TRAF6-SSRP1 axis drives glycolytic reprogramming and colorectal cancer progression through H3K18 lactylation

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Running title: TRAF6 regulates glycolytic reprogramming in CRC

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Supplement Figures

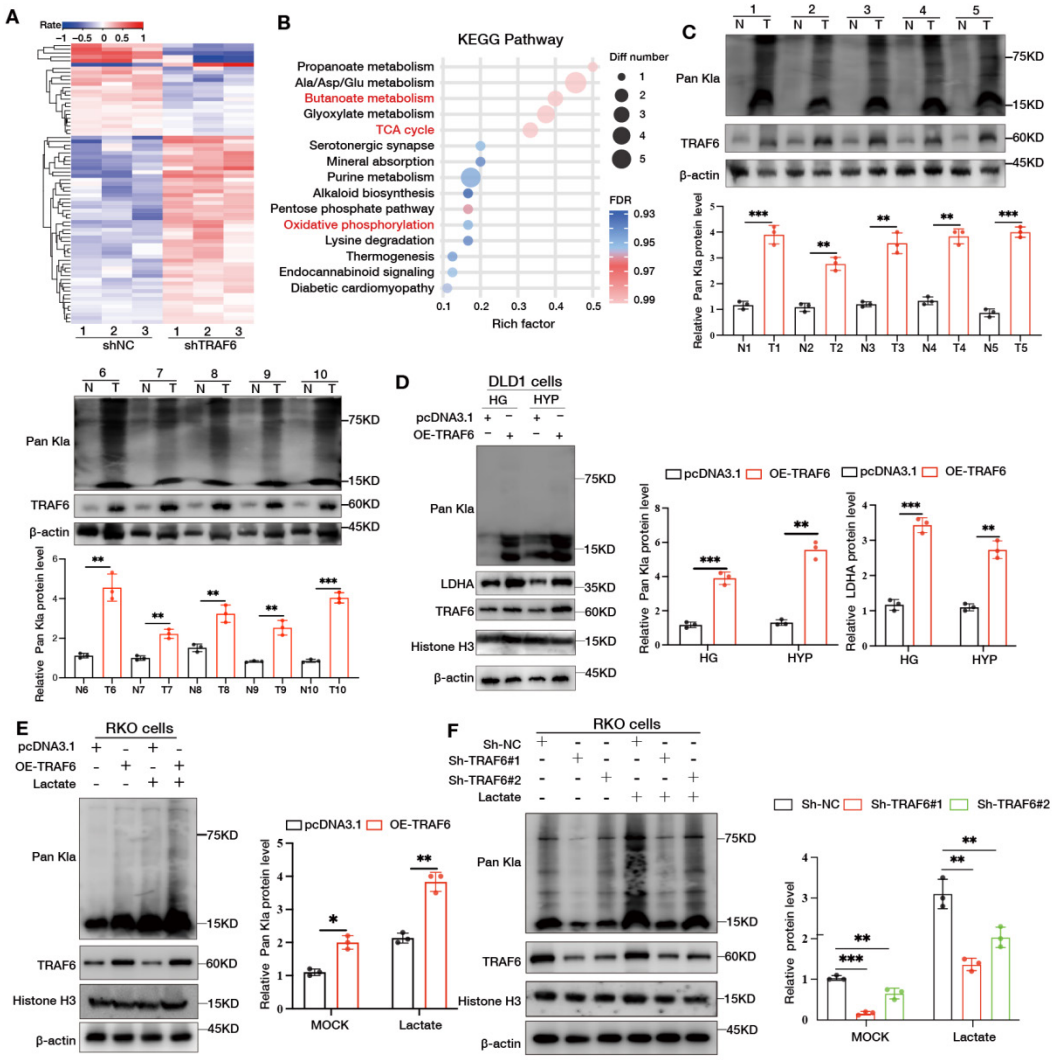


Figure S1. TRAF6 regulates glycolytic reprogramming and Pan-Kla levels in colorectal cancer. A Heatmap of differentially expressed metabolites between TRAF6-knockdown and control CRC cells. B Pathway enrichment analysis of differential metabolites identified in TRAF6-knockdown CRC cells. C Western blot analysis and quantification of Pan-Kla levels in protein extracts from 10 paired CRC tumors and matched adjacent normal tissues. D Western blot analysis of LDHA and Pan-Kla protein levels in TRAF6-overexpressing DLD1 cells cultured under high-glucose and hypoxic conditions. E Western blot analysis of Pan-Kla levels in TRAF6-overexpressing RKO cells treated with sodium lactate. F Western blot analysis of Pan-Kla levels in TRAF6-knockdown RKO cells treated with sodium lactate.

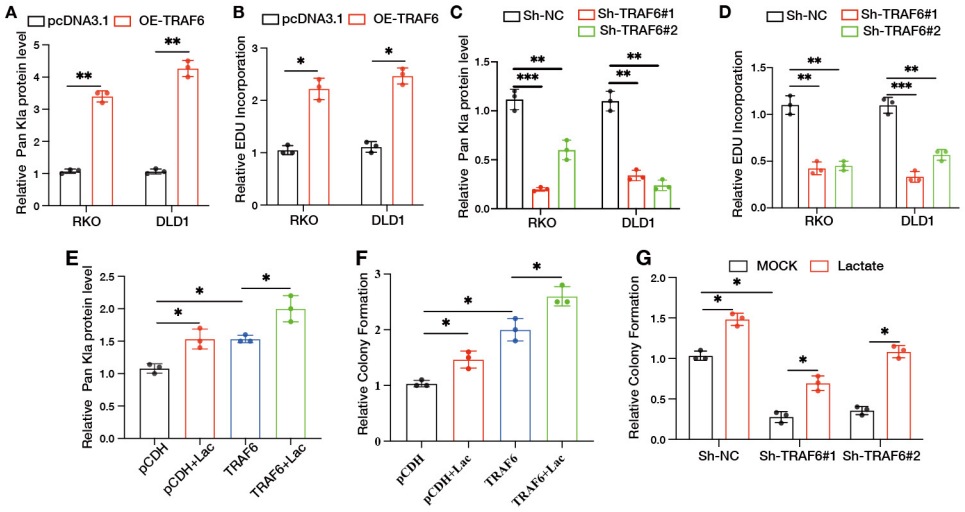


Figure S2. TRAF6 promotes CRC cell proliferation and colony formation through a lactate-dependent mechanism. A Quantification of Pan-Kla western blotting results in TRAF6-overexpressing colorectal cancer cells. B Quantification of EDU assay results in TRAF6-overexpressing cells. C Quantification of Pan-Kla western blotting results in TRAF6-knockdown cells. D Quantification of EDU assay results in TRAF6-knockdown cells. E Quantification of Pan-Kla western blotting results in TRAF6-overexpressing cells after sodium lactate treatment. F, G Quantification of colony formation assays in TRAF6-overexpressing (F) or TRAF6-knockdown (G) RKO cells following sodium lactate treatment.

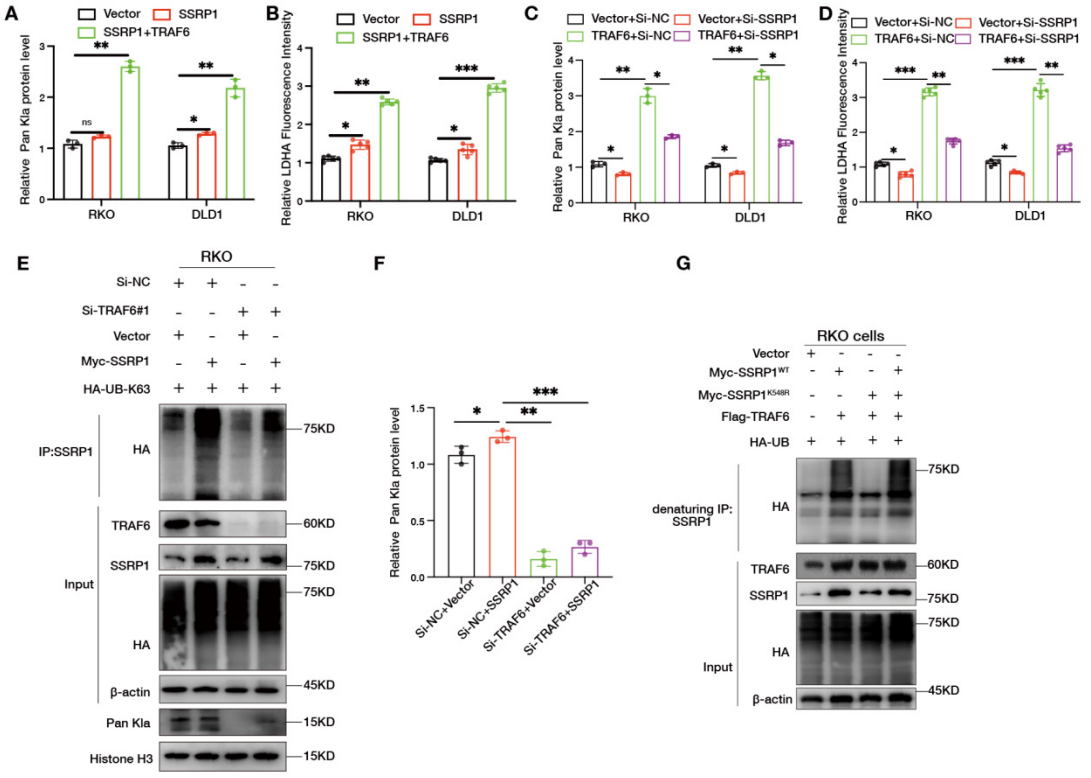


Figure S3. TRAF6 mediates ubiquitination of SSRP1 to regulate Pan-Kla levels. A Quantification of Pan-Kla levels in RKO and DLD1 cells co-transfected with Flag-TRAF6, Myc-SSRP1 and HA-Ub. B Quantification of LDHA immunofluorescence intensity in RKO and DLD1 cells co-transfected with Flag-TRAF6 and Myc-SSRP1. C Quantification of Pan-Kla levels in RKO and DLD1 cells co-transfected with Flag-TRAF6, Si-SSRP1 and HA-Ub-K63. D Quantification of LDHA immunofluorescence intensity in RKO and DLD1 cells co-transfected with Flag-TRAF6 and Si-SSRP1. E, F RKO cells were co-transfected with Si-TRAF6-1, Myc-SSRP1, and HA-UB-K63 plasmids. Immunoprecipitation was performed using an anti-SSRP1 antibody, followed by Western blot analysis to detect K63-linked ubiquitination of SSRP1 (E) and Pan-Kla levels (F), with quantification and statistical analysis. G Cell lysates were denatured with 1% SDS at 95℃ for 10 min to disrupt non-covalent protein complexes, diluted to 0.1% SDS, and subjected to immunoprecipitation with an anti-SSRP1 antibody, followed by Western blot detection of HA-tagged ubiquitin.

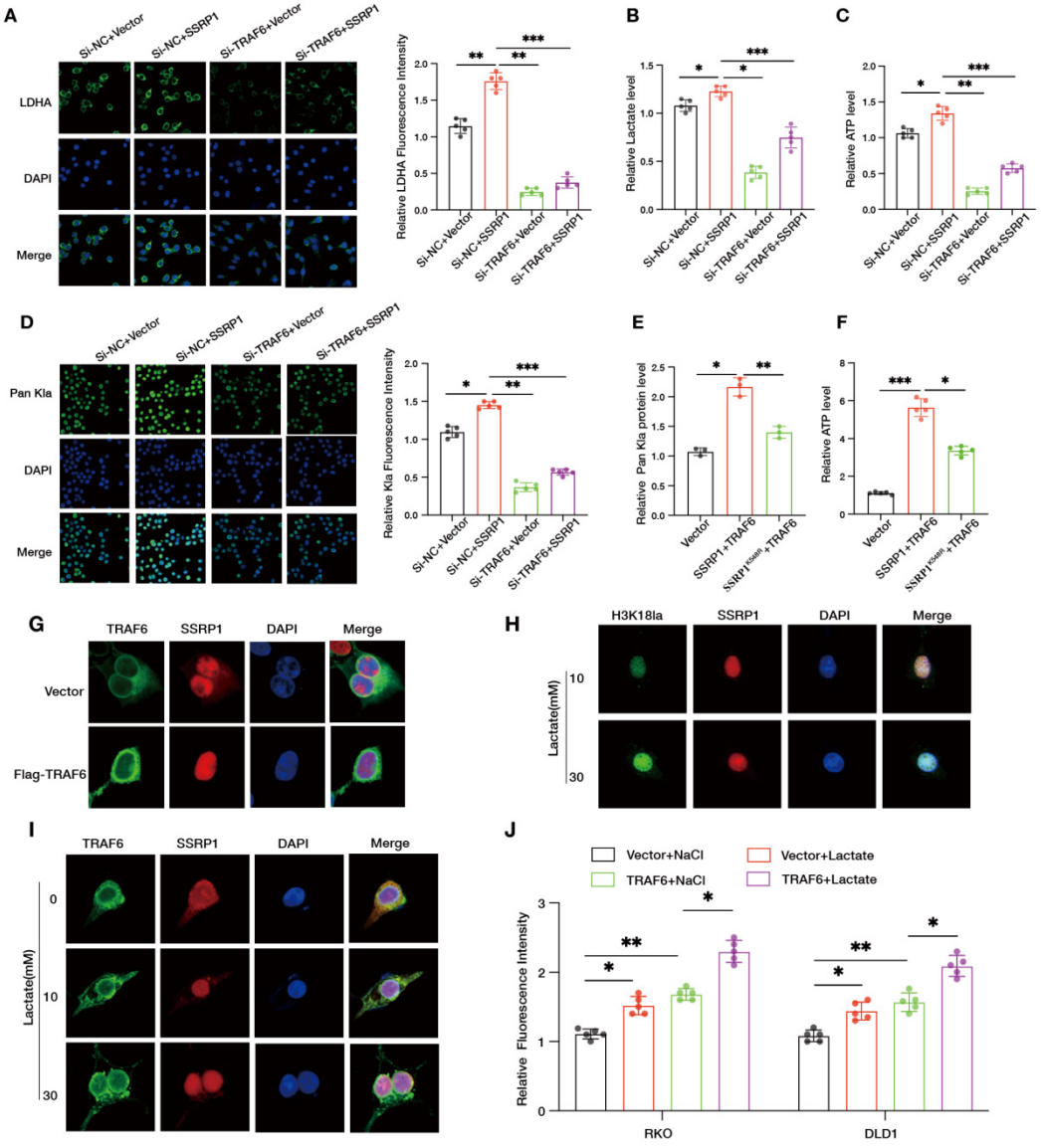


Figure S4. TRAF6 interacts with SSRP1 to regulate H3K18la and glycolytic metabolism. A-D RKO cells were co-transfected with Si-TRAF6-1 and Myc-SSRP1. Immunofluorescence staining was performed to detect LDHA (A) and Pan-Kla (D), with quantification of fluorescence intensity. Intracellular lactate levels (B) and ATP levels (C) were also measured. E, F RKO cells were co-transfected with Flag-TRAF6 and Myc-SSRP1^{K548R} plasmids. Pan-Kla protein levels were quantified (E), and intracellular ATP levels were measured (F). G RKO cells were transfected with vector or Flag-TRAF6 plasmids. Immunofluorescence co-localization of TRAF6 and SSRP1 was analyzed. H TRAF6-overexpressing RKO and DLD1 cells were treated with sodium lactate for 24 h. Immunofluorescence co-localization of H3K18la and SSRP1 was analyzed. I RKO cells were treated with increasing concentrations of sodium lactate for 24 h. Immunofluorescence co-localization analysis was performed. J TRAF6-overexpressing RKO and DLD1 cells were treated with sodium lactate for 24 h. H3K18la fluorescence intensity was quantified and statistically analyzed.

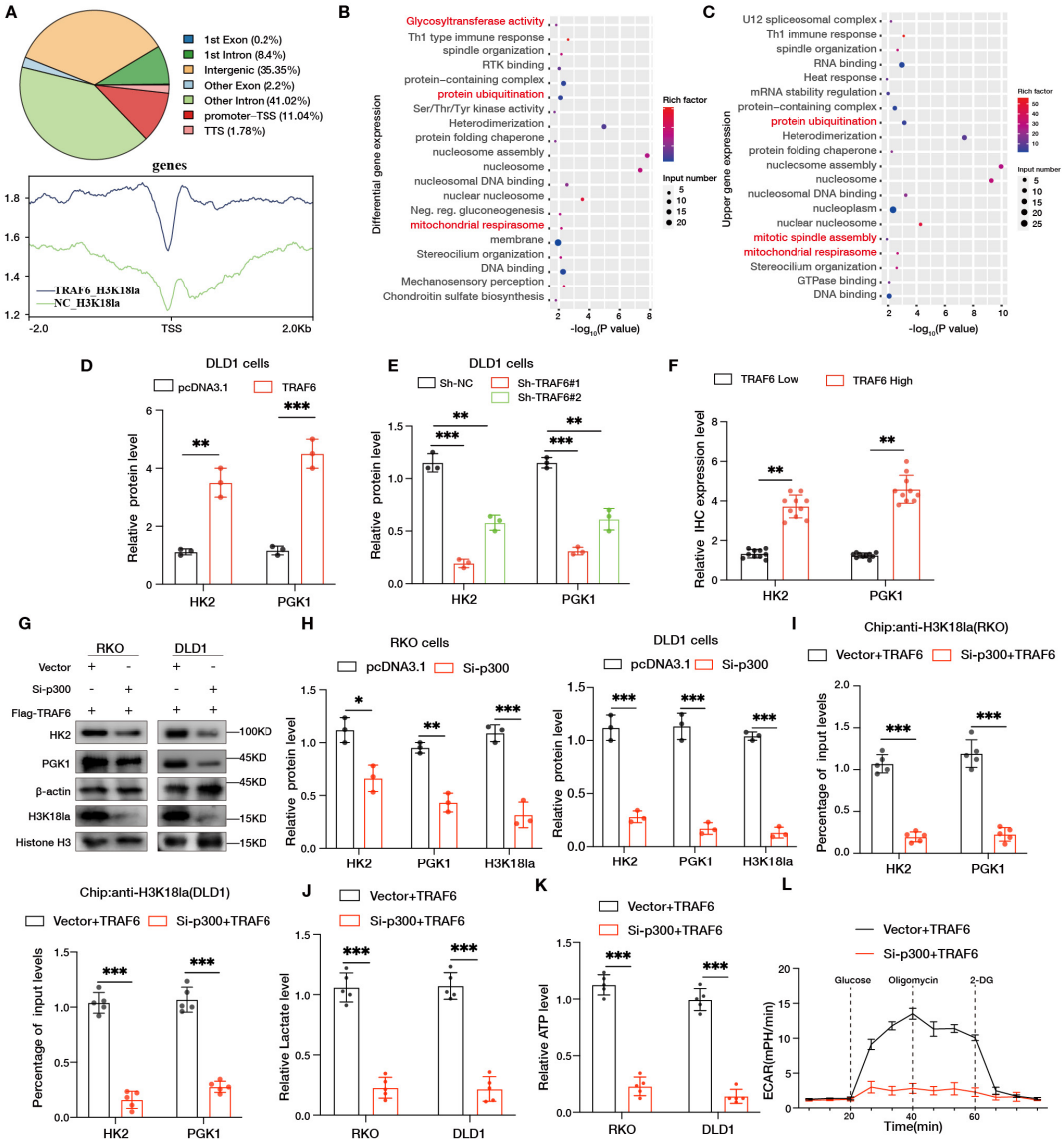


Figure S5. TRAF6 regulates the expression of key glycolytic enzymes through p300-dependent H3K18la modification. A Genomic distribution of differential H3K18la peaks identified by ChIP-seq in TRAF6-overexpressing RKO cells across different functional regions. B, C GO enrichment analysis of differentially expressed genes (B) and upregulated genes (C) from RNA-seq of TRAF6-overexpressing RKO cells. D, E Quantification of HK2 and PGK1 western blot results in TRAF6-overexpressing or TRAF6-knockdown DLD1 cells. F Statistical analysis of immunohistochemical expression of TRAF6, HK2 and PGK1 in clinical colorectal cancer specimens. G RKO and DLD1 cells were transiently transfected with Flag-TRAF6 and Si-p300 plasmids. Cells were harvested 48 h after transfection, and Western blot analysis was performed to detect HK2, PGK1, and H3K18la protein levels. H Relative HK2, PGK1, and H3K18la protein levels in RKO and DLD1 cells were quantified and statistically analyzed (n = 3). I RKO and DLD1 cells were co-transfected with Flag-TRAF6 and Si-p300 for 48 h, followed by ChIP-qPCR. Chromatin immunoprecipitation

was performed using an anti-H3K18la antibody, and qPCR was used to amplify the HK2 enhancer and PGK1 promoter regions. J RKO and DLD1 cells were co-transfected with Flag-TRAF6 and Si-p300 for 48 h, and culture medium was collected to measure lactate concentration. K Intracellular ATP levels in RKO and DLD1 cells were measured and statistically analyzed. L RKO cells were co-transfected with Flag-TRAF6 and Si-p300, and ECAR was measured.

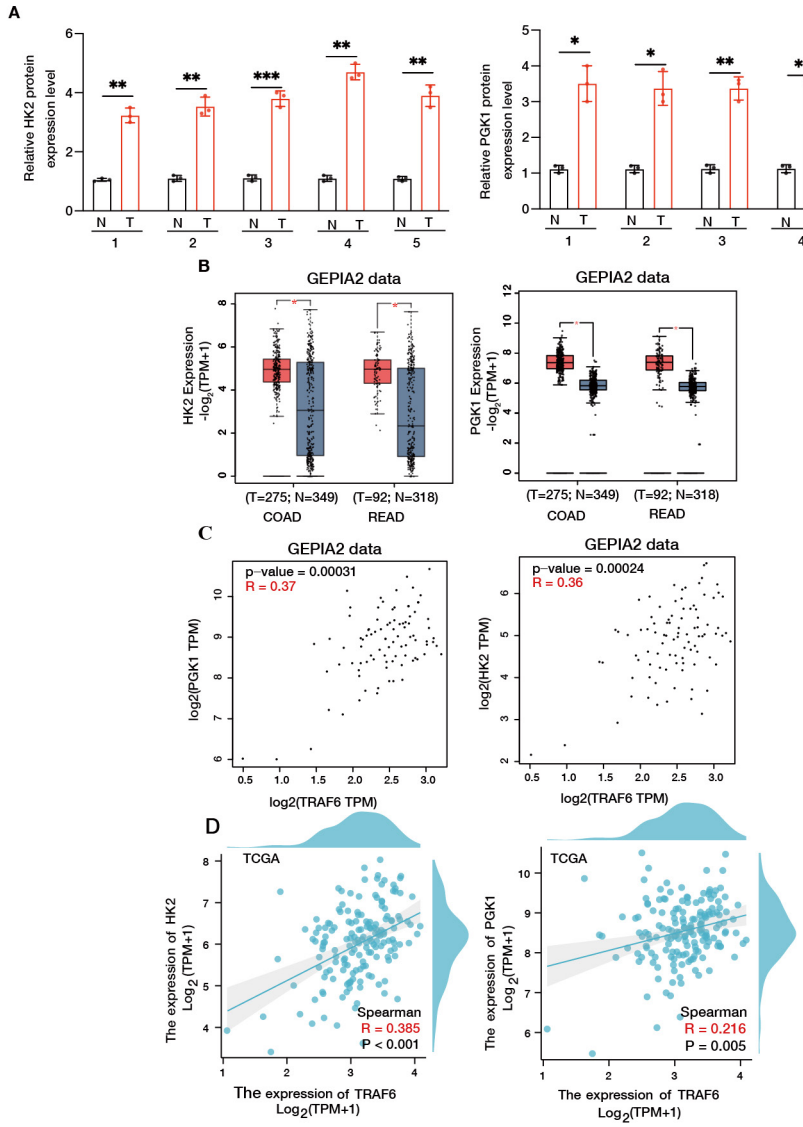


Figure S6. Validation of TRAF6 expression and its correlation with HK2 and PGK1 in CRC tissues and public databases. A Quantification of HK2 and PGK1 western blot results comparing tumor and matched normal tissues from 5 colorectal cancer patients. B Box plots of HK2 and PGK1 expression in colorectal cancer and normal tissues based on GEPIA2. C Correlation analysis between TRAF6 and HK2, and between TRAF6 and PGK1 expression in colorectal cancer samples from GEPIA2. D Correlation analysis between TRAF6 and HK2, and between TRAF6 and PGK1 expression in colorectal cancer samples from TCGA.

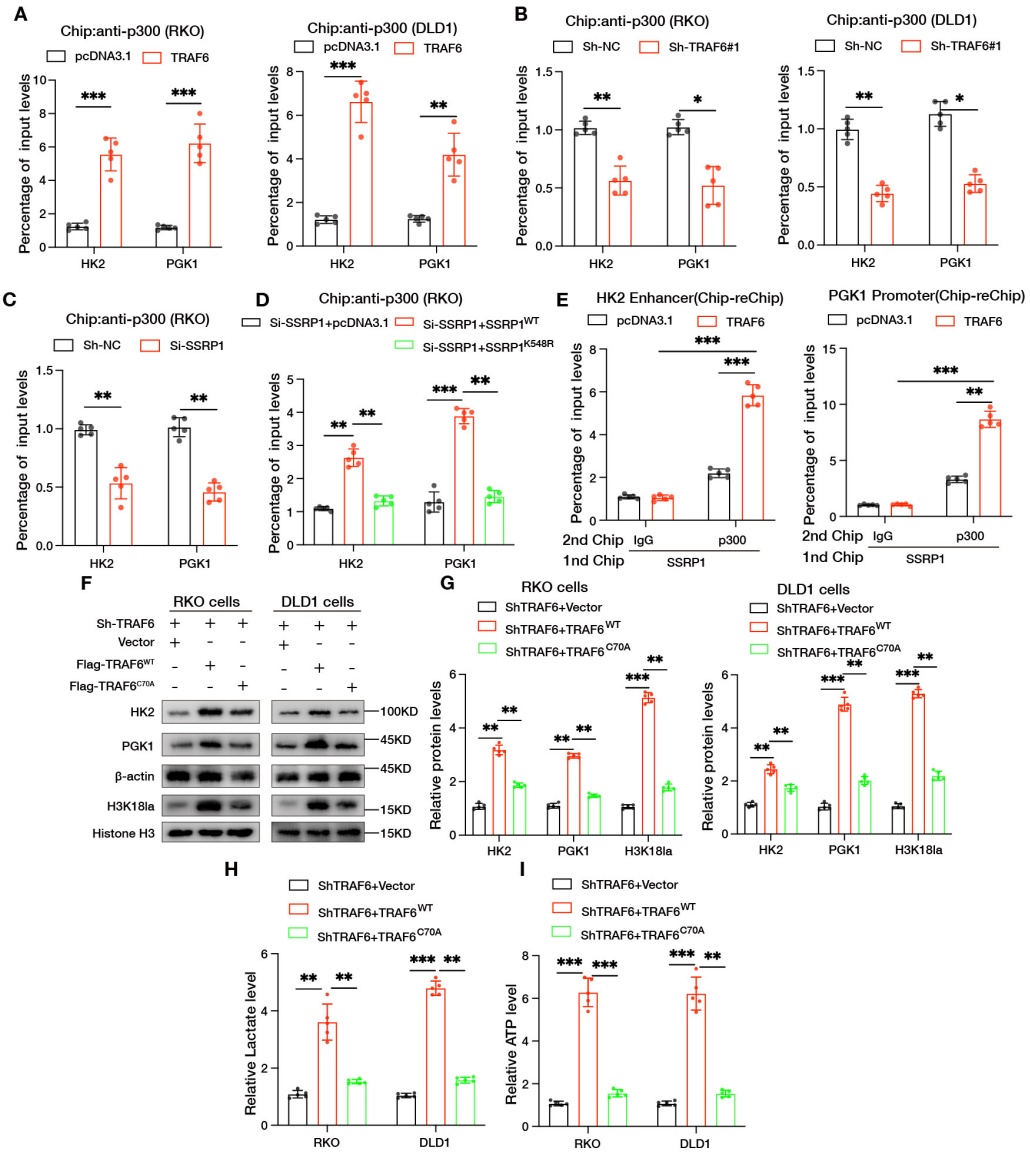


Figure S7. The TRAF6-SSRP1-p300 axis regulates histone lactylation at the HK2 and PGK1 promoter/enhancer regions. A RKO and DLD1 cells were transfected with Flag-TRAF6, and ChIP-qPCR was performed using an anti-p300 antibody to detect p300 enrichment at the HK2 enhancer and PGK1 promoter regions. B RKO and DLD1 cells were transfected with Sh-TRAF6#1, and ChIP-qPCR was performed using an anti-p300 antibody to detect p300 enrichment at the HK2 enhancer and PGK1 promoter regions. C RKO cells were transfected with Si-SSRP1, and ChIP-qPCR was performed using an anti-p300 antibody to detect p300 enrichment at the HK2 enhancer and PGK1 promoter regions. D RKO cells were transfected with Si-SSRP1+pcDNA3.1, Si-SSRP1+SSRP1^{WT}, or Si-SSRP1+SSRP1^{K548R}, and ChIP-qPCR was performed using an anti-p300 antibody to detect p300 enrichment at the HK2 enhancer and PGK1 promoter regions. E RKO cells were transfected with Flag-TRAF6 and subjected to ChIP-reChIP analysis, with an anti-SSRP1 antibody used for the first round of ChIP and IgG or an anti-p300 antibody used for the second round; qPCR was used to

detect co-enrichment at the HK2 enhancer and PGK1 promoter regions. F-I RKO and DLD1 cells with TRAF6 knockdown were reconstituted with vector, Flag-TRAF6^{WT}, or Flag-TRAF6^{C70A}. Western blot analysis was performed to detect HK2, PGK1, and H3K18la protein levels (F), with quantification of relative protein levels (G); intracellular lactate levels (H) and ATP levels (I) were also measured.

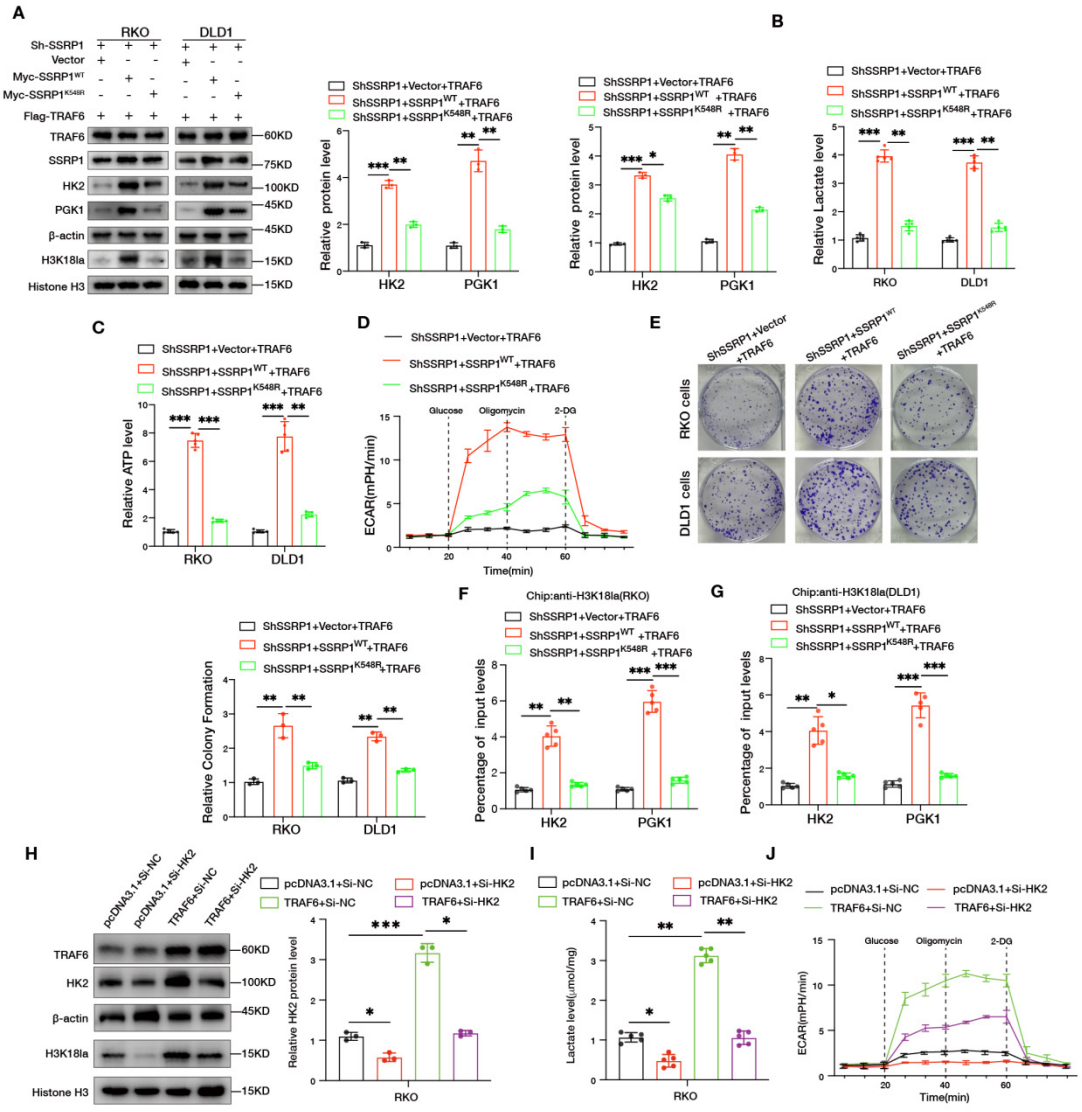


Figure S8. SSRP1 K548 ubiquitination regulates TRAF6-driven glycolysis and H3K18la enrichment at HK2/PGK1 loci. A-G RKO and DLD1 cells with SSRP1 knockdown were reconstituted with vector, Myc-SSRP1^{WT}, or Myc-SSRP1^{K548R} and co-transfected with Flag-TRAF6. Western blot analysis and quantification of HK2 and PGK1 protein levels (A); intracellular lactate (B) and ATP levels (C); extracellular acidification rate (D); colony formation assays (E). ChIP-qPCR using an anti-H3K18la antibody to detect H3K18la enrichment at the HK2 enhancer and PGK1 promoter in RKO (F) and DLD1 (G) cells. H-J RKO cells co-transfected with si-HK2 and TRAF6 were analyzed by western blotting for HK2 and related proteins (H), intracellular lactate levels (I) and ECAR (J).

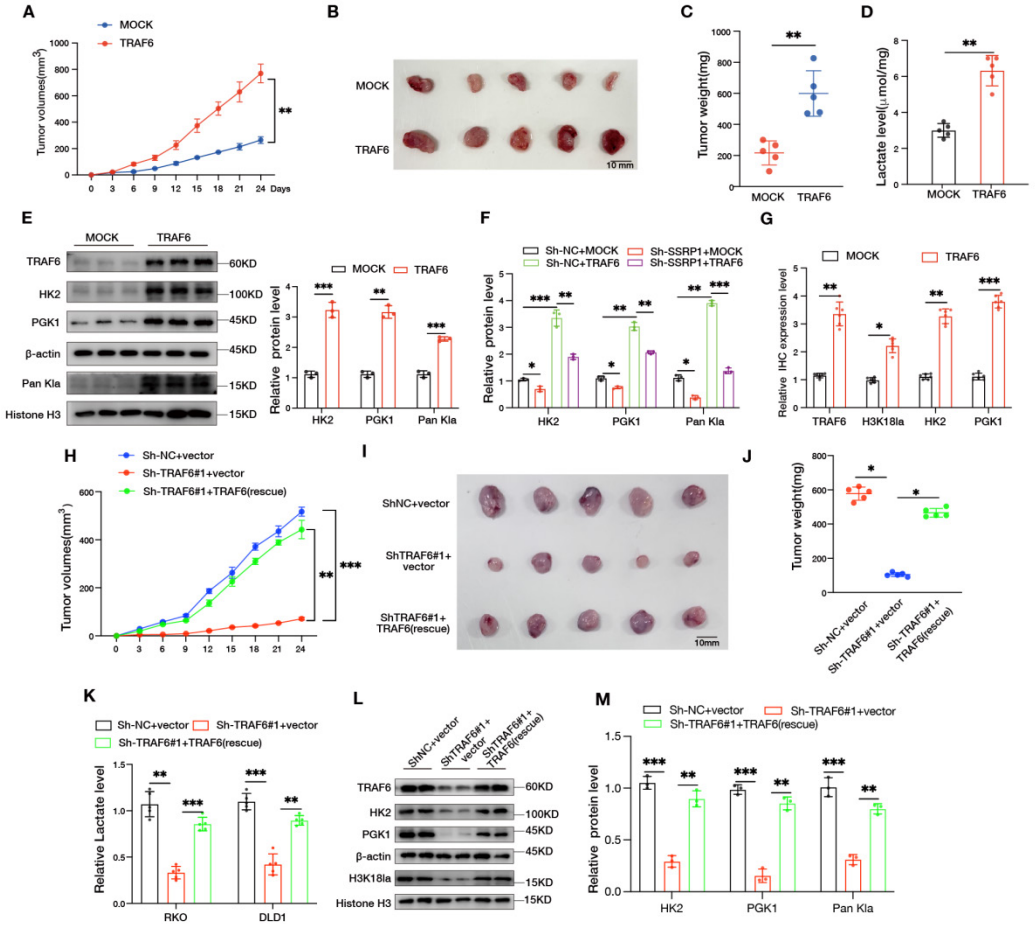


Figure S9. In vivo validation of the TRAF6-SSRP1 axis driving the glycolytic phenotype in colorectal cancer. A-E Xenograft model using BALB/c nude mice bearing subcutaneous tumors derived from TRAF6-overexpressing RKO cells; tumor volume was measured every 3 days (A), tumors from TRAF6 and control groups were photographed at sacrifice (B), tumor weight was quantified (C), and tumor lactate levels (D) and related protein expression including HK2, PGK1 and Pan-Kla (E) were assessed. F Rescue xenograft model using BALB/c nude mice bearing subcutaneous tumors derived from RKO cells co-expressing sh-SSRP1 and TRAF6, with analysis of HK2, PGK1, Pan-Kla and related protein expression in tumor tissues. G Quantification of immunohistochemical staining for TRAF6, H3K18la, HK2 and PGK1 in xenograft tumors from TRAF6-overexpressing and control groups. H-M BALB/c female nude mice were subcutaneously injected with stable TRAF6-knockdown DLD1 cells reconstituted with vector or TRAF6 (rescue), along with Sh-NC+vector control cells. Tumor volumes were measured every 3 days (H). Mice were euthanized, and representative images of xenograft tumors were shown (I), with statistical analysis of tumor weight (J). Lactate levels (K) and the expression of the indicated proteins (L) in tumor tissues were detected, with quantification (M).