Arid1a Deficiency As A Biomarker For Sensitivity To Ionizing Radiation And Atr Inhibition In Gynecologic Malignancies

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ARID1A Deficiency as a Biomarker for Sensitivity to Ionizing Radiation and ATR Inhibition in Gynecologic Malignancies

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

By

Jessie Li, Class of 2022
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Abstract

ARID1A DEFICIENCY AS A BIOMARKER FOR SENSITIVITY TO IONIZING RADIATION AND ATR INHIBITION IN GYNECOLOGIC MALIGNANCIES

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Objective: ARID1A, which encodes a protein that participates in the SWI/SNF chromatin remodeling complex, is frequently mutated in ovarian and endometrial malignancies. The emergence of the role of ARID1A in gynecologic cancer progression reveals potential implications for therapeutic targets. Our primary purpose was to investigate ARID1A deficiency as a potential biomarker conferring sensitivity to ionizing radiation and ATR inhibitors.

Methods: Paired isogenic cell lines ES2 and HCT116 were used containing wild-type ARID1A or homozygous loss of function ARID1A mutations. Ionizing radiation doses were delivered using Precision X-ray 320-kV orthovoltage unit at a dose rate of 2 Gy/45 seconds. Cytotoxicity assays were performed to evaluate cell survival following various doses of ATR inhibitor AZD6738. Clonogenic survival assays were performed with or without 50 nM of AZD6738 1 hour prior to radiation at doses of 0, 1, 2, 4, or 6 Gy. Survival curves were generated and compared using the linear quadratic equation on GraphPad Prism. gH2AX foci formation following radiation at 5 Gy was observed using immunofluorescence (IF) and Western blot. IF images were analyzed using ImageJ. Cell cycle distribution following radiation at 5 Gy was performed using flow cytometry and analysis was performed using FlowJo software.
**Results:** *ARID1A*<sup>-/-</sup> cells exhibited greater sensitivity to radiation alone compared to *ARID1A*<sup>+/+</sup> cells as measured by clonogenic survival and demonstrated a diminished DNA damage response following radiation compared to *ARID1A*<sup>+/+</sup> cells, as measured by gH2AX foci formation by IF and Western blot. *ARID1A*<sup>-/-</sup> cells exhibited an impaired G2/M cell cycle checkpoint following radiation, as exhibited by delayed cell cycle arrest at G2/M compared to wild-type cells. *ARID1A*<sup>-/-</sup> cells demonstrated sensitization to AZD6738 alone compared to wild-type cells by cytotoxicity assay. Preliminary results suggest that *ARID1A*<sup>-/-</sup> cells greater sensitization to AZD6738 treatment prior to radiation compared to radiation alone. Further evaluation of combinatorial strategies is in progress.

**Conclusions:** Our *in vitro* findings confirm that *ARID1A* deficiency confers sensitivity to radiation and ATR inhibition, suggesting a biologically informed treatment strategy for *ARID1A*-deficient malignancies. Further work regarding dosing and timing of combination treatment will be explored in the future.

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Introduction

1. Introduction to SWI/SNF complexes and ARID1A

The switch/sucrose non-fermentable (SWI/SNF) complex is a chromatin remodeling complex that modifies chromatin structure in order to alter DNA accessibility for transcriptional and regulatory machinery. The SWI/SNF complex accomplishes its role by using ATP hydrolysis to break DNA-histone contacts and to mobilize nucleosomes [1]. The complex plays a significant role in DNA replication, transcription, and repair; thus, dysfunction within the SWI/SNF pathway is a potential epigenetic mechanism for tumorigenesis. In fact, mutations within the SWI/SNF complex are found in an estimated 20% of all human cancers, making it one of the most commonly mutated tumor suppressors in cancer [2, 3].

The AT-rich interacting domain-containing protein 1A gene (ARID1A) encodes a 250-kDa protein that is a core subunit of the SWI/SNF complex. ARID1A’s role within the SWI/SNF complex is thought to be recruitment of transcriptional and co-regulatory complexes [4], with previous work demonstrating ARID1A’s role in nuclear hormone-induced transcription and cell cycle regulator expression [5-8]. Of the SWI/SNF subunit genes, ARID1A is the most frequently mutated, and ARID1A mutations have been found in many types of malignancies, among them ovarian cancer, endometrial cancer, renal cell carcinoma, stomach cancer, transitional cell carcinoma of the bladder, esophageal cancers, and hepatocellular carcinoma [9]. Mutations in ARID1A are typically either frameshift or nonsense, resulting in gene inactivation and loss of the ARID1A protein, which is generally detected using immunohistochemistry [1]. In a meta-analysis including studies across all types of malignancies, ARID1A loss was associated with reduced
recurrence-free and cancer-specific survival [10], demonstrating that \textit{ARID1A} may be a clinically impactful target to study to improve patient prognosis.

2. \textbf{ARID1A} in gynecologic malignancies

\textit{ARID1A} mutations occur with the highest frequency in gynecologic malignancies and have been shown to occur within highly specific histologic subtypes. Most strikingly, \textit{ARID1A} is the most commonly mutated gene in ovarian clear cell adenocarcinomas, an aggressive subtype of ovarian cancer that is often refractory to treatment, and is found in approximately 50\% of all clear cell cases [11, 12]. Additionally, \textit{ARID1A} mutations are linked with 30\% of ovarian endometrioid adenocarcinomas; however, \textit{ARID1A} mutations have not been found in the ovarian serous subtype [11, 12]. Among endometrial cancers, \textit{ARID1A} loss is found in approximately 40\% of uterine endometrioid adenocarcinomas, 25\% of uterine clear cell carcinomas, 18\% of uterine serous carcinomas, and 14\% of uterine carcinosarcomas [13]. \textit{ARID1A} loss more commonly occurs among endometriosis-associated cases compared to non-endometriosis-associated cases, with previous studies suggesting that \textit{ARID1A} loss occurs early in tumorigenesis, starting with endometriosis progressing to malignancy [12, 14]. Yamamoto et al. demonstrated that 86-100\% of adjacent precursor lesions to \textit{ARID1A}-deficient ovarian cancer were also found to have ARID1A loss, while distant precursor lesions in \textit{ARID1A}-deficient cancers had retained ARID1A expression. Further, adjacent precursor lesions in \textit{ARID1A}-intact cancers had retained expression [14]. However, the mechanism for which endometriosis may lead to tumor development remains unclear.

3. Mechanisms of ARID1A as a tumor suppressor
ARID1A-containing SWI/SNF complexes have been shown to play various tumor suppressor roles. Here, we discuss previous work investigating the mechanisms of ARID1A as a tumor suppressor. ARID1A is known to have both gatekeeper roles (regulation of cell cycle, apoptosis, and cellular proliferation) and caretaker roles (maintenance of genomic integrity).

First, ARID1A has been demonstrated to have roles in regulating cellular proliferation. Guan et al. demonstrated that ARID1A silencing resulted in an increased rate of cellular proliferation, while restoration of ARID1A expression suppressed cellular proliferation and tumor growth in ovarian clear cell and uterine endometrioid carcinoma cell lines [15]. Similar results have been shown in breast cancer, gastric cancer, bile duct cancer, and hepatocellular carcinoma cell lines. ARID1A has also been found to regulate expression of cell cycle genes and are thus important in cell cycle arrest. In a pre-osteoblastic cell line model, ARID1A-depleted cells were unable to undergo cell cycle arrest during differentiation, as the SWI/SNF complex is essential in repressing promoters that are critical in the induction of p21, a key regulator in cell cycle progression [5, 6]. ARID1A additionally plays a role in transcriptional regulation of telomerase reverse transcriptase (TERT), which maintains telomere length and can thus provide a survival advantage for tumor development. ARID1A serves as a negative regulator of TERT transcription and activity by binding to the TERT regulatory element and promoting a repressive chromatin structure [16].

Interestingly, ARID1A mutations in gynecologic cancers have been found to coexist with other aberrations. One such co-existing aberration occurs in p53. Previous work has demonstrated a direct interaction between ARID1A and p53, supporting a role of ARID1A in regulating transcription of p53 targets, including
CDKN1A and SMAD3. This suggests that ARID1A works in tandem with p53 by transcriptionally activating downstream genes that prevent tumor development [15]. ARID1A mutations are also found to coexist with aberrations in the PI3K/AKT pathway, a signaling pathway involved in cell cycle regulation. Overactivity of this pathway inhibits apoptosis and promotes unregulated cell proliferation and growth, promoting cancer development. Common coexisting alterations with ARID1A mutations include loss of PTEN (inhibitor of the PI3K/AKT pathway) and activating mutations of PIK3CA. Guan et al. showed that mice with ARID1A/PTEN double knockouts developed tumors; however, mice with only single deletions of either gene did not develop discernable changes [17], suggesting that ARID1A mutations alone are not sufficient for tumorigenesis, though they work in combination with other aberrations to initiate cancer development.

Additionally, ARID1A-containing SWI/SNF complexes appear to have roles in facilitating DNA repair. Thus, aberrations in the SWI/SNF complex can promote genomic instability and subsequent tumorigenesis. The specific role that ARID1A plays in SWI/SNF-mediated DNA repair has not been fully elucidated, though it is likely to play a large role in repair mechanisms given the high mutation frequency of ARID1A in human malignancy. Park et al. demonstrated that inactivation of SWI/SNF complexes resulted in defects on H2AX phosphorylation following DNA damage, having an impact on the efficiency of DNA double strand break (DSB) repair [18]. Shen et al. showed that ARID1A is recruited to DNA DSB and plays a role in sustaining ATR activation in response to DNA damage [19]. ARID1A has also been found to impact DNA mismatch repair (MMR) by recruiting MMR protein MSH2 to chromatin during DNA replication. ARID1A deficiency was found to disrupt DNA MMR, leading to microsatellite instability and increased mutagenesis [20]. Lastly,
SWI/SNF complexes have also been found to be involved with pyrimidine dimers following UV damage [21] and nucleotide excision repair [22].

4. Therapeutic strategies in ARID1A deficiency

Because the majority of ARID1A mutations are inactivating, ARID1A is a challenging therapeutic target, given current limitations in restoring tumor suppressor function in patients. Instead, studying ARID1A as a target requires investigation of therapeutic vulnerabilities created by the molecular consequences of ARID1A deficiency. Many strategies rely on the concept of synthetic lethality, in which inactivation (either by genetic or pharmacologic means) of a combination of targets leads to cell death, though inactivation of one of the two individually does not impact cell viability. Here, we discuss several synthetic lethal targets of current interest in the literature.

ARID1B, another subunit in SWI/SNF complexes that is mutually exclusive with ARID1A, has been identified as a potential vulnerability in ARID1A-deficient cells. Because ARID1A plays the dominant role in modulating chromatin accessibility for enhancers, ARID1B-containing SWI/SNF complexes only contribute significantly in the setting of ARID1A deficiency. However, ARID1B is only able to act at a certain subset of enhancers [23, 24]. Thus, inhibition of ARID1B represents an opportunity to target the remaining activity of the SWI/SNF complex provided by ARID1B in the setting of ARID1A deficiency.

As discussed previously, ARID1A mutations often co-occur with loss of PTEN or activating mutations of PIK3CA, leading to overactivation of the PI3K/AKT pathway and increased cellular proliferation. PI3K/AKT inhibitors are able to disrupt
this interdependency, and these inhibitors have been found to have increased sensitivity within ARID1A-depleted cells [25].

Several additional potential targets emerge from ARID1A’s role in DNA repair. First, as a consequence of ARID1A’s participation in DNA mismatch repair, ARID1A-deficient cells have increased microsatellite instability and subsequent elevation in mutation load, tumor-infiltrating lymphocytes, and PD-L1 expression. As a result, ARID1A-deficient tumors were found to be more sensitive to immune checkpoint blockade, with anti-PD-L1 antibody reducing tumor burden and prolonging survival of mice with ARID1A-deficient tumors but having minimal effect on ARID1A wild-type tumors [20]. Further, because ARID1A has also previously found to have a role in DNA DSB repair, PARP and ATR inhibitors have been investigated as potential synthetic lethal targets for ARID1A-mutant cancers. PARP inhibitors, which disrupt ability to repair single-strand DNA repair and eventually force DSBs to form, are effective when DSB repair pathways are impacted. PARP inhibitors are most widely known for their efficacy in BRCA1 and BRCA2-mutant cancers, though have also been found to sensitize ARID1A-deficient cancer cells as well [19]. Further, Park et al. demonstrated efficacy of combination treatment with radiation and PARP inhibitors in ARID1A-deficient cells [26]. Clinical ATR inhibitors have also been investigated and found to sensitize ARID1A-deficient cancer cells by triggering premature mitotic entry and subsequent apoptosis [27].

Other potential treatment strategies that have been investigated include inhibition of EZH2, inhibition of HDAC6, and kinase inhibitor dasatinib. EZH2 is a subunit of the Polycomb group complex, which typically acts to compact chromatin. EZH2 inhibitors have shown some efficacy in ARID1A-deficient tumors, likely because EZH2 inhibitors eliminate the activity of Polycomb complexes that is
facilitated by aberrations in the SWI/SNF complex [28]. Inhibitors of HDAC6 have also been seen to have some efficacy in ARID1A-mutant ovarian cancers. HDAC6’s function includes regulation of protein trafficking, protein degradation, and cell migration. HDAC6 is typically transcriptionally repressed by SWI/SNF complexes and thus aberrations in SWI/SNF result in de-repression of HDAC6 activity [29].

Lastly, Miller et al. used high-throughput cell-based drug screens to identify dasatinib as a synthetic lethal agent with ARID1A deficiency, as ARID1A-mutant ovarian clear cell carcinoma cells were found to be addicted to dasatinib target YES1 [30].

5. Purpose of this work

This work is most interested in the therapeutic vulnerabilities generated by ARID1A’s role in DNA repair. Specifically, data demonstrating ARID1A’s participation in facilitating DNA DSB repair suggests that ARID1A mutant tumors may be more sensitive to ionizing radiation, which directly induces DNA DSBs. Furthermore, the molecular consequences of ARID1A deficiency for the response of gynecologic cancers to ionizing radiation remain unclear. Additionally, to our knowledge, there is no data investigating ATR blockade sensitization in ARID1A-deficient cells prior to radiation therapy. A previous study found that ATR blockade in ARID1A-mutant tumors triggers premature mitotic entry [27], raising the question of whether ATR inhibition may further sensitize tumors after radiation-induced DNA damage given an increased reliance on ATR checkpoint activity. This work will thus aim to further investigate the therapeutic vulnerabilities sensitizing ARID1A-depleted cells to radiation therapy and ATR blockade in an ovarian clear cell carcinoma cell model, with the eventual goal of evaluating a combination therapy of radiation and
ATR inhibition. We believe that this work will have clinical implications in investigating novel therapeutic strategies for ARID1A-mutant malignancies.

**Statement of Purpose**

The specific aims of our work is outlined below:

- To investigate sensitivity of ARID1A-deficient cells to ionizing radiation in an ovarian clear cell carcinoma cell line model
- To investigate the DNA damage response in ARID1A-deficient cells following ionizing radiation
- To confirm sensitivity of ARID1A-deficient cells to ATR blockade in an ovarian clear cell carcinoma cell line model
- To investigate sensitivity of ARID1A-deficient cells to ionizing radiation in the setting of ATR blockade.

**Methods**

**Contributions**

JL independently completed all experiments detailed in this work, unless otherwise noted below. JL was responsible for maintaining cell cultures relevant to her work. JL was trained as a user of the Precision X-ray 320-kV orthovoltage unit for cell irradiation. JL was closely involved in the development of the project idea, as well as selecting, modifying, and troubleshooting experimental strategies and techniques. JL completed all data analysis independently. JL was also responsible for the completion of this thesis in its entirety.
ZL was responsible for generating the ARID1A-knockout cell lines. In addition, he performed the AZD6758 cytotoxicity assays.

**Methods Description**

**Cell lines**

Cell lines ES2 and HCT116 were used in this study. *ARID1A*-knockout HCT116 (homozygous truncating mutations Q456*/Q456*) and *ARID1A* wild-type HCT116 colorectal cancer lines were obtained from Horizon Discovery. *ARID1A* knockouts were generated in ovarian cancer cell line ES2 using the CRISPR-Cas9 system as described in Ran et al. [31] The CRPSIR/Cas9 vector used was pSpCas9(BB)-2A-Puro (PX459) V2.0 (ID: 62988) and was obtained from Addgene. The target sequence (5′-CACCGAGGAAGCGCTGCTGGGAAT-3′) was inserted into the cloning site of the PX459 vector. The cloned plasmid was then transfected into ES2 cells using Lipofectamine 3000, and cells were selected using puromycin (1ug/mL). Knockout clones were confirmed by Western blot and confirmed by Sanger sequencing.

**Clonogenic Survival Assay**

Cells were harvested using trypsinization and were resuspended in medium. To count the cells, a small sample of cells were mixed in equal parts with 0.4% Trypan Blue solution and 10uL was pipetted into a Countess chamber slide and inserted into the Countess 3 Automated Cell Counter. Cells diluted to the desired seeding concentration (2000, 1000, 500, or 250 cells per mL). Two mL of medium were plated in triplicate wells using 6-well plates. Cells were allowed to adhere to the plate overnight. Cells were treated with the experimental condition. For radiation treatment, ionizing radiation doses were delivered at doses of 0.5, 1, 2, 4, and 6 Gy
using the Precision X-ray 320-kV orthovoltage unit at a dose rate of 2Gy/45 seconds.

For ATR inhibitor treatment, a stock solution of 20mM AZD6738 was diluted to the desired concentration (25-1000 nM). For combination treatment, cells were treated with AZD6738 1 hour prior to radiation treatment, and the fresh medium was added 24 hours after radiation. Cells were incubated for 7 days. The medium was then removed and cells were stained with 0.5% crystal violet solution in 20% methanol.

Plating efficiency (PE) in the untreated control condition was determined with the following equation: 

\[
PE = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \times 100\%.
\]

The surviving fraction (SF) in each experimental condition was then calculated with the following equation:

\[
SF = \frac{\text{number of colonies formed after treatment}}{\text{number of cells seeded} \times \text{PE}} \times 100\%.
\]

Survival curves were generated using the linear quadratic equation:

\[
S(D) = e^{-\alpha D - \beta D^2},
\]

where S is the number of surviving cells at dose D. \(\alpha\) and \(\beta\) describe the linear and quadratic components of the curve, respectively, with the ratio of \(\alpha/\beta\) representing the radiation dose where the cells killed by the linear component is equal to cell killing by the quadratic component [32]. Curves were compared and graphed on GraphPad Prism.

**Cytotoxicity Assay**

Cells were seeded in a 96 well plate at 10,000 cells/mL (200 uL per well, 2,000 cells per well) and allowed to adhere overnight. Cells were then treated with serial dilutions of AZD6738. After 72 hours of treatment, cultures were fixed with 100uL of 20% trichloroacetic acid (TCA) on top of the culture medium to produce a final concentration of 10%. The cells were incubated at 4 degrees Celsius overnight. The plates were washed with tap water and the plate was allowed to
completely dry before adding 50 μL of 0.4% SRB dissolved in 1% acetic acid to each well and stained for 10 minutes at room temperature. Unbound dye was removed by 4 washes with 1% acetic acid, and the plate was allowed to dry completely. Protein-bound dye was dissolved with 100 μL of 10mM unbuffered Tris base solution (pH 10.5) for 5 minutes. The plate was read on a microplate reader, and the OD was measured at 510 nm. Cell growth was expressed as a percentage, defined as the ratio of the absorbance measured in the treated wells compared with the control well. Dose-effect curves were generated using Prism.

**Immunofluorescence analysis**

Cells were seeded on glass cover slips at seeding concentrations of 150,000 cells/mL and allowed to adhere overnight. Cells underwent radiation treatment at a dose of 5Gy and fixed with 4% formaldehyde at different time points after radiation (30 min, 1h, 4h, 8h, 24h). Samples were washed with 0.1% triton in PBS and blocked in 10% normal goat serum in PBS. The samples were then incubated with primary antibody (1:500, anti-phospho histone gH2AX Ser139, Cell Signaling) at 4 degrees overnight followed by the secondary antibody (1:750, Alexa Fluor 488-conjugated goat anti-rabbit IgG, Invitrogen) for 1 hour at room temperature. Samples were mounted on slides with vectashield mounting solution containing DAPI (Vector Labs). Images were captured with an inverted Zeiss LSM 510 Pascal laser confocal microscope. Images were analyzed using ImageJ.

**Cell cycle analysis**

Cells were seeded in 60mm plates and allowed to adhere overnight. Plates were irradiated at 5 Gy and fixed with ice-cold 70% ethanol at different time points.
following radiation (0, 1, 2, 8, 24 hrs). Samples were stored overnight at -20 degrees Celsius. Samples were washed with PBS twice and then incubated with 200 µL of Guava Cell Cycle Reagent (Guava Technologies) for 30 minutes in the dark. Cytofluorometric acquisitions were performed with an LSRII cytometer (BD Biosciences). About 50,000 events were analyzed per condition. Results were analyzed using FlowJo software.

**Western blot**

*SDS lysate preparation*

Cells were cultured on 60mm plates and irradiated at a dose of 5 Gy. Lysates were harvested from the cultures at several time points after radiation (0, 1, 4, 8, 24 hrs) using boiling SDS lysis buffer (1% SDS, 10 mM Tris-Cl pH 7.5). 250 µL of lysis buffer were added to the plate and lysates were scraped into Eppendorf tubes. The lysates were then boiled at 5 minutes and then homogenized by passing through a 26 G needle 6-7 times. The lysates were centrifuged at 12,000 rpm for 5 minutes, and the supernatant was transferred to a clean tube. Protein concentration was determined using the DC Protein Assay Kit (Bio-Rad). 4x sample loading buffer was added to the samples before storing at -20 degrees Celsius.

*Gel electrophoresis, transfer, and antibody incubation*

40 µg of protein were loaded into wells of a precast gel along with a protein ladder. The gel was run at 80V for 20 minutes before increasing the voltage to 250 V to complete the run. Proteins were transferred from the gel to a 0.45 um nitrocellulose membrane at transfer conditions of 300 mA for 50-60 minutes on ice. The membrane was then incubated in 5% milk in TBST for 1 hour followed by primary antibody in 5% milk in TBST (rabbit GAPDH 1:5000, Cell Signaling; rabbit ARID1A 1:2000,
Sigma-Aldrich, rabbit gH2ax 1:2000, Cell Signaling) overnight at 4 degrees Celsius. The membranes were rinsed in TBST 5 times for 5 minutes each and then incubated in anti-rabbit HRP-conjugated secondary antibody in 5% milk in TBST at 1:2000 dilution at room temperature for 1 hour. After rinsing the membranes 5 times for 5 minutes each, Pierce™ ECL Western Blotting Substrates (Thermo Scientific) were pipetted onto the membranes. Blots were captured using the CCD imaging system, and images were analyzed using Image J.

**Results**

**ARID1A-deficient cells exhibit greater sensitivity to radiation treatment**

Our first aim was to investigate the effects of **ARID1A** deficiency on sensitivity to ionizing radiation. We performed clonogenic survival assays to test the ability of **ARID1A**$^{-/-}$ and **ARID1A**$^{+/+}$ cells to survive and form colonies after treatment with radiation. Cells were treated at various doses (1 Gy, 2 Gy, 4 Gy, and 6 Gy) and allowed to incubate for 10-14 days before colonies were stained and counted. In the ES2 ovarian clear cell carcinoma cell line, the **ARID1A**$^{-/-}$ cells compared to **ARID1A**$^{+/+}$ had a decreased surviving fraction of colonies at each administered dose (Figure 1A). This same result was seen in the HCT116 colorectal cancer cell line. The survival fraction at 2Gy was decreased from 87% to 41% in ES2 cells and from 54% to 25% for HCT116 in **ARID1A**$^{+/+}$ and **ARID1A**$^{-/-}$, respectively (Figure 1B). The difference in survival was statistically significant at each dose for both cell lines (denoted by * on the curves). These findings demonstrate that **ARID1A** deficiency confers sensitivity to ionizing radiation.
ARID1A-deficient cells demonstrate impaired DNA damage response after radiation

Next, we sought to gain more mechanistic insight into the radiosensitivity exhibited by ARID1A−/− cells. We were interested in investigating the differences in DNA damage response brought on by ARID1A status following ionizing radiation.

We utilized γ-H2AX assays to observe the initial DNA damage response, as the formation of γ-H2AX is an early cellular response to DNA DSBs. First, we examined γ-H2AX foci formation using an immunofluorescence-based assay. Cells were treated with 5 Gy of radiation, fixed at various time points following radiation treatment, and incubated with γ-H2AX primary antibody. In ES2 ARID1A+/− cells, we observed strong induction of γ-H2AX foci formation at 1 hour and 4 hours after radiation. However, ARID1A−/− exhibited reduced γ-H2AX foci formation at 1h and 4 hours.

**Figure 1:** ARID1A−/− cells are more radiosensitive compared to ARID1A+/− cells. A Clonogenic assays in ES2 and HCT116 cell lines after 0, 1, 2, 4, and 6 Gy of radiation. Images are representative of three independent experiments. B Survival curves for clonogenic assays generated using linear quadratic equations. Data shown represents mean ± SD, P < 0.05.
compared to the corresponding time points in $ARID1A^{+/+}$ cells, suggesting an impaired DNA damage response (Figure 2A). The same experiment performed in HCT116 cells demonstrated similar reduction in $\gamma$-H2AX foci formation in $ARID1A^{-/-}$ cells (Figure 3A). Of note, there was some quantitative variability of $\gamma$-H2AX foci between replicates, though each replicate consistently demonstrated relative reduction of $\gamma$-H2AX foci formation in the $ARID1A^{-/-}$ condition. A representative immunofluorescence pattern is displayed for ES2 and HCT116 cells in Figures 2A and 3A, respectively.

To further validate reduced $\gamma$-H2AX expression in $ARID1A^{-/-}$ cells after radiation, we measured $\gamma$-H2AX expression by Western blot in radiation-treated cell

![Figure 2: ARID1A^{-/-} ES2 cells have diminished $\gamma$-H2AX foci formation following radiation. A Confocal microscopic images of immunofluorescence of $\gamma$-H2AX (green) with nuclear stain DAPI (blue). Images shown are representative of three independent replicates. B Percentage of cells with >15 $\gamma$-H2AX foci. Data shown represents mean ± SD.](image-url)
lysates at various timepoints following radiation. As demonstrated in Figure 4A, strong γ-H2AX expression was induced in ES2 ARID1A+/+ lysates at 30 minutes after radiation with increased expression maintained through 4 hours after radiation. ARID1A−/− lysates demonstrated some induction of γ-H2AX expression at 30 minutes and 1 hour after radiation, though expression is reduced compared to the corresponding time point in ARID1A+/+ lysates. Similar results are observed in HCT116 lysates as demonstrated in Figure 4B. All together, these results suggest that ARID1A deficiency likely generates an impaired initiation of the DNA damage response.

Figure 3: ARID1A−/− HCT116 cells have diminished γ-H2AX foci formation following radiation on immunofluorescence. A Confocal microscopic images of immunofluorescence of γ-H2AX (green) with nuclear stain DAPI (blue). Images shown are representative of three independent replicates. B Percentage of cells with >15 γ-H2AX foci. Data shown represents mean ± SD.
Next, in order to further investigate the DNA damage response after radiation, we examined the expression of CHK1, a protein kinase that is involved in the DNA damage response and cell cycle checkpoint response. Phosphorylation of CHK1 (p-CHK1) is required for activation. We performed a Western blot looking at total CHK1 and p-CHK1 expression in HCT116 cell lysates at various time points after 5 Gy of radiation (Figure 5). Total CHK1 expression was consistent between ARID1A+/+ and ARID1A−/− lysates. With regards to p-CHK1, ARID1A+/+ lysates demonstrated peak p-CHK1 expression at 1 hour after radiation with slight decreases in expression through 24 hours. ARID1A−/− lysates had a delayed peak p-CHK1 expression at 4-8 hours and additionally and diminished p-CHK1 expression at every time point compared to ARID1A+/+ lysates. Of note, ARID1A−/− lysates did appear to
have decreased baseline p-CHK1, as demonstrated by the very faint band at 0 Gy. Thus, it may be difficult to definitively comment about quantitative differences in p-CHK1 expression. Nevertheless, the delayed peak of p-CHK1 in ARID1A−/− cells further demonstrates that ARID1A deficiency confers an impairment in the DNA damage response cascade.

**Figure 5:** ARID1A−/− cells have delayed p-CHK1 expression following radiation by Western blot analysis. A Western blot showing changes in total CHK1 and p-CHK1 expression at various time points following 5Gy of radiation in HCT116 ARID1A+/+ and ARID1A−/− cell lysates.

ARID1A-deficient cells exhibit impaired G2/M cell cycle checkpoint following radiation

Given our finding of a delay in p-CHK1 expression within ARID1A−/− cells and the role of CHK1 in cell cycle checkpoint activation, we next wished to investigate the cell cycle distribution at various time points following radiation treatment. Radiation-treated HCT116 cells were fixed at the desired time points, stained with nuclear DNA stain propidium iodide, and quantitation was performed by flow cytometry. As shown in Figure 6, accumulation of cells at the G2/M checkpoint was observed in ARID1A+/+ cells after 30 min and 1 hour after radiation, demonstrating an appropriate G2/M cell cycle arrest after radiation-induced damage. However, within
ARID1A<sup>−/−</sup> cells, accumulation at the G2/M checkpoint did not occur in until 8 hours after radiation, demonstrating that ARID1A<sup>−/−</sup> cells have a delayed G2/M cell cycle arrest in response to radiation compared to ARID1A<sup>+/+</sup> cells. This result corresponds well with our previous finding of a delayed p-CHK1 peak expression at 8 hours, as the presence of activated CHK1 is an important signal transducer for the G2/M checkpoint activation.

![Figure 6](image)

**Figure 6: ARID1A<sup>−/−</sup> cells demonstrate impaired G2/M cell cycle checkpoint.** A Histograms showing DNA content determined by flow cytometry in HCT116 ARID1A<sup>+/+</sup> and ARID1A<sup>−/−</sup> cell lines. B Graph demonstrating the fold change of cells at G2/M checkpoint.

ARID1A-knockout cells demonstrate sensitization to ATR blockade alone

Previous work has identified ARID1A deficiency as a biomarker for sensitivity to clinical ATR inhibitors, such as AZD6738. We hoped to validate this phenomenon in our ES2 ovarian clear cell carcinoma cell line model and thus investigated the effects of AZD6738 on the survival of ARID1A<sup>−/−</sup> cells through cytotoxicity assays. Cells were treated with serial dilutions of AZD6738 for 72 hours, and cell survival was measured using an SRB assay. As demonstrated in Figures 6A and 6B, ARID1A<sup>−/−</sup> ES2 cells were significantly more sensitive to AZD6738 compared to ARID1A<sup>+/+</sup> ES2
cells. Similar results were observed in isogenic paired HCT116 cell lines (Figures 7C and 7D).

**Figure 7:** ARID1A deficient cells demonstrate sensitization to ATR blockade. A Relative cell survival shown following AZD6738 treatment at various concentrations for 72 hours in ES2 ARID1A+/− cells (blue/green) and ARID1A+/+ cells (black/gray). B Graph demonstrating the AZD6738 concentration resulting in 50% growth inhibitory effect (IC50) in ES2 cells. C Relative cell survival following AZD6738 treatment at various concentrations for 72 hours in HCT116 ARID1A+/− cells (blue) and ARID1A+/+ cells (pink). D Graph demonstrating the AZD6738 concentration resulting in 50% inhibitory effect in HCT116 cells.

**ARID1A**-deficient cells may be more sensitive to AZD6738 treatment prior to radiation compared to radiation alone

As detailed above, ARID1A+/− cells were nevertheless able to proceed with a DNA damage response albeit in a delayed fashion, exhibited by a delayed increase in p-CHK1 expression and delayed cell cycle arrest. This raised the possibility that the cell cycle checkpoint may be a potential target for a synthetic lethal agent. In particular, we were interested in the possibility that ATR blockade could target the
potential increased reliance on ATR checkpoint activity in ARID1A⁻/- cells treated with radiation. In order to investigate if ATR blockade may further sensitize ARID1A⁻/- cells to radiation, we tested the combination treatment of AZD6738 with radiation on clonogenic survival in HCT116 isogenic paired cell lines. Cells were treated with AZD6738 1 hour prior to radiation treatment and medium was removed 24 hours after radiation. As expected, ARID1A⁻/- cells were more radiosensitive compared to ARID1A⁺/+ cells. ATR blockade appeared to slightly sensitize ARID1A⁻/- cells to radiation compared to radiation alone, though the difference was only significant at the 6 Gy dose of radiation (Figure 8).

**Figure 8:** ATR blockade may further sensitize ARID1A⁻/- cells to radiation. A Clonogenic survival assay of HCT116 cells after 0, 1, 2, 4, and 6 Gy of radiation with and without AZD6738 treatment 1 hour prior to radiation. Images representative of 3 independent replicates. B Survival curves for clonogenic assays generated using linear quadratic equations. Data shown represents mean ± SD, P < 0.05.
Discussion

*ARID1A* is a frequently mutated gene, especially amongst gynecologic malignancies, particularly ovarian and endometrial cancers. In this work, we investigated *ARID1A* deficiency as a biomarker for treatment sensitivity to radiation therapy, ATR blockade, and combination treatment of ATR blockade prior to radiation treatment. We use cell line models of ovarian clear cell carcinoma (ES2) and colorectal carcinoma (HCT116). We found that *ARID1A*-deficient cells were more sensitive to radiation treatment as shown by decreased clonogenic survival among the *ARID1A*−/− cells. In examining the DNA damage response of *ARID1A*-deficient cells after radiation therapy, we found that *ARID1A*−/− cells have diminished gamma-H2AX foci formation, delayed p-CHK1 expression, and delayed cell cycle arrest at G2/M. In addition, our results confirm that *ARID1A*−/− cells exhibit increased sensitivity to ATR inhibitor AZD6738 in an ovarian clear cell carcinoma model. Our clonogenic assays investigating cell sensitivity to ATR blockade prior to radiation treatment demonstrated slightly increased sensitivity in *ARID1A*−/− cells compared to *ARID1A*+/+ cells, though this difference was not significant. Collectively, we believe this work provides valuable insight into how mechanistic weaknesses produced by *ARID1A* deficiency can be used to investigated more targeted therapies for *ARID1A*-mutant cancers.

As *ARID1A* has been shown to have many tumor suppressor roles, therapeutic strategies targeting *ARID1A* are more challenging compared to proto-oncogenes. Many strategies rely on the principle of synthetic lethality, the most famous example of which is the use of PARP inhibitors in BRCA-mutant cancers, in which inhibition of PARP’s single-stranded DNA repair ultimately leads to DNA DSBs that are unable to be repaired in BRCA-mutant cells [33]. *ARID1A*’s tumor suppressing roles within
maintaining genome integrity is the most relevant to our work, as we hoped to
investigate the DNA damage response and repair vulnerabilities engendered by
\textit{ARID1A} deficiency and how these may be sensitize \textit{ARID1A}-mutant tumors to
radiation and ATR blockade.

\textbf{\textit{ARID1A} deficiency as a biomarker for radiation sensitivity}

Based on previous work demonstrating defective DSB repair in \textit{ARID1A}-
deficient cells, we hypothesized that \textit{ARID1A}-deficient cells would be sensitized to
radiation-induced DNA damage.

Our first set of experiments centered around the clonogenic survival assay, a
cell culture technique to evaluate the ability of a cell to grow into a colony through
unlimited division. The clonogenic survival assay is the technique of choice to
investigate cell reproductive death after radiation treatment, though can also be used
to evaluate other agents as well [34]. We used this assay to assess the radiosensitivity
of \textit{ARID1A}^{+/} cells compared to \textit{ARID1A}^{+/+} cells. Treatment conditions included
radiation at 0 Gy, 1 Gy, 2 Gy, 4 Gy, and 6 Gy. \textit{ARID1A}^{-} cells had a diminished
surviving fraction at each dose of radiation compared to \textit{ARID1A}^{+/+} cells, and the
difference between these curves was statistically significant. We saw this result in
both ES2 and HCT116 cell lines, demonstrating that \textit{ARID1A}-deficient cells are more
sensitive to ionizing radiation compared to \textit{ARID1A}-intact cells in a colorectal and
ovarian clear cell carcinoma cell line model. The clinical implication of this finding is
the potential ability to use \textit{ARID1A} mutations as a biomarker for treatment sensitivity
to radiotherapy.
Clinical considerations for radiation therapy in \textit{ARID1A}-mutant cancers

\textit{ARID1A} mutations have high specificity for certain histologic subtypes within ovarian and endometrial cancers, with the highest frequency occurring in ovarian clear cell, ovarian endometrioid, and uterine endometrioid adenocarcinomas. The role of radiotherapy in uterine cancer is much more well-defined than in ovarian cancer, with adjuvant radiotherapy in endometrial cancer employed based on risk stratification groups based on clinicopathologic factors and recently, molecular characteristics, as seen in new European guidelines [35, 36]. However, \textit{ARID1A} mutations are not among the molecular factors currently considered in the European guidelines. Much further work elucidating the prognostic value of \textit{ARID1A} mutations within endometrial cancers would be needed before placing further deliberation into how this molecular consideration could be used to influence choices for adjuvant and novel therapies.

With regards to ovarian cancer, radiotherapy is more rarely used in adjuvant treatment. Because ovarian cancer has a tendency to disseminate via transperitoneal spread, cancer often spreads beyond the pelvis, rendering radiotherapy a less effective strategy for disease control. While whole abdominal radiotherapy was utilized for ovarian cancer prior to the cisplatin era, the lower doses required to protect the organs at risk usually resulted in poor therapeutic efficacy [37]. Thus, the mainstay of adjuvant treatment for ovarian cancer has remained platinum-based regimens for several decades, and radiotherapy is instead utilized only in some patients with refractory and resistant disease. However, more advanced radiotherapy techniques have vastly improved the potential for radiotherapy use in metastatic diseases and has inspired renewed interest in using radiotherapy for ovarian cancer. For example, in
intensity-modulated radiotherapy (IMRT), beams can be shaped and modulated, allowing for a more optimized dose to the target while minimizing dose to surrounding organs at risk [37]. A phase II trial (OVAR-IMRT-02) assessing whole abdominal IMRT after cytoreductive surgery and chemotherapy in 20 high risk ovarian cancer patients found acceptable toxicities and promising recurrence-free and overall survival [38]. This highlights future potential for IMRT as a more targeted means to treat ovarian cancer. Additional considerations for radiotherapy for the treatment of ovarian cancer include the potential for low-dose fractionated whole abdominal radiotherapy to be a sensitizer for chemotherapy agents, such as antimetabolite drugs that inhibit DNA synthesis or agents disrupting DNA repair. Additionally, the abscopal effects of radiotherapy, in which radiation leads to the release of tumor antigens and activation of immune response, is an increasing area of interest in exploring the synergy between radiation and immunotherapy.

Over 50% of clear cell ovarian cancers have ARID1A mutations, making our work to elucidate targeted therapies for ARID1A-mutant cancers especially relevant for this subtype. The clear cell subtype is known to exhibit poorer sensitivity to chemotherapy regimens compared to the more common serous subtype. While early-stage clear cell patients tend to have a better prognosis than their serous counterparts, advanced stage clear cell patients appear to have a poorer prognosis [39]. Given the paucity of data for the clear cell subtype, treatment is largely extrapolated from epithelial ovarian cancer trials, in which clear cell patients make up only a small percentage of subjects [40]. Thus, the optimal management of ovarian clear cell carcinoma remains unclear. Further, the relative resistance of the clear cell subtype to chemotherapeutic agents has raised interest in identifying other therapies that may be effective, including radiotherapy [39], particularly since the first site of recurrence for
many of these patients is in the pelvis. In a study of 241 patients with early stage ovarian clear cell patients, the addition of radiotherapy to chemotherapy significantly improved recurrence-free survival only in stage IC2/3 and II, but not in stage IA/B or IC1 [41]. In another retrospective study of 163 early-stage ovarian clear cell patients, adjuvant radiotherapy was not found to be associated with improved recurrence-free or overall survival, though this study did not include information about or stratify outcomes by ARID1A status [42] and thus may not have isolated the specific subset of patients who potentially may benefit from adjuvant radiotherapy. Given the results of our study, it would appear that ARID1A deficiency is a potential biomarker identifying clear cell carcinoma patients who may benefit from radiotherapy, and further work investigating the clinical implications of ARID1A deficiency among clear cell patients is warranted. This more sophisticated understanding of molecular biomarkers in combination with more advanced radiotherapy techniques can be utilized in the future to optimize adjuvant treatment for this patient population. Further work investigating advanced radiation techniques within the ARID1A-deficient clear cell ovarian carcinoma population would be needed to gather clinical data on this hypothesis.

**DNA damage response in ARID1A-deficient cells**

Our additional aim was to investigate the DNA damage response in ARID1A<sup>−/−</sup> cells in efforts to explore mechanisms of radiosensitivity and to explore other potential therapeutic vulnerabilities in ARID1A<sup>−/−</sup> cells following radiation. We used γ-H2AX assays as one way to observe the initial DNA damage response. The histone variant H2AX is phosphorylated at the Ser-139 residue by kinases in the PI3 family, including ATR and ATM, forming γ-H2AX [43]. Formation of γ-H2AX is an early
cellular response to DNA DSBs and thus can be used as a marker for the DNA damage response initiation. An expected response to ionizing radiation would be increased γ-H2AX foci formation. In our study, we observed γ-H2AX nuclear foci formation by an immunofluorescence-based assay and found that after 5 Gy of radiation, ARID1A−/− cells exhibited diminished γ-H2AX foci formation compared to ARID1A+/+ counterparts at each time point. While this observation of relative diminishment of γ-H2AX foci formation in ARID1A−/− was consistent across independent replicate experiments, there remained quantitative differences between replicates. For example, one replicate may have had quantitively higher γ-H2AX expression across all groups compared to another replicate; however, the ARID1A−/− group still consistently demonstrated relatively decreased foci formation compared to the ARID1A+/+ within the same replicate. Because of this, the difference in γ-H2AX foci formation did not reach statistical significance. Additional replicates of this experiment may be needed in addition to further troubleshooting of this experimental technique to standardize immunofluorescent staining. We also tested the expression of γ-H2AX by Western blot in cell lysates after 5 Gy radiation, which similarly demonstrated decreased expression of γ-H2AX in ARID1A−/− lysates compared to ARID1A+/+ lysates, most notably at 1 hour after radiation. Our finding that ARID1A−/− cells have diminished γ-H2AX expression following radiation-induced DNA damage suggests that the radiosensitivity exhibited by ARID1A−/− cells may in part be due to impaired DNA damage detection and response.

We additionally measured the expression of p-CHK1 at various time points after radiation. CHK1 is a protein kinase that plays an important role in coordinating the DNA damage response and is required for the activation of DNA damage checkpoints during the cell cycle. CHK1 is activated upon phosphorylation by ATR
We found that $ARID1A^{+/−}$ cells exhibited delayed peak expression of p-CHK1, and this finding was further evidence that $ARID1A$ deficiency conferred an impairment in the DNA damage response. Further, given the role of CHK1 in cell cycle checkpoint activation, we performed cell cycle experiments to evaluate the impact that $ARID1A$ deficiency may have on the cell cycle after radiation. Our data demonstrates that while $ARID1A^{+/−}$ cells exhibited G2/M arrest at 1 hour, $ARID1A^{-/-}$ cells did not exhibit G2/M arrest until the 8 hour mark, aligning with the delayed p-CHK1 peak expression. Thus, $ARID1A^{-/-}$ cells have aberrations in cell cycle arrest following radiation-induced DNA damage. Failure to arrest at G2 hinders the cell’s subsequent ability to repair the DNA damage [45] and may contribute to the radiosensitivity of $ARID1A^{-/-}$ cells. However, we did observe $ARID1A^{-/-}$ cells were still able to achieve cell cycle arrest albeit in a delayed manner and perhaps retain some vestige ability to undergo DNA repair prior to entering mitosis. This presents a potential vulnerability that may be targeted with additional agents that may further disrupt cell cycle checkpoint activation, such as ATR inhibitors.

**ATR inhibitors in $ARID1A$ deficiency**

ATR inhibitors have previously been investigated in $ARID1A$ deficiency, with $ARID1A$-deficient cell lines exhibiting increased sensitivity to single-agent ATR inhibitors [27]. In this work, Williamson et al. present a working model for the synthetic lethality of ARID1A and ATR, in which loss of $ARID1A$ results in defective recruitment of DNA topoisomerase TOP2A to chromatin, resulting in a DNA decatenation defect. This defect in the ability to modify the topological structure of DNA is likely to disrupt vital cell activities, such as DNA replication and transcription. The failure to ultimately resolve these issues would thus cause genomic
instability and an increased reliance on ATR checkpoint activity that can be targeted with ATR inhibitors. They found that *ARID1A* deficiency sensitized cells to ATR blockade, which triggered premature mitotic entry and ultimately cell death. Of note, this important study conducts experiments within a colorectal cancer cell line model. Thus, we validated these results in a ovarian clear cell carcinoma cell line model and confirmed the increased sensitivity of the *ARID1A*−/− cells to the ATR inhibitor AZD6738 compared to *ARID1A*+/+ cells.

Clinical ATR inhibitors have been explored in clinical trials in recent years, primarily in advanced solid tumors and in combination with other chemotherapeutic agents, most of which are ongoing [46-51]. One phase I trial with 52 patients who received ATR inhibitor VX-970 in combination with gemcitabine for resistant/refractory advanced solid tumors demonstrated that this treatment regimen was well-tolerated in patients and showed preliminary efficacy [52]. Specifically in *ARID1A*-mutant gynecologic cancers, there is a current phase II clinical trial investigating ATR inhibitors in combination with PARP inhibitors in patients with *ARID1A*-mutant cancers [53]. This proof-of-concept trial will be very useful in revealing the feasibility and efficacy of using ATR inhibitors in treatment of *ARID1A*-mutant gynecologic cancers.

As mentioned above, previous work has shown that ATR inhibition in *ARID1A*-deficient tumors causes premature mitotic entry [27]. Further, our data suggests that *ARID1A*−/− cells treated with radiation may have a delayed cell cycle arrest. We hypothesized that ATR blockade would further sensitize *ARID1A*−/− cells to radiation therapy, with the thought that the radiation-induced delayed cell cycle arrest in *ARID1A*−/− cells would force increased reliance on ATR checkpoint activity. In our clonogenic survival assay, we treated cells with AZD6738 1 hour prior to radiation
treatment and replaced the drug-containing medium with fresh medium after 24 hours. With these conditions, though there was slight sensitivity of ARID1A<sup>−/−</sup> to the combination treatment compared to radiation alone, the difference was only significant at 6 Gy of radiation. Of note, however, is that our data demonstrating increased sensitivity to ATR blockade in ARID1A<sup>−/−</sup> cells was after 72 hours of AZD6738 treatment. Thus, it is possible that a longer treatment period with ATR inhibitor before or after radiation treatment may be necessary to observe a significant effect in ARID1A<sup>−/−</sup> cells. Future experiments will explore timing of ATR blockade prior to radiation, as well as the duration of drug treatment (with re-dosing as needed) after radiation treatment to more thoroughly evaluate this combination treatment in the setting of ARID1A deficiency.

**Limitations and future directions**

Much more future work is warranted with regards to investigating ARID1A deficiency. We hope to continue gaining mechanistic insight into ARID1A deficiency’s impact on the DNA damage response to explore potential vulnerabilities and to shed light on future targeted therapies for ARID1A-mutant tumors. Specifically, we believe that further insight into how ARID1A deficiency impacts specific DNA repair pathways may reveal further synthetic lethal targets.

A limitation of this work is the use only of *in vitro* cell line models. Future experiments will seek to validate results in patient-derived models with ARID1A mutations, rather than cell lines with experimental gene knockouts. Further, we will validate radiation and ATR blockade sensitivity within *in vivo* mice models in addition to using patient-derived xenografts. In the clinical setting, further work is needed to discern the prognostic value of ARID1A deficiency in gynecologic
malignancies to identify populations who may benefit from novel adjuvant therapies, particularly among ARID1A-mutant uterine cancer populations. Among ARID1A-mutant ovarian clear cell carcinoma patients, who tend to be more resistant to current chemotherapeutic regimens, future clinical trials testing the efficacy of treatments that may be more sensitive in ARID1A-mutant tumors are warranted. Though radiotherapy is not commonly used in ovarian cancer currently, advancements in radiation techniques has generated renewed interest in the application of radiotherapy in ovarian cancer and should be considered in the setting of ARID1A deficiency.

We hope that our work has contributed to the literature exploring ARID1A deficiency. In an age of medicine in which a greater understanding of cancer genomics informs targeted therapeutics, we hope our work brings us closer to finding optimized therapies for patients with ARID1A-mutant malignancies.

Comments about the COVID-19 era

This work was completed during the time period of September 2020 – July 2021 during the COVID-19 era, which posed considerable challenges on this project. As a new laboratory trainee, I had much to learn by way of laboratory protocols, cell culture, equipment use, and experimental techniques. Given social distancing requirements, I was unable to work closely with mentors and senior members of the lab and instead learned from distanced instruction and/or independent study. In addition, I had to troubleshoot day-to-day experiments more independently without the immediate presence of more senior members and had to be more proactive with seeking assistance. Nevertheless, I am incredibly grateful to have had this opportunity to work on this fascinating project and continue to foster my lifelong interest in cancer genetics. I do believe that this time period has strengthened my ability to think
and learn more independently, to be proactive in seeking assistance, and to maintain optimism in the face of unforeseen challenges, all of which I believe are valuable skills for me, as I look forward to my aspirations of becoming a physician-scientist.
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