SUPPLEMENTARY MATERIALS
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Cell Lines and Culture Conditions

MSCs were purchased from ATCC and were maintained in MEM alpha medium with 20% FBS. The ARMS cell line RH30 was obtained from the laboratory of Anindya Dutta, University of Virginia, Charlottesville, VA. The cells were cultured in RPMI 1640 medium with 10% (vol/vol) FBS. RH4, BIRCH, RH28, MP4, RH18, RD, and RMS-13 cells were obtained from Dr. Liang Cao, NIH, Bethesda; A2243 cells were obtained from Dr. Steven Tronick at the National Cancer Institute, Bethesda; TC32 cells were obtained from Dr. Jaclyn Biegel, Children’s Hospital of Philadelphia, Philadelphia; TTC-466 cells were obtained from Dr. Timothy Triche, Children’s Hospital, Los Angeles; JN-DSRCT cells were obtained from Dr. Marc Ladanyi, Memorial Sloan Kettering Cancer Center, New York; 402-91 cells were obtained from Dr. Nils Mandahl, Lund University, Lund, Sweden; SUCCS-1 cells were obtained from Dr. Alan Epstein, University of Southern California, Los Angeles; A673 cells were purchased from ATCC; and OsACL cells were obtained from Dr. David Shapiro, St. Jude Children’s Research Hospital, Memphis, TN.

Clinical and PDX samples

RNA of Rhabdosarcoma patients were obtained from Dr. Barr. Cryo-preserved cells for implantation and flash frozen PDX samples were gifts from St. Jude Children’s Research Hospital1.

AVIL overexpression and silencing

MARS-AVIL coding region was RT-PCR amplified from RH30. AVIL cDNA clone was purchased from GeneCopoeia (GC-OG11537), and cloned into the Retrovirus vector pQCXIN. Stable cells that overexpress AVIL were selected via G418. For siRNA treatments, siAVIL1 (targeting 5′-GCTTCTGGCAAGGATATT-3′), siAVIL2 (targeting 5′-CTTCAGGGCTGTGGACAAC-3′), and control siRNA (siGL2) were purchased from Life Technologies. RNAiMAX (Invitrogen) was used for siRNA transfection, which was performed according to the manufacturer’s instructions and previous publication2. Tet-inducible shAVIL constructs were purchased from transOMIC. Stable cells were selected with puromycin. Cells are treated with doxycycline hydrochloride (final concentration 10µg/ml).

PCR and real-time PCR

RNA was extracted using RNeasy Mini Kit (QIAGEN) and quantified with Nanodrop (Thermo). cDNA was generated by Verso cDNA Synthesis Kit (Thermofisher), and a random hexamer primer3. Real-time qPCR was carried out on the StepOne Plus system from Applied Biosystems using SYBR mix (Thermo) according to previous publications4,5. Primer sequences are listed in Supplementary Table 1.

Western blotting

Cell lysates were resolved by denaturing gel electrophoresis, before performing Trans-Blot Turbo Transfer System (BIO-RAD). The membrane was subjected to western blot analysis with antibodies against the proteins of interest. The following antibodies and dilutions were used: rabbit anti-MARS (1:1000 Sigma SAB2101437), rabbit anti-AVIL (1:1000; Abcam ab72210), rabbit anti-PARP (1:1000; Cell Signaling Technology 9542), rabbit anti-Cleaved Caspase-3 (1:1000; Cell Signaling Technology 9664), MEK1/2 (L38C12) Mouse mAb (1:1000; Cell Signaling Technology 4694S), Phospho-MEK1/2(Ser217/221) Antibody (1:1000; Cell Signaling Technology 9121S), p44/42 MAPK (Erk1/2) Antibody(1:1000; Cell Signaling Technology 9102S), Phospho-p44/42 MAPK(ERK1/2) Antibody (1:1000; Cell Signaling Technology 9101S) , rabbit anti-Cleaved
Caspase-3 (1:1000; Cell Signaling Technology 9664), and mouse anti-GAPDH (1:10,000; Ambion Am4300).

FISH

DNA probes for fluorescence in situ hybridization (FISH) were labeled by nick translation with Spectrum Green or Spectrum Red 2′-deoxyuridine-5′-triphosphate (Abbott). Cells were grown in 8-chamber slides and fixed with methanol: acetone = 1:1 fixation solution for 10 min at 4 °C. BAC Fish clone, RP11-143123 was purchased from BACPAC company.

Cell migration assay

The effect of AVIL on cell migration was assayed by a wound-healing assay according to previous publication\(^6\). Briefly, cells were cultured to confluency. A wound was created by scraping the cells using a 10 ul plastic pipette tip, and the medium was replaced with fresh medium. Images were captured immediately after the scratch, and again 8h later. Cell migration was quantitatively assessed by the size of the gap within the confluent monolayer culture at the end of the experiment. Eight gaps were measured. For siRNA experiment, the measurement took place around 48–72 h after transfection.

RNA-Sequencing

RNA-Seq was performed by Axeq. Briefly, the mRNA in total RNA was converted into a library of template molecules suitable for subsequent cluster generation using the reagents provided in the Illumina TruSeq RNA Sample Preparation Kit. Paired-end sequencing was then conducted using Hiseq 2000 (Illumina). The 101-bp RNA-seq data were analyzed as before\(^7\). The circular image results of alignments were presented using Circos plot.

RNA sequencing data was preprocessed following FastQC output to check for quality issues. Alignment was done using Kallisto in paired-end mode, aligning to the cds GRCh38 build from Ensembl\(^8\). Pseudoalignments were processed for differential analysis using tximport passing off to Deseq2\(^9,10\). After Deseq2 normalization and quantitation, volcano plots were generated with EnhancedVolcano, and pathway analysis was performed using both over-representation (ORA) and gene set enrichment analysis (GSEA) using clusterProfiler with the “DOSE” and nPerm = 10000 parameters for GSEA\(^11\). Curated gene sets were taken from MsigDB with the associated reactomePA and Pathview packages\(^12,13\).

Flow cytometry

Trypsinized cells were spun down in 15ml conical tube and resuspend in 2.5ml PBS. 100% ethanol was added dropwise, and samples were put in -20°C overnight. 3uM solution of Propidium Iodide was prepared by diluting 1mg/ml (1.5mM) stock solution 1:500 in staining buffer (100mM Tris, pH 7.4, 150 mM NaCl, 1mM CaCl\(_2\),0.5mM MgCl\(_2\), 0.1%NP-40). 1mL was added to the cells, covered with dilute stain, for 15 minutes at room temperature and analyzed by flow cytometry in the presence of the dye.

Live-cell imaging

The cellular movement was analyzed by live-cell imaging. Briefly, RH30, RD, SMS-CTR or MSC were plated to 30–40% confluency in DMEM + 10% FBS, followed by transfection with siGL2 or siAVIL. 2 h prior to start of imaging media was supplemented with 0.5 μM SiR-DNA (Cytoskeleton) dye. 24 h after siRNA transfection images were collected on a Zeiss Axio-observer-Z1 epifluorescent microscope in humidified chamber in 5% CO\(_2\) environment, at 37 °C every 20 min over the period of 24 h. Resulting movies were processed using ImageJ, and cell movement was tracked semi-automatically based on SiR-DNA staining by TrackMate plugin for ImageJ\(^14\).

Tumor formation in vivo
The mouse work was performed under the study protocol approved by the University of Virginia Institutional Animal Care and Use Committee. Immunocompromised SCID/NCr BALB/c adult male mice (6–8 weeks old) were used. All animals were housed in sterilized plastic cages under specific pathogen-free conditions, at 22 °C, 12/12 light/dark cycle, 55% humidity. RH30 or RD cells were transfected with control shRNA, or shAVIL. The transfected cells were then counted, and $3 \times 10^6$ were injected subcutaneously. The tumors were harvested when most animals in the shCT group reached the human endpoint. The tumors were measured by caliber, and weighed. Since shAVIL group often had much smaller or no tumor, we waited longer before harvesting to show the survival differences.

For the tet-inducible system, mice were fed with water containing doxycycline hydrochloride (2mg/ml) and 5% sucrose. The doxycycline-sucrose solution was prepared fresh every three to four days, and kept in brown drinking bottle.

MSC stably expressing AVIL or MARS-AVIL or an empty vector were injected subcutaneously into the flanks of NIH-III Nude mice. On the same animal, the left side was injected with cells transfected with control vector, whereas the right with cells transfected with AVIL or MARS-AVIL expressing vector. Around two million cells were used per injection. The animals were monitored twice a week.

Data access

RNA-Seq data for MSC cells overexpressing AVIL, and MSC control triplicates has been deposited into GEO database, under the accession: GSE180837.

SUPPLEMENTARY FIGURE LEGENDS

**Figure S1. Detection of MARS-AVIL fusion in RMS.** a Landscape of fusion RNAs in RH30 RNA-sequencing. MARS-AVIL fusion has the highest number of reads. b MARS-AVIL was measured by qRT-PCR and normalized against that of GAPDH. Data are presented as mean values ± SD in b. c MARS-AVIL is a product of chromosomal inversion. DNA and RNA sequence flanking the junction. MARS part is in green font, and AVIL in orange. P value was calculated by standard two-tailed t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure S2. MARS-AVIL, but not AVIL, had a partial rescue of cell growth caused by siAVIL1.** a Effect of siRNAs targeting different parts of AVIL. siAVIL1 silenced both MARS-AVIL and wild-type AVIL, whereas siAVIL2 silenced only wild-type AVIL. MARS-AVIL and AVIL level was measured by qRT-PCR and normalized against that of GAPDH. b RH30 cells were transfected by siCT or siAVIL1, and further transfected with MARS-AVIL or AVIL expression vector. Microscopic images of the various groups were shown on the left, and cell counting on the right. Data are presented as mean values ± SD. P value was calculated by standard two-tailed t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

**Figure S3. MARS overexpressed had no obvious effect on RD and RH18.** a and c Cell proliferation measured by MTT in RD (a) and RH18 (c) cells stably expressing MARS or control vector. b and d Cell motility measured by wound healing in RD (b) and RH18 (d) cells stably expressing MARS or control vector. Data are presented as mean values ± SD in a-d. P value was calculated by standard two-tailed t-test.

**Figure S4. RH30 cells stably express tet-inducible shAVIL.** a Upon addition of doxycycline, GFP positive cells were detected in both shCT and shAVIL cells. b and c qRT-PCR measuring the level of MARS-AVIL (b) and AVIL (c) in the absence or presence of doxycycline.

**Figure S5. qRT-PCR measuring AVIL expression in RMS PDX models (a) and clinical; samples (b).** PAX3-FOXO1 and PAX7-FOXO1 were measured by qRT-PCR, and normalized
against that of GAPDH. RH30 is the positive control for PAX3-FOXO1, and RMZ-RC2 as the positive control for PAX7-FOXO1. a RMS PDX models include two PAX3-FOXO1 positives, one PAX7-FOXO1 positive, and two PAX3/7-FOXO1 negative cases. b RMS clinical samples include 18 PAX3-FOXO1 positives, three PAX7-FOXO1 positives, and eight PAX3/7-FOXO1 negative cases.

Figure S6. Live cell imaging tracking individual cells over 24 hours window, 24 hrs after transfection. SMS-CTR cells were transfected with siCT or siAVIL. a Mean velocities of all cells tracked in the experiment (n > 4000 cells quantified per condition) (box, 25–75 percentile; whisker, 5–95 percentile; bar in the middle, median) (two-sided Student’s t-test). b Shown are representative images depicting the starting timepoint of the experiment with overlaid lines tracking the movement of individual cells.

Figure S7. MARS-AVIL transforms MSC. A Focus formation assay. MSC cells were transfected with MARS-AVIL-expressing (MARS-AVIL) or control empty plasmid (CT). The quantitative difference of the foci number between the two groups was plotted. n=5. b MSC cells expressing control plasmid (CT) or MARS-AVIL were injected subcutaneously into the flanks of immunodeficient mice. The same animals received CT on the left side, and MARS-AVIL on the right. Representative images were shown. n=7. c and d Comparison of tumor volume (c) and weight (d) between the two groups. e Representative hematoxylin and eosin staining of the tumors harvested from the mice. Histology analysis revealed histologic features of neoplasms. Data are presented as mean values ± SD in a, c, and d. P value was calculated by standard two-tailed t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

Figure S8. RNA-Seq analysis on MSC overexpressing AVIL compared with MSC control. a Principle component analysis (PCA) plot. PCA plot is produced from shrunken log2FoldChange values obtained from Deseq2 using Approximate Posterior Estimation for GLM (apeglm) shrinkage estimation. Each dot represents a sample replicate. PC1 separation characterizes the trend exhibited by expression profiles between the two groups. b GSEA analysis. Genes bound by PAX3-FOXO1 from ChIP experiment were enriched.

Figure S9. RNA-Seq analysis on MSC cells overexpressing MARS-AVIL or AVIL. Upper part is the Venn diagram showing differential expressed genes in MARS-AVIL (a) and AVIL (b) overexpressed MSC cells. Lower part is the GO term analysis of MARS-AVIL uniquely upregulated 217 genes (c).

Supplementary Movie 1. Movie clips of RD cells transfected with control siRNA, siCT (left), and siAVIL1 (right). The same cells were imaged for 24 hours, with images taken every 20 minutes, starting 24 hours after transfection.

Supplementary Movie 2. Movie clips of SMS-CTR cells transfected with control siRNA, siCT (left), and siAVIL1 (right). The same cells were imaged for 24 hours, with images taken every 20 minutes, starting 24 hours after transfection.

Supplementary Table 1. Primers used for RT-PCR.

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