Supplementary Table 1. Clinical and histopathological features of pediatric patients with PAX3-FOXO1 alveolar rhabdomyosarcoma (n=10)

	n (%)
Sex	
Male	4 (40)
Female	6 (60)
Age (years)	
< 10	7 (70)
≥ 10	3 (30)
Localisation	
Orbit-Genitourinary tract-Head and Neck[$]	5 (50)
Cranial paramenigeal-Extremity-Other[$$]	5 (50)
Tumor volume	
< 5 cm	4 (40)
≥ 5 cm	6 (60)
IRS stage	
I	1 (10)
II	0 (0)
III	5 (50)
IV	4 (40)
Metastasis	
No	6 (60)
Yes	4 (40)
Recurrence	
No	4 (40)
Yes	6 (60)
Outcome	
Alive	5 (50)
DOD	5 (50)

Abbreviations: DOD, dead of disease; IRS, Intergroup Rhabdomyosarcoma Study Group staging system; RFI, Recurrence-Free Interval. [$]Favorable and [$$]Unfavorable tumor localization

SUPPLEMENTARY MATERIALS AND METHODS:

Cell lines culture. The PAX3-FOXO1 alveolar RMS cell lines RH30, RH41 and RH4 were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FCS, 1% glutamine and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5%CO_2/95% air. Human myoblasts, SkMC (C-12530 PromoCell GmbH, Heidelberg, Germany), were maintained in proliferating condition in PromoCell Cell Growth Medium (GM) supplemented with growth factors (C-23060 and C23160, PromoCell GmbH, Heidelberg, Germany). A human skeletal muscle differentiation model was obtained treating SkMC myoblasts for at least 10 days with a differentiating medium (DM) containing appropriate supplements (C-23161 and C-39366, PromoCell GmbH, Heidelberg Germany). Several aliquots of the first culture for each RMS cell line were stored in liquid nitrogen at -80°C for subsequent assays. Each aliquot was passaged for a maximum of 5 months. ATCC genomics core utilizes scientific knowledge and technical expertise to design and perform numerous authentication and confirmatory assays (such as DNA barcoding and species identification, quantitative gene expression and transcriptome analyses) for ATCC collections. See www.lgc.standards-atcc.org. The DSMZ authenticates all human cell lines prior to accession by DNA-typing, while the species-of-origin of animal cell lines are confirmed by PCR-analysis ("speciation"). Independent evidence of authenticity is also provided by cytogenetic and immunophenotypic tests of characterization which are particularly informative among human tumor cell lines which form the bulk of the collection (see www.dsmz.de). Four different batches of SkMC were obtained, each from a different healthy donor, and immediately cultured and assayed in specific experiments as reported. The cell factory departments tested cells for cell morphology, adherence rate, and cell viability; immunohistochemical tests for cell-type specific markers are carried out for each lot and, furthermore, the capacity to differentiate into multinucleated syncytia is routinely checked for each lot (see www.promocell.com).

Western blotting details and antibodies: Cells were lysed in RIPA buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 1% D.O.C. (Na), 0,1% SDS, 1% Triton X-100) containing protease inhibitors (Sigma Chemical Co., St Louis, MO, USA). Lysates were sonicated, incubated on ice 30 min and centrifugated at 10,000 g 20 min at 4°C. Supernatants were used as total lysates. Protein concentrations were estimated with the BCA protein assay (Pierce, Rockford, IL). Supernatants were boiled in reducing SDS sample buffer and 30 μg of protein lysate per lane were run trough 7 to 15% SDS-PAGE gels, transferred to Hybond ECL membranes (Amersham, GE HEALTHCARE BioScience Corporate Piscataway, NJ, USA). Membranes were blocked 1h in 5% non-fat dried milk in TBS and incubated overnight with the appropriate primary antibody at 4°C. Membranes were then washed in TBS and incubated with the appropriate secondary antibody. Both primary and secondary antibodies were diluted in 5% non-fat dried milk in TBS. Detection was performed by ECL Western Blotting Detection Reagents or by ECL Plus Western Blotting Detection Reagents (Amersham, GE HEALTHCARE BioScience Corporate Piscataway, NJ, USA). Nuclear fraction-enrichment assays were performed as described in Materials and Methods. EZH2 (612666), activated BAX (556467) and PARP-1 (556494) were detected using antibodies from BD (Transduction Laboratories™, BD, Franklin Lakes, NJ, USA). Antibodies against Myogenin (F5D) and Myosin Heavy Chain (Meromyosin, MF20) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (DSHB, Iowa City, IA, USA). Antibodies against FBXO32 (sc-33782), BCL2 (sc7382), MyoD (sc-760), cMyc (sc-40), BAX (sc-493) and β-actin (sc-1616) and all secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Antibodies against BIM (2819), Troponin I (4002) and GAPDH (D16H11) were obtained from Cell Signaling (Beverly, MA, USA). The antibody against the Topoisomerase IIβ was obtained from Sigma Aldrich (Sigma Chemical Co., St Louis, MO, USA). Antibody against against Histone 3 (H3), H3K27me3 (Lys27), H3K4me3 (Lys4) and H3K9me3 (Lys9) were obtained from Millipore (EMD Millipore Corporation, Billerica, MA, USA). Antibody against α-tubulin (ab4074) was from Abcam (Cambridge, UK). All the antibodies

were used in accordance with the manufacturer's instructions. Images of radiograms were acquired through the HP Precision ScanJet 5300 C Scanner (Hewlett-Packard, Palo Alto, USA) and densitometric analysis was performed by using Quantity One Basic 1-D Software (Bio-Rad Laboratories, Inc., Hercules, CA).

Transient EZH2 silencing with an additional oligo siRNA. Cells were sequentially transfected by 2 subsequent rounds (24h) with a siRNA targeting the 5'-UTR of EZH2 mRNA (Ref. n. 22) with the following sequence 5'-CGGTGGGACTCAGAAGGCA-3' (100 nM final concentration each round) (Sigma-Proligo, St Louis, MO) using Oligofectamine (Invitrogen, Carlsbad, CA), according to manufacturer's recommendations. A non-targeting siRNA was used as control (Sigma, St Louis, MO).

Lentiviral-mediated RNA interference with a short hairpin (sh)RNA against EZH2. The shRNA-mediated EZH2 silencing was performed by infecting RH30 cells with a Smart Choice Lentiviral shRNA particles expressing 19 nucleotide shRNAs against EZH2 (VSH5417, SH-004218-01-01; EZH2 target sequence TTTGCAAATCATTCCGGTAA) (Dharmacon, Thermoscientific, Waltham, MA, USA). A non-targeting shRNA (SV 2.0; HV221111) was used as negative control (Dharmacon, Thermoscientific, Waltham, MA, USA). Cells were infected with 10 multiplicity of infection (MOI) particles per cell of lentivirus using polybrene (Invitrogen, Carlsbad, CA, USA) at final concentration of 0.6 µg /ml for 16 hours in medium supplemented with 10% FCS. EZH2 silencing was assessed by both Western blotting and qRT-PCR 48h post-infection.

Supplementary Figure Legends:

Supplementary Figure 1. EZH2 depletion by an additional siRNA targeting the 5'-UTR of EZH2 mRNA or by lentiviral-mediated EZH2 short-hairpin (sh) RNA expression in PAX3-FOXO1 alveolar RMS cells cultured in proliferating medium (*i.e.*, supplemented with 10% fetal calf serum). RH30 and RH4 cells were transfected (double round) with either a previous tested siRNA from Sigma-Proligo (EZH2 siRNA§) or with a non-targeting control siRNA (CTR siRNA§) or left untreated and analyzed 24h after the second siRNA treatment. (**a**) Western blot showing levels of EZH2, H3K27me3, H3, FBXO32, Myogenin, PARP-1, BCL2, BAX** (activated form), BAX, BIM and α-tubulin (as loading control) (black arrow indicates cleaved PARP-1). Representative of three independent experiments. (**b**) Cells were stained for Annexin V and 7-AAD 24h after the second round of silencing and the frequency of Annexin V and 7-AAD-positive labeling (% cell death) was recorded by flow cytometry. Samples were analyzed within 1h. Representative cytofluorimetric plots are shown. Annexin V^+/7-AAD^- events (lower right quadrants) represent early stages of apoptosis, whereas Annexin V^+/7-AAD^+ events (upper right quadrants) stand for late apoptotic cells. Representative of three independent experiments run in duplicate. (**c**) RH30 cells were infected with lentiviral vectors expressing either a shRNA against EZH2 (EZH2 shRNA) or a non-targeting control shRNA (CTR shRNA). Western blot showing levels of EZH2, Myogenin, BCL2, PARP-1 and GAPDH (as loading control) in RH30 cells 4 days after lentiviral infection (black arrow indicates cleaved PARP-1). Representative of three independent experiments. (**d**) mRNA levels (real time qRT-PCR) of EZH2 and FBXO32 in EZH2 shRNA RH30 cells 4 days after lentiviral infection were normalized to GAPDH levels and expressed as fold increase over CTR shRNA values (1 arbitrary unit, not reported). Columns, means; Bars, SD. Results from three independent experiments performed in duplicate.

Supplementary Figure 2. Effects of EZH2 depletion on SKMC cultured in proliferating (growth) medium (GM) (*i.e.* supplemented with 10% of fetal calf serum). SKMC were transfected (double round) with either a siRNA against EZH2 (EZH2 siRNA) or with a non-targeting control siRNA (CTR siRNA) or left untreated. (**a**) Western blot showing levels of EZH2, H3K27me3 and H3 24h after the second siRNA treatment, and levels of Myogenin and α-tubulin (as loading control) 72h after the second siRNA treatment. Representative of three independent experiments. (**b**) SKMC were stained for Annexin V and 7-AAD 24h after the second round of silencing and the frequency of Annexin V and 7-AAD-positive labeling (% cell death) was recorded by flow cytometry. Samples were analyzed within 1h. Representative cytofluorimetric plots are shown. Annexin V^+/7-AAD^- events (lower right quadrants) represent early stages of apoptosis, whereas Annexin V^+/7-AAD^+ events (upper right quadrants) stand for late apoptotic cells. Representative of two independent experiments run in duplicate. (**c**) mRNA levels (real time qRT-PCR) of EZH2, Myogenin and FBXO32 in EZH2 siRNA or CTR siRNA SKMC 24h after the second round of silencing were normalized to GAPDH levels and expressed as fold increase over Untreated SKMC (1 arbitrary unit, not reported). Columns, means; Bars, SD. Results from two independent experiments performed in duplicate.

Supplementary Figure 3. FBXO32 induction after EZH2 silencing is counteracted by enforced expression of murine Ezh2. (**a**) RH30 cells were infected (double round) with pMSCV-GFP retroviral vector expressing murine Ezh2 (mEzh2) or with an empty pMSCV-GFP control vector (Vector) and 24h after the second round of infection were transfected (double round) with EZH2 or CTR siRNA. mRNA levels (real time qRT-PCR) of endogenous EZH2 and FBXO32 were

analyzed 36h after the second round of silencing, normalized to GAPDH levels and expressed as fold increase over CTR siRNA (1 arbitrary unit, not reported). Results are the average of three independent experiments. (**b**) pMSCV-mEzh2 (mEzh2) and pMSCV control vector (Vector) RH30 cells were harvested 24h after the second round of infection and analyzed for GFP expression by flow cytometry. Dot-plots report the percentage of GFP-positive population in each sample. (**c**) mRNA levels of exogenous murine Ezh2 that confirms murine Ezh2 overexpression in RH30 cells; murine Ezh2 mRNA levels (real time qRT-PCR) are reported and expressed as fold increase over Uninfected condition (1 arbitrary unit, not reported). *P <0.05 (Student's t-test). (**d**) RH30 cells were infected (double round) with pMSCV-GFP retroviral vector expressing murine Ezh2 (mEzh2) or with an empty pMSCV-GFP control vector (Vector) and 24h after the second round of infection were harvested and analyzed for the expression of EZH2 and H3K27me3. Ezh2 (D2C9) XP antibody (5246, Cell Signaling, Beverly, MA, USA) was used to detect EZH2 levels since it has been confirmed to reveal both murine and human forms of the protein. GAPDH and H3 were the loading controls. Representative of two independent experiments.

Supplementary Figure 4. Efficiency of infection of FBXO32-expressing vector in RH30 and RH4 cells. Cells were infected (double round) with either pMN-GFP control (Vector) or pMN-GFP myc-FBXO32 (FBXO32) over-expressing retroviral vectors and analyzed 24h after the second round of infection. Representative immunofluorescence of GFP-positive cells compared to phase-contrast images of total cell population is shown (50X Magnification).

Supplementary Figure 5. ChIP assays on RMS cells after JARID2 or CTR siRNA transfection showing the levels of histone H3 trimethylation on Lys27 (H3K27me3) at the *FBXO32* Polycomb dependent regulatory region. Normal rabbit IgG was used as negative control for IP. Graphs represent the percent of immunoprecipitated relative to input DNA. Results are the average of three independent experiments. (Ref. n. 30 on Main Maunscript) **P*<0.05 (Student's t-test).

Supplementary Figure 6. Inactivation of EZH2 in RH30 cells by using the catalytic inhibitor MC1948. (**a**) RH30 cells were treated with 5μM MC1948 or DMSO (vehicle) in proliferating medium (i.e. supplemented with 10% of fetal calf serum), harvested and counted at the indicated time points starting at 24h (day 1) from the onset of treatment. **P*<0.05 (Student's t-test); Bars, Standard Deviation (SD). (**b**) Western blot showing levels of EZH2 along with histone H3 trimethylation on Lys27 (H3K27me3) after 72h of 5μM MC1845 treatment in RH30 cells. Total Histone3 (H3) served as loading control. Representative of 3 independent experiments.