AWARD NUMBER: W81XWH-19-1-0108

TITLE: Exploit Dimethyl Fumarate to Uncover Druggable Vulnerabilities and Prevent Recurrence of ER+ Breast Cancers

PRINCIPAL INVESTIGATOR: Dr. Irida Kastrati

CONTRACTING ORGANIZATION: Loyola University, Chicago, Maywood, IL

REPORT DATE: March 2023

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
March 2023	Annual	01Mar2022-28Feb2023

4. TITLE AND SUBTITLE

Exploit Dimethyl Fumarate to Uncover Druggable Vulnerabilities and Prevent Recurrence of ER+ Breast Cancers

5a. CONTRACT NUMBER
W81XWH-19-1-0108

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Irida Kastrati, PhD

E-Mail: ikastrati@luc.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Loyola University Chicago
Dept. of Cancer Biology
2160 S 1st Ave,
Maywood, IL 60153

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Our *in vivo* tumor studies using two complementary approaches, RNA transcriptomic analysis and chemoproteomics converged on identifying the IRF9/interferon (IFN) axis as critical to tumor recurrence and a therapeutic target of DMF in breast cancer. Next, we focused on two questions: (i) how does IRF9/IFN axis promote tumor recurrence, (ii) the molecular basis of IRF9 inhibition by DMF. We found that IRF9 expression is higher in luminal tumors compared to other subtypes or normal breast tissue using the TCGA database. IFN signaling is higher in mammospheres (MS), which enrich for cancer stem cells. We found that IRF9 expression is elevated and required for MS formation. DMF treatment inhibits IFN signaling in MS measured by ISGs (interferon stimulated genes) and results in succination of IRF9. We also found that DMF inhibits cytokine-stimulated transcription of ISGs. Based on modeling, we hypothesized that succination of IRF9 disrupts its interaction with STAT2 and was demonstrated using co-IP studies. We conclude that higher IRF9 bestows stem-like properties to promote tumor recurrence. IRF9 can be developed into a new target against tumor recurrence.

15. SUBJECT TERMS
DMF, Recurrence, ER+ breast cancer, Interferon signaling, Tamoxifen tolerance.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	10	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

Introduction

We have reported that co-treatment with DMF+Tamoxifen effectively prevented tumor recurrence compared to Tamoxifen treatment alone[1]. DMF as a small molecule is expected to covalently modify target proteins via succination of protein thiols. Therefore, by identifying the succination reactome of DMF in the breast cancer landscape, new critical drivers of tumor recurrence will emerge. In turn, this mechanistic insight will be harnessed therapeutically to prevent tumor recurrence.

Subtasks and Milestones for this Period According to SOW.

Our efforts were focused on Aim 2 subtasks and milestones of SOW shown in black in the table below.

Aim 2	Identify DMF's succination targets that are critical for tamoxifen persister cell survival.	Timeline (in months)
Subtask 1	Isolate tissue for proteomics from CLX and PDX tumors treated for 4 weeks with Veh, DMF, Tam and Tam+DMF	6-17
Subtask 2	Conduct proteomics using the isoTOP-ABPP method: prepare samples, run mass spectrometry	7-18
Subtask 3	Analyze isoTOP-ABPP proteomics data: define common and unique targets modified by DMF *in vivo*	9-20
Subtask 4	Prioritize targets for novelty in breast cancer and correlation to patient outcome	10-21
Subtask 5	Validate top 10 targets by using DMF probe to IP	21-28
Subtask 6	Generate CRISPR/Cas9 KO cell lines for top 10 candidates	28-32
Subtask 7	Screen of KO cell lines using clonogenic and re-growth assay	32-36
Milestone 1	Define the total succination reactome of DMF in tumor cells	
Milestone 2	Identify and validate unique targets modulated by DMF *in vivo*	
Milestone 3	Determine relevance of targets (either individually, or as a signature) on patient outcome	
Milestone 4	Establish DMF's molecular mechanism of action	
Milestone 5	Establish relevance of targets on recurrence	

Body

Subtask 4: Prioritize targets for novelty in breast cancer and correlation to patient outcome.

Our earlier studies using transcriptomics and activity-based proteomics on tumor samples converged on the interferon pathway (IFN) and the interferon regulated factor 9 (IRF9) as the likely primary mediators of DMF actions on tumor recurrence (please see the report from previous year). Type I IFNs, such as IFNα/β, bind their cognate IFN-α/β receptor (IFNAR) complex, which is associated with tyrosine kinases. The activation of both JAK1 (Janus kinase 1) and TYK2 (Tyrosine Kinase 2) results in the downstream phosphorylation of both STAT1 and STAT2. The phosphorylated STAT1-STAT2 heterodimer associates with a third transcription factor, IRF9, leading to the formation of the IFN-stimulated gene (ISG) factor 3 (ISGF3) complex. The ISGF3 complex then translocates to the nucleus and binds DNA sequences referred to as IFN-stimulated response elements (ISRE) within the ISG promoter regions, leading to ISG transcription. While IFN signaling plays a critical role during viral infections, emerging data suggest an important role for IFN in tumorigenesis through modulation of immune surveillance. Independent of viral infection, both IFNs and ISG expression have been found in human tumors. While the effects of IFN signaling and ISG phenotypes vary in cancer biology, it remains clinically relevant to gain a deeper understanding of IFN signaling regulation in this context.

<u>IRF9 levels are elevated in luminal breast tumors and predict poor patient outcome.</u> Investigation into patient databases such as The Cancer Genome Atlas (TCGA) shows that IRF9 is elevated in breast tumors compared

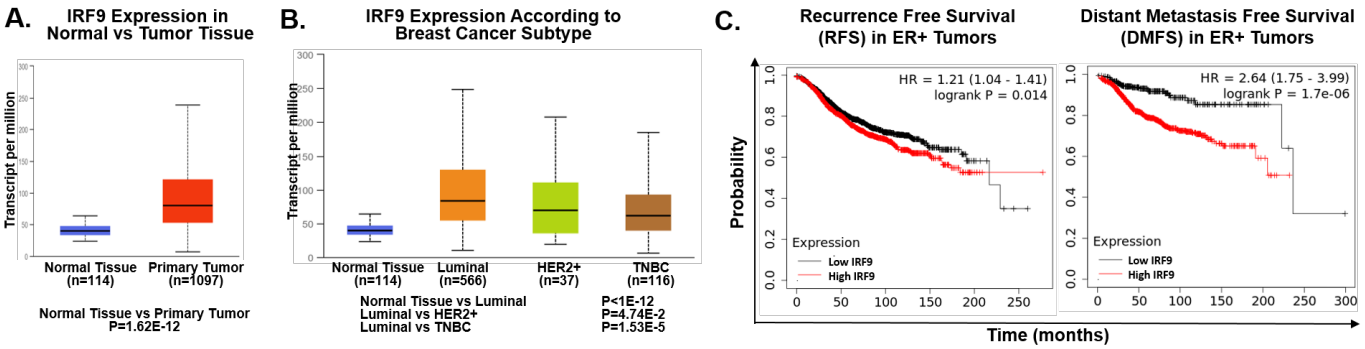

Figure 1. IRF9 expression levels and outcome data in breast cancer patient databases. (A-B) IRF9 mRNA expression in normal breast tissue versus primary breast tumors (A) or stratified according to breast cancer subtype (B) from the TCGA database. IRF9 expression levels are highest in Luminal tumors. P-values are indicated below. (C) Recurrence free survival or distant metastasis free survival in ER+ breast cancer according to IRF9 expression. High IRF9 predicts poor patient outcome. Data plotted with kmplot.com.

to normal tissue (Fig. 1A); IRF9 expression is highest in luminal or estrogen receptor (ER)+ tumors compared to the other subtypes (Fig. 1B), and specifically in these luminal ER+ tumors, high IRF9 predicts poor patient outcome (Fig. 1C). A report from 2001 showed that IRF9 overexpression confers resistance to antimicrotubule chemodrugs in MCF-7 cells, but the impact on endocrine therapy was not investigated[2]. The mechanisms by which IRF9 may drive aggressiveness and ER+ tumor recurrence remain unknown. **Therefore, based on its novelty and correlation to patient outcome, we prioritized IRF9 as the DMF target going forward.**

Subtask 5: Validate top 10 targets by using DMF probe to IP.

So far we have validated the oncogenic transcription factor ZNF217 using the DMF probe as recently published (see Sharma T., et al, BCRT 2023). To validate IRF9 succination by DMF identified in residual tumor cells *in vivo* via the isoTOP-ABPP methodology, we used a DMF chemical probe bearing an alkyne handle (Fig. 2A) as we have previously reported[3]. We synthesized this DMF-probe designed to replicate the biological

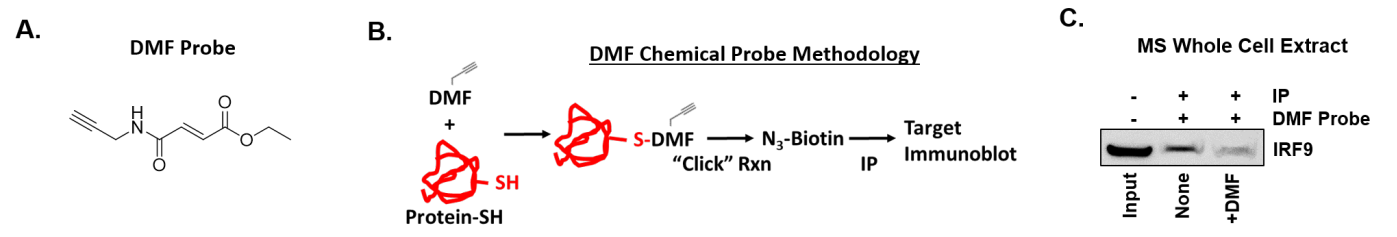

Figure 2. IRF9 is validated as a DMF target using immunoprecipitation by the DMF-Probe. (A) The chemical structure of DMF Probe. (B) A schematic representation of DMF chemical probe methodology to identify DMF succination targets. (C) Lysates from MCF-7 MS were reacted with DMF-Probe at 50μM. Pretreatment with DMF (3^{d} lane) is used to determine specificity of DMF-Probe. The 'click chemistry' was used to crosslink the azido-biotin tag to DMF-Probe. IRF9 protein levels were measured by immunoblotting and compared between IPs and inputs (1st lane, 10% of total protein lysate).

activity of DMF. The small alkynyl modification to DMF serves as a 'handle' to allow visualization and immunoprecipitation (IP) of succinated proteins (Fig. 2A-B) without loss of the specific bioactivity of DMF[3,4]. This method has an advantage over the chemo-proteomics approach because the IP by DMF-probe does not rely on tryptic digest or mass spectrometry for protein identification. **Using this DMF-chemical probe, we were able to identify and validate IRF9 as a succination target of DMF in mammospheres (MS) from ER+ cells (Fig. 2C).** The validation of additional target will be pursued later.

Subtask 6: Generate CRISPR/Cas9 KO cell lines for top 10 candidates.

We have recently reported the deletion of ZNF217 in breast cancer cells using CRISPR/Cas9 gene editing (see Sharma T., et al, BCRT 2023). We applied the same strategy to IRF9, but so far we have been unsuccessful to select cells after transduction. This may indicate that IRF9 is an essential factor in ER+ breast cancer cells**. In the meantime, we utilized an siRNA approach to interrogate the role of IRF9 in recurrence models as shown in Figs. 3E and 5E.** Deletion of additional targets will be pursued later.

Subtask 7: Screen of KO cell lines using clonogenic and re-growth assay.

DMF inhibits elevated IFN-IRF9-ISGs signaling in breast cancer stem cells (CSCs) via succination of IRF9. To model residual persister tumor cells *in vitro*, as an alternative to clonogenic assay, we utilized the mammosphere (MS) assay, which enriches for stemness, a predominant feature of residual disease[5]. MCF-7 cells were seeded at single cell density and grown in undifferentiating detached conditions for 7 days which results in MS formation and enrichment for CSCs (Fig. 3A). Transcriptomics and bioinformatics analysis indicates that IFN signaling is upregulated in MS (Fig. 3B).

The expression of ISGs is significantly upregulated in MS compared to standard adherent 2D culture even in

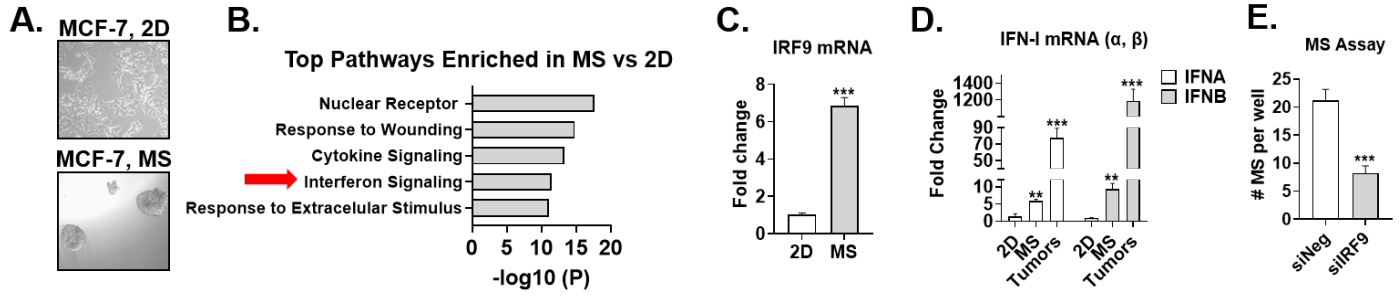

Figure 3. IFN-IRF9 signaling is upregulated and required in mammospheres. (A) Representative images of adherent 2D bulk culture (up) versus mammosphere (MS, bottom) of MCF-7 cells. (B) RNAseq and bioinformatics for top-enriched pathways in MS compared to 2D. (C) IRF9 expression was measured by RT-QPCR. (D) Type I IFNα and β expression were measured by RT-QPCR in 2D, MS and in MCF-7 xenograft tumors. Data normalized to 2D conditions. (E) Cells were transfected with 20nM siNeg or siRNA for IRF9 for 48hrs, then trypsinized and seeded for MS formation assay for 7 another days. ** p<0.01, ***p<0.001.

the absence of any exogenous IFN addition into the culture (Fig. 4A, white vs grey bars). Moreover, IRF9 expression (Fig. 3C) and protein levels (data not shown) are elevated in MS. The expression of cytokine genes IFNα and β is elevated in a similar fashion in MS compared to standard 2D and in xenograft tumors (Fig. 3D). When IRF9 is silenced with an siRNA, it significantly blocks mammosphere formation (Fig. 3E). *Altogether, these data indicate that IRF9 is elevated and required in CSCs from ER+ cell lines.*

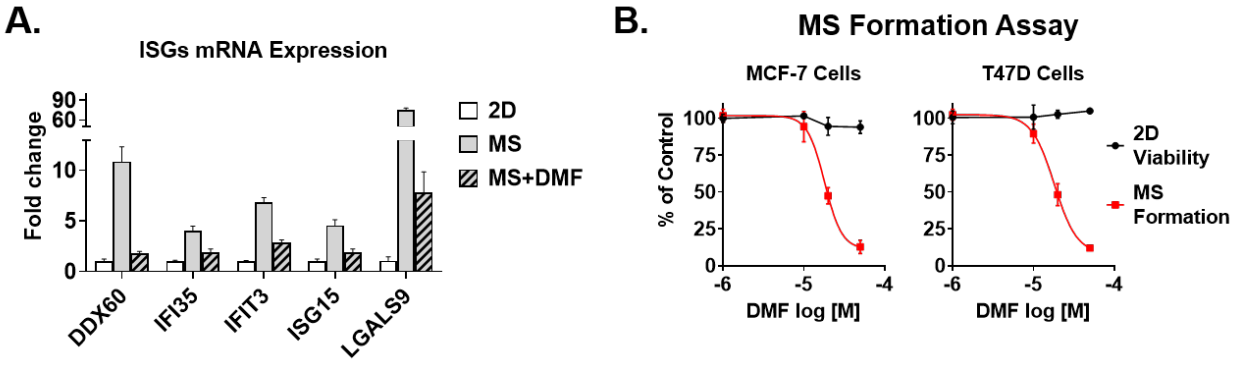

Figure 4. DMF inhibits IFN signaling in mammospheres (MS) which may contribute to its anti-CSC activity. (A) The expression of interferon stimulated genes (ISGs) is elevated in MS and is inhibited by DMF. DMF, 50μM, was added to MS for the last 24hrs in culture. Gene expression was measured by RT-QPCR and normalized to 2D conditions. (B) Dose response curves for DMF's activity on 2D (black line) vs MS formation(red line) in ER+ cell lines, MCF-7 and T47D.

DMF addition for the last 24hrs in MS culture results in inhibition of ISGs' expression, indicating that DMF inhibits elevated IFN signaling in MS (Fig. 4A, gray vs hashed bars). We have previously shown that DMF inhibits MS growth of ER+ cells at doses that do not affect adherent 2D culture (Fig. 4B)[3]. This may be due to DMF's inhibition of IRF9, which is elevated and required in MS culture.

To summarize, our data indicates that IFN-IRF9 axis is elevated and required in mammospheres (MS); DMF inhibits IFN signaling in MS via succination of IRF9, and this may contribute to DMF's anti-CSC activity.

DMF inhibits cytokine induced IFN-IRF9 signaling. While MS culture displays intrinsic activation of IFN-IRF9 signaling, adherent standard 2D culture displays no basal activation. For this reason, ER+ MCF-7 breast cancer cells in standard culture were treated with IFNα cytokine, which results in a significantly increased

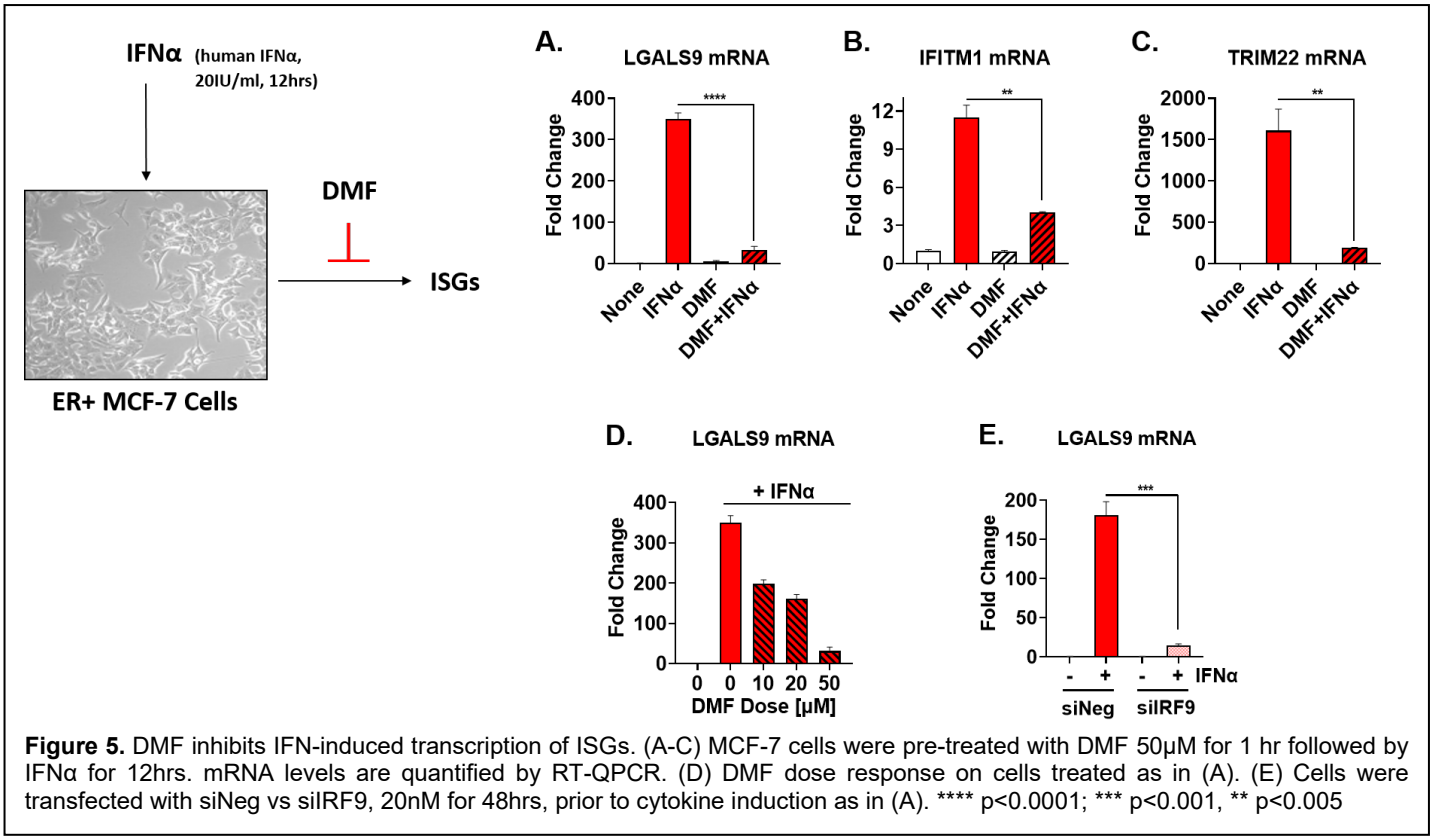

Figure 5. DMF inhibits IFN-induced transcription of ISGs. (A-C) MCF-7 cells were pre-treated with DMF 50μM for 1 hr followed by IFNα for 12hrs. mRNA levels are quantified by RT-QPCR. (D) DMF dose response on cells treated as in (A). (E) Cells were transfected with siNeg vs siIRF9, 20nM for 48hrs, prior to cytokine induction as in (A). **** p<0.0001; *** p<0.001, ** p<0.005

expression of a number of ISGs, such as LGALS9, IFITM1 and TRIM2 (Fig. 5A, B, C, second bar in red), indicative of pathway activation. Co-treatment with DMF significantly inhibited the transcription of all ISGs in a similar pattern (Fig. 5A, B, C, last bar in hashed). Next, we conducted a DMF-dose response study and estimated that the IC_{50} for DMF inhibition of ISGs is around 20μM as illustrated by LGALS9 gene (Fig. 5D). Lastly, we confirmed that the ISGs profiled above are *bona fide* IRF9-target genes by using an siRNA against IRF9 (Fig. 5E). *To summarize, DMF effectively inhibits IFN cytokine-induced transcription of IGSs in ER+ breast cancer cells that are regulated by the IRF9 transcription factor.*

IRF9 succination disrupts the IRF9-STAT2 complex. To determine the molecular basis of IRF9 inhibition by DMF, we used molecular modeling and predicted that covalent modification of cysteine 319, which was identified by mass spectrometry as the succination site of IRF9, may disrupt the interface with STAT2 (Fig. 6A-B). To test this hypothesis, we used co-immunoprecipitation of STAT2 and IRF9. DMF either alone or in combination with IFN results in reduced pull-down of STAT2 and IRF9 (Fig. 6C, see last 2 lanes). This indicates that succination of IRF9 by DMF disrupts the IRF9-STAT2 complex.

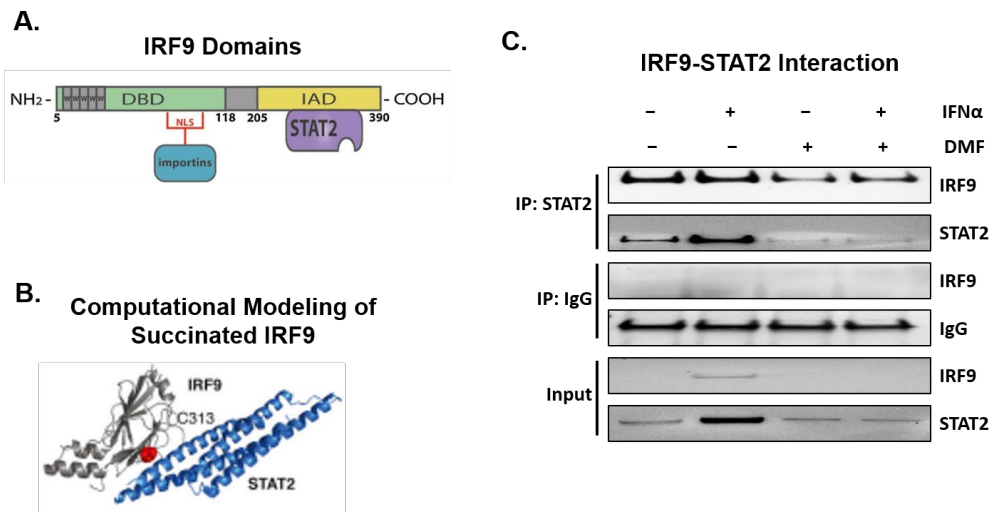

Figure 6. Succination of IRF9 disrupts the IRF9-STAT2 complex. (A) IRF9 domains relevant for the interaction with STAT2. (B) Molecular modeling based on the crystal structures of murine IRF9 (PDB: 5OEN) and STAT2. Cysteine C313 in murine IRF9 is orthologous to C319 in human IRF9 that was succinated by DMF in our studies. (C) MCF-7 cells were treated as in Fig. 5A. Cell lysates were IP-ed using STAT2 and probed for IRF9 levels. IgG IP was used as a control.

Conclusion: This work is establishing IRF9 as e new target against tumor recurrence. The mechanistic insights pursued here to better understand the role of IRF9 succination and IRF9 signaling can be used to generate a new class of inhibitors or drugs with implications in oncology and other immune pathologies. IRF9 levels can be used to stratify patients at high risk of recurrence that are most likely to benefit from DMF.

Key Research Accomplishments

- We followed up on a top target/pathway identified by both tumor transcriptomics and activity-based proteomics, the interferon/IRF9 axis, as a target of DMF and relevant to tumor recurrence.
- We demonstrated in two models, IFN cytokine-induced and in intrinsically activated IFN axis in MS, that DMF can inhibit this pathway effectively by succination of IRF9 and disruption of IRF9-STAT2 interaction.
- Our work indicates that IRF9 drives tumor recurrence at least in part by bestowing stem-like properties on tumor cells.
- There are no known inhibitors for IRF9. DMF can be used as the starting point to generate more specific IRF9 inhibitors.
- IRF9 can be developed into a therapeutic biomarker to identify patients at high risk of recurrence that are most likely to benefit from DMF.

Reportable Outcomes

1. A new paper on identifying ZNF217 as a druggable target relevant to endocrine resistance and recurrence has been recently published (Sharmal et al, BCRT, 2023).
2. We have made progress in identifying critical signaling pathways and druggable targets of DMF driving tumor recurrence.

Conclusion

Our progress is on track and follows the approved SOW.

References

1. Kastrati, I.*, et al.* The NF-kappaB Pathway Promotes Tamoxifen Tolerance and Disease Recurrence in Estrogen Receptor-Positive Breast Cancers. *Mol Cancer Res* **18**, 1018-1027 (2020).
2. Luker, K.E., Pica, C.M., Schreiber, R.D. & Piwnica-Worms, D. Overexpression of IRF9 confers resistance to antimicrotubule agents in breast cancer cells. *Cancer Res* **61**, 6540-6547 (2001).
3. Kastrati, I.*, et al.* Dimethyl Fumarate Inhibits the Nuclear Factor kappaB Pathway in Breast Cancer Cells by Covalent Modification of p65 Protein. *J Biol Chem* **291**, 3639-3647 (2016).
4. Speers, A.E., Adam, G.C. & Cravatt, B.F. Activity-based protein profiling in vivo using a copper(i)-catalyzed azide-alkyne [3 + 2] cycloaddition. *Journal of the American Chemical Society* **125**, 4686-4687 (2003).
5. De Angelis, M.L., Francescangeli, F., La Torre, F. & Zeuner, A. Stem Cell Plasticity and Dormancy in the Development of Cancer Therapy Resistance. *Front Oncol* **9**, 626 (2019).

Appendices

See pdf of recently published paper in the journal of *Breast Cancer Research and Treatment (BCRT)*.