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TITLE: Differential ADAR1 Dependency in Breast Cancer Reveals Therapeutic Opportunities Through Regulation of MDM2 and Ferroptosis

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14. ABSTRACT

We aim to examine hypotheses that the ADAR1-MDM2 signaling axis could **1)** mediate TNBC-associated ADAR1-dependency through ferroptotic cell-death; **2)** induce MDM2-addiction and sensitize breast cancer cells to MDM2-inhibition therapy, **depending on breast cancer subtypes**. To maximize the potential clinical utility and translational impact, we will incorporate high-throughput drug screening to identify drug candidates, from FDA-approved drug libraries, to provide proof-of-concept of ferroptosis-based therapeutic strategies against breast cancer. We hope to achieve both the **short-term-goal** of re-purposing existing drugs to demonstrate and amplify the clinical effect, and the **long-term-goal** of establishing a sustainable research program to highlight an innovative strategy against hard-to-treat breast cancers. In this first year, we have shown that ADAR1 protects TNBC from iron-dependent metabolic cell death. We have discovered that ADAR1 loss sensitizes TNBC to ferroptosis and MDM2 is a potential contributor to ADAR1-regulated ferroptosis sensitization.

15. SUBJECT TERMS
ADAR1, triple-negative breast cancer, MDM2, ferroptosis, drug screening, drug repurposing

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Table of Contents

Page

1. INTRODUCTION

We recently demonstrated that the expression of an RNA-editing enzyme, ADAR1, is essential for TNBC tumorigenesis. This TNBC-centric phenomenon suggests distinct mechanisms separating the biology and vulnerability between TNBC and non-TNBC. To identify genetic factors involved in this pathway through RNA sequencing, we recognized MDM2 as the most prominently overexpressed gene upon ADAR1 loss, in both TNBC and non-TNBC cell lines. In this proposal, we provide rationales, supported by preliminary data, to investigate potential roles of ADAR1-repressed MDM2 expression in the survival of breast cancer cells. We aim to examine hypotheses that the ADAR1-MDM2 signaling axis could **1)** mediate TNBC-associated ADAR1-dependency through ferroptotic cell-death; **2)** induce MDM2-addiction and sensitize breast cancer cells to MDM2-inhibition therapy, **depending on breast cancer subtypes**. To maximize the potential clinical utility and translational impact, we will incorporate high-throughput drug screening to identify drug candidates, from FDA-approved drug libraries, to provide proof-of-concept of ferroptosis-based therapeutic strategies against breast cancer. We hope to achieve both the **short-term-goal** of re-purposing existing drugs to demonstrate and amplify the clinical effect, and the **long-term-goal** of establishing a sustainable research program to highlight an innovative strategy against hard-to-treat breast cancers.

2. KEYWORDS

ADAR1, triple-negative breast cancer, MDM2, ferroptosis, drug screening, drug repurposing

3. ACCOMPLISHMENTS

Major Goals of the Project

There was one major goal for the first year of the grant proposal: determine if TNBC-associated ADAR1-dependency can be attributed to MDM2-mediated ferroptosis.

Goals Accomplished

***Major Task 1*.** To determine if TNBC-associated ADAR1-dependency can be attributed to MDM2-mediated ferroptosis (Months 1-18):

Subtask 1: To determine if ADAR1 loss induce ferroptosis (Months 1-6).

Subtask 2: To determine the level to which ferroptosis contributes to TNBC cell death caused by ADAR1 loss (Months 1-6).

Subtask 3: To determine if ferroptosis-related genes and lipid profiles are regulated in ADAR1-Deficient TNBC cells (Months 6-12)

To determine the level to which ferroptosis contributes to reduced proliferation and induced cell death in TNBC in response to ADAR1 loss, ADAR1-deficient TNBC cells were treated with ferroptosis inhibitors, including Ferrostatin-1 (Fer-1), Liproxstatin-1, or Deferoxamine (DFO) for 24 hours, and cells were subjected to CellTiter-Glo® Luminescent Cell Viability Assay. Ferroptosis inhibitors failed to restore ADAR1-protected cell viability, indicating that ferroptosis is not responsible for reduced proliferation and cell death induced by the loss of ADAR1 (data not shown).

Interestingly, ADAR1-deficient TNBC cell line MDA-MB231 displayed increased sensitivity to ferroptosis inducer RSL3 (**Fig. 1A**). Several breast cancer cell lines were then tested to determine IC50 of RSL3 between ADAR1-Intact and ADAR1-Deficient cells (**Fig. 1B**). Similar to MDA-MB231, ADAR1 knockdown also sensitized two other TNBC cell lines, HCC1806 and MDA-MB468, to RSL3. This elevated sensitivity to ferroptosis was further corroborated by a using a separate ferroptosis inducer, ML-162 (data not shown). Loss of ADAR1, however, did not sensitize Non-TNBC cell lines (MCF7, ZR75.1, T47D) we tested to RSL3 treatment.

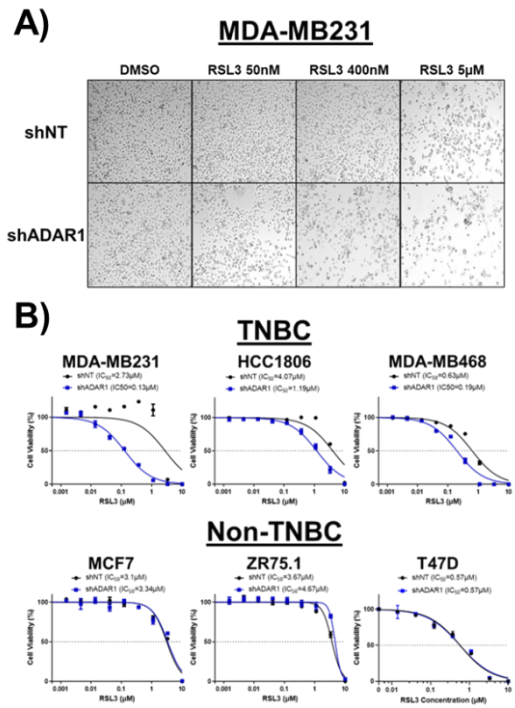

Figure 1. ADAR1 loss sensitizes TNBC to ferroptosis. **A)** ADAR1-Deficient MDA-MB231 is more sensitive to ferroptosis inducer RSL3. **B)** ADAR1 loss sensitizes TNBC, but not Non-TNBC, to RSL3. Black dots/trendline represent ADAR1-Intact (shNT) cells; Blue dots/trendline represent ADAR1-Deficient (shADAR1) cells. IC50, half maximal inhibitory concentration.

A form of metabolic cell death, ferroptosis is regulated by metabolic factors and mechanisms including Glutathione/Redox pathway, labile iron availability, and lipid compositions. To determine if ADAR1 is involved in the regulation of polyunsaturated fatty acid phospholipids (PUFA-PL) level to predispose TNBC cells to ferroptosis, ADAR1-Intact and ADAR1-Deficient MDA-MB231 cells were subjected to single-reaction monitoring-based liquid chromatography coupled to mass spectrometry (LC-MS) to measure the steady-state abundance of PUFA-PC (phosphatidylcholine), the main PUFA species contributing to ferroptosis regulation.

Upon ADAR1 loss, significant lipid remodeling was detected in MDA-MB231 cells (**Fig. 2**). Both the length (**Fig. 2B-C**) and level of unsaturation (**Fig. 2D-E**) for PC-FA were found to be significantly increased in ADAR1-Deficient (shADAR1) MDA-MB231 compared to ADAR1-Intact (shNT) cells, suggesting that ADAR1 loss results in downstream signaling pathways leading to lipid remodeling favoring ferroptosis sensitivity.

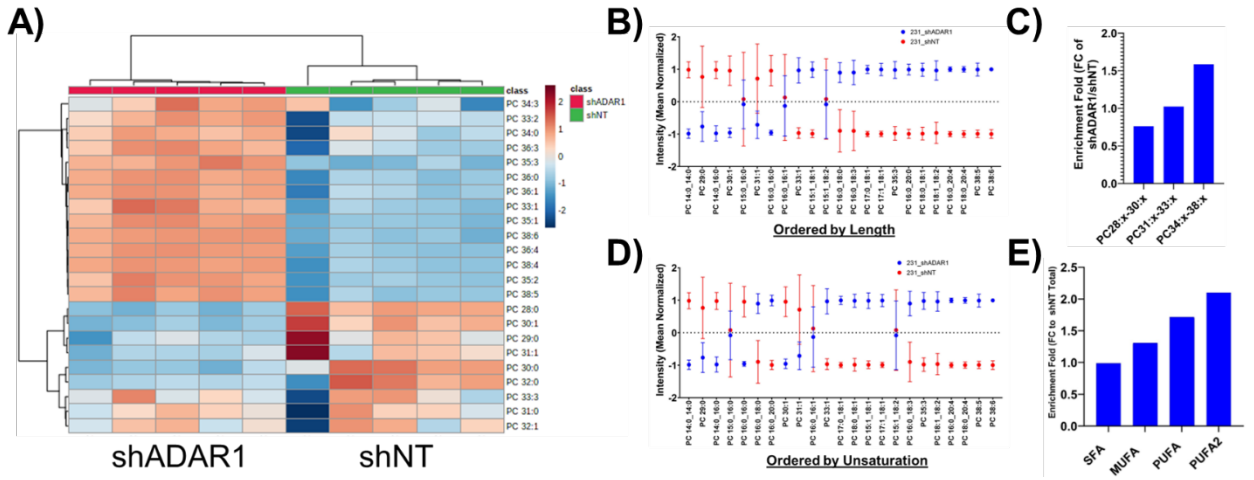

Figure 2. ADAR1 loss results in lipid remodeling favoring ferroptosis. **A)** Heat map of lipid profiling for phosphatidylcholine-fatty acid (PC-FA) between ADAR1-Intact (shNT) and ADAR1-Deficient (shADAR1) MDA-MB231 cells. **B)** Relative abundance of PC-FA listed in the order of carbon-chain length (from left to right: shorter to longer). **C)** Enrichment fold (shADAR1/shNT) of PC-FA for short (PC28-30), medium (PC31-33) and long (PC34-38) carbon-chain length. **D)** Relative abundance of PC-FA listed in the order of carbon-chain double-bond number (from left to right: lower to higher). **E)** Enrichment fold (shADAR1/shNT) of PC-FA for saturated (SFA), mono-unsaturated (# of double-bond = 1; MUFA), poly-unsaturated (# of double-bond > 2; PUFA), and hyper-poly-unsaturated (# of double-bond > 4; PUFA2) species.

To determine if ADAR1-loss results in molecular changes that predispose TNBC cells to lipid remodeling and ferroptosis, total RNA were extracted from ADAR1-Intact and ADAR1-Deficient MDA-MB231 cells, and quantitative real-time PCR (RT-PCR) was performed to determine relative expression levels of genes regulating lipid metabolism and ferroptosis (**Fig. 3**). Several genes we tested so far, including GCH1 and MFSD2A, were found to be expressed at significantly different levels between ADAR1-Intact and ADAR1-Deficient MDA-MB231 cells. Since these genes have been reported to play roles in regulating lipid metabolism pathways, including maintaining stability (GCH1) and transportation (MFSD2A) of PUFA, further investigation will determine if these alterations contribute to ADAR1-controlled ferroptosis sensitivity in TNBC.

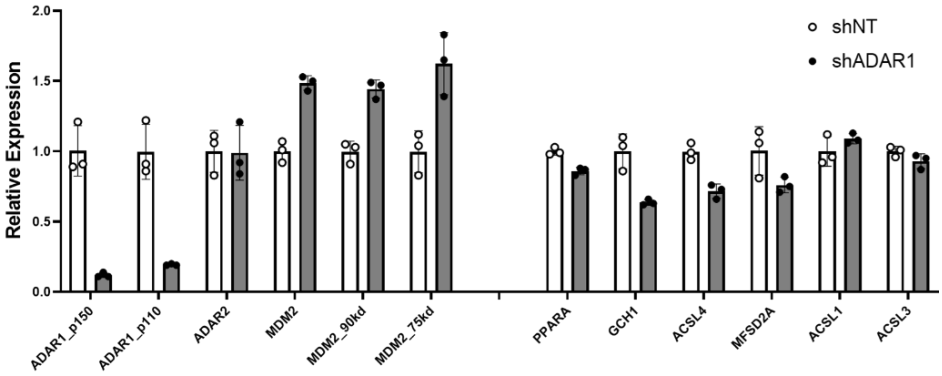

Figure 3. ADAR1 loss results in altered expression of genes involved in lipid metabolism. RNA was extracted from ADAR1-Intact (shNT) and ADAR1-Deficient (shADAR1) MDA-MB231 and subjected to qRT-PCR. ADAR1_p150, ADAR1_p110, and ADAR2 are controls for shADAR1 treatment. MDM2 induction was previously shown in ADAR1-Deficient TNBC cells.

To test if induction of MDM2 upon ADAR1 loss contributes to increased sensitivity to ferroptosis, we first decided to determine if overexpression of MDM2 is sufficient to sensitize TNBC cells to ferroptosis. We found that MDA-MB231 cells overexpressing MDM2 to similar level compared to ADAR1-Deficient cells are significantly more sensitive to RSL3 treatment (**Fig. 4A-B**). This result suggests that MDM2 induction upon ADAR1 loss partially contributes to elevated sensitivity to ferroptosis. To further corroborate this result, we will reverse the MDM2 induction in ADAR1-Deficient cells to determine if it is enough to reverse the sensitization phenotype. This experiment will be performed by

pharmacologically downregulating MDM2 by MD224, a proven MDM2 Proteolysis targeting chimeric (PROTAC) inhibitor in MDA-MB231 cells (**Fig. 4C**). These results will determine if ADAR1-mediated regulation of MDM2 is one of the mechanisms contributing to ferroptosis sensitization.

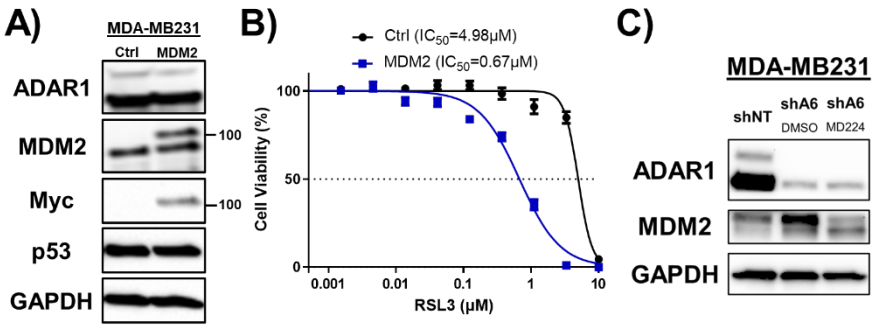

Figure 4. ADAR1-mediated regulation of MDM2 contributes to ferroptosis sensitization. **A)** Overexpression of MDM2 in MDA-MB231 cells shown by western blot analysis. Myc antibody detects MDM2 due to the presence of a myc-tag. **B)** MDM2-overexpressing MDA-MB231 cells are more sensitive to RSL3 treatment. **C)** MD224, a MDM2 PROTAC inhibitor, is capable of reducing MDM2 expression in MDA-MB231 cells. shA6 represents an effective shADAR1.

KEY RESEARCH ACCOMPLISHMENTS

- ADAR1 protects TNBC from iron-dependent metabolic cell death, ferroptosis.
- ADAR1 loss sensitizes TNBC to ferroptosis and results in significant lipid remodeling.
- MDM2 is a potential contributor to ADAR1-regulated ferroptosis sensitization.

REPORTABLE OUTCOMES

None

CONCLUSIONS

We have demonstrated that although ferroptosis does not account for reduced proliferation and induced cell death in ADAR1-Deficient TNBC, ADAR1 does protect TNBC from ferroptosis and we can significantly sensitize TNBC to ferroptosis by reducing ADAR1 expression. Investigations of relevant metabolic parameters have uncovered a novel lipid remodeling phenotype upon ADAR1 loss that favors sensitization of TNBC to ferroptosis. Further understanding of involved mechanisms will allow us to develop highly effective combinatorial therapeutic strategies by combining ADAR1 inhibition and ferroptosis induction.

Training Opportunities

Nothing to Report

Results Disseminated to the Community

I participated this past year in disseminating our initial findings to three independent groups of large donors to the American Cancer Society. These donors visited my laboratory at Washington University where I discussed the research in this grant proposal and how our results were moving the field of breast cancer research forward. We engaged in a question-and-answer session where the donors queried me on the clinical impact of this work. I anticipate doing this laboratory tour again next year and have already been asked by the American Cancer Society to do so. I also was the keynote speaker at the American Cancer Society Strides Against Breast Cancer event.

Plans for Next Reporting Period
In the second year, we will focus on determining whether inhibition of ADAR1 and MDM2 provide synthetic lethality in non-TNBC cells. We will also focus on re-purposing existing drugs that are capable of inducing ferroptosis or sensitizing TNBC cells to ferroptosis inducer, RSL3.

4. IMPACT

Impact on Principal Discipline
Our current work will be incredibly impactful for those studying breast cancer aggression in vitro and in vivo. We have uncovered a novel pathway underlying the ability of breast cancer epithelial cells to proliferate at a high rate and readily form transformed colonies in soft agar. These are all hallmarks of aggressive tumors. In year 2, we will move these findings into a more relevant in vivo model system, hoping to underscore the importance of ADAR1 and ferroptosis in breast tumor aggressiveness and metastasis.

Impact on Other Disciplines
Nothing to Report

Impact on Technology Transfer
Nothing to Report

Impact on Society
We have disseminated the data and ideals from this grant proposal to several groups in the St. Louis community including the American Cancer Society and Bridget's Brigade for breast cancer. They were encouraged by our progress and excited about the future clinical impact our work might provide.

5. CHANGES/PROBLEMS

Changes in Approach
Nothing to Report

Anticipated Problems or Delays
Nothing to Report

Changes in Human, Animal Biohazards and/or Selective Agents
Nothing to Report

6. PRODUCTS

Publications, Conference Papers and Presentations
Nothing to Report

Internet Sites
Nothing to Report

Technologies or Techniques
Nothing to Report

Inventions, Patents and/or Licenses
Nothing to Report

7. PARTICIPANTS
Individuals That Have Worked on Project

Name:	Jason D. Weber
Project Role:	PI
Nearest person month worked:	0.6
Contribution to Project:	Dr. Weber served as the mentor for Dr. Kung in planning all experiments and overseeing the final data analysis.
Funding Support:	NIH R01CA190986, NIHR01CA174743, W81XWH-15-1-0528

Name:	Maxene Ilagen
Project Role:	Co-Investigator
Nearest person month worked:	0.6
Contribution to Project:	Ms. Ilagen will provide expertise and consultation in assay development for drug screenings and access to drug libraries.

Name:	Che-Pei Kung
Project Role:	Co-Investigator and Staff Scientist
Nearest person month worked:	12
Contribution to Project:	Dr. Kung will perform all of the experiments described in the proposal.
Funding Support:	None

Name:	Miriam Sindelar
Project Role:	Staff Scientist
Nearest person month worked:	0.6
Contribution to Project:	Ms. Sindelar will perform all of the free fatty acid measurementds and quantification using LC/MS.
Funding Support:	None

Changes in Active Other Support for PD/PI
Nothing to Report

Other Organizations Involved as Partners
Nothing to Report