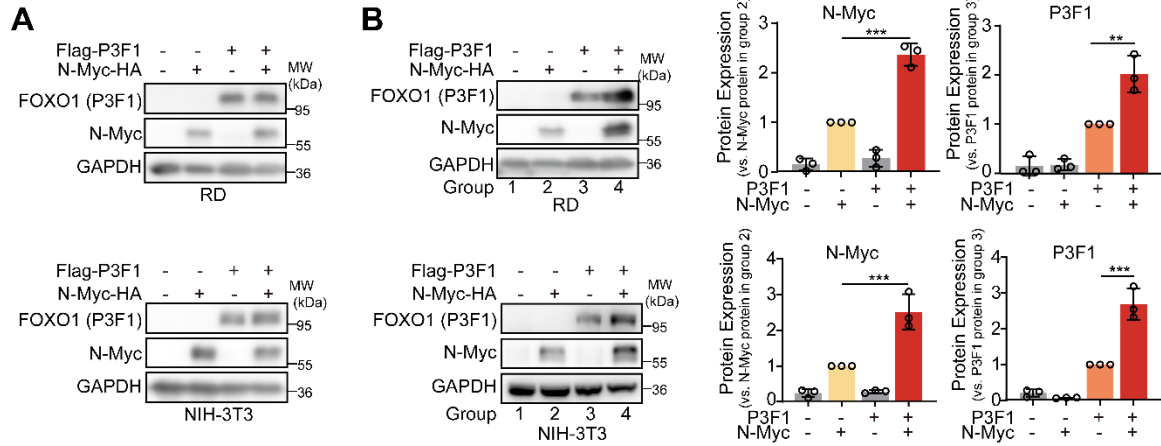
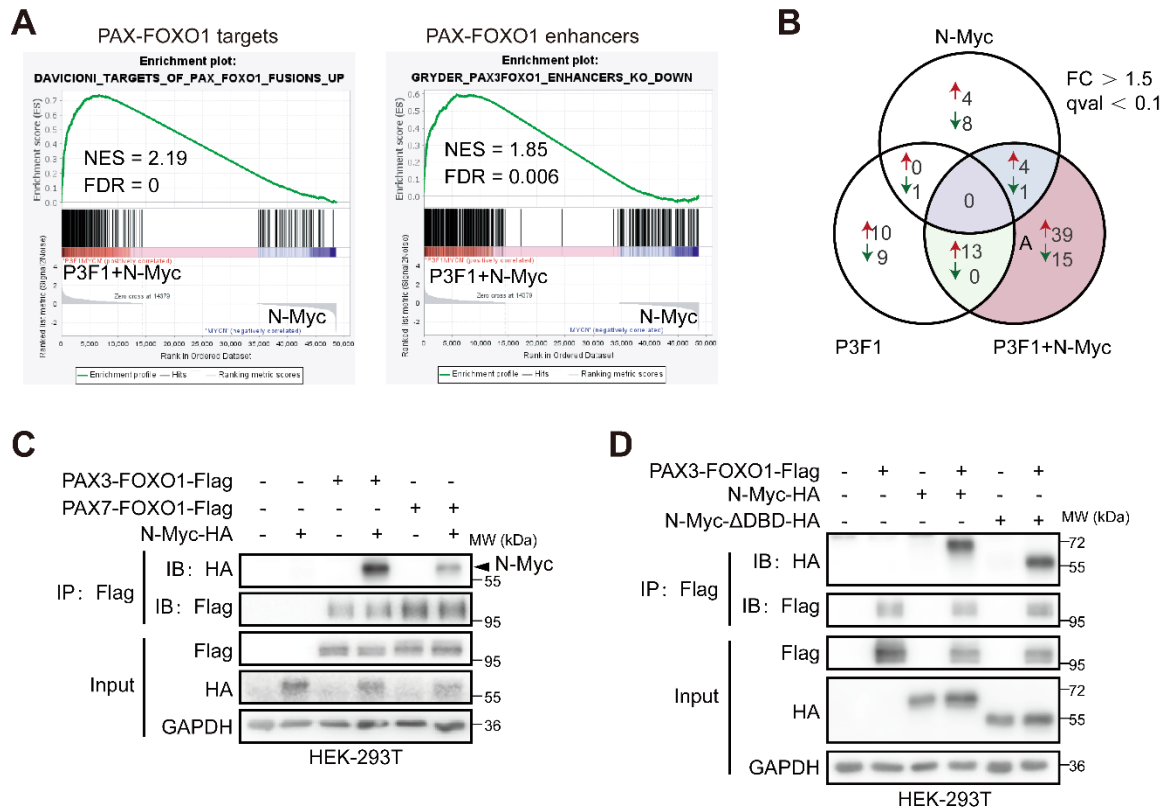


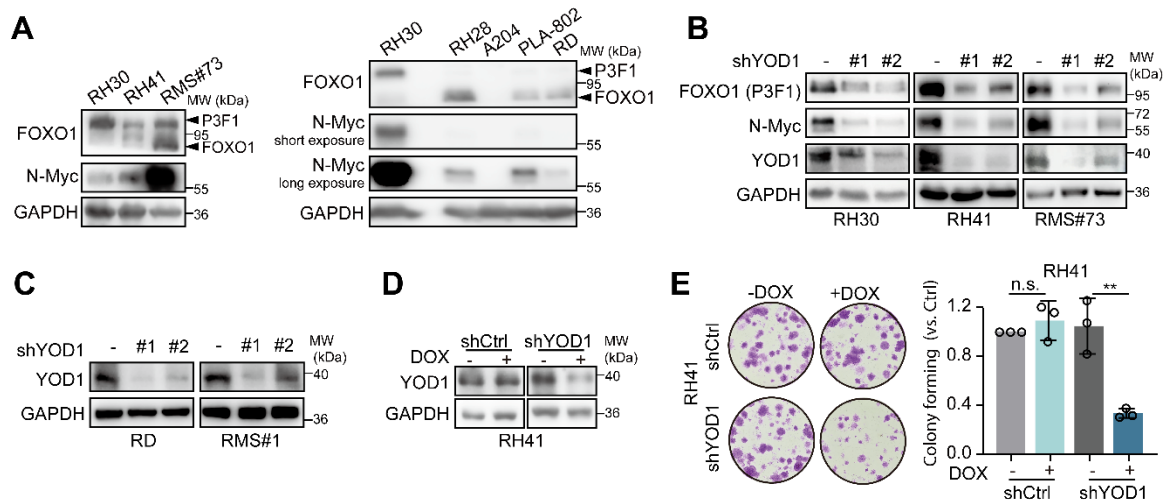


Supplementary Figure 1. Co-expression of PAX3-FOXO1 and N-Myc regulates their mutual expression levels. (A) After lentivirus infection Day3, protein levels of P3F1 and N-Myc were evaluated by Western blotting. (B) Western blot analysis of protein lysates from pooled cell colonies (harvested from a single well). Protein expression levels were normalized to the loading control (GAPDH). The bar graph shows the fold change in protein expression in the co-expression group relative to the single overexpression group. Data are presented as mean $\pm$ SD ($n = 3$). Data represent the mean $\pm$ SD. 1-way ANOVA with Tukey's multiple comparisons test; ** $P < 0.01$, *** $P < 0.001$.

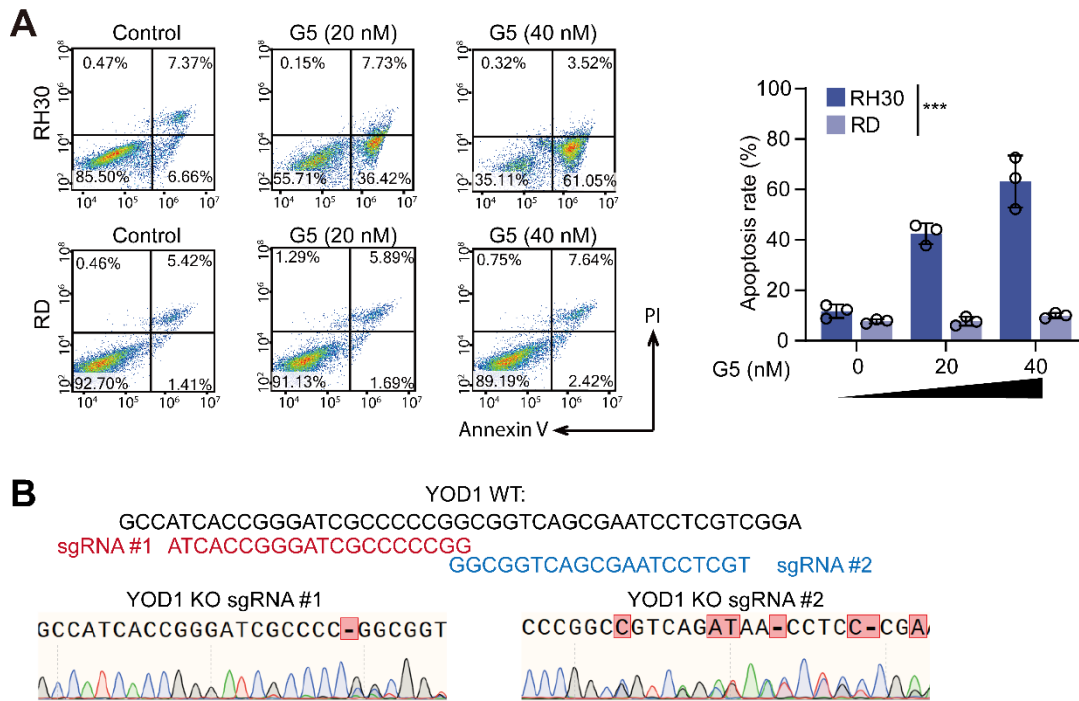


Supplementary Figure 2. Reciprocal Regulation between PAX3-FOXO1 and N-Myc.

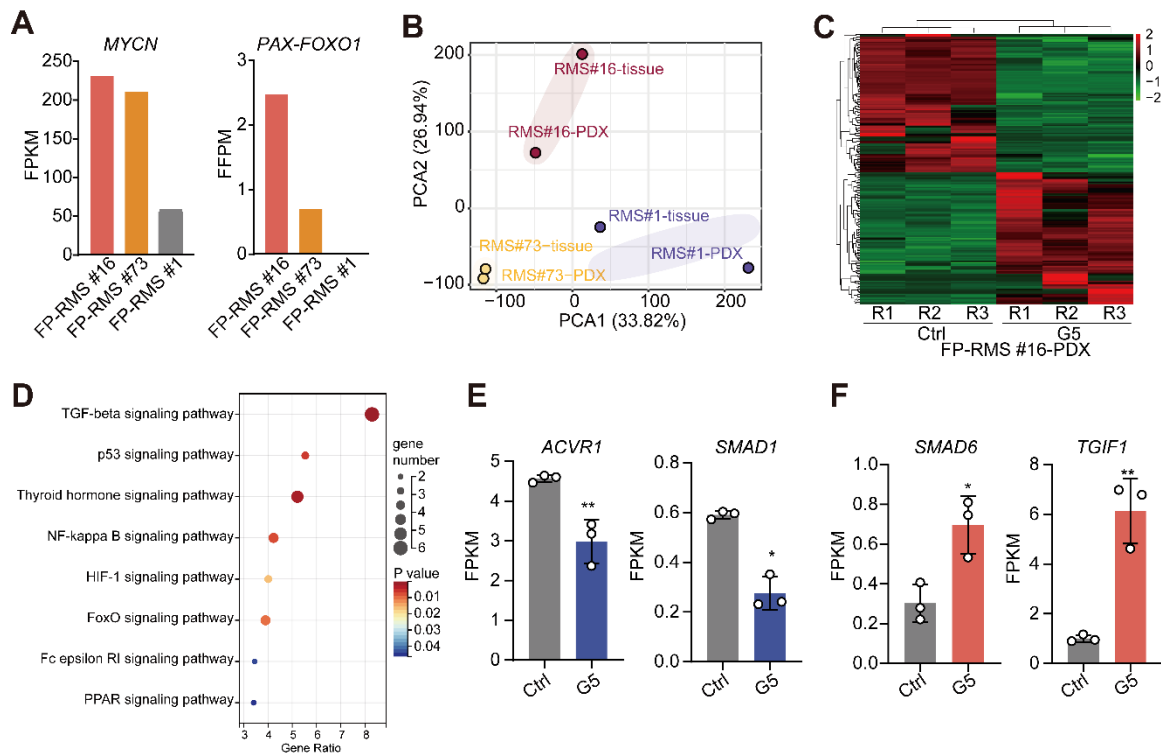
(A) GSEA of transcriptomes from P3F1+N-Myc co-expressing cells versus N-Myc-only cells. Analysis was performed using a defined PAX3-FOXO1 (P3F) target and enhancer gene set to assess pathway enrichment. (B) Venn diagram of differentially expressed genes from the comparison of N-Myc, P3F1, and P3F1+N-Myc groups with the empty vector control. (C) IPs by Flag beads from HEK-293T cells transfected with vector or N-Myc-HA along with vector, P3F1-Flag or P7F1-Flag for 48 h were subjected to IB. (D) IPs by Flag beads from HEK-293T cells transfected with vector, N-Myc-HA or N-Myc-ΔDBD-HA along with vector, P3F1-Flag or P7F1-Flag for 48 h were subjected to IB. Data are representative of three independent experiments.



Supplementary Figure 3. YOD1 plays a crucial role in the development of rhabdomyosarcoma. (A) Characterization of RMS cell line models. Western blot showing endogenous expression levels of PAX3-FOXO1 and N-Myc, which delineates fusion-positive (FP-RMS) from fusion-negative (FN-RMS) models. (B) YOD1 knockdown in FP-RMS models. Western blot analysis of P3F1, N-Myc, and YOD1 protein levels following shRNA-mediated YOD1 knockdown in FP-RMS cell lines (RH30, RH41) and patient-derived cells (PDCs, RMS#73). (C) YOD1 knockdown in FN-RMS models. Protein levels of YOD1 was assessed by Western blot after shYOD1 knockdown in FN-RMS cell lines (RD) and PDCs (RMS#1). (D) Inducible YOD1 knockdown in RH41 cells. YOD1 expression was analyzed by Western blot with or without doxycycline (DOX, 2 μ g/mL) induction. (E) Clonogenic assay in RH41 cells with inducible YOD1 knockdown. Colonies were stained with sulforhodamine B (SRB) and quantified using ImageJ. The bar graph represents the fold change in colony formation capacity relative to the shCtrl-DOX group. Student's *t* test. *n* = 3. n.s: *P* > 0.05, ***P* < 0.01.



Supplementary Figure 4. G5 exhibits anti-tumor activity in FP-RMS cell. (A) G5 induces a time-dependent apoptotic response. FP-RMS (RH30) and FN-RMS (RD) cells were treated with G5, and the induction of apoptosis was analyzed by flow cytometry following Annexin V and PI staining at the indicated time points. The histogram summarizes the quantitation of total apoptotic cells (mean $\pm$ SD, $n = 3$). 2-way ANOVA. *** $P < 0.001$. **(B)** Genotypic confirmation of YOD1 knockout. Sanger sequencing traces of the genomic region targeted by the YOD1-specific sgRNA, confirming the introduction of indels that disrupt the YOD1 coding sequence.



Supplementary Figure 5. Analysis of related gene levels in PDX. (A) RNA-seq analysis of MYCN and PAX3/7-FOXO1 fusion transcript expression in RMS-PDX samples. MYCN expression levels are quantified as FPKM, while the specific PAX3-FOXO1 or PAX7-FOXO1 fusion transcripts, identified by STAR-Fusion, are quantified as FFPM. **(B)**, Principal component analysis comparing DNA methylation profiles of RMS PDXs, PDCs, and patient tissue. **(C)** Unsupervised hierarchical clustering was performed to compare the transcriptome profiles after G5 and the transcriptome profiles of Ctrl group. **(D)** Pathway enrichment analysis by RNA sequencing in G5-treated FP-RMS PDX models. **(E and F)**, RNA sequencing analysis showed the messenger RNA changes of the TGF-beta signaling pathway. Student's *t* test. *n* = 3. **P* < 0.05; ***P* < 0.01.

Supplementary Table 1. RNA-seq gene expression data for RD cells shown in Figure 2C.xlsx (Microsoft Excel, xlsx format)

Supplementary Table 2. Clinical Characteristics of RMS-PDC

Sample ID	Gender	Age at Surgery in years	Histological category	Subtype	Fusion type
RMS#1	female	<10Y	RMS	ERMS	FN
RMS#10	male	>10Y	RMS	ARMS	PAX3-FOXO1
RMS#15	male	<10Y	RMS	ARMS	PAX3-FOXO1
RMS#16	male	<10Y	RMS	ARMS	PAX7-FOXO1
RMS#19	female	>10Y	RMS	ARMS	PAX3-FOXO1
RMS#24	male	>10Y	RMS	ERMS	FN
RMS#31	male	<10Y	RMS	ERMS	FN
RMS#32	male	<10Y	RMS	ERMS	FN
RMS#33	male	<10Y	RMS	unknown	FN
RMS#34	female	<10Y	RMS	ERMS	FN
RMS#36	male	>10Y	RMS	ERMS	FN
RMS#39	female	>10Y	RMS	unknown	FN
RMS#40	male	<10Y	RMS	ERMS	FN
RMS#42	female	>10Y	RMS	unknown	FN
RMS#44	female	<10Y	RMS	ERMS	FN

RMS#54	male	<10Y	RMS	unknown	FN
RMS#73	female	>10Y	RMS	ARMS	PAX3-FOXO1
RMS#75	male	<10Y	RMS	ERMS	FN
RMS#95	female	>10Y	RMS	ARMS	PAX3-FOXO1

Supplementary Table 3. Hairpin sequences of shRNA

	shRNA	Hairpin Sequences
1	sh <i>PAX3-FOXO1</i> #1	GGCCTCTCACCTCAGAATTCA
2	sh <i>PAX3-FOXO1</i> #2	TCTCACCTCAGAATTCAATTC
3	sh <i>MYCN</i> #1	CAGCAGCAGTTGCTAAAGAAA
4	sh <i>MYCN</i> #2	CGGACGAAGATGACTTCTACT
5	sh <i>YOD1</i> #1	GAGTACTGTGACTGGATCAAA
6	sh <i>YOD1</i> #2	CCAGAAGTTCACCTGCATTTA

Supplementary Table 4. Sequences of RT-PCR primers

	Primers	5'-3' sequences
1	<i>PAX3-FOXO1</i> -F	CCTCTCACCTCAGAATTCAATT
	<i>PAX3-FOXO1</i> -R	TCTGGATTGAGCATCCACCAAG
2	<i>PAX7-FOXO1</i> -F	AGCTTCTCCAGCTACTCTGAC
	<i>PAX7-FOXO1</i> -R	CTCTGGATTGAGCATCCACC
3	<i>MYCN</i> -F	ACCACAAGGCCCTCAGTACC
	<i>MYCN</i> -R	TCTCCACAGTGACCACGTCGATT

	Primers	5'-3' sequences
4	<i>GAPDH</i> -F	CTGGGCTACACTGAGCACC
	<i>GAPDH</i> -R	AAGTGGTCGTTGAGGGCAATG
5	<i>YOD1</i> -F	CAGCGTAACTTCCCTGATCCA
	<i>YOD1</i> -R	TGGCATGTTCCCTTGCTTC

Supplementary Table 5. Sequences of sgRNA

sgRNA	Clone Sequence
sgRNA#1	Sense (5' to 3'): CACCGATCACCGGGATCGCCCCCGG
	Antisense (5' to 3'): AAACCCGGGGGCGATCCCGGTGATC
sgRNA#2	Sense (5' to 3'): CACCGGCGGTCAGCGAATCCTCGT
	Antisense (5' to 3'): AAACACGAGGATTCGCTGACCGCC