Characterizing a USP9X-COP1 regulatory axis of RIT1 protein abundance

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Summary

Standard care of lung cancer is moving away from chemotherapy in favor of personalized approaches based on specific mutations in each tumor. Genome-sequencing studies have identified somatic mutations in the small GTPase *RIT1* (Ras-like in all tissues) in lung adenocarcinoma patients. Thousands of patients per year are diagnosed with *RIT1*-driven cancer, but treatment options are limited. A targeted therapy for *RIT1*-driven disease could greatly improve patient outcomes.

Little is known about how *RIT1* drives cellular transformation. To genetically dissect signaling pathways downstream of RIT1, we performed a genome-wide CRISPR/Cas9 screen in isogenic PC9 lung adenocarcinoma cells in which cell survival is dependent on expression of RIT1^{M90I}. In this system, *RIT1*-driven drug resistance provides a selectable phenotype to assess the growth effects of individual gene knockouts. Using this approach, we can identify genetic dependencies (genes that, when knocked out, confer a growth disadvantage) and cooperating factors (genes that, when knocked out, confer a growth advantage) in *RIT1*-mutant lung cancer cells. From this analysis, we identified the deubiquitinase *USP9X* and the E3 ubiquitin ligase *COP1* as genetic regulators of RIT1 function. Previous work suggests that the protein abundance of RIT1 is essential for its pathogenic function; given this, we proposal a regulatory axis of RIT1 protein abundance mediated by USP9X and COP1. I hypothesize that USP9X positively regulates RIT1 protein levels while COP1 counteracts this effect. This work is poised to not only expand our understanding of RIT1 biology and oncogenic mechanisms, but it also identifies USP9X as a potential druggable target for the treatment of *RIT1*-driven cancers and other diseases characterized by *RIT1* mutations and amplifications.

RIT1 is mutated in lung adenocarcinoma

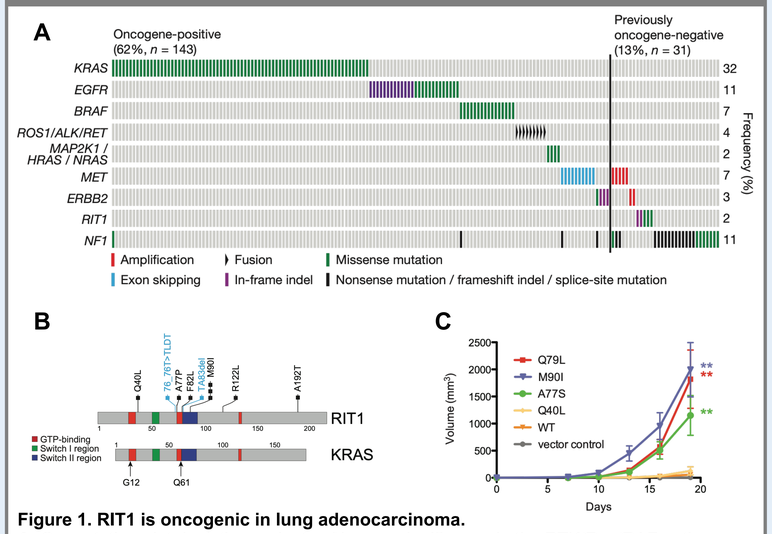

Figure 1. RIT1 is oncogenic in lung adenocarcinoma.
A. Co-mutation plot depicting variants of known significance in the RTK/Ras/RAF pathway. *RIT1* mutations are mutually-exclusive with other known driver mutations in this pathway. (TCGA, 2014) **B.** RIT1 is homologous in sequence and domains to KRAS. The 'hot spot' of transformative mutations in RIT1 reside in the switch II domain of RIT1, including at amino acid 90. **C.** Tumor growth of xenografts of NIH3T3 cells. Data shown is mean +/-s.e.m. of nine replicates per construct. *P<0.01 by two-tailed t-test. (Berger et al., 2014)

Assay system to identify genetic dependencies of *RIT1*-mutant cells

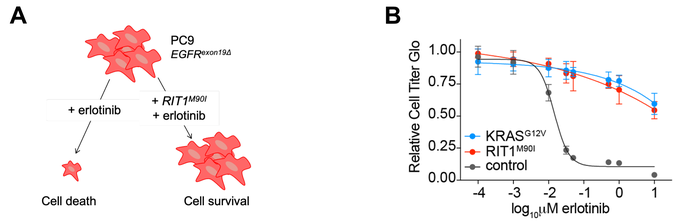

Figure 2. RIT1^{M90I} confers resistance to EGFR inhibition.
A. Schematic representation of erlotinib resistance in PC9 cells expressing RIT1^{M90I}. **B.** Dose-response of erlotinib in PC9-Cas9 parental cells (control) and PC9-Cas9 cells expressing RIT1^{M90I} or KRAS^{G12V}. Data shown is the mean and standard deviation of 8 technical replicates per cell line.

Genome-wide CRISPR/Cas9 screen in isogenic lung adenocarcinoma cell lines

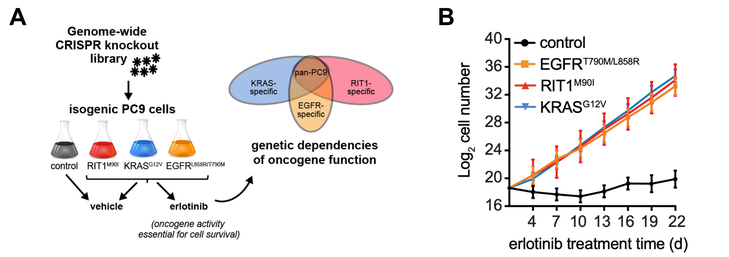

Figure 3. CRISPR/Cas9 screen in PC9-Cas9 cells expressing known oncogenes.
A. Schematic of CRISPR screens performed. Abundance of guide RNAs was compared between early time point and final (day 22 for DMSO and day 28 for erlotinib) time points. **B.** Proliferation assay of isogenic cell lines grown in 50nM erlotinib. Error bars indicate +/- 95% confidence interval of two replicates per cell line. All oncogene-expressing cell lines proliferate in erlotinib at a similar rate.

USP9X and *COP1* are key genetic players in *RIT1*-driven cell survival

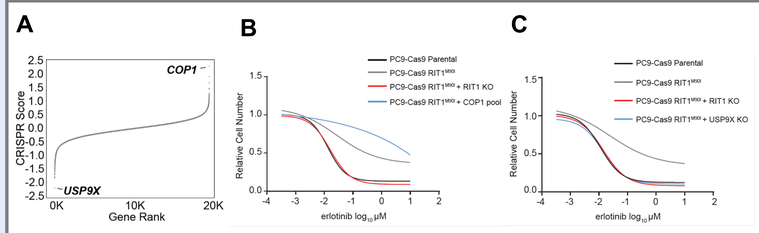

Figure 4. Identification of USP9X and COP1 as RIT1 regulators.
A. Rank plot of differential CRISPR score (normalized $\log_2$(fold change) of the average of 4 sgRNAs per gene in two biological replicates) in PC9-Cas9-RIT1^{M90I} cells between erlotinib-treated and DMSO-treated screens. **B.** Dose response curve of control PC9 cells or pooled PC9-Cas9-RIT1^{M90I} cells with *RIT1* or *COP1* knockout (KO). **C.** Dose response curve of control PC9 cells or pooled PC9-Cas9-RIT1^{M90I} cells with *RIT1* or *USP9X* KO.

USP9X loss reduces RIT1 protein abundance and stability

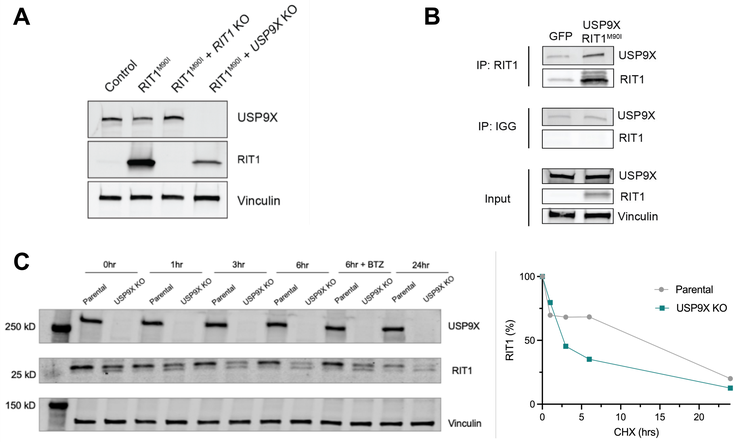

Figure 5. USP9X interacts with RIT1 and regulates RIT1 protein abundance.
A. Western blot of RIT1 and USP9X in parental (control) PC9 cells or *RIT1* knockout (KO) and *USP9X* KO cells derived from PC9-Cas9-RIT1^{M90I}. Vinculin serves as loading control. **B.** Co-IP of RIT1^{M90I} and USP9X in HEK293T cells. Immunoprecipitation was performed with a control IGG antibody or RIT1 antibody. Vinculin serves as a loading control. **C.** *left*, PC9-Cas9 parental and *USP9X* KO cells were treated with 20μg/mL cycloheximide (CHX) for indicated amounts of time. 100nM of the proteasome inhibitor bortezomib (+BTZ) was used to rescue degradation. Vinculin serves as a loading control. *right,* half-life of RIT1 based on quantitative analysis of relative band intensity in western blot on left.

USP9X as a novel druggable target in *RIT1*-driven diseases

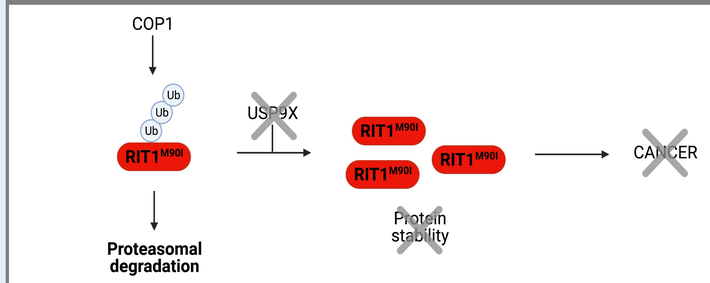

Figure 6. Proposed model of USP9X as a therapeutic vulnerability in *RIT1*-mutant cells.
USP9X knockout or inhibition is predicted to reduce RIT1 protein stability and abrogate *RIT1*-driven cancer.

References

Vichas, A.*, Riley A.K.*, Nkinsi, N.T., Kamlapurkar, S., Parrish, P.C.R., Lo, A., Duke, F., Chen, J., Fung, I., Watson, J., et al. (2021). Integrative oncogene-dependency mapping identifies RIT1 vulnerabilities and synergies in lung cancer. Nat. Commun. *12*, 4789. *equal contribution

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