Supplementary Information

The Wnt pathway corepressor TLE3 interacts with the histone methyltransferase KMT1A to inhibit differentiation in Rhabdomyosarcoma

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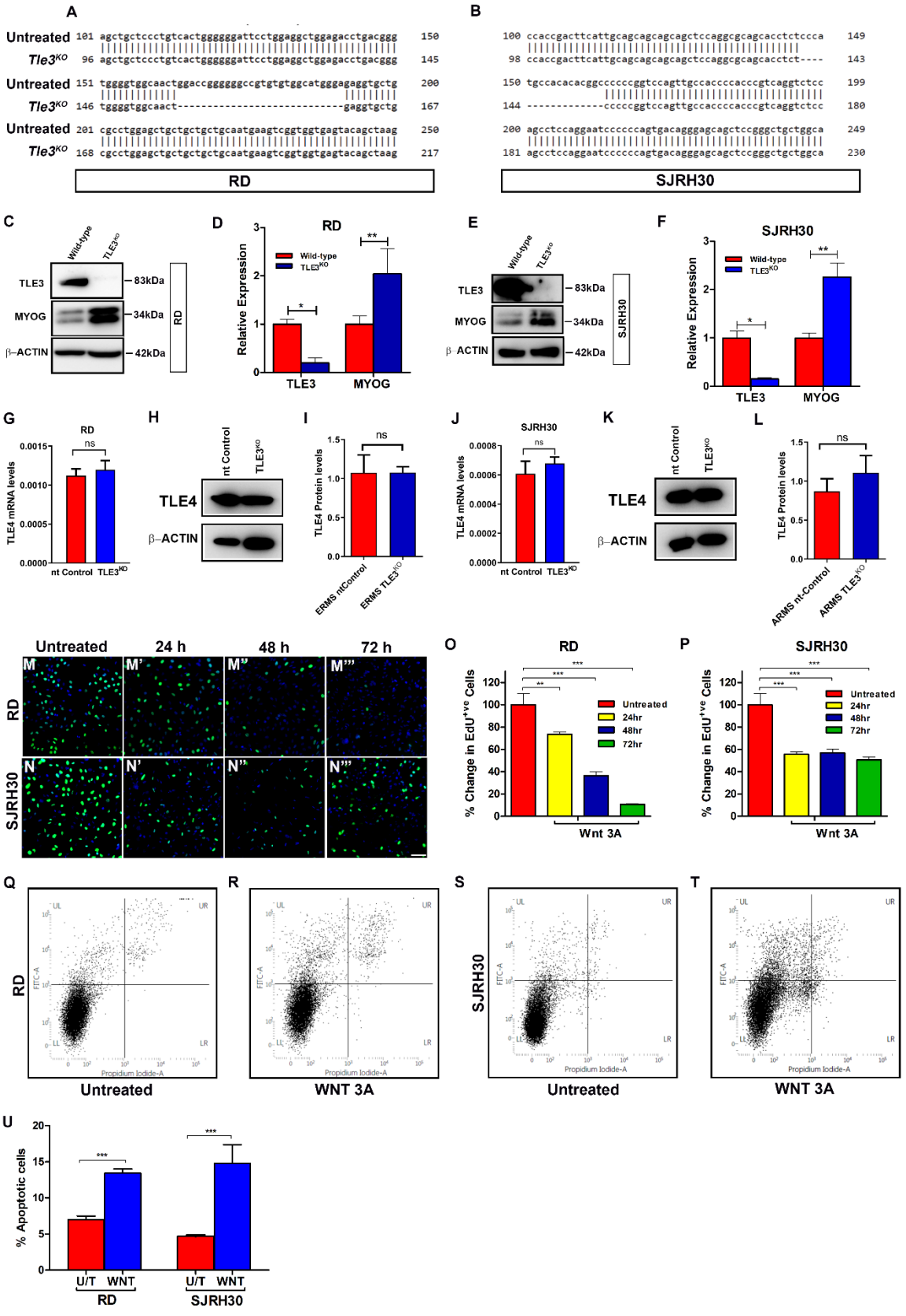
Figure S1


A
Untreated 101 agctgctccctgtcactgggggattcctggaggctggagacctgacggg 150
Tle3KO 96 agctgctccctgtcactgggggattcctggaggctggagacctgacggg 145
Untreated 151 tggggtggcaactggaccgggggccgtgtgtggcatgggagaggtgctg 200
Tle3KO 146 tggggtggcaact---------- gaggtgctg 167
Untreated 201 cgcctggagctgctgctgctgcaatgaagtcggtggtgagtacagctaag 250
Tle3KO 168 cgcctggagctgctgctgctgcaatgaagtcggtggtgagtacagctaag 217
RD
B
100 ccaccgacttcattgcagcagcagcagctccaggcgcagcacctctccca 149
98 ccaccgacttcattgcagcagcagcagctccaggcgcagcacctct---- 143
150 tgccacacacggccccccggtccagttgccaccccacccgtcaggtctcc 199
144 ----------ccccggtccagttgccaccccacccgtcaggtctcc 180
200 agcctccaggaatccccccagtgacagggagcagctccgggctgctggca 249
181 agcctccaggaatccccccagtgacagggagcagctccgggctgctggca 230
SJRH30
C
Wild-type
TLE3KO
TLE3
MYOG
β–ACTIN
83kDa
34kDa
42kDa
RD
D
RD
Wild-type
TLE3KO
Relative Expression
TLE3
MYOG
E
SJRH30
F
SJRH30
G
RD
ns
TLE4 mRNA levels
nt Control
TLE3KD
H
nt Control
TLE3KO
TLE4
β–ACTIN
I
ns
TLE4 Protein levels
ERMS ntControl
ERMS TLE3KO
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ARMS nt-Control
ARMS TLE3KO
Untreated
24 h
48 h
72 h
RD
SJRH30
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M'
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O
RD
% Change in EdU+ve Cells
Untreated
24hr
48hr
72hr
Wnt 3A
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SJRH30
% Change in EdU+ve Cells
Untreated
24hr
48hr
72hr
Wnt 3A
Q
R
S
T
RD
SJRH30
UL
UR
LL
LR
FITC-A
Propidium Iodide-A
Untreated
WNT 3A
U
% Apoptotic cells
U/T
WNT
RD
SJRH30

Figure S1: Wnt3A treatment reduces cell proliferation and enhances apoptosis in rhabdomyosarcoma. A-B: Sequence alignment of the *TLE3* exon 7 genomic region in control and $TLE3^{KO}$ clones following CRISPR-Cas9-based genomic editing in RD (**A**) and SJRH30 (**B**) cells. **C-F:** Western blots for TLE3, MYOG, and β-ACTIN on control and $TLE3^{KO}$ RD **(C)** and SJRH30 **(E)** cells and their respective densitometric quantification (n=3) **(D, F). G-L:** Quantification of human *TLE4* transcript levels and protein expression in nt control and $TLE3^{KO}$ RD **(G-H)** and SJRH30 **(J-K)** cells and their respective densitometric quantification (n=3) **(I, L). M-P:** Representative immunofluorescence micrographs labelling 5-ethynyl-2'-deoxyuridine (EdU) (green) and Hoechst (blue) in RD (**M-M'''**) and SJRH30 (**N-N'''**) cells following Wnt3A treatment for the indicated time points, and their quantification showing the percentage of EdU^+ nuclei (n=3) (**O, P**). **Q-U:** Annexin V, FITC-PI dual staining of rhabdomyosarcoma cells following Wnt3A treatment. Representative FACS plots showing the distribution of stained cells in RD (**Q, R**) and SJRH30 (**S, T**) sub-types and their quantification as a percent of total cells (n=3) (**U**). Data are mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001, ANOVA, Tukey's test. Scale bar: 100 µm (**N'"**).

Figure S2

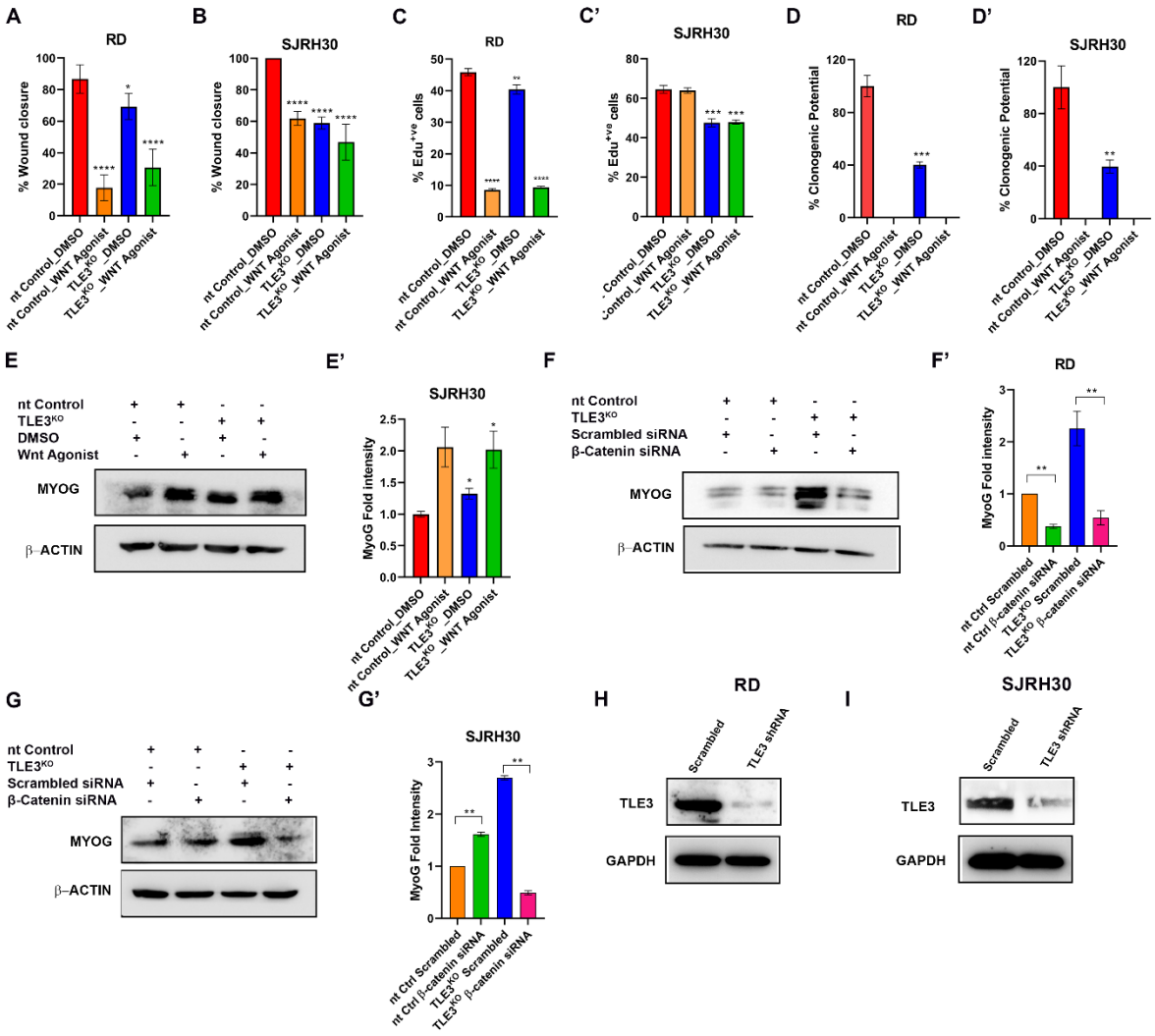

Figure S2: Treatment with the Wnt agonist CHIR99021 recapitulates the effect of TLE3 deletion in rhabdomyosarcoma cells. A-B: Graphs depicting percent wound closure in nt control and *TLE3*^{KO} RD (n=4) **(A)** and SJRH30 (n=5) **(B)** cells treated with DMSO or Wnt Agonist at 24 hours after treatment. **C-C':** Quantification of the percentage of EdU^{+} nuclei in nt control and *TLE3*^{KO} RD **(C)** and SJRH30 **(C')** cells treated with DMSO or the Wnt agonist CHIR99021 (n=4). **D-D':** Graphs quantifying the clonogenic potential in nt control and *TLE3*^{KO} RD (n=4) **(D)** and SJRH30 (n=6) **(D')** cells treated with DMSO or the Wnt agonist CHIR99021. **E-E':** Western blots for MYOG and β-ACTIN on nt control and *TLE3*^{KO} SJRH30 cells treated with DMSO or the Wnt

Agonist CHIR99021 **(E),** and densitometric quantification **(E')**. **F-G':** Western blots for MYOG and β-ACTIN on nt control and *TLE3*^{KO} RD (n=4) **(F)** and SJRH30 (n=3) **(G)** cells treated with control siRNA or *β-catenin* siRNA and their respective densitometric quantification **(F', G'). H-I:** Representative western blots for TLE3 and GAPDH (loading control), on lysates from scrambled and *TLE3* shRNA stably integrated RD (**H**) and SJRH30 (**I**) cells, following treatment with doxycycline. Data are mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001, ANOVA, Tukey's test.

Figure S3

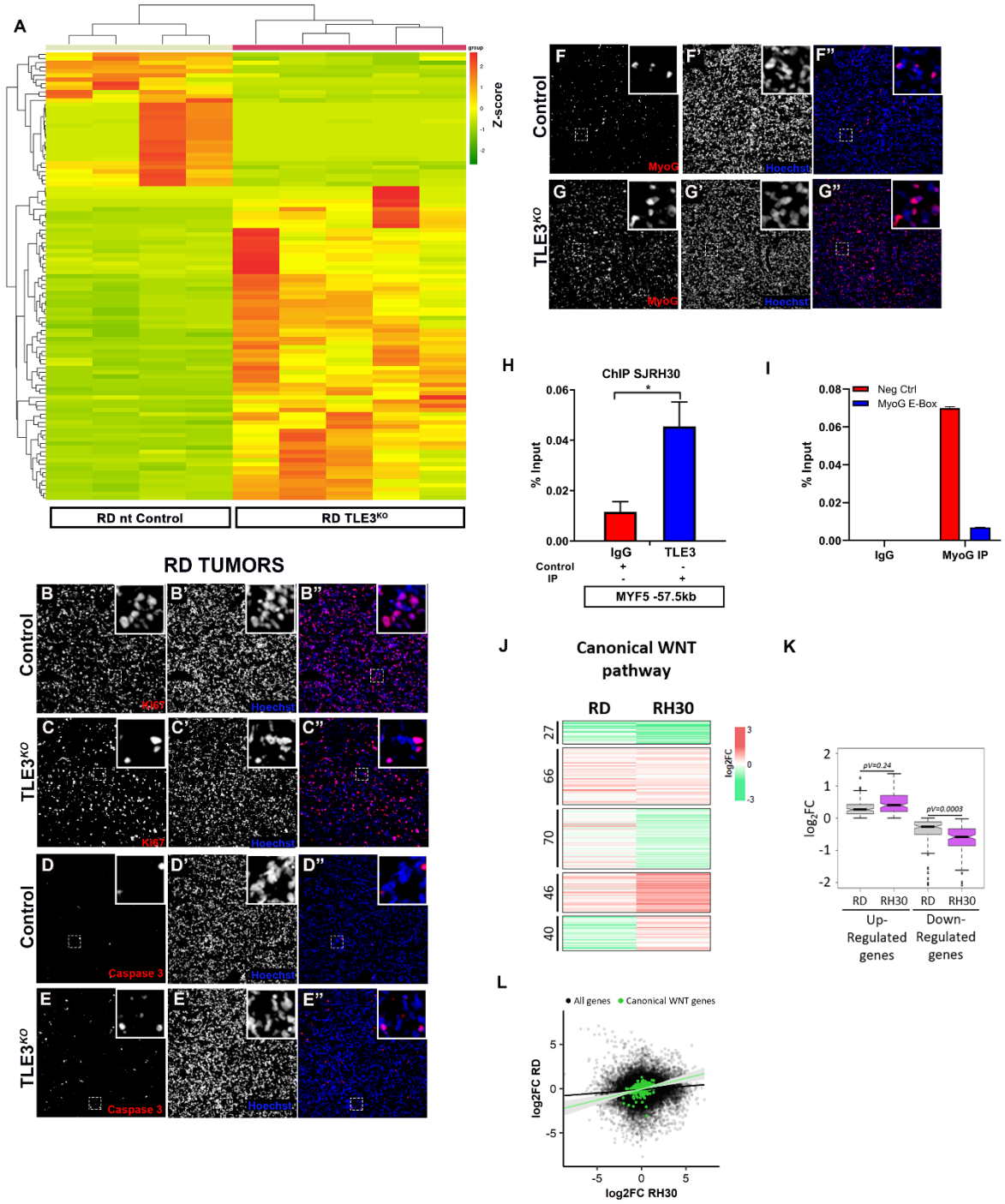

Figure S3. Loss of TLE3 function deregulates Wnt pathway genes and leads to reduced tumorigenic potential in rhabdomyosarcoma. A: A complete linkage hierarchical cluster analysis of differentially expressed genes identified between nt Control and *TLE3*^{KO} mutant

libraries of RD tumor xenografts by RNA-seq (n=5). **B-G''**: Representative immunofluorescence of sections through nt control or *TLE3*^{KO} RD xenografts labeled for Ki67 (**B-C''**), Cleaved Caspase 3 (**D-E''**), or MYOG (**F-G''**) in red and Hoechst (blue). Insets are magnifications of the boxed areas within the panels. **H-I:** Chromatin immunoprecipitation (ChIP) using IgG control or TLE3 antibodies for the *MYF5* -57.5kb region, with red bars indicating IgG control and blue bars indicate TLE3 IP **(H)** and IgG control or MYOG antibodies for the *MYOGENIN* E-Box region, with red bars indicating regions with no antibody binding and blue bars MYOG IP **(I)** in SJRH30 cells. **J:** K-mean clustering of Log2 fold changes in RD and SJRH30 cells for *TLE3*^{KO} compared to their nt controls, for the canonical WNT signaling pathway (GO:0060828) gene set (n=5). Negative values indicate downregulation whereas positive values indicate upregulation in the *TLE3*^{KO} group. Left annotation indicates the number of genes in each cluster, for which gene details are provided in Supplementary Table S5. **K:** Boxplot of log2 fold change (FC) in RD and SJRH30 cells for *TLE3*^{KO} compared to their nt controls, categorized for up- or down-regulated genes associated with the canonical WNT pathway. **L:** Correlation plot of log2FC in RD and SJRH30 *TLE3*^{KO} compared to their nt controls, with regression lines. Pearson correlation is calculated for the canonical WNT signaling pathway genes (green) and for the rest of the expressed genes (black). Correlation value of all genes (minus WNT signaling pathway) is 0.103; correlation of WNT pathway genes (green) is 0.315. The WNT genes showed a significantly higher correlation (P value of 0.0002) compared to all genes calculated using the R package "cocor". Data are mean ± SEM. *P < 0.05, two-tailed Student's t test.

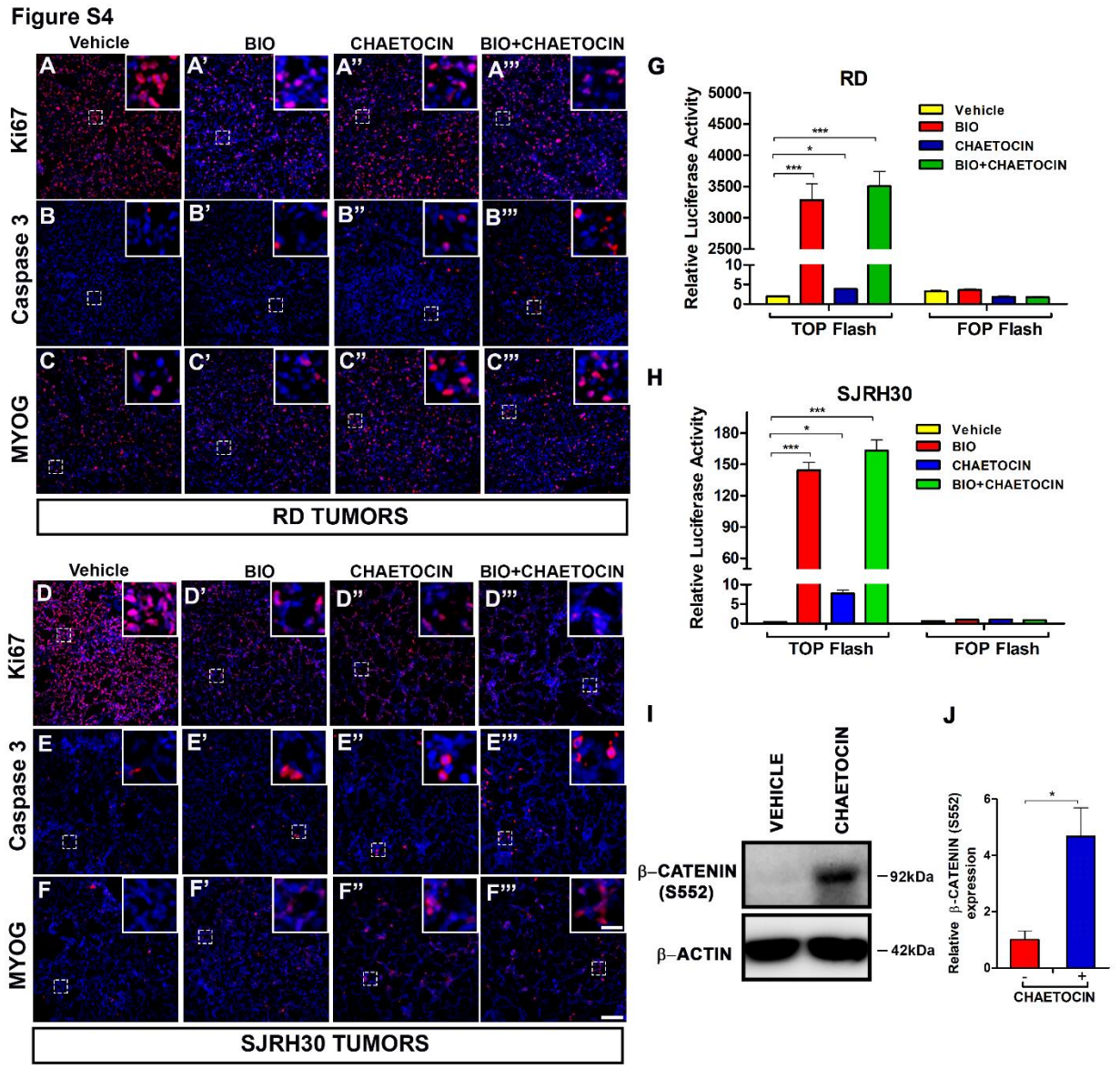

Figure S4: Chaetocin enhances the anti-tumorigenic effect of BIO in rhabdomyosarcoma. A-F'''': Representative immunofluorescence of sections through RD (**A-C'''**) and SJRH30 (**D-F''''**) xenografts following vehicle, or BIO, or chaetocin, or BIO+chaetocin treatment, labeled for Ki67 (**A-A''', D-D'''**), Cleaved Caspase 3 (**B-B''', E-E'''**), or MYOG (**C-C''', F-F'''**) in red and Hoechst (blue). Insets are magnifications of the boxed areas within the panels. **G-H:** Relative luciferase activity for the Wnt pathway upon vehicle or BIO (2 µM), or chaetocin (200nM), or BIO+chaetocin treatment, measured by transfecting the TOP-FLASH or FOP-FLASH reporter in

RD **(G)** and SJRH30 **(H)** cells (n=3). **I-J**: Western blots for phospho-β-Catenin (S552) and β-ACTIN on vehicle or chaetocin treated SJRH30 cell lysates (**I**) and the densitometric quantification (**J**). Data are mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001, one-way ANOVA, Tukey's test. Scale bars: 100 µm (**F'''**), Inset 25 µm.

Figure S5

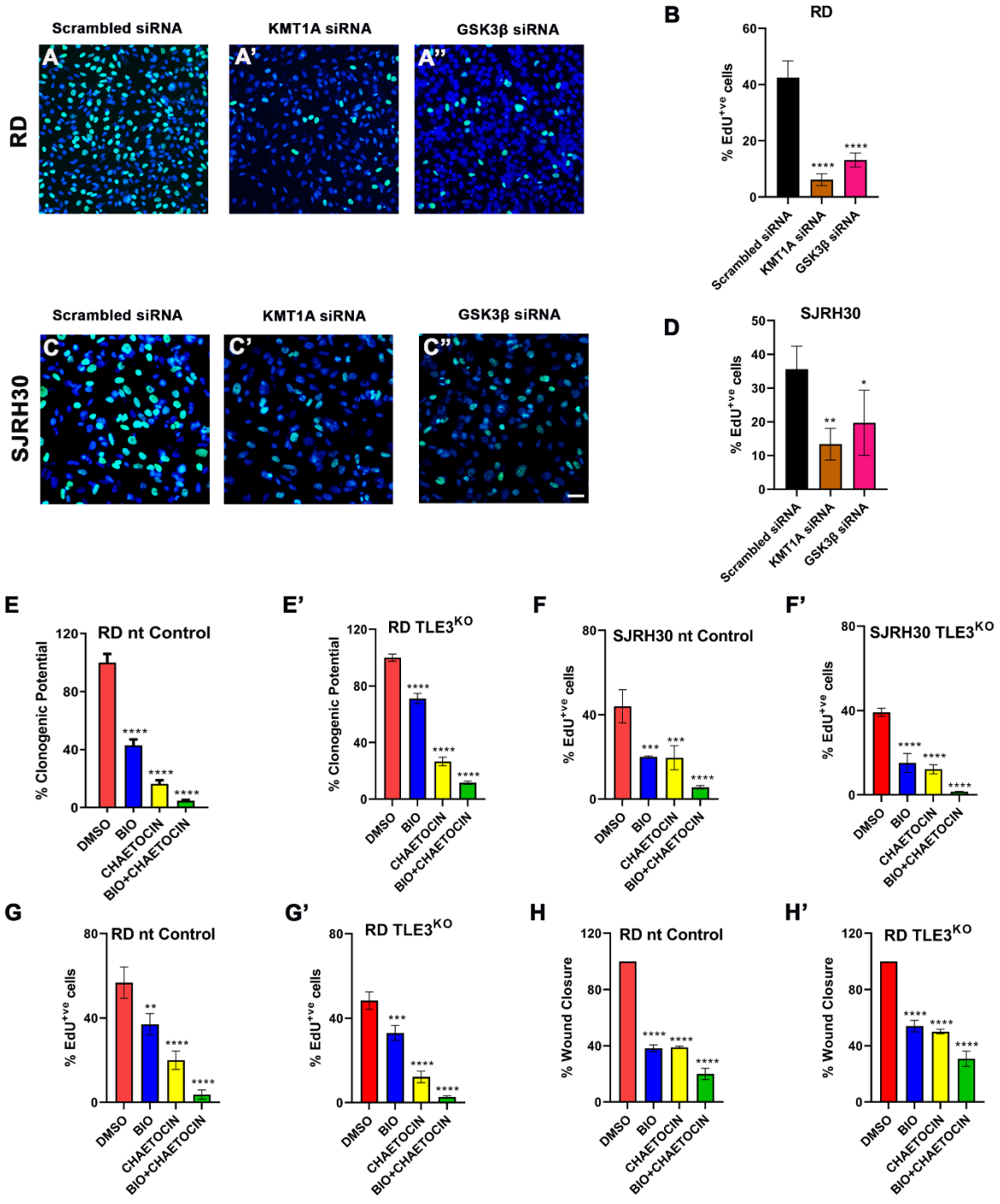

Figure S5. Knockdown of *GSK3β* and *KMT1A* leads to reduced proliferation in rhabdomyosarcoma cells. A-D: Representative immunofluorescence micrographs labelling 5-

ethynyl-2'-deoxyuridine (EdU) (green) and Hoechst (blue) in RD (**A-A''**) and SJRH30 (**C-C''**) cells (n=3) following treatment with scrambled (**A, C**), or *KMT1A* (**A', C'**) or *GSK3β* **(A'', C'')** siRNAs, and their quantification showing percentage of EdU^+ nuclei **(B, D**). **E-E':** Quantification of the clonogenic potential in nt control **(E)** and $TLE3^{KO}$ **(E')** RD cells (n=3) treated with DMSO or BIO or Chaetocin or BIO+Chaetocin drugs. **F-G':** Quantification of the percentage EdU^+ nuclei in nt control and $TLE3^{KO}$ SJRH30 **(F, F')** and RD **(G, G')** cells (n=3) treated with DMSO or BIO or Chaetocin or BIO+Chaetocin drugs. **H-H':** Graphs depicting percent wound closure in nt control **(H)** and $TLE3^{KO}$ **(H')** RD cells (n=3) treated with DMSO or BIO or Chaetocin or BIO+Chaetocin drugs. Data are mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001, ANOVA, Tukey's test. Scale bar: 100 µm (**C"**).

Supplementary Materials and Methods

Transfection of cells

For plasmid transfections, ~1x10^5 RMS cells were plated per well in 24-well plates, cultured overnight, and transfected the following day with 1µg of luciferase reporter plasmid and 10ng of the *pRL-TK* vector (Promega), using Lipofectamine 2000 (ThermoFisher Scientific; Cat# 11668-019) according to manufacturers' instructions. For experiments involving Wnt3A, 6-bromo-indirubin-3′-oxime (BIO), chaetocin, or BIO+chaetocin treatment, the medium was replaced with Wnt3A-conditioned or control-conditioned medium or the drugs, 24 hours after transfection and subsequently cultured for 48 hours. Cells were then washed with DPBS (Gibco Cat# 14190-144), lysed in PBB buffer (1M K_2HPO_4, 1M KH_2PO_4, Triton-X) and processed for luciferase assay.

For transient knockdown of *TLE3*, reverse transfection was performed. Briefly, ~50,000 cells were seeded in each well of a 24-well plate that was layered with transfection mix comprising 100µl Opti-MEM (Gibco; Cat# 31985070), 10nM of *TLE3* siRNA (ThermoFisher Scientific; Cat# 4390816) or control siRNA (ThermoFisher Scientific; Cat# 4390847) and 2µl Lipofectamine RNAiMAX (ThermoFisher Scientific; Cat# 13778150). Cells were cultured for 72 hours after transfection and knockdown efficiency verified. All treatments were identical between control and *TLE3* siRNA-transfected cells. For co-transfection with plasmid DNA, transfection mix was prepared using Lipofectamine 2000.

For doxycycline-induced stable knockdown of TLE3, human RMS cells (SJRH30, RD) were infected using lentiviral doxycycline-inducible construct particles to knockdown the expression of TLE3 in ARMS cells. TLE3-directed shRNA expression was achieved by adding Doxycycline (Sigma-Aldrich; Cat# D9891-25G, final concentration 1 µg/ml), and then maintained throughout the protocol by replacing the media (including doxycycline) every 2 days. Cells were expanded

using the same media with doxycycline for 3 or 4 additional days before harvesting them for analysis.

Drugs and Reagents

6-bromo-indirubin-3′-oxime (Merck; Cat# B1686) and chaetocin (Cayman Chemicals; Cat# 28097-03-2) were reconstituted in DMSO according to manufacturer's recommendations.

Plasmids *4RE-Luc* [1], and *Myogenin-Luc* [2] were generous gifts from Dr. Asoke Mal (Roswell Park Comprehensive Cancer Center, Buffalo, USA). Wnt reporter plasmids *TOPFLASH* and *FOPFLASH* [3] were kind gifts from Dr. Charles Murtaugh (University of Utah, Salt Lake City, USA). The *pCMV3tag-3b-Tle3-FLAG* plasmid was generated by cloning *Tle3* into the *pCMV3tag-3b-FLAG* vector (Agilent). *pCMV (myc)3 SUV39H1* [4] was gifted by Dr. Thomas Jenuwein (Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany). For stable CRISPR-Cas9-based *TLE3* genomic deletion, gRNAs *pPN218* (Cat# 91677), *pPN219* (Cat# 91678) and *pSpCas9n(BB)-2A-Puro* (*PX462*) V2.0 (Cat# 62987) were purchased from Addgene [5].

Animal Studies

A conditional knockout mouse for *Tle3*, $Tle3^{floxed/+}$ [6], was a kind gift from Dr. Claudio Villanueva (University of Utah, USA). $Pax3^{cre/+}$ (stock # 005549) [7], and NOD/SCID (stock # 001303) mice are from Jackson Laboratories. Genotyping of $Tle3^{floxed}$ and $Pax3^{cre}$ was performed by PCR using recommended primers on genomic DNA extract prepared using Hot SHOT lysis method [8] from mouse ear clips. Primer sequences used for genotyping are listed in Supplementary Table S1.

For human RMS cell line xenograft experiments, $5x10^6$ cells were resuspended in PBS, mixed 1:1 with Matrigel (Corning, Cat# 356234), and injected subcutaneously between the flank and mid-abdomen region of 6 to 8-week-old female NOD/SCID mice. RMS tumor volumes were determined using the formula $\text{width}^2 \times \text{length} \times 0.5$ [9]. At a tumor volume of 50 to 100 mm^3, mice

were randomly assigned to treatment with vehicle (PBS) or BIO or chaetocin or BIO+chaetocin. Tumors were excised and flash frozen for further analysis when the control tumor volume reached roughly 10% baseline body weight.

For in vivo drug treatments, reconstituted BIO was formulated in PBS and administered intraperitoneally at a dose of 10mg/kg for four consecutive doses over 2 weeks. Reconstituted chaetocin was prepared in PBS and administered intra-peritoneally at a dose of 1mg/kg for four consecutive doses over 2 weeks. In the combination (BIO+chaetocin) treatment regime, a time gap of 8-12 hours was kept between the two individual drug doses.

For experiments involving TLE3 knockdown in developing RMS xenografts, RD and SJRH30 cells with stable integration of *scrambled shRNA* or *TLE3 shRNA* were implanted in NOD/SCID mice as described above. Once the tumors reached a size of $100mm^3$, doxycycline (1mg/ml) was added to the drinking water and replenished every alternate day till the experimental end-points.

All the animals used in the study were included in the analysis and interpretation of the results. The animals used in the study were randomly chosen for xenografting of non-targeting and TLE3 knockout cell lines and drug treatments were randomly given to the animals. The investigator measuring the tumor size of the xenografted animals was blind to the drug treatment given. All the animal experiments were carried out according to the Institutional Animal Ethics Committee (IAEC) approved protocols of the Regional Centre for Biotechnology (Protocol numbers: RCB/IAEC/2016/008 and RCB/IAEC/2018/027).

RNA isolation and quantitative PCR (qPCR)

Total RNA was extracted from RMS cells using RLT lysis buffer (Qiagen) according to manufacturer's instructions (Qiagen RNeasy mini kit; Cat# 74106), followed by cDNA synthesis using reverse transcriptase and oligo-dT primer (Clontech Takara Cellartis; Cat# 6110A). The

cDNA samples were then used as template for qPCR on an ABI 7500 Fast Real-Time System (Applied Biosystems) using TB green premix Ex Taq (Clontech Takara Cellartis; Cat# RR420A) and specific primers for each gene (Supplementary Table S1).

For in vivo studies, total RNA was extracted from hind limbs (E9.5 to E18.5) or tibialis anterior muscle (P0 onwards) of C57Bl/6 wild type mice using RLT lysis buffer according to manufacturer's instructions, followed by cDNA synthesis using reverse transcriptase and oligo-dT primer. The cDNA samples were used as template for qPCR. Expression levels of the genes examined were normalized to *Gapdh* expression for each sample. Taqman probes were used to quantitate the expression of *Tle3* (Applied Biosystems; Cat# Mm00437097_m1) and *Gapdh* was used as normalizing control (Applied Biosystems; Cat# 99999915_g1).

RNA-sequencing and analysis:

Control and TLE3 knockout RD and SJRH30 cells were cultured and xenografted into NOD/SCID mice as described above. They were excised when control tumors reached the maximum permitted size. Tumors were flash frozen in liquid nitrogen. Total RNA was extracted using RNeasy mini kit (Qiagen or Zymo research). RNA quality was assessed using Tapestation (Agilent) and quantified using Qubit (ThermoFisher Scientific). 250ng of total RNA was used to enrich the mRNA using NEBNext Poly (A) mRNA magnetic isolation module (New England Biolabs; Cat# E7490) by following the manufacturers' protocol. The enriched mRNAs were further taken for library preparation using the NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs; Cat# E7775S) and sequencing was done using HiSeq X Ten (Illumina).

The quality of sequence data was checked using fastqc. The data was processed to remove low quality bases and adapter sequences using fastp v 0.20.1 with default parameters. The presence of rRNA reads were checked and removed using bbduk package in bbmap software and sortmeRNA

rRNA database. The reads were mapped onto indexed Human reference genome (GRCh38.p7) using STAR aligner v2.7. Gene level expression values were obtained as read counts using featureCounts. Expression similarity between biological replicates was checked by spearman correlation and Principal Components Analysis using R. Differential expression analysis was carried out using edgeR package after normalizing the data based on trimmed mean of M (TMM) values. The genes that showed significant differential expression were used for Gene Ontology and pathway enrichment analysis. The secure reviewer access token to access the RNA-seq data record GSE202614 while it remains in private status is otazsuwqhnypban.

Genes with absolute log2 fold change ≥ 2 and adjusted P value ≤ 0.05 were considered significant. The genes that showed significant differential expression were used for Gene Ontology and pathway enrichment analysis. The log2 fold change of genes associated with the regulation of the canonical WNT pathway (GO:0060828) was used to compare SJRH30 and RD tumor data in nt Control and TLE3 knockout tumors in a heatmap using the R package ComplexHeatmap.

For the human patient derived RNA-seq analysis, TLE3 FPKM values were obtained from 5 ARMS patient samples (SJRHB038_R, SJRHB009_D, SJRHB010_D, SJRHB007_D, SJRHB008_D) and 12 ERMS patient samples (SJRHB003_D, SJRHB005_D, SJRHB006_D, SJRHB012_R, SJRHB012_Z, SJRHB001_D, SJRHB013_D, SJRHB012_D, SJRHB012_S, SJRHB004_D, SJRHB011_D, SJRHB002_D) as described in [10], using data available at the public online portal at the St. Jude Children's Research Hospital (https://pecan.stjude.org/proteinpaint/study/RHB2018). The values are represented using box-plot module of GraphPad Prism software.

Mass Spectrometry:

Cell lysates were prepared from control and *TLE3* knockout RD and SJRH30 cells, solubilized in 4X Laemmli buffer and boiled for 10 min to denature the proteins. Protein lysates were separated by 10% SDS-PAGE, stained with Coomassie Brilliant Blue R-250 and protein bands excised using sterile blades. The excised gel pieces were de-stained, dehydrated and digested using mass spectrometry grade trypsin (Pierce Trypsin protease; Thermo Scientific, 90057) at 37°C overnight. Peptide extraction was carried out by ultrasonication for 20 minutes in extraction solution (50% Acetonitrile, 0.1% Formic acid) and the extract was dried in a speed vacuum concentrator at 25°C. The samples after desalting were further purified with Pierce C18 Zip-Tips (Thermo Scientific, 87782) and loaded on a LC-MS/MS mass spectrometer (Triple TOF 5600 SCIEX). The raw files of MS-MS run were used to generate Mascot generic files (mgf), then processed and subjected to database searches using Search GUI to confer protein identification. Protein identifications were accepted when at least two peptides with Mascot scores above the statistically significant threshold ($p < 0.05$) matched the identified protein. Search results were processed, combined and interpreted in the form of emPAI (exponentially modified Protein Abundance Index) using MASCOT software, run with a false discovery rate of 1%. The values are represented in the form of heatmap using rainbow module of GraphdPad Prism software. The mass spectrometry results are provided in Supplementary Tables S3 and S4.

Immunoblotting

Protein lysates were prepared from RMS cells that were plated, cultured, and harvested at indicated time points as explained above in the cell culture section. Briefly, cells were lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (Sigma; Cat# R0278-50) containing protease inhibitor (Sigma; Cat# P8340) at 1:100 dilution. Total protein was isolated from the hind limb muscles of $Pax3^{+/+}$, $Tle3^{fl/fl}$ and $Pax3^{Cre/+}$, $Tle3^{fl/fl}$ neonatal mice at P0 by homogenization using

the Precellys 24 homogenizer (Bertin Technologies). Quantification of the protein samples was done using Pierce BCA Protein Assay kit (Thermo Scientific; Cat# 23225) as per manufacturer's protocol. The protein samples were quantified using BCA Protein Assay kit (ThermoFisher Scientific; Cat# 23225) as per manufacturer's protocol. Equal amount of protein extracts was separated by 10% or 12% SDS-PAGE, depending on the molecular weight of the protein to be detected, followed by transfer to PVDF membrane (Millipore; Billerica, MA, USA; Cat# iPVH00010) at 4°C overnight. Western blot was performed using standard procedures, with blocking in 5% skimmed milk (Himedia; Cat# RM1254-500GM) for 3 to 5 hours, washes in PBS containing 0.1% Tween 20 (Sigma; Cat# P7949-100ML), incubated overnight in primary antibody at 4^0C, and 2 hours in secondary antibody at room temperature. HRP-conjugated secondary antibodies were used, and signal was detected using the Luminata Forte HRP substrate (Millipore; Cat# WBLUF0100). Blots were imaged using ImageQuant LAS 4000 (GE Healthcare Life Sciences). Blots were stripped using standard procedures and re-probed for β-actin. Densitometric analysis was performed by quantifying the amount of target protein normalized to β-actin levels using ImageQuant TL v7.0 software (GE Healthcare Life Sciences). Data from a minimum of three independent replicates were used for analysis, with the error bar representing the standard error of the mean. Antibodies and the concentrations used are listed in Supplementary Table S2.

Co-immunoprecipitation

RMS cells plated on 6-well plates were co-transfected with *pCMV3tag-3b-Tle3-FLAG* and *pCMV (myc)3 SUV39H1* vectors and cultured as mentioned previously. Briefly, 48 hours post-transfection, RMS cells were lysed in ice-cold IP Lysis Buffer (Thermo Fisher; Cat# 87787) supplemented with 1x protease inhibitor (Sigma; Cat# P8340). Pre-clearing of the protein lysates was performed by incubating with Protein G Sepharose 4 Fast flow beads (GE Healthcare Life

Sciences, Chicago, IL, USA; Cat# 17-0618-01) for 1 hour at 4°C on a tube mixer. Subsequently, the pre-cleared lysates containing Myc-conjugated proteins were immunoprecipitated using anti-Myc antibody overnight at 4°C on a tube mixer. IgG raised in rabbit was used as control group. The antibody bound proteins were then incubated with Protein G Sepharose 4 Fast flow beads for 6 hours at 4°C on a tube mixer to capture the immunocomplexes. Immunoprecipitates were washed five times using ice-cold lysis buffer, boiled in 2X Laemmli sample buffer at 95°C for 15 min, and the immunoprecipitates subjected to western analysis as described above.

The C2C12 cell lysates were immunoprecipitated using 2µg of the respective antibody and 50µl of protein G sepharose 4 fast flow beads. Capturing of immunocomplexes was performed on a rotator at 4°C overnight. After the beads were washed five times with IP lysis buffer and eluted, the immunoprecipitates were analyzed by western blot.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described previously [11]. Briefly, RMS cells were crosslinked in 1% formaldehyde solution, followed by quenching using 0.125M glycine. These cells were then subjected to sonication by Bioruptor (Diagenode) to obtain DNA fragments in the size range of 300-500 bp. Sonicated chromatin was precleared with a solution mix of protein G slurry (Dynabeads, Invitrogen; Cat# 10003D) and BSA. The solubilized fragmented chromatin was immunoprecipitated using TLE3 and KMT1A antibodies. Respective IgG antibodies were used as isotype controls. Antibody-chromatin complexes were pulled down using protein G beads, washed and eluted in elution buffer. Reverse crosslink was performed subsequently at 65^0C overnight. Fragmented DNA was then isolated using phenol-chloroform and precipitated with chilled ethanol. DNA fragments acquired were subjected to qPCR using MYOG E box site and MYF5 -57.5kb enhancer specific primers and control primers [10].

Cell survival assay

The effect of BIO, chaetocin or BIO+chaetocin on the viability of RD, SJRH30 and HEK cells was examined using the MTT assay. Briefly, 10,000 cells were plated in flat-bottomed 96-well microplates and cultured overnight as mentioned above. Starting the following day, the cells were incubated in different concentrations of BIO, chaetocin or BIO+chaetocin for 18 hours, 24 hours and 48 hours. Thereafter, the medium was replaced with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) dissolved at a final concentration of 1 mg/ml in serum-free, phenol red-free medium. Following incubation of 2 hours, the formed formazan crystals were then dissolved by DMSO and the absorbance of the solution was measured at 570 nm wavelength. Survival of control cells was set to 100%.

CRISPR Cas9-based generation of *TLE3* knockout cells

Non-targeting (nt) gRNAs and *CRISPR* guide plasmids targeting exon 7 of *TLE3* and *Cas9* plasmids were purchased from Addgene [5]. RMS cells were plated in 6-well plates at a seeding density of $3x10^5$ per well. Following overnight incubation, cells were co-transfected with non-targeting or targeting guide RNAs and the Cas9 plasmids using Lipofectamine 2000. Following transfection, after 48 hours, cells were treated with 1 μM puromycin (Sigma Aldrich; Cat# P8833) for 10 days to select successfully transfected cells. A total of 384 individual cells (192 for ARMS and 192 for ERMS sub-types) were manually picked and seeded into 96-well plates. Colonies were allowed to expand for 14 days and clones in 96-well plates were then duplicated for cell freezing, genomic DNA extraction and western blotting for estimating TLE3 levels. Genomic DNA from RMS cells was extracted using Lysis Buffer (100 mM NaCl; 20 mM Tris, pH 7.6; 10 mM EDTA; 0.5% SDS) supplemented with Proteinase K (Roche; Cat# 03115879001) at 37°C for 4 hours without shaking [12]. Primers specific to exon 7 of TLE3 were used to amplify a 160bp amplicon

spanning the Cas9 cleavage sites, cloned into TA vector (TOPO TA Cloning Kit, Invitrogen; Cat# 45-0641) and sequenced.

Generation of stable shRNA cell lines

The short hairpin RNA (shRNA) sense strand sequences used were as follows - scrambled: CCTAAGGTTAAGTCGCCCTCG and TLE3: CCTATGGCTTGAACATTGAAA [13]. Primers encoding the scrambled or TLE3 shRNA sequences were annealed and cloned into the doxycycline inducible shRNA plasmid EZ-Tet-pLKO-puro (Addgene; Cat # 85966) as described [14]. Scrambled and TLE3 shRNA lentivirus particles were generated by co-transfection of the shRNA constructs along with the envelope plasmid pMD2.G (Addgene; Cat # 12259) and packaging plasmid psPAX2 (Addgene; Cat # 12260) into HEK293T cells. The lentiviral particles were harvested after 48 hours and used to infect RD and SJRH30 cells in the presence of 8 µg polybrene (Sigma Aldrich; Cat # H9268). The media was replaced with fresh media containing 0.5-0.8 µg/mL of puromycin over 4-7 days for selecting puromycin-resistant stable shRNA cell clones, which were used for further experiments.

Wound Closure Assay

Briefly, nt Control or *TLE3*^{KO} RMS cells were seeded in 6 well plates at a density of $3x10^5$ cells per well in complete media and incubated overnight as mentioned above. For drug studies, cells were treated with DMSO, or BIO, or Chaetocin, or BIO+Chaetocin, or Wnt Agonist for 24 hrs. After attaining 90% confluency, cells were wounded by scraping with a sterile pipette tip. The scratched area was imaged at indicated time intervals using an inverted microscope (Nikon Eclipse TS100, Minato Tokyo, Japan). A reference point was made on the plates for acquisition of image at the same position for all the time-points of the study [15]. Imaging was performed till wound closure was observed in the respective control groups. The images were used to measure the

scratched area at zero-time point (A_0) and at the time point of closure (A_t), using ImageJ software [16]. Percentage wound closure was calculated using the formula $[(A_0 - A_t)/A_0]\times100$.

Clonogenic Assay

For anchorage-dependent colony formation assay, nt Control or *TLE3*^{KO} RMS cells were plated in 6 well plates at 4000 cells/well and cultured for 9 days. For drug studies, cells were treated with DMSO, or BIO, or Chaetocin, or BIO+Chaetocin, or Wnt Agonist for 24 hrs. Colonies were washed twice with ice cold 1×PBS, fixed in 10% neutral buffered formalin (30 min) and subsequently stained with 0.5% crystal violet (~30 min). Respective wells were imaged using a digital camera and colony forming units or colonies comprising >50 cells were counted manually under a stereomicroscope. The clonogenic assays were performed independently a minimum of three times and graphical data is represented as mean ± SEM.

Luciferase Assay

For TOPFLASH luciferase assay, about 1x 10^5 RMS cells were plated per well in a 24-well plate and allowed to grow overnight. The following day, cells were transfected with 1µg of *TOPFLASH* or *FOPFLASH* luciferase reporters, and 10ng of the *pRL-TK* vector (Promega) using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific; Cat# 11668-019). For experiments involving Wnt3A or drug treatment, the medium was replaced with Wnt3A- or control-conditioned medium, or drug, 24 hours after transfection and subsequently cultured for 48 hours. Following 48 hours of incubation, cells were washed with DPBS (Gibco; Cat# 14190-144), lysed with PBB buffer (1M K_2HPO_4, 1M KH_2PO_4, Triton-X) and luciferase activity measured using Dual-Glo Luciferase Assay System (Promega; Cat# E2920).

For *4RE-Luc* based luciferase assay, RMS cells seeded in 24 well plates were transfected with *4RE-Luc* alone, or *4RE-Luc + pCMV3tag-3b-Tle3-FLAG*, or *4RE-Luc + pCMV (myc)3 SUV39H1*,

or *4RE-Luc + pCMV3tag-3b-Tle3-FLAG + pCMV (myc)3 SUV39H1* vectors using Lipofectamine 2000. Similarly, for *Myogenin-Luc* based assay, RMS cells were transfected with *Myogenin-Luc* alone, or *Myogenin-Luc + pCMV3tag-3b-Tle3-FLAG*, or *Myogenin-Luc + pCMV (myc)3 SUV39H1*, or *Myogenin-Luc + pCMV3tag-3b-Tle3-FLAG + pCMV (myc)3 SUV39H1* vectors. *phRL-CMV* (Promega) was used to normalize transfection efficiency. After 48 hours, cells were washed with PBS, lysed and luciferase assays performed using the Dual-Glo Luciferase Assay system.

Immunofluorescence and microscopy

RMS tumor xenografts were harvested, fixed in 4% paraformaldehyde (PFA) and embedded in Optimal Cutting Temperature (OCT) (Tissue-Tek). Tumor tissues were sectioned at 8µm thickness using a cryomicrotome (Thermo Scientific; Micron HM 550) on Poly-L-Lysine (Sigma, P8920) coated slides. Sections were fixed in 4% PFA for 5 minutes at RT, subjected to antigen retrieval in citrate buffer (1.8 mM citric acid and 8.2 mM sodium citrate in water) at 121^0 C for 10 minutes in a 2100 PickCell Retriever (Aptum Biologics). Tissue sections were blocked with blocking buffer containing 5% goat serum (BioAbChem; Cat# 72-0480) and 0.1% Triton X-100 (MP Biomedicals; Cat# 194854) for 1 hour at room temperature, incubated overnight with MyoG, Ki67, or Caspase3 primary antibodies at 4^0 C, followed by three washes in PBS. The signals were amplified by incubating with a biotin-conjugated anti-mouse or anti-rabbit secondary antibody for 2 hours at room temperature, followed by incubating with streptavidin-Cy3 fluorophore for 1 hour at room temperature and PBS washes. The slides were counterstained with Hoechst 3342 (Invitrogen, 1399) for nuclear staining for 5 min at room temperature and mounted using Fluoromount-G (Southern Biotech; Cat# 0100-01). The sections were imaged using an Olympus

epi-fluorescence microscope (Olympus BX63F) with an ORCA-Flash4 monochrome camera (Hamamatsu).

Cell proliferation and apoptosis assays

RMS cells were plated on cover slips at a density of 50,000 per well in 24 well plates, reverse transfected using *TLE3*, or *KMT1A*, or *GSK3β*, or *β-CATENIN* siRNA or control siRNA and incubated for 72 hours. Cells were then treated with 10μM EdU (Thermo Scientific; Cat# 10337) and incubated for 4-6 hours. For drug studies, cells were treated with DMSO, or BIO, or Chaetocin, or BIO+Chaetocin, or Wnt Agonist for 24 hours prior to EdU incorporation. For Wnt3A treatment studies, cells were treated with Wnt3A conditioned media or control media for the indicated time periods; EdU was added 4-6 hours prior to harvesting. Thereafter, cells were fixed by 3.7% formaldehyde (Millipore; Cat# 1.04003.2.500), and permeabilized with 0.5% triton X-100 (Millipore; Cat# 194854). EdU incorporation was detected by adding Click-iT reaction cocktail followed by incubation at room temperature for 30 minutes. Nuclear staining was performed using 1x Hoechst solution, images captured using a Nikon microscope and maximally projected. Total number of EdU^+ nuclei were counted using spot and annotation functions in Imaris software (http://www.bitplane.com/).

Cellular apoptosis in RMS cells treated with Wnt3A conditioned media or control media was detected using AnnexinV-PI dual staining followed by flow cytometric analysis as per manufacturer's protocol (Thermo Fisher; Cat # V13242). The stained cells were analyzed by three laser BD FACSVerse (Becton Dickinson Biosciences, USA).

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