THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF SCIENCE

Effects of a SETD2 mutation on DNA damage response in renal cell carcinoma

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A thesis
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ABSTRACT

SETD2 is a histone methyltransferase that trimethylates histone H3K36. Normally, *SETD2* functions as a tumor suppressor gene and is involved in homologous recombination (HR) DNA repair of double stranded breaks (DSBs), however mutations in *SETD2* arise in about 10% of renal cell carcinomas (RCC). Labs showed that there are HR defects associated with *SETD2* loss. We aimed to characterize these HR defects in *Setd2*-mutated vs WT RENCA cells. We aimed to determine if HR deficiency is caused by a lack of DSB detection or a lack of repair after a DSB is detected. Nuclear foci formation at DSBs marks their detection and early repair and could thus be used to determine if DSBs were detected. Western blotting was used to determine DNA damage protein activation (DSB repair). We utilized the HR-GFP plasmid to determine if DSBs are repaired as well. We hypothesized *Setd2*-mutant cells would be more sensitive to PARP inhibitors and evaluated that using cell viability. We show that *Setd2* mutant cells do not die upon buildup of DNA damage, which indicates an impaired DNA damage repair system. This could be from a lack of p53 expression found in *Setd2* mutant cells. Furthermore, *Setd2* mutant vs WT cell lines showed similar effects to Olaparib PARP inhibitor, indicating no therapeutic susceptibility. Other DNA damaging agents like Etoposide also indicated no therapeutic susceptibility.

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Furthermore, I would like to thank Dr. Thomas McGuire for providing feedback on my thesis and Dr. David Ruth for providing guidance with the thesis process.

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Chapter 1

Introduction

1.1 Renal cell carcinoma

Kidney cancer continues to be a widespread disease with 81,800 cases predicted to be diagnosed in 2023.[1] Renal cell carcinoma (RCC) is the most common type of kidney cancer with clear cell renal cell carcinoma (ccRCC) making up about 80% of all RCC cases.[2] Common risk factors include smoking, obesity and genetics. Some genetic mutations in ccRCC are VHL, PBRM1, BAP1, SETD2, PIK3CA, and TSC1/2.[3] A SETD2 mutation is found in about 10-20% of ccRCC cases.[4]

The chance of having renal cell carcinoma increases with age and is typically seen more in males than females. The disease can manifest itself through paraneoplastic syndromes (PNS), which result from tumor hormones or the body's attack on the tumor. 20% of patients with RCC will develop PNS, which is why it is valuable to recognize features such as hypertension, hypercalcemia, or amyloidosis as possible signs of RCC.[5]

This research aimed to characterize the homologous recombination defect associated with SETD2 loss in ccRCC. By understanding the molecular basis, new targeted therapy and immunotherapy options can arise.

1.2 Histone modifications

The study of changes in gene expression through epigenetics has been a primary focus of cancer research. DNA is packaged as chromatin and wraps around histones with the core

histones being H2A, H2B, H3, and H4. Histone modifications like methylation repress or activate transcription based on the histone residue that is methylated. Acetylation, on the other hand, only activates genes. DNA methylation has been shown to be involved in tumorigenesis and cancer progression.[6] DNA methylation also leads to gene silencing when occurring in the regulatory region of the promoter.

1.3 SETD2 and role in cancer

SETD2 is a histone methyltransferase that trimethylates histone H3K36. SETD2 and H3K36 methylation play a role in DNA damage repair through homologous recombination (HR), and a loss of SETD2 results in HR loss at sites of double stranded breaks (DSBs).[7] Through recruitment of RAD51 to DSBs, SETD2 is essential for HR DNA repair.[8] There are a variety of cancer types associated with a SETD2 mutation; most notably ccRCC with 15.6% mutation found in 418 samples.[7] Besides transcriptional regulation, SETD2 functions in DNA repair. It is involved in homologous recombination (HR) DNA repair of double stranded breaks and acts as a tumor suppressor gene. HR defects exist with SETD2 loss.[8] We aimed to characterize the HR defect as either a lack of DNA DSB detection or lack of repair after detection.

1.4 DNA damage response proteins

SETD2 is involved in HR-mediated repair of DSBs by recruiting other DNA damage repair proteins to arrive on the scene. Some proteins in the DNA damage repair pathway include p53, γH2AX, RAD51, and BRCA1. p53 is a tumor suppressor gene and when activated can promote DNA repair or cause cells to undergo apoptotic mechanisms. γH2AX is a useful marker of DNA damage, especially in immunofluorescence where it is expressed as foci (dots) on the

cells. The phosphorylation of H2AX by ATM is one of the first changes to occur to the site of double stranded breaks. ATM is activated by DSBs and regulates DNA repair proteins. RAD51 is a DNA damage repair protein involved in strand exchange during homologous recombination. It is activated later in the DNA repair mechanism. BRCA1 is also a later stage protein involved in DNA repair, specifically through HR.

1.5 DNA repair mechanisms

DNA is repaired by either homologous recombination (HR) or nonhomologous end joining (NHEJ). HR involves a homologue template strand which fuses to the broken strand of DNA while NHEJ connects the broken strands together which could result in errors. The H3K36me3 activity of SETD2 is necessary for HR repair.[9]

1.6 PARP and PARP Inhibitors

PARP (poly ADP ribose polymerase) is an enzyme that helps repair DNA damage by recognizing single and double stranded DNA breaks. Proteins are recruited to the site of DNA damage which allow repair. PARP inhibitors inhibit repair in damaged DNA of cancer cells. A mechanism of PARP trapping leads to DSB accumulation and cell death. DSBs are repaired primarily by one of two pathways, homologous or nonhomologous end repair (HR or NHEJ). When cancers are deficient in HR-mediated repair (as is common) and PARP is inhibited, both pathways of DSB repair are blocked, causing synthetic lethality. Normal cells have another repair pathway, so PARP inhibition is expected to be selective to cancer. PARP inhibitors, such as Olaparib or Niraparib compete with NAD+ for the active site of PARP molecules.[10] NAD+ is an important coenzyme in metabolism. These classes of drugs were chosen for experimentation

in this project because of their activity in HR-deficient scenarios. Previous studies showed that a BRCA mutation leads to HR deficient contexts, in which PARP inhibitors are used clinically.[11] A cancer cell which is both BRCA-mutated and subjected to PARP inhibition dies by way of synthetic lethality, where the loss of both repair mechanisms leads to cell death. However, normal cells are not affected by PARP inhibitors as they have an active BRCA 1 /2 pathway which allows survival even when PARP is inhibited. With this reasoning, SETD2 mutant cells are expected to have a more sensitive response than SETD2 WT cells in the presence of PARP inhibition because SETD2 has been shown to be required for efficient HR-mediated repair.

Chapter 2

Materials and Methods

2.1 Cell Culture

RENCA cells are derived from a spontaneously occurring mouse renal tumor and were purchased from ATCC. Setd2 mutant and WT cells were generated by Alex Metz in the Abbosh lab. 4 clones of Setd2 WT cells were expanded from RENCA cells infected with a sham Cas9 lentivirus: P.1, P.2, P.3, and P.4. Setd2 mutant cell lines were generated through CRISPR knockout. The expanded clones are 2.1-2.8 and 3.1-3.8. Setd2 was knocked out using a p-LentiCRISPR-e (addgene #78852) in RENCA cells. SETD2 ko clones have a variety of mutations that lead to a insertion or deletion frameshift. Sterile cell culture techniques such as 70% ethanol sprayed on materials entering the hood and personal protective equipment were used to avoid contamination. Cell culture media for the RENCA cell line was prepared with RPMI culture media, 10% FBS, 1% Sodium Pyruvate, 1% L-Glutamine, and 1% Non-Essential

Amino Acids. Prepared media and 0.05% Trypsin-EDTA were warmed before passaging cells. Cells were taken from a 37°C and 5% CO2 incubator and inspected under the microscope to check for confluency. Then, cells were transferred to the culture hood and the old media was aspirated. Cells were washed with 1x dPBS (Dulbecco's Phosphate Buffered Saline) to remove debris. After washing and aspirating the dPBS, trypsin-EDTA was added to allow the cells to unstick from the surface. Trypsinizsed cells were incubated at 37° for 5 minutes. Fresh RENCA cell media was combined usually in a 1:10 dilution with trypsin. Extra cell suspensions were then plated, frozen, or discarded. Cells were then placed in the incubator to grow until ready for use.

2.2 Western blotting

To determine DNA damage protein expression in SETD2 WT and mutants, cells were first treated with 20 uM Etoposide for 15 minutes with a 30 minute recovery. Cell pellets were collected, kept on ice, and lysed in cold RIPA buffer infused with protease and phosphatase inhibitors. Tubes were vortexed, sonicated, and centrifuged. A 96 well plate was used to determine sample protein concentrations. Known BSA concentrations and the RENCA cell samples were put on the plate in duplicates. Using the BCA protein assay kit, BCA reagent A and B were mixed in a 50:1 ratio and distributed to each well turning the solution purple. The plate was placed on a shaker for 2 minutes, and then incubated at 37° for 30 minutes. The OD_{600} absorbance data was read and used to calculate protein concentration of each sample. The tubes were vortexed and boiled at 95° for 10 minutes. Equal amounts of protein were loaded in each well of a NuPAGE 4-12% Bis-Tris polyacrylamide gel and resolved via PAGE. Proteins were then transferred to a membrane and blocked in 5% milk made with TBST. The desired primary

antibody (ATM, p53, RAD51, γH2AX or Cyclophilin B) was diluted 1:1000, and the secondary matching the primary species was diluted 1:10,000. Both antibodies were made with 5% BSA. Proteins were developed using a blotting substrate and imaged.

2.3 Immunofluorescence

Fluorescence microscopy was performed using a Leica confocal microscope at 63x. Cells were plated in each well of an 8-well chamber slide. Samples included *Setd2* WT and mutants with and without Etoposide treatment. γH2AX stained cells were treated with 50 uM Etoposide for 15 minutes with a 30 minute recovery. RAD51 stained cells were treated with 50 uM Etoposide for 8 hours. After the allotted time, the drug was taken off and cells were washed quickly with PBST. Cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilized using 0.2% TritonX for 10 minutes. Permeabilized cells were blocked for 30 minutes using Licor blocking buffer. Afterwards, cells were incubated with the primary antibody for 1 hour at room temperature: γH2AX at 1:400 dilution or RAD51 at 1:500 dilution. Cells were incubated with anti-rabbit secondary antibody conjugated to Alexa Fluor 568 or Alexa Fluor 546 for 1 hour at room temperature in the dark. Cells were counterstained with Hoechst, a blue nuclear dye.

2.4 HR-GFP plasmid transfection

The HR-GFP plasmid can be restriction digested and only expresses GFP when the plasmid is repaired by HR. The HR-GFP plasmid was digested with I-SceI restriction enzyme which produced a double stranded break. Restriction enzymes recognize restriction sites and cut

out a specific nucleotide sequence. To confirm the cut, samples were resolved on agarose gel and visualized with ethidium bromide. For the transfection, 80 uL of serum-free RPMI media, 1 ug linearized HR-GFP plasmid and 1 ug of RFP plasmid were mixed well in a 1.5 ml Eppendorf tube and incubated at room temperature for 5 minutes. 6 uL of Lipofectamine 2000, a common transfection agent, was mixed well with 80 uL of serum-free RPMI media in another 1.5 ml Eppendorf tube and incubated at room temperature for 5 minutes. After 5 minutes, both tubes were combined and incubated at 37° for 20 minutes. Meanwhile, the old media was aspirated off the cells getting transfected and replaced with 500 uL of serum-free RPMI media in each well. After 20 minutes, the plasmid-lipofectamine-media suspension was transferred into each well and incubated at 37° for 6 hours. Then, the transfection media was replaced with normal RENCA cell media. Cells were measured 3 days post transfection by FACS flow cytometry. If the double stranded break was repaired by HR, we detected a green fluorescence in the cells. FlowJo was used to analyze the number of GFP and RFP positive cells in each SETD2 WT and mutant cell line to calculate repair efficiency.

2.5 Cell viability

To quantify SETD2 WT and mutants' responses to Olaparib or Etoposide, cellular metabolism was assessed through Cell-Titer Glo. The IC_{50} dose for each cell line was calculated. Cells were plated in triplicates on a 96 well plate and left to grow overnight. Olaparib or Etoposide was diluted in RENCA media from a high to low concentration. RENCA media was taken off the cells and the drug media was placed on the cells. Cells were read 3 days later with Etoposide treatment and 4 days later with Olaparib. Cell Titer Glo reagent was added to each

well during the reading and shook for 5 minutes. 8 minutes later, the cell plates were taken to the computer to be read. Values were normalized using Excel and graphed using Prism software.

Chapter 3

Results

3.1 *Setd2* mutants accumulate more DNA damage than *Setd2* WT

Following Etoposide treatment, lysates of *Setd2* WT and mutant cell lines were subjected to western blot. The blot was stained using primary and secondary antibodies conjugated to four proteins: ATM, p53, RAD51, and γH2AX. Cyclophilin B was the loading control. γH2AX expression is similar between *Setd2* WT and mutant clones which may suggest optimal detection signaling in both cell lines. Protein expression is seen in **Figure 1**.

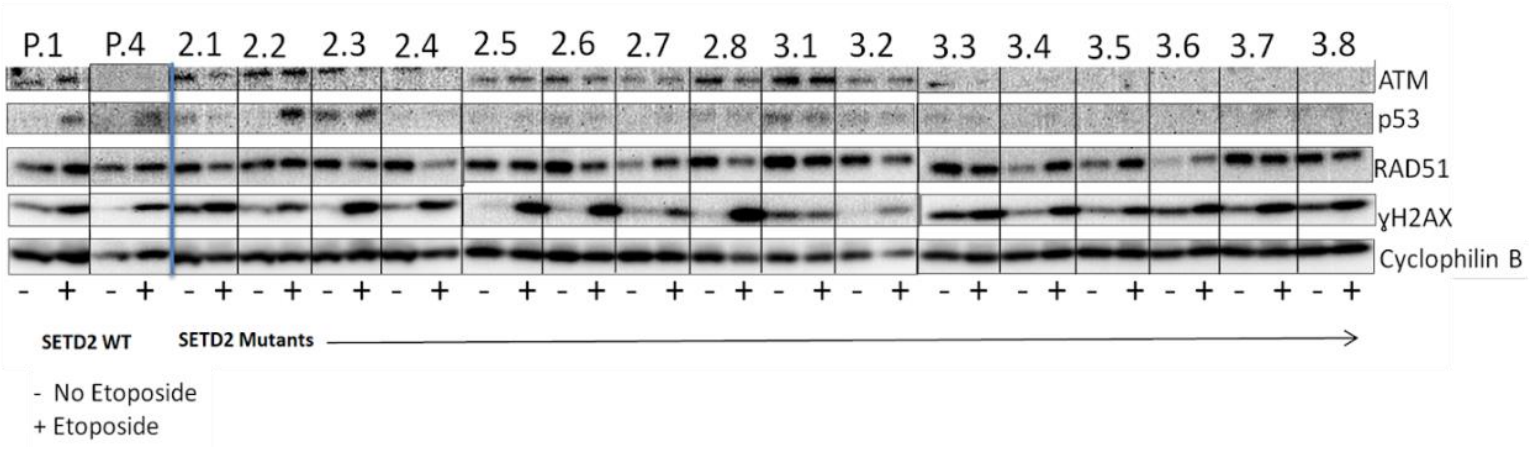

Figure 1: *Setd2* mutants may sense DSBs better than *Setd2* WT yet have impaired DNA DSB repair. p53 loss may accompany *Setd2* deficient cells. Cells were treated with 20 uM Etoposide for 15 mins with a 30 min recovery.

We hypothesized *Setd2*-mutant cells will display more DNA damage than *Setd2* WT cells. To test this hypothesis, the cell lines were subjected to γH2AX confocal immunofluorescence microscopy as shown in **Figure 2**. The integrated signal intensity shown in **Figure 3** indicates that *Setd2*-mutant cells accumulated more DNA damage than *Setd2* WT.

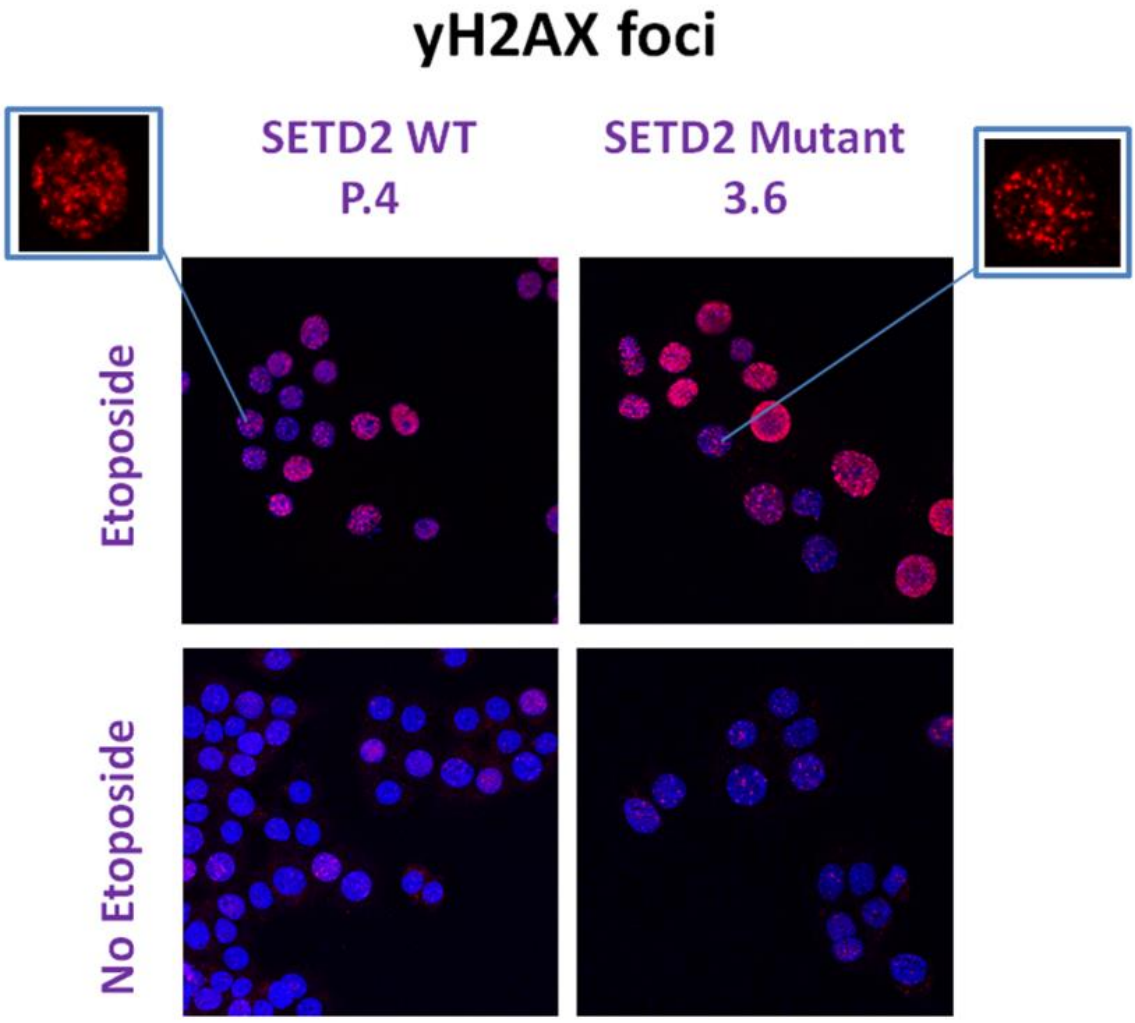

Figure 2. Cells treated with 50 uM Etoposide for 15 mins with a 30 min recovery. Images obtained using Leica confocal microscope at 63x.

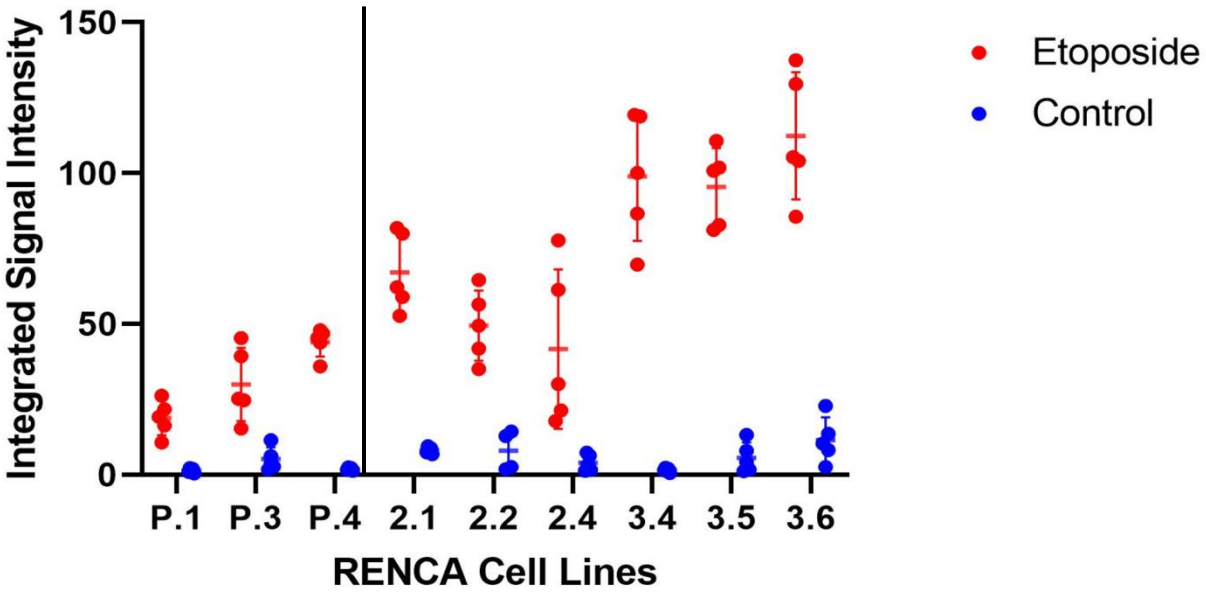

Figure 3. *Setd2* mutants show greater levels of DNA detection through γH2AX signaling upon DSB induction.

3.2 *Setd2* loss impairs DNA repair through HR

Visually, more RAD51 foci were detected in *Setd2*-WT cells as seen in **Figure 4**. This ties into the main function of *SETD2* to recruit RAD51 to aid in HR DNA repair.[8]

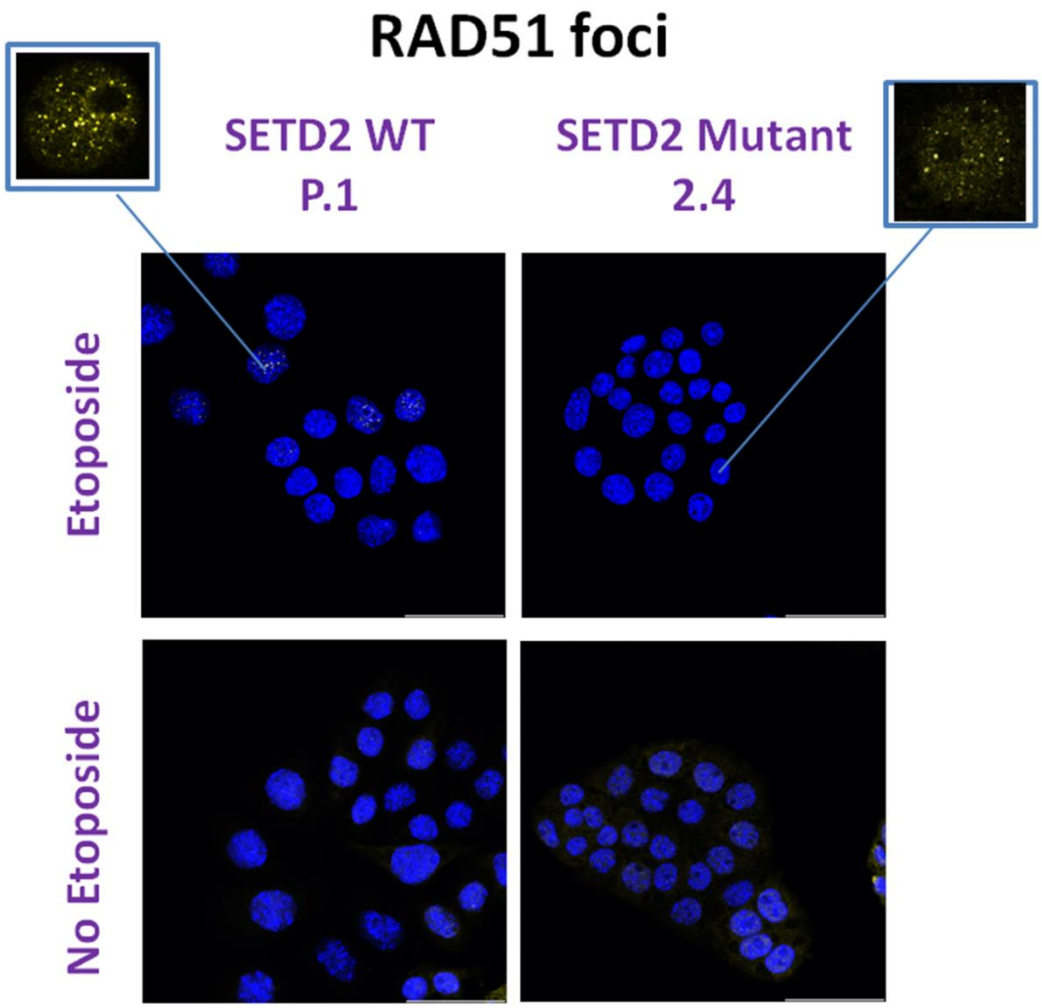

Figure 4. Cells treated with 50 uM Etoposide for 8 hours. Images obtained using Leica confocal microscope at 63x.

SETD2 WT had a higher average number of RAD51 foci/nucleus when compared to mutant clones, **Figure 5**. This suggests that *SETD2* WT are better at repairing DSBs.

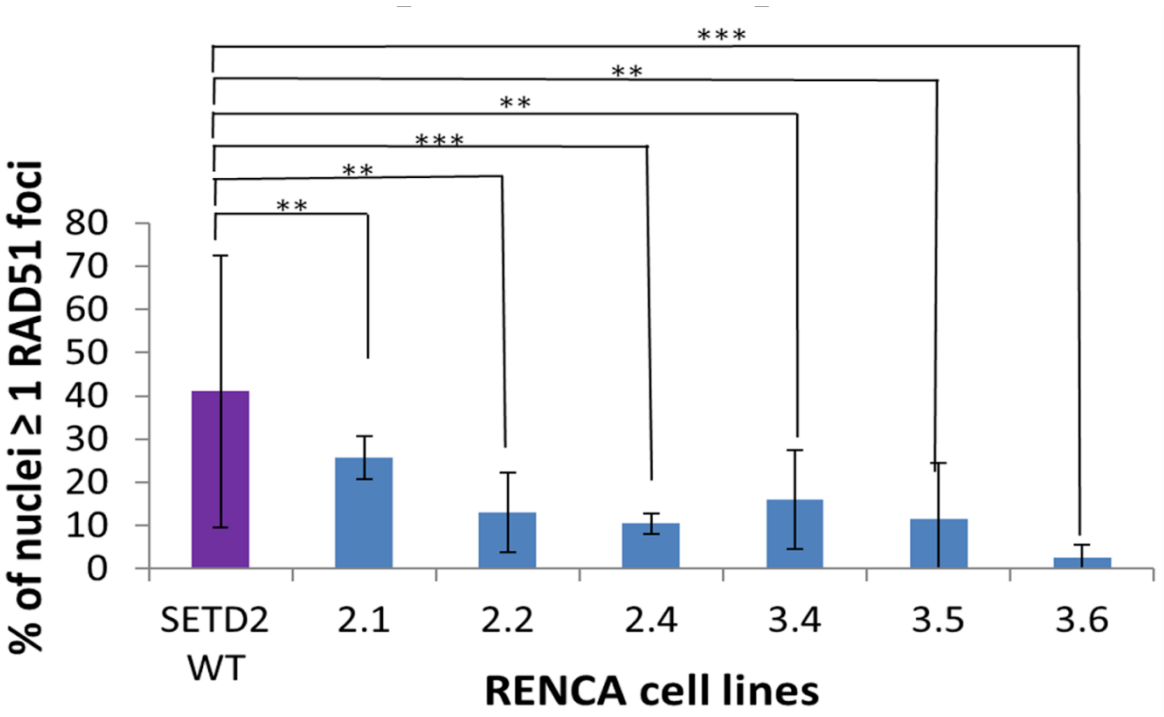

Figure 5. *Setd2* WT show significantly more RAD51 foci per nuclei in the presence of Etoposide.

3.3 *Setd2* WT have lower HR DNA repair capacity than *Setd2* mutants

To compare DSB repair in *Setd2* WT and mutant clones, we introduced the HR-GFP plasmid and analyzed the results by flow cytometry. If HR repair occurs then intact GFP is translated and can be detected by FACS. If no repair occurs, cells do not fluoresce and this can also be detected by FACS. With the HR deficient nature of *Setd2* mutants, we hypothesized

Setd2 mutants would express less GFP while *Setd2* WT will express more GFP as an indication of DSB repair. **Figure 6** depicts efficiency of HR repair with *Setd2* WT expressing lower levels than *Setd2* mutants.

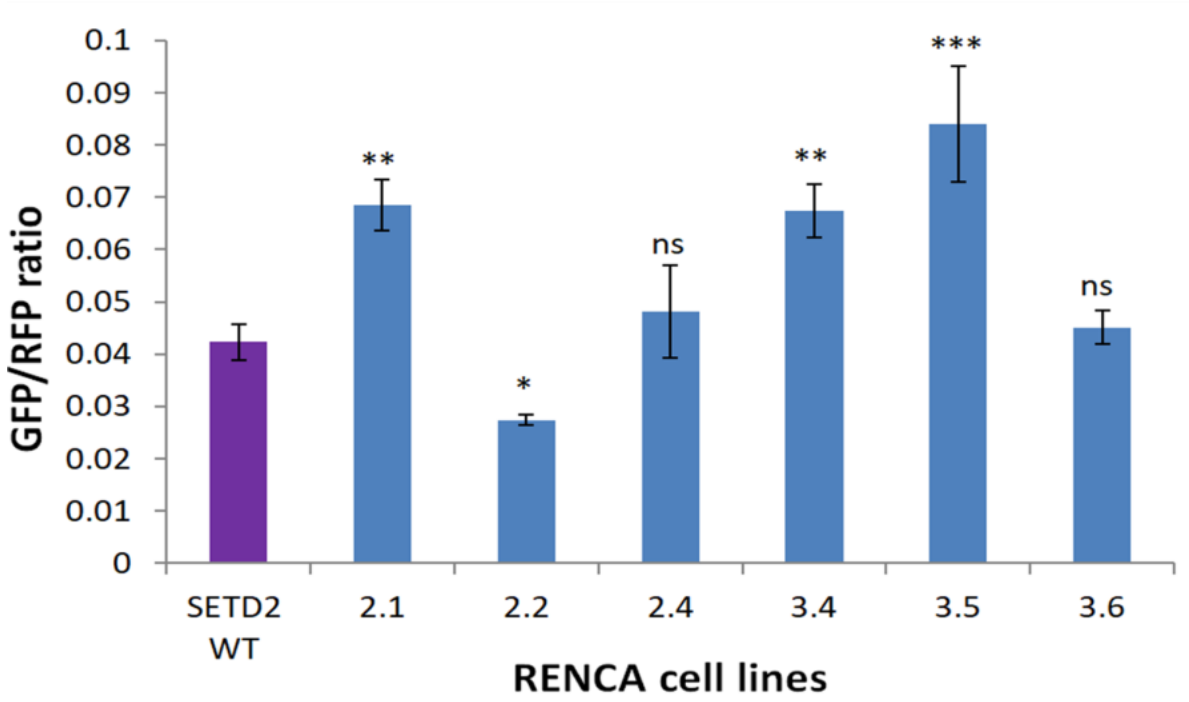

Figure 6. Efficiency of HR repair in *Setd2* WT and mutant cells was measured by the fraction of HRGFP + cells to RFP. DSB was induced by I-sceI restriction enzyme. Cells were analyzed 3 days post transfection. p53 inhibits HR DNA repair which may account for *Setd2* WT expressing a lower GFP/RFP ratio.

3.4 Olaparib PARP inhibitor shows no effect between *Setd2* WT and mutant cell lines

Cell viability assays were used to calculate the IC_{50} dose and cells were treated with Olaparib. Olaparib targets HR deficient cells, and thus we hypothesized *Setd2* mutant cells should be more sensitive to the drug. As shown in **Figure 7**, *Setd2* mutants do not show increased sensitivity to Olaparib.

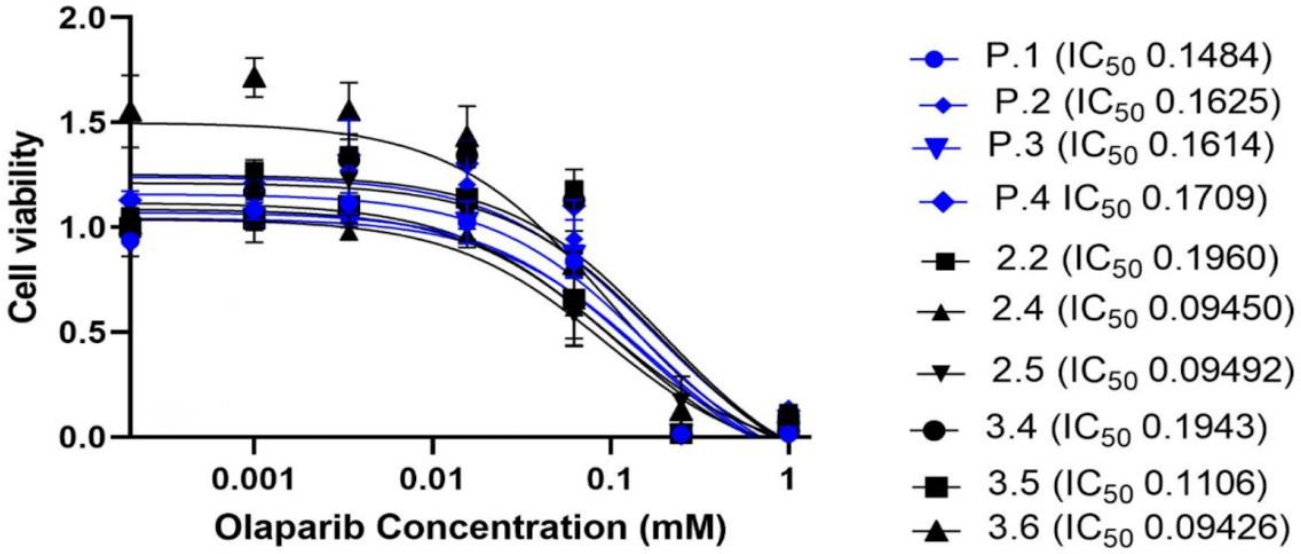

Figure 7. *Setd2* WT and mutants were exposed to increasing doses of Olaparib PARP inhibitor. Cell viability was determined 4 days after treatment. *Setd2* mutants do not show increased sensitivity to Olaparib.

3.5 *Setd2* mutant cells are more resistant to Etoposide

Etoposide is a DNA damaging agent and functions as topoisomerase II inhibitor.[8] In other words, Etoposide causes DSBs which lead to cell death. **Figure 8** shows a trend of *Setd2* WT being more sensitive to Etoposide, while *Setd2* mutants appear to be resistant.

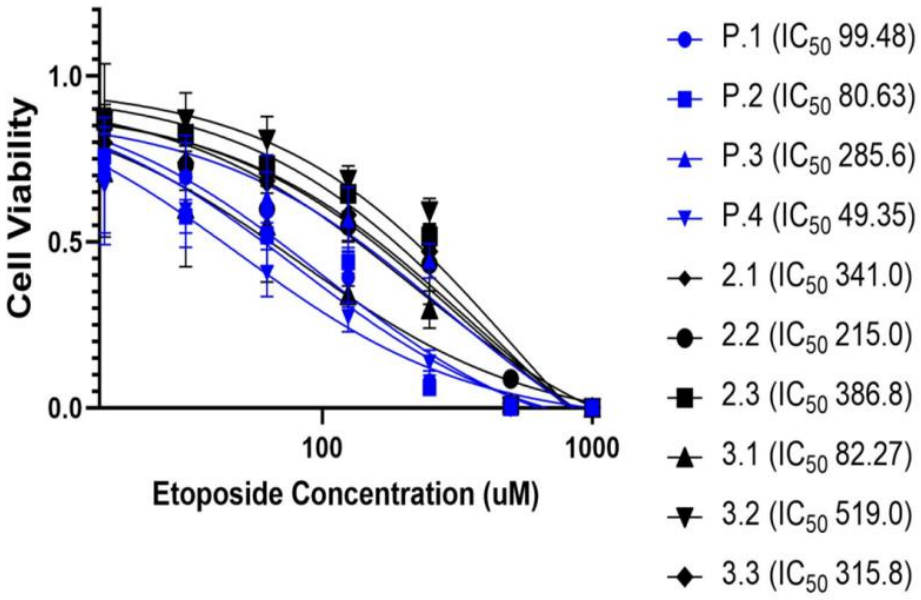

Figure 8. *Setd2* WT and mutants were exposed to increasing doses of Etoposide. Cell viability was determined 3 days after treatment. *Setd2* WT appear to be more sensitive to Etoposide which may suggest their better ability to undergo the DNA damage repair pathway up to and including apoptosis.

Chapter 4

Discussion and Conclusion

In this study, by focusing on DNA damage and repair in *Setd2* WT and mutant RENCA cells, we show that *SETD2* mutant cells are able to detect DSBs but they paradoxically repair DSBs even better than *SETD2*-WT cells. Based on the western blot in figure 1, we believe that when DNA is damaged, mutant cells do not induce p53 like *SETD2*-WT cells to start the apoptotic cascade. *Setd2* mutant cells appear to have increased DNA damage repair through HR and do not die upon the accumulation of such DNA damage. p53 is known to mediate cell death and is induced with DNA damage in *Setd2* WT settings but not in *Setd2* mutant settings, possibly explaining the lack of cell death in mutant cells. With therapeutics, *Setd2*-deficient cells do not harbor therapeutic susceptibility to PARP inhibitors or other DNA damaging agents such as Etoposide. By researching DNA damage and repair, we hope to benefit kidney cancer patients by understanding the SETD2 mechanism and exploring new avenues of therapeutics.

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Experience

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- Help prevent delirium and cognitive decline in patients

Undergraduate Summer Research Fellowship, Fox Chase, *Philadelphia, PA* May 2021- August 2021
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- Researched SETD2 mutation commonly found in renal cell carcinoma
- Performed experiments to test the hypothesis
- Attended weekly lab meetings

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- Western blotting
- Immunofluorescence
- Flow cytometry
- Cell passaging

Leadership

Penn State Abington Hillel August 2020-May 2023
President
- Connect with students through monthly meetings and events
- Collaborate with other student organizations to develop group events
- Delegate responsibilities to executive board

Penn State Abington Hillel September 2019 – August 2020
Treasurer
- Submit form requests for event funding
- Oversee budget and manage expenses by itemizing costs
- Coordinate with executive board to plan events to get campus community involved

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