotechnology-based transfection reagent (System Biosciences). Retrovirus-containing supernatants were collected at 48 hours after transfection, and filtered through 0.45-μm filter. Similarly, 293FT cells were co-transfected with lentiviral construct along

with packaging Gag-Pol and Env constructs followed by collection of virus-containing supernatants at 48 hour time-point and filtered. Both retro- and lenti- viruses were diluted with growth medium and transduced 3 consecutive days in the presence of 8 μg/ml of freshly prepared polybrene (Sigma-Aldrich). Where applicable, virus-transduced cells were subjected to appropriate antibiotic selection until the un-transduced cells died.

Small molecule libraries and high throughput screening

To screen small molecule inhibitors of PAX3-FOXO1, two chemical libraries (the 2000 compounds in the Spectrum Collection and LOPAC^{TM} 1280, which are available at Small Molecule Screening Core), containing a total of 3,280 compounds were used. These compounds include FDA and internationally approved drugs, bioactive and natural compounds with described biological activity. The library compounds were dissolved in 100% DMSO at ~ 10 mM concentration and stored at -30^{0}C in 384-well plates. Prior to screen the above two small molecule libraries, the U20325-PRSLuc cells were validated for strong reporter luciferase signal with minimal well-to-well variability. In addition, these cells were optimized for cell seeding density, automation in cell plating and liquid handling, length of incubation before treatment of libraries, and the half-life of reporter luciferase. Once established, 3x10^3 cells per well in 40 μL growth medium were seeded in 384-well plates and incubated for 24 hours at 37^{0}C. Then, 40 nL of each compound or DMSO from the stock plate was added into assay plates (final concentration of each compound and DMSO were 10μM and 0.1% respective) using a JANUS robotic liquid handler (PerkinElmer) equipped with 384-pinn tool (V&P Scientific). The cells were incubated for 24 hours at 37^{0}C and luciferase activity for each well was assayed using SteadyGlo kit (Promega) in the luminescence mode with the use of Envision multilabel plate reader (PerkinElmer). Primary hits selected based on the decreased luciferase activity were subsequently subjected to a secondary screen in the same manner as the initial screen. For this screen, primary hits from the stock plates diluted in growth medium in such a manner that the

final concentrations of each compound lie in a range below (four 3-fold dilution) except one concentration above 10 μM used in the initial screening. Vehicle DMSO control was adjusted similarly, and then both diluted compounds and solvent were added to cells and incubated similarly to that of initial screening. While the luciferase activity of the treated plates were assayed using the SteadyGlo kit, similarly treated plates were also assayed to measure cell viability for each well in parallel using CellTiterBlue kit (Promega).

Luciferase assays

ARMS-derivative luciferase reporter cells (5 $x10^4$) or (1 x 10^4) in growth medium were seeded per well in 12-well and 96-well plates respectively and incubated overnight. Subsequently, these cells were incubated with compounds at the times and concentrations or transduced with viruses that were specified for each experiment. Cells were then lysed and assayed for luciferase activity as described previously (2, 3) using the luciferase assay system (Promega) according to manufacturer's instruction. Luciferase activity was determined in triplicate and normalized by total protein. To evaluate the IC_{50} of TG on the suppression of PRSLuc reporter luciferase expression, cells were treated with TG that ranged from 0.006 to 1000 nM for 24 hours, luciferase activity was measured and a dose-response curve was generated. IC_{50} value was determined from the dose-response curve using GraphPad Prism software (GraphPad, San Diego, CA, USA).

Immunoblot analysis

Cells were plated in tissue cultures dishes and grown to 60-70% confluency. The medium was then aspirated and cells were incubated with freshly diluted compound(s) in growth medium at the times and concentration specified for each experiment. Subsequently, cells were washed with PBS, harvested by centrifugation, and processed for the preparation of total, nuclear and cytoplasmic cell extracts, and immunoblot analysis as described previously (5). Briefly, total cell extracts cells were prepared by suspending cells in lysis buffer containing protease inhibitor

cocktail (Roche), followed by sonication and clarified by centrifugation. To prepare nuclear cytoplasmic fractionation, cells were suspended in hypotonic buffer to swollen followed by douncing in a glass homogenizer and centrifuged to receive nuclear pellet and clear supernatant (cytoplasmic cell extract). Nuclear cell extract were obtained by suspending nuclei in lysis buffer, followed by sonication and clarified by centrifugation. Total, nuclear and cytoplasmic cell extracts were assayed for protein concentration by the Bio-Rad procedure. For immunoblot analysis, equivalent amount of protein extracts was separated by 10% SDS-PAGE, followed by transfer to PVDF membranes. Following blocking in 5% milk, the membranes were incubated with primary antibodies overnight at 4^0C. All primary antibodies were used at 1:1000 except peroxidase-conjugated β-action at 1:50,000 dilutions. The membranes were subsequently incubated with appropriate secondary peroxidase-conjugated antibodies (1:5000) at room temperature for 1 hour. Signals were detected by enhanced chemiluminescent reagent (GE Healthcare), and the image was retrieved and analyzed by Alpha Innotech FluorChem® HD2 Imager (R&D systems).

RT-PCR Analysis

RNA was isolated from cells using Trizol reagent (Invitrogen), treated with DNaseI, and reverse transcribed into cDNA using Superscript III reverse transcriptase kit (Invitrogen) according to the manufacturer's instruction. Detection of *PAX3* was achieved by semi-quantitative PCR analysis of cDNA product using forward (5'-AGACATTTACACCAGGGAGGA-3') and reverse (5'-TAGCCTGCGGTGCTATAGGT-3') primers. Detection and expression of *PAX3-FOXO1, MYOD* and *SKP2* were achieved by real-time PCR analysis of the respective cDNA products using specific primer pairs, followed by quantitation using $\Delta\Delta C_T$ method as described in our recent study (2). Briefly, mRNA level of *PAX3-FOXO1, MYOD and SKP2* were normalized to that of *ACTB.* The cycle threshold value (C_T) for *PAX3-FOXO1/MYOD/SKP2* and *ACTB* mRNAs for each sample was calculated. A normalized target value (ΔC_T) was then derived by subtracting

the amount of *PAX3-FOXO1/MYOD/SKP2* mRNA by that of *ACTB* and the changes of *PAX3-FOXO1, MYOD* and *SKP2* mRNA expression were quantified by the equation: $2^{-\Delta\Delta C}{}_T$. All experiments were performed in triplicate. The primer pairs were used for *PAX3-FOXO1*: *PAX3* forward (5'-AGACATTTACACCAGGGAGGA-3'), *FOXO1* reverse (5'-AACTGGAAAGAGTTCTTGGT-3'); *MYOD*: forward (5'-CCGCCGCCTGAGCAAAGTGA-3'), reverse (5'-CCGGAGGCGACTCTGGTGGT-3'); *SKP2:* forward (5'-GTGGTACCGCCTCTCGCTCG-3'), reverse (5'-GCAGAGTCGCCACGGCAGAT-3').

ChIP Assay

ChIP was performed on HA-epitope tagged PAX3-FOXO1 overexpressing Rh30-PAX3-FOXO1-HA cells according to the method as described in previous study (4). In short, cells treated with TG or vehicle control were fixed with formaldehyde for 10 min at room temperature, sonicated, followed by preparation of soluble chromatin. Subsequently, DNA was derived from anti-HA immunoprecipitates of chromatin solution and subjected to real-time PCR analysis of enhancer regions of *MYOD* and *IGF1R* using specific primers (for *MYOD*; forward, 5'-CCTTGGTTGCGCTTACTGC-3' and reverse 5'-ACAAACTGGAAAGCCCTCTCT-3', and for *IGF1R*; forward, 5'-AGTCTAGCCTGCTGTGATTGTGCT-3' and reverse, 5'-AAACCTGCACTGA

GGTCAACTGGT-3'). Relative enrichment was calculated as the amount of the amplified DNA normalized to input.

Cell proliferation Assay

The growth of ARMS cells in the presence and absence of TG was determined by Trypan Blue Exclusion method in the following way: Cells were plated in a 12-well plate ($1x10^4$ cells per well) and incubated overnight. The following day, medium was removed and the cells were incubated with fresh growth medium containing with TG at its IC_{50} concentration (8.0 nM and 2.3 nM for human and mouse ARMS cells respectively) or vehicle control at the time indicated in the

experiment. To evaluate cell growth, cultures were trypsinized at indicated time intervals, stained with Trypan Blue (Invitrogen) followed by counting viable cells using hemocytometer.

Clonogenic assay

ARMS cells were plated at low density ($1x10^3$ cells per well) in 6-well plates and incubated overnight. Next day, cells were treated with TG at the concentration specified in cell proliferation assay or vehicle control and incubated by refreshing every two days with medium containing with TG or vehicle until visible colony forms. Cells were subsequently stained with 1% methylene blue and counted in a stereomicroscope and documented.

Colony formation in soft agar

Anchorage-independent cell growth in soft agar was performed as described in our previous study (3). Briefly, TG or vehicle control treated Rh30 and U20325 cells ($2x\ 10^5$ cells per well) were suspended in Nobel agar (0.3% final) in growth media and plated on top of 0.5% bottom agar and cultured at 37°C with 5% CO_2. The final concentration of TG exposed to Rh30 and U20325 cells were 8.0 nM and 2.3 nM respectively. Growth media containing TG or vehicle was replaced every 2 days on respective well. After 20 days, plates were stained with 0.005% of crystal violet and colonies were counted with stereomicroscope.

Xenograft experiments

First, we determined the maximum tolerated dose of TG (single intravenous dose) (ie, the maximum dose of TG that was not associated with appreciable toxicity, where body weight and mortality were the limiting criteria. The maximum tolerable dose of TG was determined to be 0.2 mg/kg body weight. Having determined the maximum tolerable dose of TG, Rh28 cells were injected subcutaneously into each flank (left and right) of thirty 8-10 weeks old CIEA NOG mice (male and female) (Taconic, Germantown, New York). In all mice, palpable tumors on both

flanks were observed 20 days after inoculation. Mice were then randomly assigned into two TG (TG-1 and TG2) (n= 10 for each group) or control (n= 10, PBS) treatment groups. The following treatments were administrated via intravenous injection (single treatment): (PBS), TG-1 (0.1 mg/kg) and TG-2 (0.15 mg/kg). Tumor size was determined every 3 days by measuring tumor length and width with calipers up to 15 days. In the control groups, some tumors exceeded the IACUC maximal size (2 cm in any dimension) prior to day 15 and were euthanized by CO_2 asphyxiation and thus not included in analysis. After treatment, mice were sacrificed and tumors extracted for further analysis. Tumor volumes for all mice in each Xenograft-treatment group were averaged to the mean tumor volume for the corresponding group.

Immunohistochemistry of Xenograft tumor tissue

Tumors obtained from sacrificed mice by dissection were fixed in 10% formalin and embedded in paraffin. Paraffin sections were cut at 4-5 μm and stained with hematoxylin-eosin (H&E). For immunohistochemical analysis, 4-5 μm paraffin sections were placed on charged slides and deparaffinized, and endogenous peroxidase was quenched with aqueous 3% H_2O_2. Slides were then loaded on a DAKO autostainer, followed by incubation with serum-free protein blocker (DAKO) for 5 minutes and subsequently incubation with primary rabbit antibody Ki-67 (1:200 dilution) or activated caspase 3 (1:158 dilution) in citrate buffer for 1 hour. Biotinylated anti-rabbit IgG were used as secondary antibody. Staining was visualized using DAB chromogen, slides were counterstained with hematoxylin, dehydrated, mounted, and images were captured. Immunohistochemistry staining of tumor sections and capturing of stained images were carried out in Pathology Resource Network.

References

1. Keller C, Arenkiel BR, Coffin CM, El-Bardeesy N, DePinho RA, Capecchi MR. Alveolar rhabdomyosarcomas in conditional Pax3:FOXO1 mice: cooperativity of Ink4a/ARF and Trp53 loss of function. Genes & development. 2004;18:2614-26.
2. Jothi M, Nishijo K, Keller C, Mal AK. AKT and PAX3-FOXO1 cooperation enforces myogenic differentiation blockade in alveolar rhabdomyosarcoma cell. Cell Cycle. 2012;11.
3. Lee MH, Jothi M, Gudkov AV, Mal AK. Histone methyltransferase KMT1A restrains entry of alveolar rhabdomyosarcoma cells into a myogenic differentiated state. Cancer research. 2011;71:3921-31.
4. Mal AK. Histone methyltransferase Suv39h1 represses MyoD-stimulated myogenic differentiation. The EMBO journal. 2006;25:3323-34.
5. Mal A, Sturniolo M, Schiltz RL, Ghosh MK, Harter ML. A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program. The EMBO journal. 2001;20:1739-53.

Supplementary Figure Legends

Supplementary Figure S1. Validation of PAX3-FOXO1-mediated reporter luciferase activity in U20325-PRSLuc reporter cells and evidence displaying U20325 cells does not express PAX3. (**A**) shRNA mediated knock-down of PAX3-FOXO1 diminishes luciferase activity in U20325-PRSLuc reporter cells. Lentivirus expressing PAX3-FOXO1 shRNA or scrambled control shRNA was transduced into cells and cultured for 2 days. Luciferase activity was evaluated in cell extracts and values were expressed after protein normalization. Error bar, ± SEM (n=3) (top). Immunoblot of reporter cell extracts were probed with FoxO1/FOXO1 and β-actin antibodies to detect PAX3-FOXO1 and for loading control respectively (bottom). (**B**) PAX3-FOXO1-positive Rh28 and Rh30 ARMS cells express low levels of wild-type (non-fused) PAX3 protein.

Immunoblot of extracts from these cells, probed for PAX3-FOXO1 (FoxO1/FOXO1 antibody), PAX3, and β-acting served as a loading control. (**C**) Immunoblot of extracts from U20325 ARMS cells and C2C12 myoblast cells shows the expression of both PAX3-FOXO1 and MyoD in the former but only MyoD in the latter cells, β-actin was used as a loading control. (**D**) Immunoblot of extracts from U20325 cells, probed for PAX3, and β-acting as loading control (top). Semi-quantitative RT-PCR analysis for *PAX3* mRNA performed in these cells, *ACTB* served as a loading control (bottom). 293A cells expressing ectopic *PAX3* cDNA were used as a positive control for both immunoblot and RT-PCR analyses.

Supplementary Figure S2. Screen of primary hits for the identification of PAX3-FOXO1 inhibitors. (**A**) The primary hits were evaluated for luciferase activity in parallel cell viability in a dose-response study of U20325-PRSLuc reporter cells. Compounds that have passed both criteria in dose-response assay were sorted and named as PFI-1 to PFI-9. Values for both luciferase activity and cell viability were normalized to vehicle control and expressed as percentage survival. (**B**) Above PFI compounds were evaluated for their effect on luciferase activity in the indicated reporter cells. Cells were incubated with each compound (at a concentration that has displayed ≤ 30 percent survival of luciferase activity in dose-response analysis as in (A) for 8 hours. Values expressed as percentage luciferase activity compared to vehicle control. Error bars, ± SEM (n=3). (**C**) Luciferase activity assay was performed in two additional PAX3-FOXO1-positive U20497T-PRSLuc and Rh30-PRSLuc reporter cells incubated with indicated PFI compounds (concentration used here is identical to that described in B) or vehicle for 24 hours. Luciferase activity expressed as percentage compared to vehicle control. Error bars, ± SEM (n=3).

Supplementary Figure S3. Thapsigargin but not dithiothreitol (DTT) decreases the expression levels of PAX3-FOXO1 protein in ARMS cells. PAX3-FOXO1 protein levels were determined by immunoblot analysis of extracts from Rh28 and Rh30 cells treated with 8.0 nM TG or 1.0 mM

DTT or vehicle control for 24 hours. As indicated, PAX3-FOXO1 was detected with FoxO1/FOXO1 antibody, and β-actin used as loading control.

Supplementary Figure S4. Thapsigargin induces apoptotic DNA fragmentations in ARMS cells. Indicated ARMS cells were treated with 8.0 nM TG or vehicle control for 4 days followed by collection of both floating and adhere cells. Subsequently, DNA was isolated from these cells and analyzed by agarose gel electrophoresis.