THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF SCIENCE

EVALUATION OF MECHANISMS UNDERLYING THERAPEUTIC SUSCEPTIBILITY AND IMMUNOTHERAPEUTIC POTENTIAL IN SETD2 MUTANT KIDNEY CANCERS

ROBERT N. UZZO
FALL 2021

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Science
with honors in Science

Reviewed and approved* by the following:

David Degraff, PhD
Assistant Professor of Pathology and Laboratory Medicine
Division of Experimental Pathology
Assistant Professor, Department of Surgery
Penn State Cancer Institute
Thesis Supervisor

Ronald Markle, PhD
Professor of Biology, Director Pre-Medicine and Science Majors
Department of Biology
Pennsylvania State University
Honors Advisor

Philip Abbosh, MD, PhD
Assistant Professor
Molecular Therapeutics Research Program
Fox Chase Cancer Center – Temple University
Principle Investigator

* Electronic approvals are on file.

ABSTRACT

OBJECTIVE: *SETD2* mutations occur in approximately 10% of all renal epithelial malignant neoplasms. As a histone H3 lysine methyltransferase, *SETD2* is implicated in the proper splicing of mRNA. Its loss induces intron retention. We hypothesized that *SETD2* deficient renal cell carcinomas (RCC) retain introns, which are aberrantly translated, resulting in protein misfolding. We hypothesize that this would trigger the unfolded protein response (UPR). This mechanism may offer two potential therapeutic approaches, a synthetically lethal strategy as well as mechanisms based on increased immunogenicity secondary to upregulated neopeptide expression.

METHODS: We analyzed KIRC tumors from TCGA with gene set enrichment analysis, comparing transcriptomes of *SETD2* mutant versus wild type (WT) tumors. Next, we established *SETD2* knockout cell lines in RENCA, a naturally occurring murine RCC line, using CRISPR edits. Sanger and next generation sequencing were performed to confirm *SETD2* deficient isogenic cell lines. Western blotting was then used to measure three sensors of the UPR pathway (PERK, IREα, ATF6) to investigate pathway activation. This was further investigated using immunofluorescence microscopy. Finally, we analyzed a panel of UPR inhibitors to determine if *SETD2* loss results in increased therapeutic susceptibility.

RESULTS: In *SETD2* deficient TCGA tumors, the UPR gene set was significantly enriched (q=0.01). Sanger sequencing and immunoblot confirmed successful knockout of *SETD2* at the gene and protein levels. In addition, H3-K36 trimethylation was lost, confirming loss of *SETD2* function. *SETD2* deficiency induced activation of UPR through upregulation of protein kinase PERK and the enzyme IREα. Additionally, transcription factor ATF6 was noted to be

cleaved and localized to the nucleus using immunofluorescence microscopy in *SETD2* deficient cells indicating that UPR pathway was activated. These results demonstrate a potential therapeutic sensitization that occurs with *SETD2* mutations in RCC.

CONCLUSION: We show that *SETD2* loss in RCC induces activation of the UPR and offers a potential therapeutic mechanism which may be druggable using synthetic lethality. Furthermore, candidate therapeutic susceptibilities could lead to a biomarker-based clinical trial using repurposed FDA-approved therapies.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This work was made possible through the Molecular Therapeutics Undergraduate Research Fellowship Program at Fox Chase Cancer Center – Temple University Health System. This work was supported by DoD grant CA181178 (P. Abbosh) and the Fox Chase Cancer Center NCI Comprehensive Cancer Support Grant CA006927 (all investigators).

I would like to thank Drs. David Degraff and Ronald Markle for their critical reading of this work and invaluable support and insights. Additionally, I would like to thank Uttam Satyal, PhD, a post-doctoral candidate in the Abbosh lab for his guidance, support, and transfer of knowledge, as I learned the complex techniques and critical thinking required to complete many of these molecular based experiments.

Most importantly, I would like to thank my Principal Investigator, Philip Abbosh, MD, PhD. His mentorship and trust have profoundly impacted my thought processes. He taught me to ask good questions, formulate testable hypotheses, and investigate them rigorously using sound scientific principles and methods. He trusted my thoughts and skills, even when I wasn't sure I was worthy of that trust. This translational research experience has transformed my desire to make meaningful discoveries and contribute to scientific advancement with purpose. By allowing me to work in his lab and on this project, he has provided me a lifelong foundation in scientific inquiry. Finally, I am also indebted to my many colleagues in the Abbosh lab and the Molecular Therapeutics Instructional Program at Fox Chase Cancer Center for their relentless encouragement and support.

Chapter 1

Introduction

1.1 The Genetic Basis of RCC

Metastatic renal cell carcinoma (mRCC) remains an incurable disease for most patients. Over the last two decades, significant progress has been made in our understanding of the molecular basis of RCC leading to revolutionary targeted immunotherapy options. These systemic therapies have profoundly improved progression free and overall survival rates for patients with mRCC.

RCC represents a molecularly heterogeneous group of epithelial neoplasias of the kidney, each with unique molecular signatures. The predominant histologies are clear cell RCC (ccRCC), which accounts for approximately 60-70% of all kidney tumors, and papillary RCC, which accounts for 10-15%. The most commonly mutated region of the genome in RCC is the short arm of chromosome 3 (3p), where the tumor suppressor genes *VHL* (3p25.3), *BAP1* (3p21.1), *PBRM-1*(3p21.1) and *SETD2* (3p21.31) are located.[1] These four genes are the most commonly mutated genes in ccRCC, with *SETD2*-mutant tumors occurring in about 10% of total ccRCC cases and ~30% of metastatic ccRCC cases.[2]

The aim of this research is to investigate new molecular insights and therapeutic options for a well-defined population of patients with advanced RCC, specifically patients with deleterious mutations in the *SETD2* gene.

1.2 Chromatin Access and Histone Modifications

The nature of epigenetic modifications, specifically the modifications of histones, is highly relevant to the investigations of this thesis. The cellular DNA in eukaryotes is organized around histone octamers. These protein complexes are critical to DNA condensation into chromatin and chromosomes. Within each histone octamer, there are four distinct proteins – H2A, H2B, H3, and H4 – arranged in dimers with one another. These histone-DNA complexes have protein tails that can be modified post-translationally, impacting all DNA-based processes.[3] Modifications to these histone tails such as methylation or acetylation can therefore loosen or condense the DNA bound to them, thus regulating transcriptional activity. When a section of DNA is tightly packed, called heterochromatin, specific genes encoded within that sequence experience limited expression. When the DNA is loosened from the histone, called euchromatin, corresponding sections of genes have greater expression.[4] The rate of transcription is highly dependent on these actions and counteractions, which result in the ability or inability of biological machinery to gain access to and interact with the gene intended to be transcribed.

Epigenetic modification mechanisms have the ability to enact broad, genome-wide changes in transcription and subsequent translation.[5] With regard to cancer cells, studies have demonstrated genome-scale changes in covalent modifications at specific histone marks during oncogenesis and metastatic events including intravasation, colonization, and subsequent stage progression of tumors.[6,7] These covalent modifications can alter the extent to which regions of DNA are available for transcription. Two primary mechanisms include histone methylation, which is typically associated with loss of gene expression, and acetylation, which is more often associated with gain of gene expression. If, for example, the histones bound to a region of the

genome encoding for specific cellular genes are modified, the expression of neighboring genes may also be modified. Therefore, histone modification could positively or negatively affect expression of an oncogene or tumor suppressor with significant implications on cellular transformation. Whether these epigenetic changes are driver events and therefore required to induce carcinogenesis or passenger events that occur as the result of genetic modifications is not yet fully understood.

1.3 SETD2 and Histone Methylation

Methylation is the chemical modification of histone tails of lysine and/or arginine amino acid residues. This directly impacts chromatin compaction around histone proteins and therefore transcriptional activation of affected genes.[4] The gene of interest for this research, *SETD2*, encodes a 300 kDa histone methyltransferase that, while mutated in 10-15% of all ccRCCs and ~10% of papillary renal cell carcinomas, is dysfunctional in 30% of patients with metastatic disease.[8–10] The gene carries out its function via trimethylation of lysine within histone octamer H3, specifically on the 36th amino acid, a lysine residue (H3K36). The enzymatic activity of *SETD2* and histone mark H3K36 methylation is an evolutionarily conserved mechanism that has been firmly implicated in the proper splicing of mRNAs during transcription.[11–14] When undergoing transcription and translation, non-coding portions of the mRNA (introns) must be spliced out of the transcript before the protein is made. However, if *SETD2* is mutated and does not perform its function correctly by trimethylating histone mark H3K36, proper transcript splicing does not occur. Importantly, this could result in aberrant protein expression, structure, and function of *SETD2* dependent genes due to retained introns.

1.4 Synthetic Lethality and the Unfolded Protein Response

Similar to *SETD2*-mutant RCC tumors, other cancers such as melanoma also retain introns and have been shown to generate mutant peptides.[15,16] Recent advances in anti-tumor immunity have focused on directing T cells toward these misfolded proteins, which hold great promise in harnessing the immune system to fight cancer.[17,18]

One evolutionarily conserved pathway, the unfolded protein response (UPR), is responsible for detecting aberrant proteins made during translation. UPR then slows down protein synthesis and degrades misfolded proteins to maintain cellular homeostasis. In the absence of a functioning UPR, the cell will collect non-functional proteins in its cytosol and endoplasmic reticulum, ultimately affecting overall cell function. If these cellular stressors persist, apoptosis will occur to prevent proliferation of cells with aberrant function. In *SETD2*-mutant kidney cancers, the protein structure of aberrantly translated peptides would generate an expected increase in the amount of misfolded and unfolded proteins within the cell. This offers a potential therapeutic vulnerability with a mechanism to intervene by inducing synthetic lethality.

Synthetic lethality occurs when the loss of function of two independent signaling or metabolic pathways results in lethality, whereas the loss of function of either pathway alone is not lethal. When two genes exhibit a synthetically lethal relationship, candidate therapeutic inhibitors can be used to commit the cell to an apoptotic pathway.[19] Synthetic lethality can be used as a therapeutic rationale when loss of function of one pathway occurs due to mutation and the other pathway is inhibited pharmacologically. PARP inhibition in *BRCA1/2* inherited breast and ovarian cancer syndromes is based on synthetic lethality. In the case of *SETD2* deficiency, there is compensatory activation of the UPR pathway to maintain cellular function. If these

mutant cells are subjected to UPR inhibitors, they would be unable to clear aberrant protein buildup quickly enough inducing apoptosis within *SETD2*-mutant cells with minimal off target effects.

1.5 The CRISPR/Cas9 System

As gene editing technologies become more sophisticated, our ability to sequence and manipulate the cellular genome at specific sites and with greater accuracy is now possible. These mechanisms are currently being explored to gain insight into gene function and exploit them for therapeutic benefit. *In-vitro* manipulation of genes can model mutations commonly found to cause cancer, including insertion and deletion mutations (indels). This can help us understand how a cell acts under specific sets of genetic conditions and environmental stresses which can lead to mapping of mechanistic pathways, responses to approved treatments, and development of novel therapeutic approaches. Perhaps the most revolutionary tool to be developed to date for gene manipulation and functional studies is the CRISPR/Cas9 system.

The CRISPR/Cas9 system leverages an evolutionarily conserved defense mechanism that prokaryotes evolved to defend against viral infection. The system employs clustered regularly interspersed short palindromic repeats (CRISPR) arranged in an array of RNA sequences, each ~20 base pairs long.[20,21] When this array works in cooperation with a CRISPR associated (Cas) endonuclease, specific RNA sequences can detect possible viral genomic loci and target them for cleavage, defending the cell against viral infection.[21] There are two distinct RNAs required for CRISPR-Cas9 mediated cleavage of DNA: (i) CRISPR-RNA (crRNA), which recognizes foreign DNA through the 20-base pair complementary region called the protospacer adjacent motif

(PAM), and (ii) trans-acting CRISPR-RNA (tracrRNA) which is responsible for hybridizing with the crRNA.[22,23] When used in conjunction, these RNA sequences will guide the Cas9 endonuclease to a targeted gene of interest and generate a homologous double-stranded DNA break, mediated by the Cas9 enzyme. To facilitate the CRISPR-Cas9 system, the crRNA–tracrRNA can be combined into a chimeric single-guide RNA (sgRNA).[24] Because the sgRNA contains a region complementary to a DNA sequence of interest, the Cas9 enzyme can be directed to any genomic locus with an appropriate PAM sequence. After the sgRNA tracks the targeted sequence and induces a break, naturally occurring repair mechanisms can provoke indel mutations, causing a genetic knock-out through frameshift mutations or allowing researchers to insert desired sequences to study various manipulations of gene expression[25] In experiments detailed below, we use CRISPR gene editing technology to establish isogenic *SETD2* knock-out cell lines with a high degree of accuracy.

1.6 Preliminary Data

We began our exploration of *SETD2* mutant ccRCC cases by analyzing data from *The Cancer Genome Atlas* (TCGA) to understand the relationship between *SETD2*-mutant tumors and immune signaling pathways such as the UPR. The goal of these preliminary data was to understand whether our hypothesis was worth pursuing. Specifically, we hypothesized that if aberrantly retained introns were translated, they would form misfolded proteins that would trigger the UPR. To evaluate our hypothesis, we used hallmark gene set enrichment analysis (**GSEA**) - a statistical ranking method that identifies groups of genes that are activated or repressed across different conditions. By ranking the expression of genes found within a specific

genetic signature, gene sets with outlier rankings (high or low) can then be inferred to have been activated or repressed. Once gene expression has been ranked, a comparison between conditions (in this case, *SETD2* mutant and wildtype tumors) can identify signatures that are differentially expressed between the groups. Using this approach, our group found that the UPR signature was strongly upregulated in *SETD2*-mutant tumors (q=0.012, Figure 1) providing the basis for our subsequent work detailed below. Furthermore, several immune signaling pathways were ac , inf sig sh

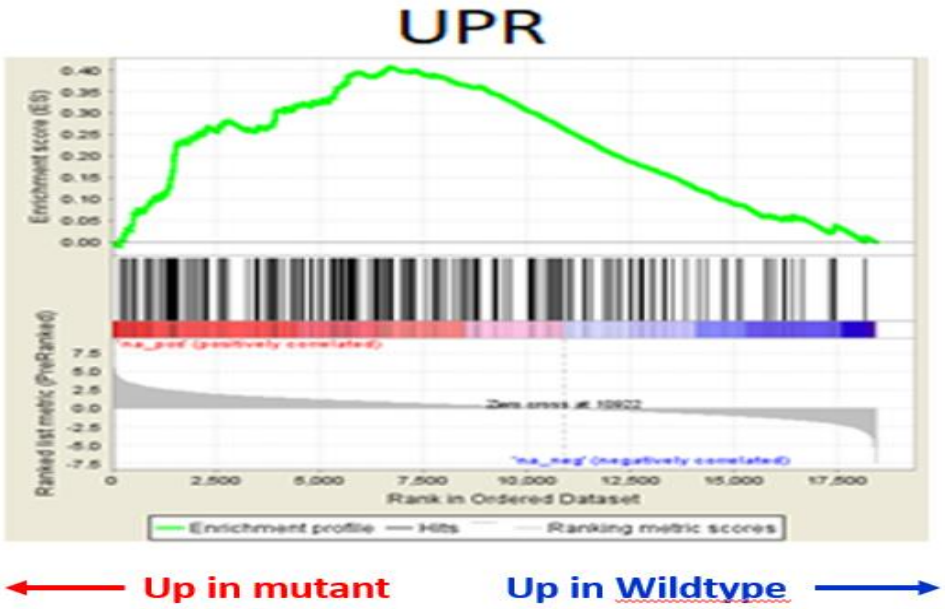

Figure 1: GSEA Demonstrating Relationship Between *SETD2* Mutations and Activated UPR

TCGA ccRCC tumors underwent Hallmark GSEA as grouped by SETD2 mutation status. Genes within each gene set are represented by black hatch marks. Concentration of hatch marks and the shift of the green curve to the left indicate that these genes are upregulated in SETD2-mutant tumors.

1.7 Concluding Comments

mRCC is a lethal and incurable disease in most cases. While the mutational burden and genotypes vary among patients, *SETD2* is mutated in ~10-15% of ccRCCs and 10% of pRCCs.[8–10] Our preliminary data using the TCGA demonstrate that *SETD2* mutant tumors are associated with activation of the UPR and highly active immune signaling pathways.

Here we hypothesize that these findings are due to intron retention during transcription, leading to subsequent translation of aberrantly folded peptides with disrupted function. These aberrantly folded proteins cause multiple cellular events including increased immune signaling and activation of the UPR pathway to minimize dysregulated growth. Unfortunately, these pathways appear insufficient to prevent *SETD2* mutant ccRCC progression.

Our specific aim was to demonstrate that *SETD2* loss in ccRCC causes a cellular dependency on the UPR. If this evolutionarily conserved mechanism were subjected to pharmacological inhibitors, we anticipate rapid accumulation of misfolded proteins, thereby stressing the endoplasmic reticulum and inducing apoptosis under conditions of synthetic lethality. Here, we develop a *SETD2*-mutant RENCA cell line and investigate this novel approach as a potential targeted therapy for patients with *SETD2* mutant RCC. The overall objective of this project is to evaluate the candidacy of *SETD2* mutation as a therapeutic vulnerability. Findings from this study could lead to future clinical trials targeting patients with *SETD2* mutant mRCC.

Chapter 2

Materials and Methods

2.1 Cell Culture

Standard sterile cell culture techniques were used throughout this project. 70% ethanol was used to sterilize all materials entering the cell culture hood. In addition, appropriate personal protective equipment was used to ensure no cell lines were contaminated as a result of passaging. Cell culture media for the RENCA cell line was prepared (RPMI Culture Media, 10% FBS, 1% Sodium Pyruvate, 1% L-Glutamine, 1% Non-Essential Amino Acids). Before passaging, culture media and 0.05% Trypsin-EDTA were warmed to approximately 37°C. Cells were removed from the incubator (37°C and 5% CO_2) and inspected under a microscope. After inspection, the cells were placed in the culture hood, old culture media was aspirated, cultures were washed with 1x dPBS(Dulbecco's Phosphate Buffered Saline (- Ca^{2+}, - Mg^{2+})) to remove residual debris. After aspiration of dPBS, trypsin-EDTA was added to remove cells from the growing surface. Trypsinizsed cells were incubated at 37° for 5 minutes. The appropriate cell culture media was added in a minimum of 2:1 ratio with trypsin-EDTA to stop trypsinization. Excess cell suspension was removed while leaving a fraction of cells behind to continue cell growth. Cell suspensions were then frozen, plated, or isolated for ongoing and future experimentation. Fresh media was added to the remaining cell culture for continuation of cell culture growth. Cells were then placed back in the incubator and left to grow until confluent.

2.2 Guide Design, Plasmid Cloning, and Restriction Enzyme Digest

Single guide RNA (sgRNA) sequences were chosen using sgRNA Designer (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design). Forward and reverse oligos were hybridized for each sgRNA and duplexed in a 20 µL reaction (16 µL of nuclease free H_2O, 2 µL T4 Ligase Buffer, 1 µL of 100 mM forward oligo, 1 µL of 100 mM reverse oligo). The duplex program helped the oligos to anneal as double stranded inserts by heating the samples to 95°C for 2 minutes and gradually cooling the sample down to 25°C over 45 minutes. Next, the duplexed oligos were phosphorylated using a 20 µL kinase reaction (10 µL nuclease free H_2O, 2 µL ATP, 1 µL PNK Enzyme, 2 µL PNK Buffer A, 5 µL duplexed oligo insert). Kinase reactions were incubated at 37°C for 20 minutes followed by 70°C for 10 minutes. The kinase/duplex product was then diluted 1:100 in nuclease free H_2O. The annealed, kinased, diluted insert was then ligated into the pre-digested pLCV2E vector backbone in a 10 µL ligation reaction (7 µL nuclease free H_2O, 1 µL T4 ligase buffer, 0.5 µL T4 Ligase, 0.5 pLCV2E vector backbone). The ligation reactions were incubated at 16°C for 20 minutes. 1 µL of ligation product was transformed into 50 µL of *E. coli* bacteria and streaked out onto pre-heated agar plates with ampicillin. Colonies formed overnight and were plucked, propagated, and purified using a QIAprep Spin Miniprep Kit (Qiagen 27106).

Restriction enzymes were purchased from New England Bio Labs.[26] For plasmid vector digestion and confirmation of inserts, a 50 µL reaction was used (5 µL 10x Cutsmart Buffer, 1µL BamHI, 1 µL Esp3I, 1 µg plasmid vector, 42 µL Nuclease Free H_2O). Each reaction was mixed with DNA loading dye (Thermo Fisher R0611) and digested at 37°C for >1 hr. Samples were resolved on 1% agarose gel containing ethidium bromide at a concentration of 0.5 µg/mL.

Samples were visualized using a FluorChem E system. Once inserts were visually confirmed they were sent for sequencing.

2.3 Sanger Sequencing

Plasmids confirmed to contain the desired insert were diluted to a concentration of 200 ng/µL. Samples were sent to Genewiz[27] for sequencing (800 ng plasmid product, 5 µL primer, 15 µL nuclease free H_2O). Results were confirmed using Chromas software.

2.4 Lentivirus Production, CRISPR Edits, DNA Extractions, and ICE Analysis

Lentiviruses were produced in HEK293T cells by transfection. Confluent HEK293T cells were transfected in a 10 cm dish using 3.2 µg of CMV-VSVG , 6.4 µg of psPAX2, 0.8 µg of pLentiCRISPR-E (Addgene #78852) containing sgRNAs directed to SETD2 or a GFP insert used as a negative control, and 30 µL of Lipofectamine 2000. pLentiCRISPR-E contains a cloning site for one sgRNA, *pyogenes* espCas9 v1.1, and a puromycin antibiotic resistance cassette. After two days' time, cell culture media containing lentiviral particles was collected and clarified by centrifugation.

RENCA cells were infected by adding lentiviral infected media with 8 µg/mL of polybrene. Cells were re-seeded and treated with an empirically determined dose of puromycin for one week following 48 hours of lentiviral infection. Qualitatively stable cell cultures were grown and collected for indel mutation quantitation. For indel mutation quantitation, DNA was isolated from pooled cells. The isolated DNA was subject to PCR-amplification. PCR products

were run on a 4%-20% TBE polyacrylamide gel to ensure amplification and monitor for heteroduplex formation, purified using QIAgen PCR cleanup kit, and subjected to Sanger sequencing as described above.[28] Analysis was done using ICE by Synthego.[29] The sequencing data was compared to the unedited DNA from negative control cells to confirm successful edits.

2.5 BCA Protein Assay

Protein concentration of cell pellets for immunoblotting was normalized using the Pierce BCA Protein Assay Kit (Thermo Scientific #23227). Manufacturer's instructions were followed, and subsequent dilutions and protein concentrations were normalized using Microsoft Excel.

2.6 Western Blotting

Cells were harvested, kept on ice, and lysed in cold RIPA buffer.[30] RIPA buffer was supplemented with protease and phosphatase inhibitors. After lysis of cell pellets, lysate samples were centrifuged for clarification. Sample protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher #23227). Lysates were denatured through the addition of DTT and LDS loading buffer. Samples were then boiled at 100°C for 10 minutes. Equal amounts of protein were loaded into each well of a NuPAGE 4-12% Bis-Tris polyacrylamide gel and separated via gel electrophoresis. Resolved proteins were then transferred onto a polyvinylidene difluoride membrane, blocked in 5% milk made in TBST (0.1% Tween 20, Tris Buffered Saline Sigma #T-6664) and probed for the desired primary antibody. The appropriate species-matched HRP-labeled secondary antibodies were used

(antibody information found in Supplementary Tables 1&2). Both primary[31] and secondary[32] antibodies were made with 5% BSA (Fisher Bioreagents #BP1600-100) in TBST. Primary antibody was diluted 1:1000 while secondary antibody was diluted 1:100. Proteins were developed/visualized using ECL prime substrate (GE Healthcare #RPN2232).

2.7 Fluorescence Microscopy

All fluorescence microscopy was performed using the Leica SP8 confocal microscope at 630x. *ATF6 Localization:* Approximately 40,000 cells were plated in each well of multiple 8-well chamber slides. Samples included an unedited negative control, a negative control CRISPR edited without an insert, and two *SETD2*-mutant samples that were genetically modified using CRISPR sgRNAs inserted into a backbone plasmid. Cells were washed in ice cold PBS, fixed in 4% paraformaldehyde for 10 minutes, and permeabilized using 0.2% TritonX-100. Permeabilized cells were blocked for 30 minutes using 1% BSA containing 25 mg/mL glycine in PBST (PBS + 0.01% Tween-20). Cells were subsequently incubated with anti-ATF6 antibody (1:100) diluted in blocking solution for 1 hour at room temperature. Cells were incubated in donkey anti-rabbit secondary antibody conjugated to Brilliant Violet 510 for 1 hour at room temperature and counter stained using DRAQ5, a far-red nuclear DNA dye.

Chapter 3

Results

3.1 CRISPR Target, sgRNAs, and Primer Design

The area of the *SETD2* gene targeted for Cas9 repression was located in Exon 10 along the coding region for the SET domain (nucleotides 1561-1667). This section of DNA is important for *SETD2* function as it is homologous to the human gene and this functional domain is key for the enzyme's activity.[33] Additionally, this section of DNA contained no sites which could be targeted by the restriction enzymes BamH1 and Esp3I, which was located right at the end of the U6 promoter. These restriction enzymes were used to determine if inserts were present in the vector backbone plasmid after ligation as shown below in **Figure 2**. Given that the target is contained within an exon, disruption of this region was expected to disrupt *SETD2* activity. The oligos and primers as ordered are listed in **Supplementary Tables 1 & 2**.

3.2 Restriction Enzyme Digest and Plasmid Verification

The plasmids containing negative control and sgRNA inserts were digested using BamH1 and Esp3I restriction enzymes. The restriction digests were resolved on an agarose gel with two controls, a single cut digest and a double cut digest. The customized vector backbone has an Esp3I restriction site associated with the location of insertion for the sgRNAs utilized. This site should be destroyed when the insert is present, but present when the insert is absent. Therefore, an empty ligation product would result in the plasmid being cut at two sites, resulting in the

observation of two bands following gel electrophoresis. On the other hand, presence of only one band would indicate that the insert has interrupted the Esp3I cut site and the only remaining restriction site is BamH1. **Figure 2** shows the vector backbone used for insertions. **Figure 3** shows an effective restriction enzyme digest for all samples excluding sgRNA-1B. This demonstrates that nearly all plasmid products contained an insert due to the removal of the Esp3I restriction enzyme site.

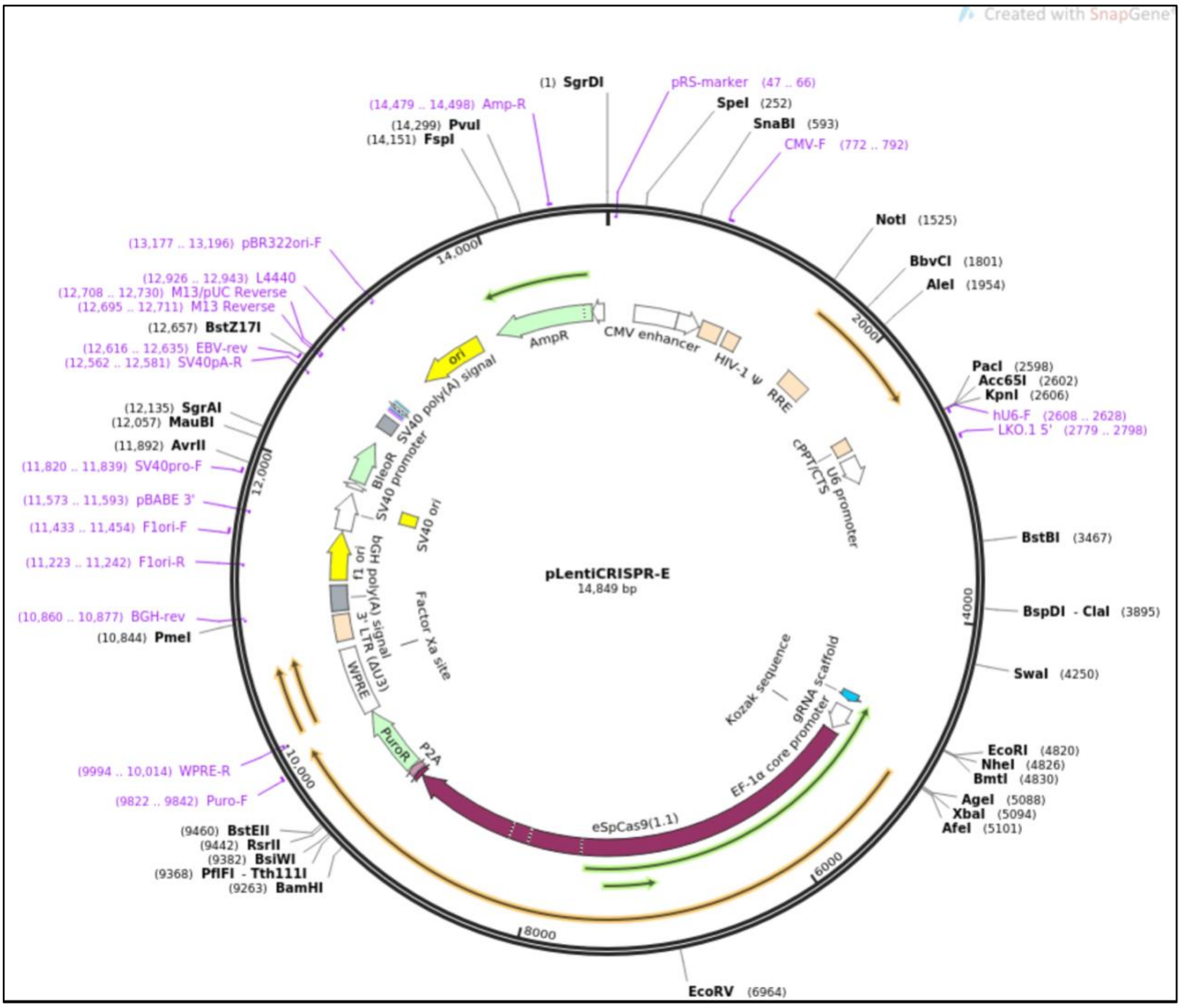

Figure 2: pLentiCRISPRV2-E Empty Backbone Plasmid

Supplied by Addgene, this graphic demonstrates the general organization of an empty lentiviral backbone vector (pLCV2E) used to introduce sgRNAs. This vector contains eSpCas9 and a puromycin resistance cassette.

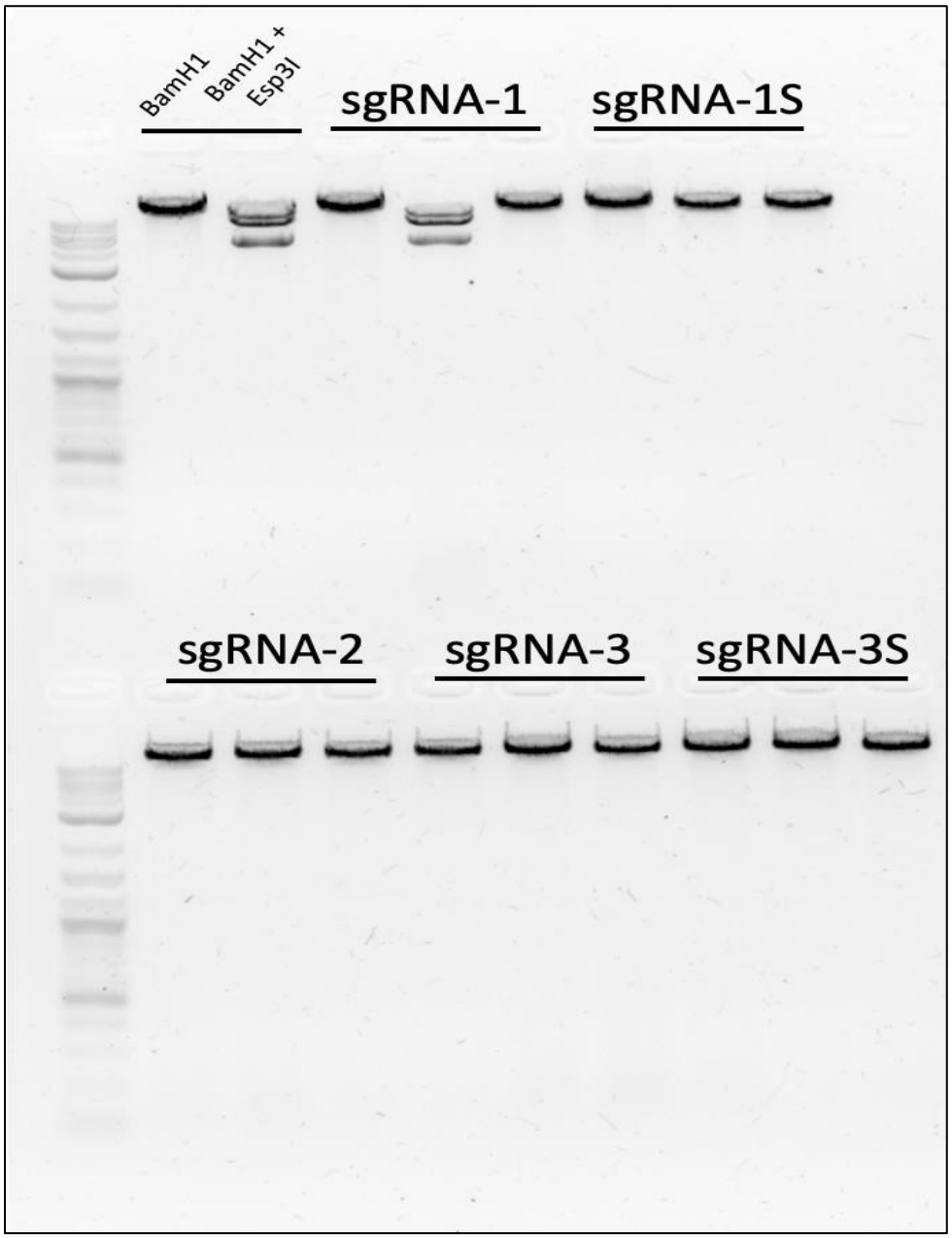

Figure 3: Agarose Gel Demonstrating Successful Ligation Products

Products of the plasmid ligation were confirmed visually on the agarose gel above. Each mmSETD2 guide was isolated from bacterial colonies in triplicates to increase probability of a successful insert being selected. Single bands indicate the presence of the desired insert while double bands indicate an unsuccessful ligation of the insert into the vector backbone.

3.3 Cloning, Sanger Sequencing, and Selection of CRISPR Guides

Before infection of the pLCV2E *Setd2* guide-containing plasmids into 293T cells, we confirmed the inserts using Sanger sequencing. The confirmed plasmid constructs were transformed into *E. coli* bacteria for preparation of plasmid adequate for transfection (i.e. endotoxin-free plasmid) using the QIAgen MidiPrep kit. Following plasmid preparation, lentivirus was constructed and used to generate stable cell RENCA lines as described in methods, samples were sent for Sanger sequencing. These sequences were consistent with that of the intended plasmid, thereby indicating that the cloning of these plasmids with their respective guide RNAs was successful and could therefore be transformed into the murine RCC line, RENCA.

3.4 Confirmation of *SETD2* Knockout

Following generation of stable RENCA cells, DNA from the newly established cell line was isolated, and used to amplify a PCR product whose primers flanked the anticipated Setd2 CRISPR targeting site. The amplicons were sent for Sanger sequencing. The sequencing results were broken down into two categories to identify which sgRNA insert was the most effective in editing our gene of interest. After analyzing the indel % and knockout score of the five total CRISPR guides, two of the guides, SETD2-sgRNA1 and SETD2-sgRNA3s, exhibited an indel % and knockout score of 0, indicating an unsuccessful CRISPR edit. The remaining three cell lines – SETD2-sgRNA1s, SETD2-sgRNA2, and SETD2-sgRNA3 – exhibited an indel incidence of 20%, 70%, and 67% respectively, indicating that the targeted gene of interest was partially ablated.

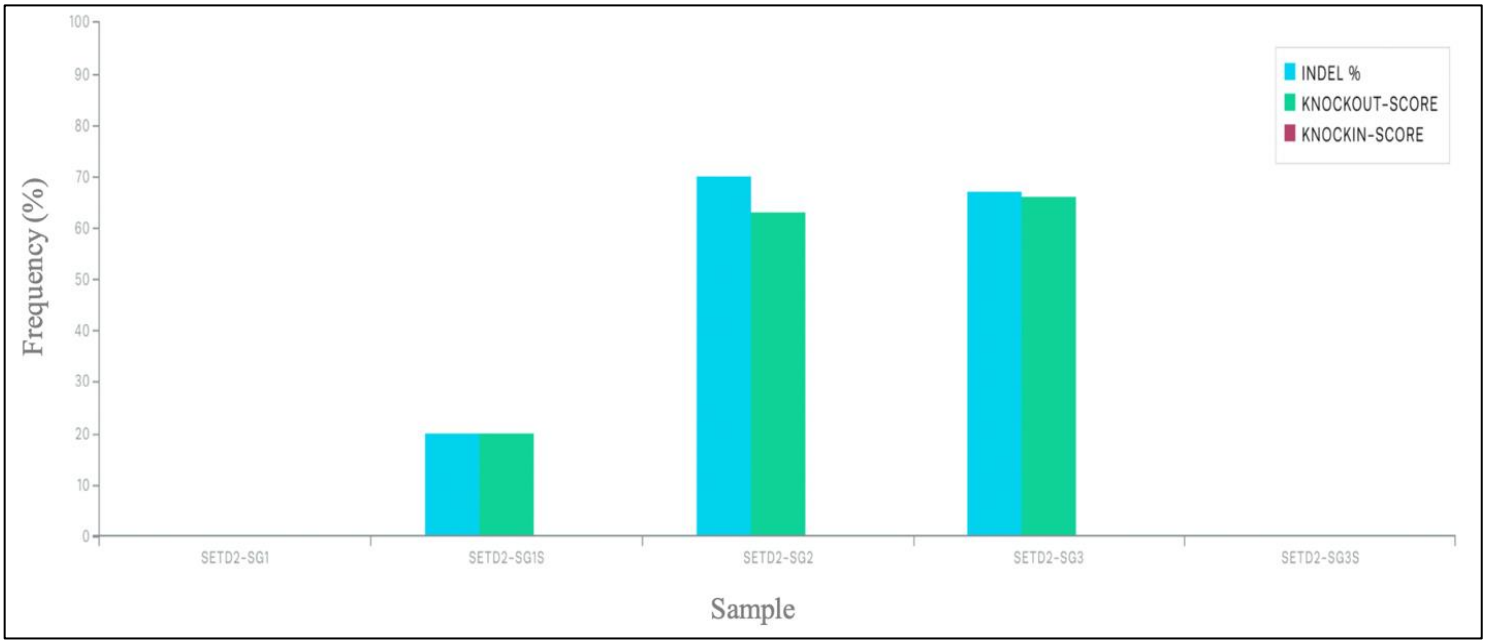

Figure 4: Indel Percentage and Knockout Efficiency of *SETD2* CRISPR Pooled Cells

Supplied by Synthego[29], this graphic depicts the efficacy of each sgRNA following transfection and puromycin selection. sgRNA2 and sgRNA3 both show an indel percentage exceeding 65%, which confirms partially successful ablation of the SETD2 gene.

3.5 Isogenic Knockout Immunoblot

Following knockout confirmation via sanger sequencing, lysates of *Setd2* CRISPR pooled cells and negative controls were subjected to western blot. The blot was probed using primary and secondary antibodies conjugated to three proteins: *SETD2*, Actin, and H3trimethylK36. The sgRNA1 pooled cell line was blotted to validate the pLCV2E negative control pooled cells and the two cell lines with the highest knockout scores were compared. Furthermore, HEK293T pooled cells were blotted as a positive control as these cells were expected to express *SETD2* properly. Protein expression for these five cell lines is shown in **Figure 5.**

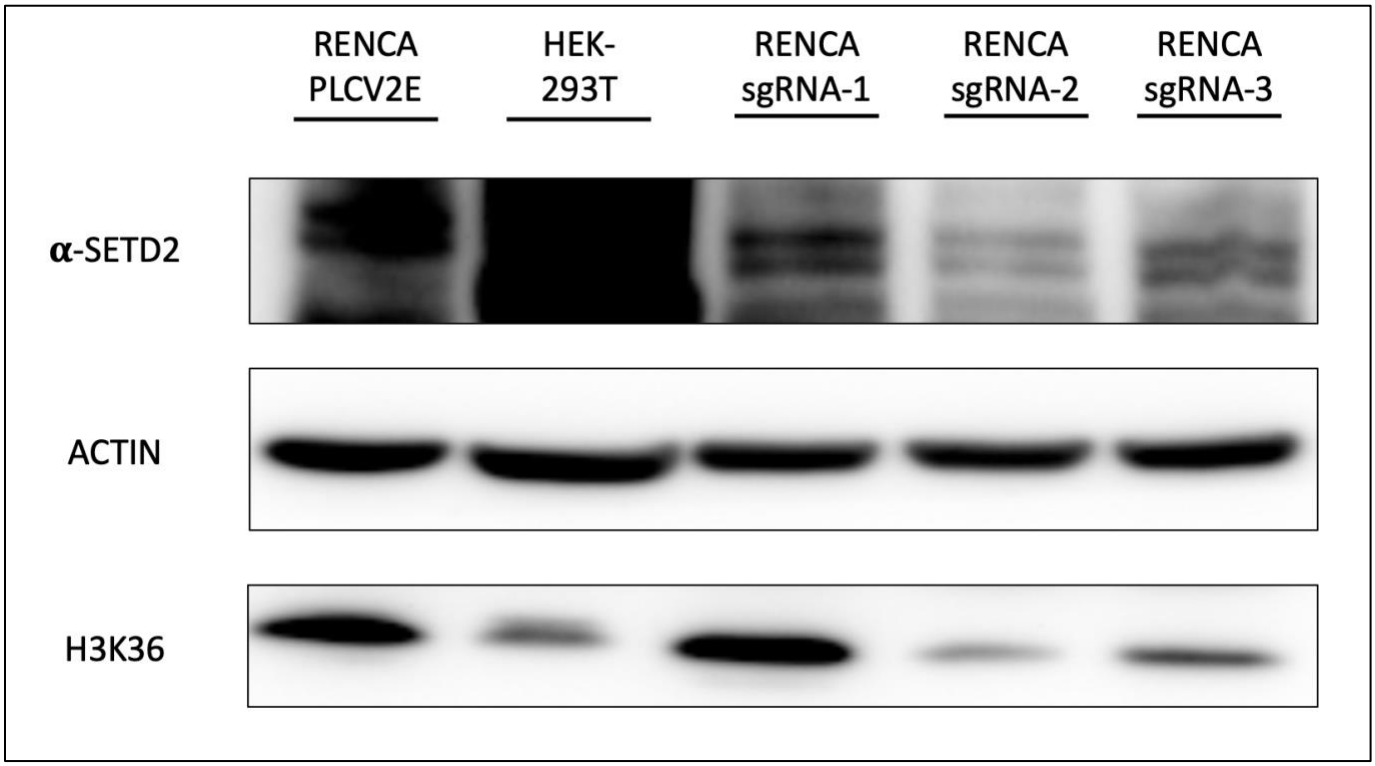

Figure 5: Loss of *SETD2* Results in Decreased H3K36me3 Expression in Isogenic Knockout Cells

There is a direct relationship between the presence of SETD2 and trimethylation of the histone mark H3K36. This is consistent with previous findings, indicating that intron splicing may be affected in the isogenic knockout cell lines.

3.6 Measurement of the UPR

Three transmembrane proteins are responsible for activation and subsequent regulation of the UPR mechanism. These proteins, Inositol Requiring 1α (IRE1α), PKR-like ER kinase (PERK), and Activating Transcription Factor 6 (ATF6), become upregulated as part of an adaptive response to ER stress.[34] As described in **Section 1.6**, the UPR transcriptional signature is prominently enriched *SETD2*-mutant tumors (q=0.012). We hypothesis that this enhancement of the UPR activity is thought to be the result of misfolded proteins that have been targeted for degradation. After successfully knocking out *SETD2* and reducing H3K36 trimethylation, the activity of the UPR in edited cell lines was measured via western blot. **Figure 6** depicts two

negative controls, including the unedited RENCA and pLCV2E empty vector backbone cell lines, in comparison to the two most successful knockout cell lines, RENCA-mutant sg2 and RENCA-mutant sg3.

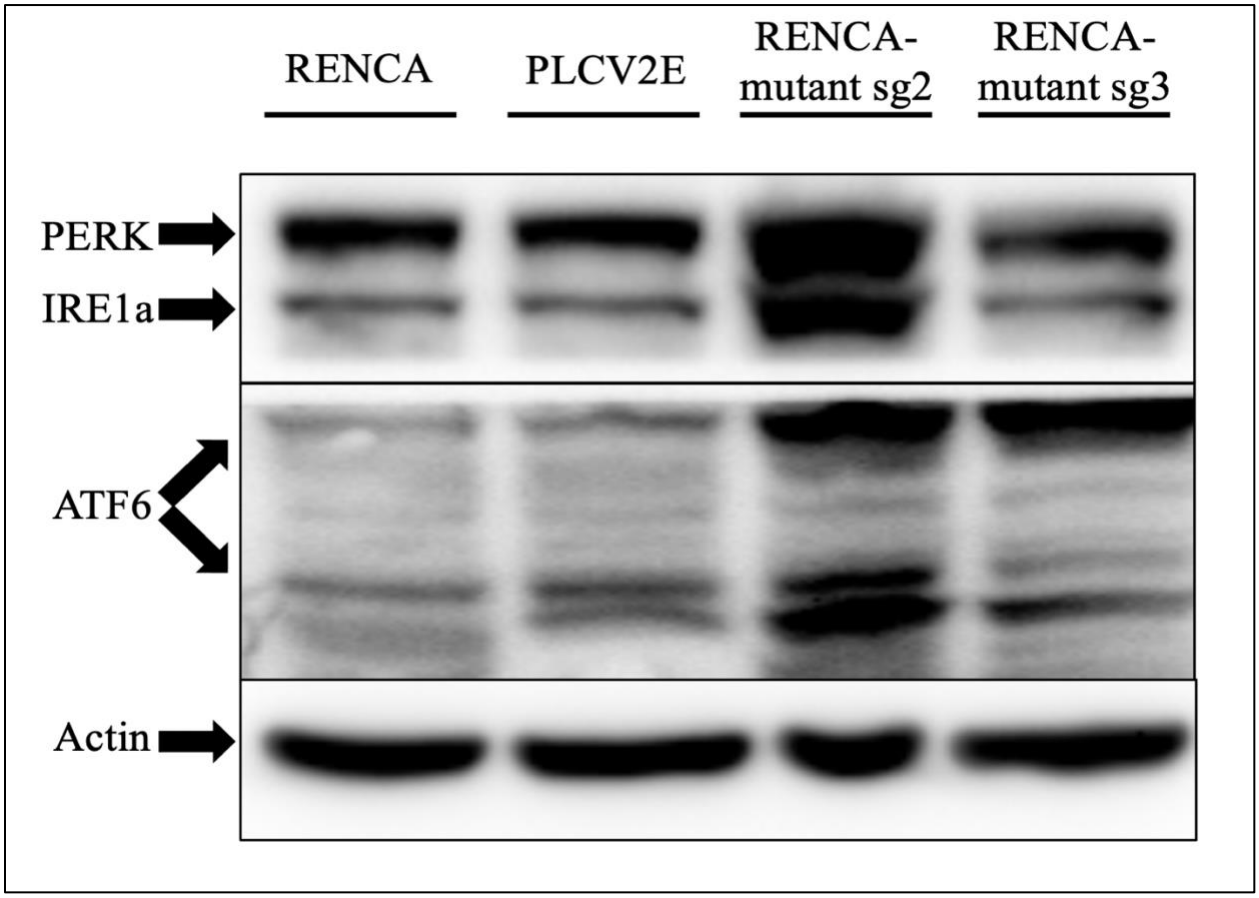

Figure 6 Loss of *SETD2* Results in Activated Arms of the UPR

Increased expression of three master regulators in the UPR mechanism were observed to be upregulated in pooled cell lines that showed a decreased expression of SETD2 and H3K36 trimethylation. This is consistent with GSEA results.

3.7 Immunofluorescent Validation of ATF6 Subcellular Localization

Upon activation of the UPR, the alpha isoform of the transmembrane transcription factor ATF6 will travel to the Golgi, where it is cleaved and subsequently transported to the nucleus to regulate transcription of UPR genes.[34] Given that ATF6α demonstrates nuclear localization

when the UPR is activated, we hypothesized that unedited RENCA cell lines would exhibit cytoplasmic ATF6α due to an inactive UPR. We further hypothesized that *SETD2*-mutant RENCA cell lines would exhibit nuclear localization, further corroborating activation of the UPR. To test this hypothesis, the aforementioned cell lines were subjected to confocal immunofluorescence microscopy as shown in **Figure 7.**

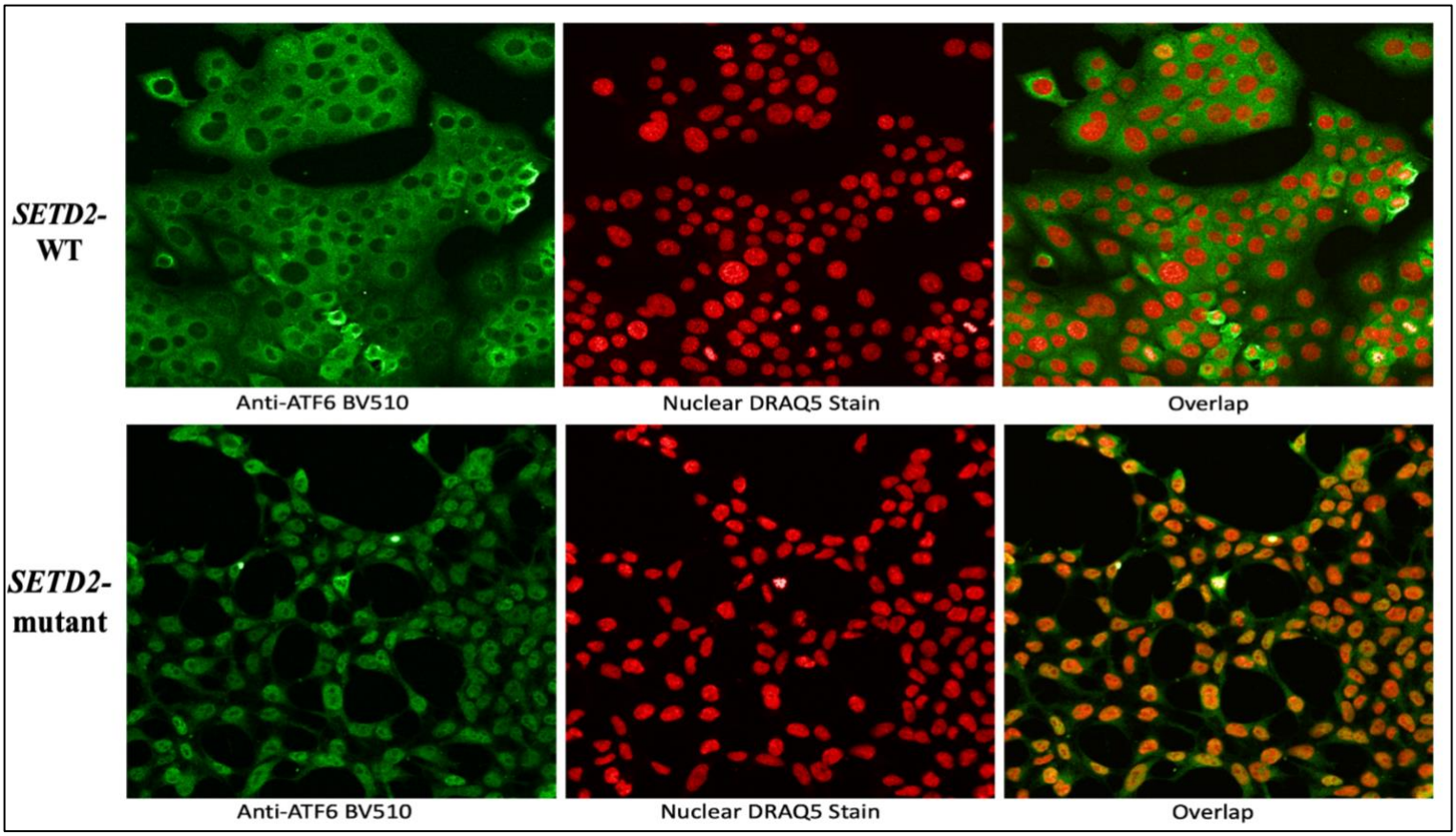

Figure 7: Loss of *SETD2* Results in Nuclear Localization of ATF6α

ATF6α is cytoplasmic when SETD2 is intact (SETD2-WT) and localizes to the nucleus upon CRISPR induced KO

Chapter 4

Discussion

Renal cell carcinomas (RCC) are epithelial tumors arising from the metabolically active renal tubules. The incidence of RCC is approximately 76,000 per year in the U.S. resulting in roughly 14,000 cancer specific deaths.[35] Across all races, ages, and genders, RCC occurs in nearly 16/100,000 individuals per year. Risk factors include age (median at diagnosis = 64 years), male gender, and a host of putative potentially modifiable risk factors including smoking, obesity, and hypertension.[36] Overall survival rates for mRCC have historically been measured in months, such that a patient presenting with mRCC prior to 2006 had a median survival of only 10-12 months.[37] Today, five-year relative survival rate across all stages and types of RCC is approximately 75%, meaning only one in four patients with RCC will die of disease within five years of diagnosis. In 2021, thanks to significant advances in our understanding of the molecular basis of RCC and systemic therapeutic interventions, those patients who present with or develop metastatic disease have a median overall survival that exceeds 40 months.[38]

While great progress has been made in the treatment of stage IV disease, mRCC remains incurable for most patients. Until 2003, there were few effective systemic treatment options due to chemotherapeutic resistance. Additionally, the only effective forms of immunotherapy (interferon-alpha and Interleukin-2) had overall response rates which were consistently <10%.[38] Given significant advancements in the understanding of the molecular causes of mRCC, advanced therapies targeting the VEGF pathway became available in the mid-2000s including oral receptor tyrosine kinase inhibitors (TKI), mTOR inhibitors, and monoclonal antibodies against VEGF. Dozens of randomized clinical trials led to incremental improvements in progression free and overall survival using single and combination therapies inhibiting tumor-

dependent, but not mutation-specific pathways.[38] More recently, second generation immunotherapies including checkpoint inhibitors have significantly improved survival rates in mRCC. When combined with TKIs, the effect is profound, with 70% of patients achieving at least partial response in most trials reported so far and median overall survival rates measuring >48 months for TKIs in combination with PD1 inhibitors.[39]

Importantly, all currently available therapies are used to treat any histologic subtype of RCC, regardless of their genetic makeup and driver events. Data from the TRACERx trial have demonstrated that mRCC is comprised of an ecosystem of malignant clones with multiple branchpoints that are constantly evolving in a struggle for survival and dominance.[40] Stochastic and therapeutic pressures change the malignant ecosystem in both primary and metastatic RCC cones. This is a potential cause of therapeutic escape and progression for the most lethal clones.

Targeting specific mutant pathways is an evolving strategy. Recently, a new therapy targeting a VHL mutant pathway was developed and approved by the FDA as a novel mechanism for patients with ccRCC due to VHL disease. The drug, belzutifan (previously MK-6482), is an oral inhibitor of hypoxia-inducible factor (HIF) 2α. It is associated with a 36% response rate in treatment naïve ccRCC associated with VHL as well as other VEGF driven VHL tumors of the pancreas, retina, and central nervous system.[41] Belzutifan is the first therapy developed and approved with an indication to treat kidney cancers with a specifically defined mutation. Here, we explored the mechanisms of *SETD2* mutant kidney cancers to understand potential therapeutic vulnerabilities of these more aggressive RCC clones in a similar manner.

Recent studies have linked the pathophysiology of various cancers to the variable functionality of histone modifying enzymes, including methyltransferases.[42] Novel mutations of histone modifying and chromatin remodeling genes have been identified in RCC. Located near

VHL on chromosome 3p, mutations in several genes, *PBRM1*, *BAP1*, and *SETD2* have been strongly implicated in RCC development and progression. Specifically, mutations to *SETD2* along with mutations in *BAP1*- have been associated with poor overall survival.[1]

Our histone modifier of interest, *SETD2,* has a mutational incidence of 10-15% of human ccRCC primary tumors and approximately 30% in mRCC patient samples.[8–11] Collectively, the observation of a significant increased prevalence of *SETD2* mutations in mRCC foci suggests a role for *SETD2*-mutations in promoting progression.[2] While the precise mechanism whereby *SETD2* mutations provide a permissive ecosystem for mRCC is unknown, clues may be derived based on the known role of the *SETD2* in proper intron splicing. Multiple investigators have demonstrated that *SETD2*-mutant tumors retain introns that otherwise would not have been transcribed. In *SETD2*-mutant RCC, it has been estimated that one out of every four transcripts is aberrantly spliced.[11] The loss of *SETD2* may therefore be a significant cause of widespread genomic instability, aberrant splicing processes, whole cell RNA processing deficiencies, and may therefore impact multiple protein based biological processes ranging from cell division to differentiation to apoptosis.[2,11] We sought to extend these observations and use them to explore additional mechanisms of *SETD2* mutant RCC in an effort to provide potential therapeutic insights.

Our first step was to create a *SETD2* knockout RCC cell line to facilitate subsequent mechanistic studies. We designed and developed a vector backbone plasmid for insertion of sgRNA oligos using the CRISPR-Cas9 system. This was then used to generate lentivirus to apply to a well-established RENCA murine cell line. Sanger sequencing was used to confirm successful transfection before subjecting CRISPR knockout cells lines to immunoblot. Edited cell lines displayed marked decreases in H3K36 trimethylation, indicating successful knockout

of *SETD2* function. It should be noted that we observed minimal residual H3K36 trimethylation in knock out cell lines, suggesting incomplete ablation of *SETD2* function in a minority of cells. This was likely due to heterogeneity of pooled cell knockouts. In the remainder of our studies, we isolated single cell clones with high indel rates as measured by Sanger sequencing. Given a consistent actin loading control and the fact that *SETD2* is solely responsible for this modification, we believe our resultant cell line successfully ablated *SETD2* function and thereby created an isogenic cell line, with *SETD2* knockout being responsible for differential effects in protein expression.

Once we had a working *SETD2* mutant RCC cell line, we were able to explore the mechanisms underlying cellular function under various conditions. Luco et al (2010) identified and demonstrated a critical relationship between distinctive histone modifications and the outcomes of alternative splicing. Their findings illustrated that epigenetic transcriptional modifications, including trimethylation of specific histone marks, directly impact gene splicing outcomes through active modulation of the transcriptional machinery.[13] Simon et al (2014) substantiated these findings by linking mutations in *SETD2* with H3K36me3 loss, leading to variations in chromatin accessibility and intron retention. Our understanding of these mechanisms, in combination with our preliminary analysis of TCGA *SETD2* mutant tumors, led us to hypothesize that *SETD2* mutant RCC cells retain introns during transcription, leading to misfolded proteins and subsequent UPR upregulation. We further hypothesized that these mechanisms may be responsible for *SETD2* mutated RCC cell survival or potentially offer targetable strategies using a synthetic lethal approach or enhanced immunogenicity.

It has been previously demonstrated in melanoma models that retained introns may be translated.[16] These aberrantly translated introns can stimulate a cell survival mechanism termed

the unfolded protein response (UPR) which recognizes and degrades the aberrant proteins in an effort to avoid cellular death from accumulating ER stress. The UPR is a three-step survival mechanism. First, the UPR will halt the production of proteins in the ER to avoid increasing cellular stress levels and potentially inducing apoptosis. Next, the UPR will work to degrade unfolded or misfolded proteins. Finally, the UPR will produce chaperones to facilitate proper protein folding and reestablish cellular homeostasis. We hypothesized that if the UPR is upregulated in *SETD2* mutant RCC to maintain homeostasis and promote cell survival, then inhibitors of the UPR may facilitate cellular death via apoptosis using a synthetic lethal approach. One possible therapeutic scenario is to combine UPR inhibitors with angiogenesis inhibitors in *SETD2* mutant RCC. Angiogenesis inhibitors, which are commonly used agents to treat kidney cancer, may therefore enhance a mutation-specific response by promoting apoptosis.[43]

In our experiments, we confirm that *SETD2* mutant RCC cells exhibit upregulation of the UPR. Pooled mutant cell lines that exhibited significant decreases in H3K36 trimethylation concurrently showed marked increased UPR activity. This is in line with our hypothesis as well as mechanistic data from multiple other investigators.[11,13] Cellular survival and propagation of these isogenic *SETD2* mutant cells may be the result of upregulated UPR activity. The activated UPR may therefore be a therapeutic vulnerability in *SETD2*-deficient tumors. If proper inhibitors of this cell survival mechanism are used in conjunction with other targeted and immunotherapies, this sensitization could lead to enhanced apoptosis in *SETD2*-mutant cancer cells. Currently, the most effective strategies for mRCC in the clinic is a combination of tyrosine kinase (angiogenesis) inhibitors and immunotherapies.[38] The mechanisms described here may offer a synergistic approach by inhibiting the UPR to induce synthetic lethality as well as enhance

neopeptide expression and thereby immunotherapy recognition and activation. For example, this vulnerability could leverage synthetic lethality by decreasing cell viability following treatment with pharmacological UPR inhibitors such as sunitinib[44], STF-083010[45], MKC-3946[46], KIRA6[47], and GSK2656157.[48]

While provocative, there are limitations to our data and our findings. One potential concern is that *SETD2* may not be the sole contributor of UPR pathway activation in our cell model. In other words, redundant and overlapping pathways in *SETD2* mutant cells may contribute more profoundly to UPR upregulation. If this were the case, other candidate vulnerabilities could be explored using inhibitors of chromatin or microtubule structures, which would subsequently cause genomic instability and cellular apoptosis.[49] Additionally, residual H3K36 trimethylation despite strong ablation of *SETD2* could be a result of heterozygous knockouts. According to the two-hit hypothesis, both alleles in the gene pool would have to undergo successful CRISPR edits to result in complete loss of H3K36 trimethylation. To minimize this risk, we later selected, sequenced, and propagated single cell clones with the 100% indel rate for study. Future explorations here would require isolation and establishment of single cell clones confirmed to have homozygous *SETD2* knockouts. These experiments are currently underway.

To further substantiate our results, our next steps are to interrogate multiple *SETD2* knockout cell line and single cell clones with pharmacological inhibitors of the UPR. We hypothesize that proteasome or UPR pathway inhibitors will eliminate the ability for cells to clear intron retaining peptides that have been misfolded. Cell viability assays will be used in future experiments to evaluate a small panel of inhibitors that investigates *SETD2* mutant response to loss of UPR functionality. Additionally, samples will be sent for RNASeq to measure

the specific rate of intron retention. This will help fill in the mechanistic gap between transcription and UPR activation. Finally, to determine if retained introns persist through translation, lysates from *SETD2*-deficient cell lines will be subjected to proteomic analysis via liquid chromatography and mass spectrometry (LC/MS/MS) and compared to expected protein sequences given RNASeq results. Comparison between proteomics data and inferred peptide structures from *in silico* translation of retained introns would further substantiate our hypothesis. These data would then form the basis for in vitro and *in vivo* testing of synergistic mechanism to treat *SETD2* mutant RCC with the hope of initiating a clinical trial using combination therapy to include a UPR inhibitor, an anti-angiogenic and an immunotherapeutic in a well-defined population of patients with mRCC and *SETD2* clonal expansion. To date, the Abbosh lab continues to investigate the mechanisms described in this thesis to further validate and extend our findings.

Chapter 5

Conclusion

Here we present our data showing that ccRCCs with mutation of *SETD2* have distinct immunological and biological features that may have exploitable vulnerabilities. We designed and created a *SETD2* mutant RCC cell line using CRISPR/Cas9 techniques. Using in vitro isogenic models, we explored potential mechanisms of *SETD2* mutant cell survival and demonstrate activation of the UPR thereby demonstrating a unique therapeutic susceptibility. The experiments outlined in this thesis indicate a potential novel mechanism and treatment approach to *SETD2* mutant RCCs clonotypes.

Patients with deleterious mutations in *SETD2* may retain introns which are subsequently translated. We have shown that the knockout of *SETD2* significantly impacts trimethylation of histone mark H3K36. Furthermore, our results illustrate UPR upregulation in the same CRISPR edited cell lines. Although the mechanism is not yet fully understood, *SETD2*-deficiency could contribute to UPR activation because of intronic peptides that the cell is trying to degrade for its own survival. This can be exploited as a therapeutic weakness, leveraging a synthetic lethal and/or enhanced immunogenic approach. If the UPR is rendered non-functional, unfolded proteins will accumulate stressing the cell to the point of apoptosis. This could be the basis of a personalized targeted therapeutic approach with less off target toxicity. By targeting the UPR mechanism with approved pharmacological inhibitors, we hope to use this novel approach to exploit candidate therapeutic susceptibilities in genetically definable subsets of kidney cancer patients. As with all foundational mechanistic science, we hope to bring this strategy forward into a clinical trial for the benefit of those afflicted with advanced RCC.

Appendix A
Supplementary Tables

Table 1: sgRNAs used for CRISPR Transfection

Oligos were designed as RNA to be duplexed, kinased, diluted, and ligated into a custom pLCV2E backbone.

Guide Name	sgRNA Sequence	Length (bp)
mmSetD2 sg1F	CACC GAAGTACTGTAAGAACCCCG	20
mmSetD2 sg1R	AAAC CGGGGTTCTTACAGTACTTC	20
mmSetD2 sg1sF	CACC GTACTGTAAGAACCCCG	17
mmSetD2 sg1sR	AAAC CGGGGTTCTTACAGTAC	17
mmSetD2 sg2F	CACC GAATGTGAAACACAAAACCA	20
mmSetD2 sg2R	AAAC TGGTTTTGTGTTTCACATTC	20
mmSetD2 sg3F	CACC GAAGTACATGCAGAAGTTTG	20
mmSetD2 sg3R	AAAC CAAACTTCTGCATGTACTTC	20
mmSetD2 sg3sF	CACC GTACATGCAGAAGTTTG	17
mmSetD2 sg3sR	AAAC CAAACTTCTGCATGTAC	17

Table 2: Primers Used for SETD2 PCR

Primers were designed and used to amplify the targeted insertion within the ligated CRISPR plasmid.

Primer Name	Sequence	Length (bp)
mmSetD2 survF	GCACAGTTGAGCAGCATGTC	20
mmSetD2 survR	GCTCAGGATGGTGGGTCATC	20

Bibliography

1. Piva, F. *et al.* BAP1, PBRM1 and SETD2 in clear-cell renal cell carcinoma: Molecular diagnostics and possible targets for personalized therapies. *Expert Rev. Mol. Diagn.* **15**, 1–10 (2015).
2. González-Rodríguez, P. *et al.* SETD2 mutation in renal clear cell carcinoma suppress autophagy via regulation of ATG12. *Cell Death Dis.* **11**, 69 (2020).
3. Lawrence, M., Daujat, S. & Schneider, R. Lateral Thinking: How Histone Modifications Regulate Gene Expression. *Trends Genet.* **32**, 42–56 (2016).
4. Strahl, B. & Allis, C. Strahl, B. D. & Allis, C. D. The language of covalent histone modifications. Nature 403, 41-45. *Nature* **403**, 41–45 (2000).
5. Yuan, S. *et al.* Global Regulation of the Histone Mark H3K36me2 Underlies Epithelial Plasticity and Metastatic Progression. *Cancer Discov.* **10**, 854 LP – 871 (2020).
6. Cardenas, H. *et al.* TGF-β induces global changes in DNA methylation during the epithelial-to-mesenchymal transition in ovarian cancer cells. *Epigenetics* **9**, 1461–1472 (2014).
7. McDonald, O. G., Wu, H., Timp, W., Doi, A. & Feinberg, A. P. Genome-scale epigenetic reprogramming during epithelial-to-mesenchymal transition. *Nat. Struct. Mol. Biol.* **18**, 867–874 (2011).
8. Sato, Y. *et al.* Integrated molecular analysis of clear-cell renal cell carcinoma. *Nat. Genet.* **45**, (2013).
9. Network, C. G. A. R. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* **499**, 43–49 (2013).
10. Network, C. G. A. R. *et al.* Comprehensive Molecular Characterization of Papillary

Renal-Cell Carcinoma. *N. Engl. J. Med.* **374**, 135–145 (2016).

11. Simon, J. M. *et al.* Variation in chromatin accessibility in human kidney cancer links H3K36 methyltransferase loss with widespread RNA processing defects. *Genome Res.* **24**, 241–250 (2014).
12. Pradeepa, M. M., Sutherland, H. G., Ule, J., Grimes, G. R. & Bickmore, W. A. Psip1/Ledgf p52 binds methylated histone H3K36 and splicing factors and contributes to the regulation of alternative splicing. *PLoS Genet.* **8**, e1002717–e1002717 (2012).
13. Luco, R. F. *et al.* Regulation of alternative splicing by histone modifications. *Science* **327**, 996–1000 (2010).
14. de Almeida, S. *et al.* Splicing enhances recruitment of methyltransferase HYPB/Setd2 and methylation of histone H3 Lys36. *Nat. Struct. Mol. Biol.* **18**, 977–983 (2011).
15. Giannopoulou, A. F. *et al.* Gene-Specific Intron Retention Serves as Molecular Signature that Distinguishes Melanoma from Non-Melanoma Cancer Cells in Greek Patients. *Int. J. Mol. Sci.* **20**, 937 (2019).
16. Smart, A. C. *et al.* Intron retention is a source of neoepitopes in cancer. *Nat. Biotechnol.* **36**, 1056–1058 (2018).
17. Keskin, D. B. *et al.* Neoantigen vaccine generates intratumoral T cell responses in phase Ib glioblastoma trial. *Nature* **565**, 234–239 (2019).
18. Ott, P. A. *et al.* An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* **547**, 217–221 (2017).
19. Brough, R., Frankum, J. R., Costa-Cabral, S., Lord, C. J. & Ashworth, A. Searching for synthetic lethality in cancer. *Curr. Opin. Genet. Dev.* **21**, 34–41 (2011).
20. Lentsch, E. *et al.* CRISPR/Cas9-Mediated Knock-Out of Kras(G12D) Mutated Pancreatic

Cancer Cell Lines. *Int. J. Mol. Sci.* **20**, 5706 (2019).

21. Barrangou, R. *et al.* CRISPR Provides Acquired Resistance Against Viruses in Prokaryotes. *Science (80-.).* **315**, 1709 LP – 1712 (2007).

22. Hale, C. R. *et al.* RNA-guided RNA cleavage by a CRISPR RNA-Cas protein complex. *Cell* **139**, 945–956 (2009).

23. Gasiunas, G., Barrangou, R., Horvath, P. & Siksnys, V. Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **109**, E2579–E2586 (2012).

24. Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).

25. Wang, H., La Russa, M. & Qi, L. S. CRISPR/Cas9 in Genome Editing and Beyond. *Annu. Rev. Biochem.* **85**, 227–264 (2016).

26. Restriction Endonucleases Products | NEB. https://www.neb.com/products/restriction-endonucleases.

27. Sanger Sequencing - GENEWIZ. https://www.genewiz.com/Public/Services/Sanger-Sequencing?utm_term=genewiz sanger sequencing&utm_campaign=Sanger+Sequencing+2018&utm_source=adwords&utm_medium=ppc&hsa_tgt=kwd-403850111190&hsa_grp=47208260374&hsa_src=g&hsa_net=adwords&hsa_mt=p&hsa_ver=3&hsa_ad=430669392213&hsa_acc=8363678060&hsa_kw=genewiz sanger sequencing&hsa_cam=973612930&gclid=Cj0KCQjwvIT5BRCqARIsAAwwD-So1BXYHV0rnx6_-2IAQ4Z7dW3S4YXqjAJfXrJ-3QGqIwnu1y4hoIwaArAUEALw_wcB.

28. Chen, J. *et al.* Efficient Detection, Quantification and Enrichment of Subtle Allelic Alterations. *DNA Res.* **19**, 423–433 (2012).

29. Synthego - CRISPR Performance Analysis. https://ice.synthego.com/#/.

30. RIPA Buffer. https://www.sigmaaldrich.com/US/en/product/SIGMA/R0278.

31. Anti-SETD2 antibody produced in rabbit Prestige Antibodies® Powered by Atlas Antibodies, affinity isolated antibody, buffered aqueous glycerol solution. https://www.sigmaaldrich.com/US/en/product/sigma/hpa042451.

32. IgG (H+L) Goat anti-Rabbit, HRP, Invitrogen 1 mL; HRP:Antibodies | Fisher Scientific. https://www.fishersci.com/shop/products/goat-anti-rabbit-igg-hrp/656120.

33. Hacker, K. E. *et al.* Structure/Function Analysis of Recurrent Mutations in SETD2 Protein Reveals a Critical and Conserved Role for a SET Domain Residue in Maintaining Protein Stability and Histone H3 Lys-36 Trimethylation. *J. Biol. Chem.* **291**, 21283–21295 (2016).

34. Oslowski, C. & Urano, F. Measuring ER Stress and the Unfolded Protein Response Using Mammalian Tissue Culture System. *Methods Enzymol.* **490**, 71–92 (2011).

35. Kidney and Renal Pelvis Cancer — Cancer Stat Facts. https://seer.cancer.gov/statfacts/html/kidrp.html.

36. Campbell, S. *et al.* Renal Mass and Localized Renal Cancer: Evaluation, Management, and Follow-Up: AUA Guideline Part I. *J. Urol.* **206**, (2021).

37. Motzer, R. *et al.* Survival and Prognostic Stratification of 670 Patients With Advanced Renal Cell Carcinoma. *J. Clin. Oncol.* **17**, 2530–2540 (1999).

38. Motzer, R. *et al.* NCCN Guidelines Insights: Kidney Cancer, Version 1.2021. *J. Natl. Compr. Canc. Netw.* **18**, 1160–1170 (2020).

39. Bedke, J. *et al.* Updated European Association of Urology Guidelines on Renal Cell Carcinoma: Nivolumab plus Cabozantinib Joins Immune Checkpoint Inhibition Combination Therapies for Treatment-naïve Metastatic Clear-Cell Renal Cell Carcinoma. *Eur. Urol.* **79**, (2020).

40. Mitchell, T. & Stewart, G. Timing the Landmark Events in the Evolution of Clear Cell Renal Cell Cancer: TRACERx Renal. (2018) doi:10.17863/CAM.20294.

41. Hasanov, E. & Jonasch, E. MK-6482 as a potential treatment for von Hippel-Lindau disease-associated clear cell renal cell carcinoma. *Expert Opin. Investig. Drugs* **30**, (2021).

42. Duns, G. *et al.* Histone Methyltransferase Gene SETD2 Is a Novel Tumor Suppressor Gene in Clear Cell Renal Cell Carcinoma. *Cancer Res.* **70**, 4287–4291 (2010).

43. Karali, E. *et al.* VEGF Signals through ATF6 and PERK to promote endothelial cell survival and angiogenesis in the absence of ER stress. *Mol. Cell* **54**, (2014).

44. Ali, M. *et al.* Structure of the Ire1 autophosphorylation complex and implications for the unfolded protein response. *EMBO J.* **30**, 894–905 (2011).

45. Papandreou, I. *et al.* Identification of an IRE1alpha endonuclease specific inhibitor with cytotoxic activity against human multiple myeloma. *Blood* **117**, 1311–1314 (2010).

46. Volkmann, K. *et al.* Potent and Selective Inhibitors of the Inositol-requiring Enzyme 1 Endoribonuclease. *J. Biol. Chem.* **286**, 12743–12755 (2011).

47. Ghosh, R. *et al.* Allosteric Inhibition of the IRE1a RNase Preserves Cell Viability and Function during Endoplasmic Reticulum Stress. *Cell* (2014) doi:10.1016/j.cell.2014.07.002.

48. Axten, J. *et al.* Discovery of GSK2656157: An Optimized PERK Inhibitor Selected for Preclinical Development. *ACS Med. Chem. Lett.* **4**, 964–968 (2013).

49. Chiang, Y. *et al.* SETD2 Haploinsufficiency for Microtubule Methylation Is an Early Driver of Genomic Instability in Renal Cell Carcinoma. *Cancer Res.* **78**, canres.3460.2017 (2018).

ACADEMIC VITA
ROBERT N. UZZO

State College, PA 16802 • rvu5021@psu.edu

EDUCATION

Penn State Smeal College of Business — University Park, PA
Master of Business Administration — *Anticipated* 5/23
- Concentration: Supply Chain Management

Penn State Eberly College of Science — University Park, PA
Bachelor of Science in Business Administration — 12/21
- Schreyer Honors College

EXPERIENCE

Janssen Pharmaceuticals of Johnson and Johnson — Spring House, PA
Procurement Co-Op | Discovery/Pre-Clinical Biomarkers — 1/21 – 12/21
- Managed spend profile of $167MM supporting research of clinically relevant biomarkers
- Connected key business partners to ensure prompt execution of outsourced R&D services
- Identified bottlenecks to implement key efficiencies to enable 12% increase in case volume

Temple Health System at Fox Chase Cancer Center — Philadelphia, PA
Undergraduate Research Fellow — 5/19 – 12/21
- Pioneered 3-dimensional organoid project to culture urothelial murine bladder cancer models
- Evaluated synthetic lethality of SETD2-mutant cancers via custom CRISPR/Cas9 vectors
- Partnered with Harvard Institute of Health to study DNA repair after UV induced damage

Clot Not (U.S. Patent #15/722,077) — Philadelphia, PA
Co-founder | Vice President | Developer/Designer — 1/16 – 12/19
- Engineered a compression device for prevention/treatment of deep vein thrombosis
- Collaborated with industrial design company to 3D print prototypes for less than $10

Tech Serve — Multiple Locations
President — 1/16 – 9/18
- Directed donation and installation of 170+ fully licensed computers valued at over $50k to provide educational computer labs at four schools spanning eastern U.S.
- Organized, sourced, and physically executed set-up of educational computer lab for underprivileged school in Bayamón, Puerto Rico

Centre for Volunteers in Medicine — State College, PA
Medical Liaison | Data Entry | Community Relations — 7/19 – 12/20
- Discussed lab results to improve medical literacy for patients lacking medical insurance
- Enabled transition from paper to medical records to fully automate electronic medical records

SKILLS

Epic (EHR), Salesforce, Tableau, Emotional Intelligence, Public Speaking, Coachability

ACTIVITIES

Entrepreneurship, Translational Research, Investing, Scuba Diving, Playing Squash