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Supplemental information

The deubiquitinase USP9X regulates RIT1 protein abundance and oncogenic phenotypes

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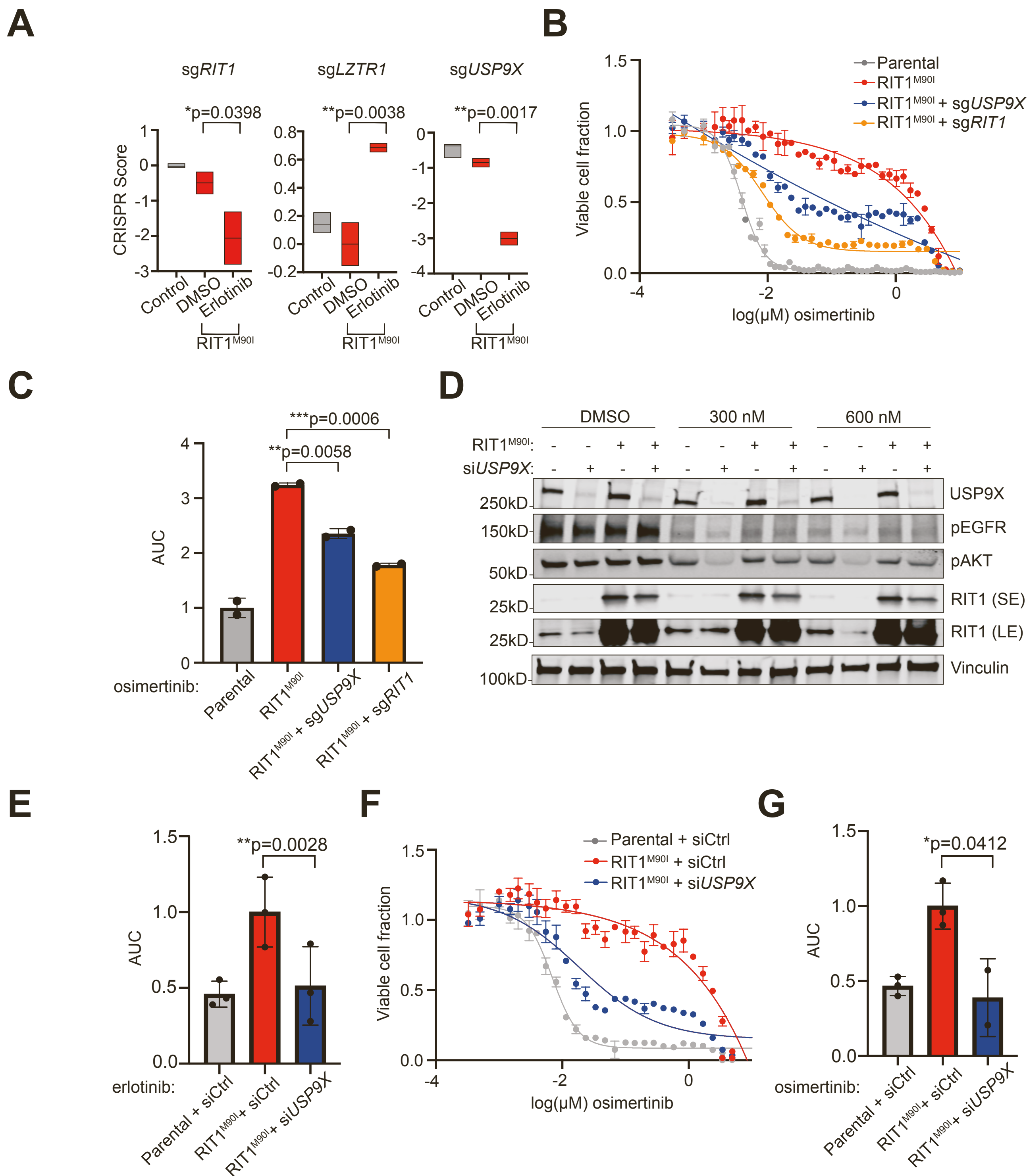
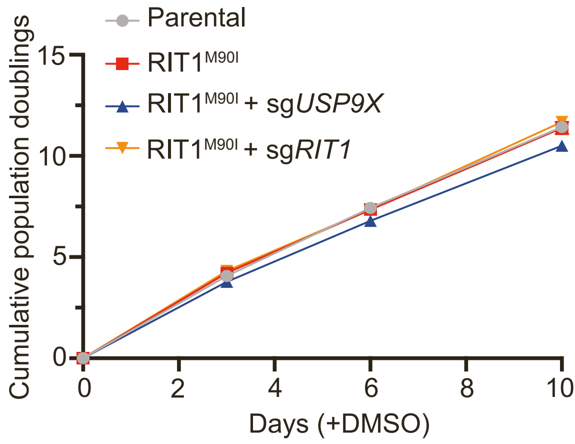
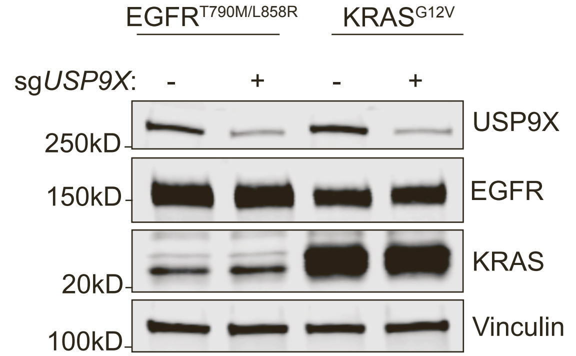
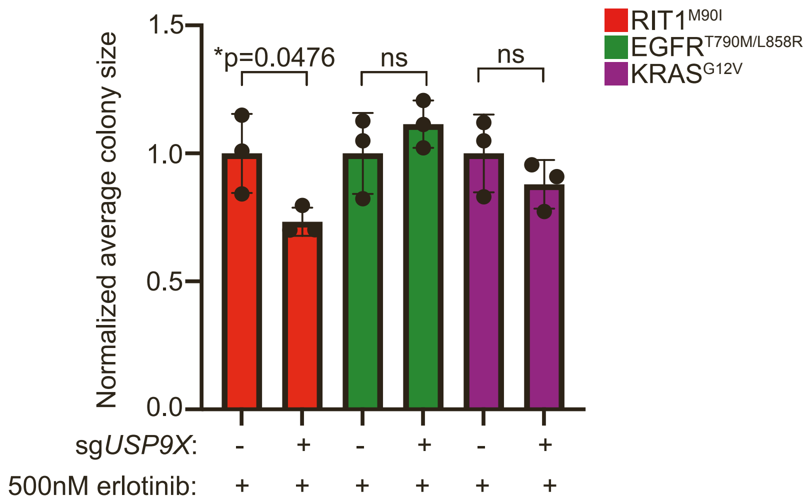
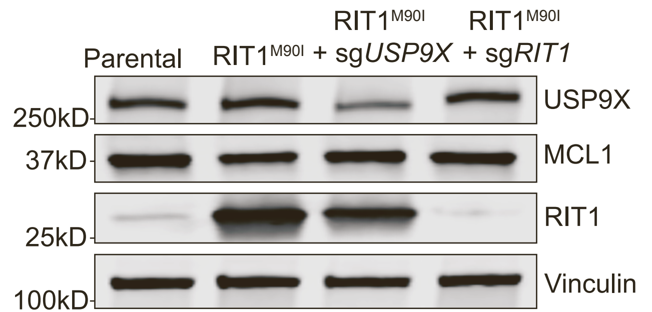


Figure S1. *RIT1^{M90I}* requires USP9X to promote drug resistance in PC9 cells, related to Figure 1. A, Box plots showing average CRISPR score of indicated sgRNAs in parental PC9-Cas9 (+Luciferase/Control) cells or RIT1^{M90I}-mutant PC9-Cas9 cells. Box plots show the median (center line) and the min and max range of replicates. For Control conditions, n = 3 biological replicates. For RIT1^{M90I}-mutant cells, n = 2 biological replicates. p-values calculated by unpaired two-tailed t-tests. **B,** Dose-response curves of parental PC9-Cas9 cells and RIT1^{M90I}-mutant PC9-Cas9 cells with indicated gene knockouts (sg*RIT1* and sg*USP9X*) treated with osimertinib for 72 hours. CellTiterGlo was used to quantify viable cell fraction determined by normalization to DMSO control. Data shown are the mean ± s.d. of two technical replicates. Data are representative results from n = 2 independent experiments. **C,** Area-under-the-curve (AUC) analysis of dose response curves shown in (D). p-values calculated by unpaired two-tailed t-tests. **D,** Western blot of parental and RIT1^{M90I}-mutant PC9-Cas9 cells transfected with siCtrl or si*USP9X*. 24 hours later, DMSO or osimertinib were added and lysates were collected after 72 hours. SE = short exposure. LE = long exposure. Vinculin serves as a loading control. **E,** Area-under-the-curve (AUC) analysis of dose response curves in Figure 1F. p-value calculated by unpaired two-tailed t-test. **F,** Dose-response curve of RIT1^{M90I}-mutant PC9-Cas9 cells treated with siCtrl or si*USP9X* for 48 hours, prior to treatment with osimertinib for 72 hours. CellTiterGlo was used to quantify viable cell fraction determined by normalization to DMSO control. Data shown are the mean ± s.d. of two technical replicates. Data are representative results from n = 3 independent experiments. **G,** Area-under-the-curve (AUC) analysis of dose response curves shown in (F). p-value calculated by unpaired two-tailed t-test. Data are representative results from n = 3 independent experiments.









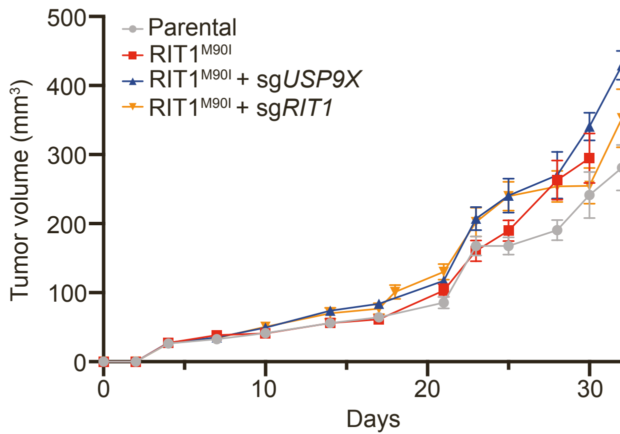


Figure S2. RIT1^{M90I} expression in PC9 cells, related to Figure 2. A, Proliferation of parental and RIT1^{M90I}-mutant PC9-Cas9 cells with indicated gene knockouts (sg*RIT1* and sg*USP9X*) treated with DMSO. Data shown are the mean ± s.d. of three technical replicates per cell line. Data are representative results from n = 2 independent experiments. **B,** Western blot of *EGFR*- and *KRAS*-mutant PC9-Cas9 cells with or without sg*USP9X*. "-" lanes represent cells harboring sgNTC. Vinculin serves as a loading control. **C,** Normalized average colony area of all colonies formed by indicated cell lines treated with 500 nM erlotinib for 10 days. All data were normalized to DMSO control for each cell line, and then normalized to 'no sg*USP9X*' conditions. Data shown are the mean ± s.d. of three technical replicates per cell line. p-values calculated by unpaired two-tailed t-tests. ns = not significant. **D,** Western blot of parental and RIT1^{M90I}-mutant PC9-Cas9 cells with sg*USP9X* or sg*RIT1*. Vinculin serves as a loading control. **E,** Xenograft assay of PC9-Cas9 cells (Parental, RIT1^{M90I}, RIT1^{M90I} + sg*USP9X*, and RIT1^{M90I} + sg*RIT1*) in immunocompromised mice treated with DMSO vehicle daily. Data shown are the mean ± s.e.m. of n = 8 tumors per group.

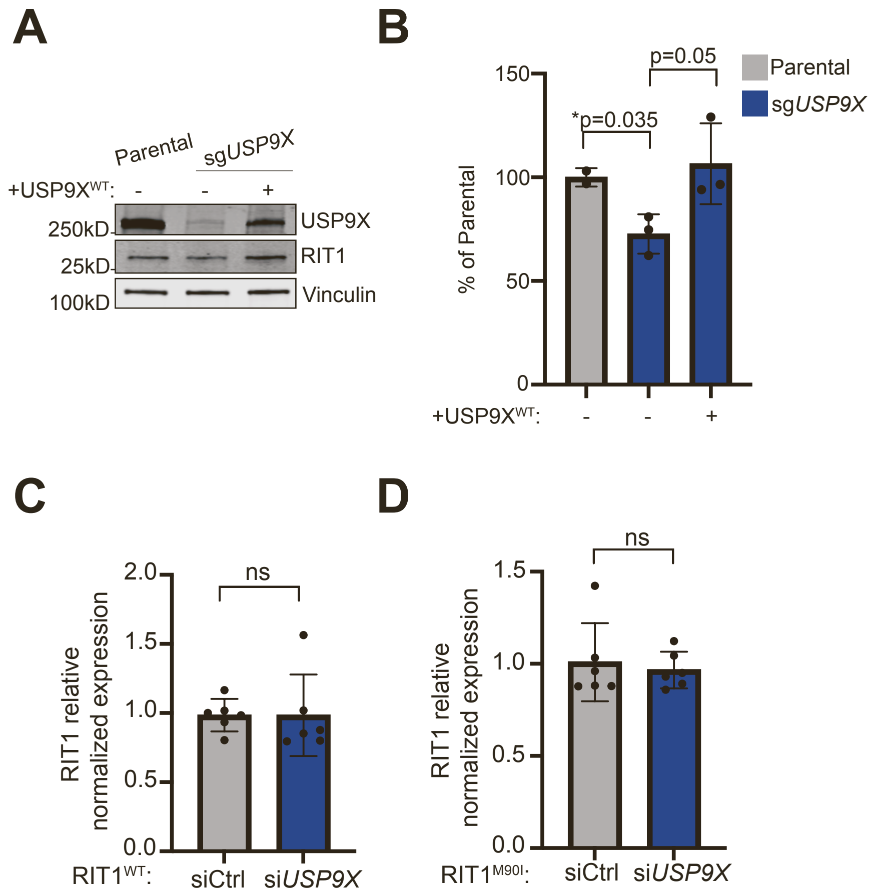


Figure S3. USP9X regulates RIT1 protein abundance and not mRNA expression, related to Figure 3. A, Western blot of parental PC9-Cas9 cells and *USP9X* knockout (sg*USP9X*) cells transfected with control GFP or $USP9X^{WT}$ plasmids. Vinculin serves as a loading control. **B,** Quantification of RIT1 band intensity from (A) and additional replicates. Data compared to parental PC9-Cas9 cells transfected with control GFP plasmid. p-values calculated by unpaired two-tailed t-tests. **C,** Relative mRNA expression of *RIT1* as determined by qPCR and standard curve-based quantification. Parental PC9-Cas9 cells were treated with siCtrl or si*USP9X* for 48 hours before RNA collection. Relative normalized expression calculated in BioRad software. Each dot represents a biological replicate (individual cDNA preparation). Each qPCR sample was run in triplicate for housekeeping (18S) and RIT1 probes. ns = not significant by unpaired two-tailed t-test. **D,** Relative mRNA expression of *RIT1* as determined by qPCR and standard curve-based quantification. $RIT1^{M90I}$-mutant PC9-Cas9 cells were treated with siCtrl or si*USP9X* for 48 hours before RNA collection. Relative normalized expression calculated in BioRad software. Each dot represents a biological replicate (individual cDNA preparation). Each qPCR sample was run in triplicate for housekeeping (18S) and RIT1 probes. ns = not significant by unpaired two-tailed t-test.

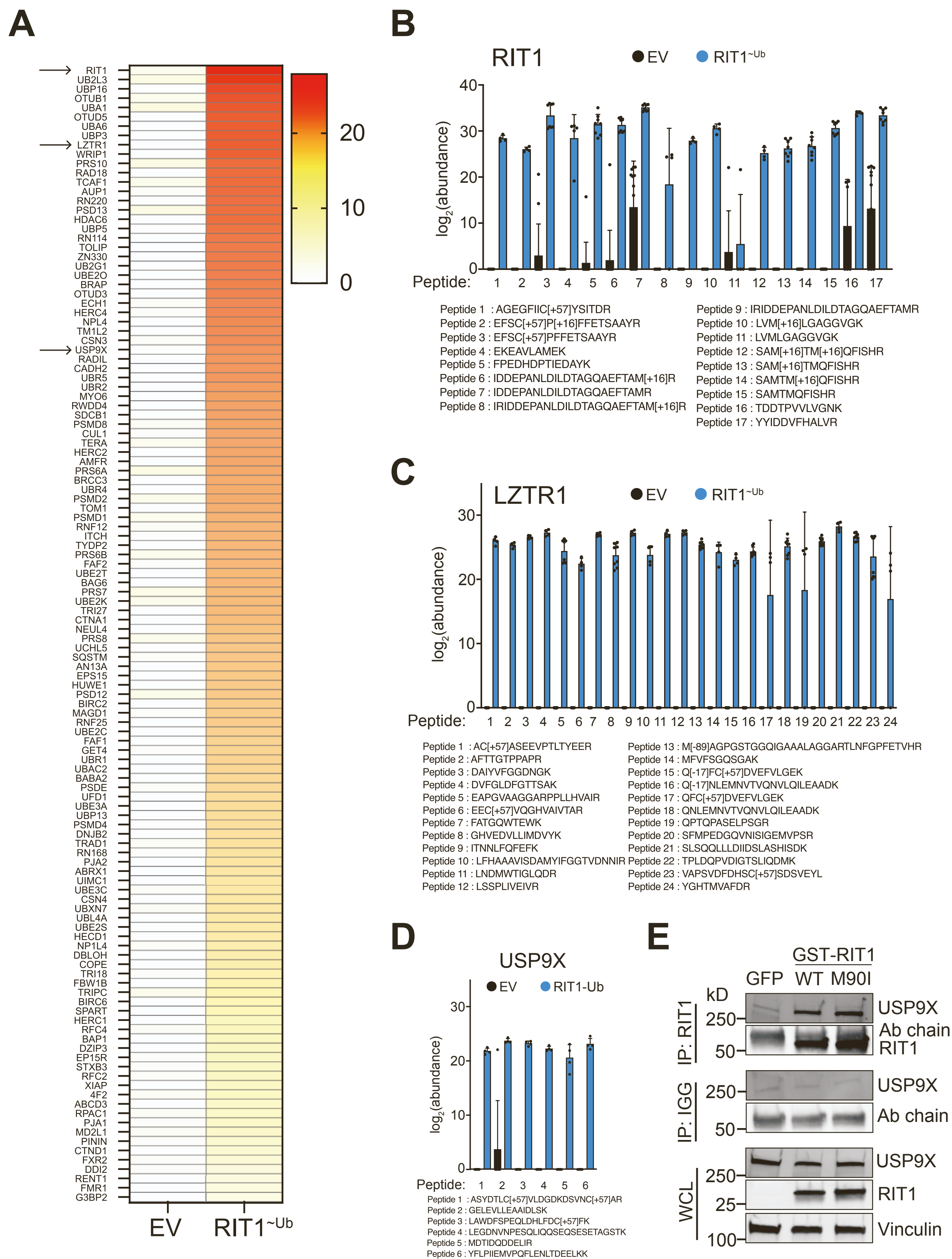


Figure S4. Protein-protein interaction of USP9X and RIT1 identified by mass spectrometry, related to Figure 5.
A, Heatmap of peptides detected in affinity purification/mass spectrometry (AP/MS) experiment. Data were filtered for proteins that were at least 5 times higher in RIT1$^{\sim Ub}$ condition compared to EV (Empty Vector). Abundance values are the log_2-based number of the peak intensity from the MS. The mean of all peptides was combined across biological replicates. For EV samples, n = 7 biological replicates. For RIT1$^{\sim Ub}$ samples, n = 4 biological replicates. **B,** Abundance (log_2-transformed) of individual RIT1 peptides in Empty Vector (EV) control and RIT1$^{\sim Ub}$ conditions from AP/MS. **C,** Abundance (log_2-transformed) of individual LZTR1 peptides in EV and RIT1$^{\sim Ub}$ conditions from AP/MS. **D,** Abundance (log_2-transformed) of individual USP9X peptides in EV and RIT1$^{\sim Ub}$ conditions from AP/MS. For B-D, some peptides were detected in all replicates while other peptides were only detected in some replicates. **E,** Co-immunoprecipitation experiment in HEK293T cells transfected with indicated GST-tagged RIT1 variants or a GFP transfection control. Vinculin serves as a loading control. WCL = whole cell lysate. Data shown are representative of n = 2 independent experiments.

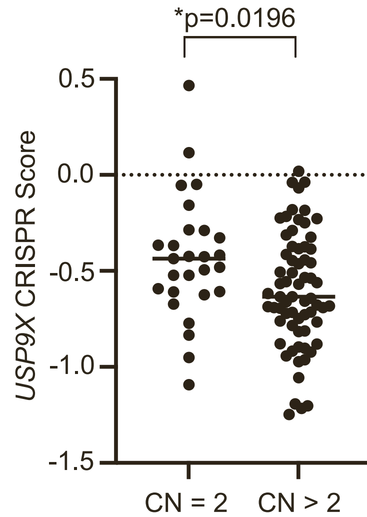


Figure S5. Altered *USP9X* dependency in cells with elevated *RIT1* copy number, related to Figure 6. Data from the Cancer Dependency Map (DepMap) comparing *USP9X* CRISPR score (DepMap Public 23Q4+ Score, Chronos) in lung cancer cells with normal copy number of *RIT1* (CN = 1) versus cells with high copy number of *RIT1* (CN > 1). Copy Number data based on the Copy Number (Absolute) data set. p-value calculated by unpaired two-tailed t-test.