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TITLE: BUD31 and Lipid Metabolism: A New Potential Therapeutic Entry Point for Myc-Driven Breast Cancer

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14. ABSTRACT Myc activation is common in breast cancer, correlated with triple negative disease, and associated with mortality. Thus, understanding Myc-driven breast cancer will facilitate knowledge of triple negative disease, a subtype of breast cancer with poor outcome and limited treatment options. Our laboratory has performed a genome-wide RNAi screen to identify genes that are required to tolerate Myc activation. Through this screen, we have identified BUD31, a poorly understood gene, and components of the fatty acid oxidation pathway (FAMs) as required for tolerance of Myc driven stress. Our goal herein is to test the hypothesis that BUD31 and FAMs may be putative new therapeutic entry points for Myc-driven breast cancer. Within this period of performance, we have published a manuscript in Nature detailing a role for BUD31 in splicing and shown more broadly that splicing is a viable therapeutic intervention point for myc-driven breast cancer. The connection between BUD31 and FAMs still remains unclear. Nevertheless, in support of our hypothesis and data, others have more recently published and presented mechanisms whereby fatty acid oxidation is a point of treatment sensitivity in triple negative breast cancer (TNBC). Their and our initial findings suggest that FAMs and downstream pathways may be particularly important for metastatic TNBC. As metastasis is the leading cause of breast cancer patient mortality, we believe this avenue warrants further attention.				
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1. INTRODUCTION:

Background: Myc activation is common in breast cancer, correlated with triple negative disease, and associated with mortality. Thus, understanding Myc-driven breast cancer will facilitate knowledge of triple negative disease, a subtype of breast cancer with poor outcome and limited treatment options. Myc confers both pro- and anti-tumorigenic effects on cells suggesting a sensitive balancing act for survival downstream of Myc activation. Supporting pathways, while not oncogenic by themselves, are necessary to help cells tolerate Myc driven stresses. Since direct pharmacological targeting of Myc has been shown to be largely unsuccessful, our laboratory has performed a genome-wide RNAi screen to identify supporting genes that are required to tolerate Myc activation in human mammary epithelial cells (Kessler et al.). Through this screen, we have identified BUD31, a poorly understood gene, and components of the fatty acid oxidation pathway (hereafter FAMs- fatty acid oxidation Myc synthetic lethal genes) as required for tolerance of Myc driven stress and as putative new therapeutic entry points for Myc-driven breast cancer. Our preliminary data indicate that BUD31 physically interacts with FAMs suggesting a functional relationship between the two. **Objective/Hypothesis:** Based on these preliminary data, we hypothesize that Myc activation confers a dependence on BUD31 and FAMs for breast cancer survival through a mechanism involving physical and functional interaction between BUD31 and fatty acid oxidation. **Specific Aims and Study Design:** To directly test the hypothesis above, we propose the following specific aims. AIM1: To determine if BUD31 interactions with lipid metabolism putative binding partners are necessary for the dependence of Myc-driven breast cancer on BUD31. We will perform structure-function analysis using the orthogonal methods of bimolecular fluorescence complementation and co-immunoprecipitation to confirm and map BUD31 interaction with lipid metabolism components and to test whether specific disruption of this binding alters the function of confirmed binding partners. AIM2: To confirm the Myc-induced reliance on FAMs in breast cancer and identify the mechanism of dependence of Myc-dependent breast cancer on fatty acid oxidation. We will test if depletion of FAMs in Myc dependent and independent cell lines induces toxicity in vitro. To identify Myc-driven changes to lipid metabolism, we will use mass spectrometry and Biolog analysis of metabolites. Analysis of publically available datasets for correlations between FAM gene expression and patient outcome will be performed. AIM 3: To systematically examine BUD31/FAMs requirements for Myc-driven breast cancer in vivo. We will utilize innovative in vivo screening technology and a panel of orthotopic syngeneic transplant mouse models both developed by our laboratory to test if BUD31/FAMs are necessary for tolerance of Myc. Genetic and pharmacological approaches to inhibit these targets will determine if this strategy presents a viable option for the treatment of Myc-driven breast cancer. **Impact:** Overall, this proposal identifies a novel therapeutic entry point for patients with Myc-driven and triple negative breast cancer. Our work will provide the pre-clinical data necessary for translating this strategy to the clinic with the ultimate goal of reducing both non-specific toxicities associated with treatment and overall mortality.

2. **KEYWORDS:** breast cancer, Myc synthetic lethality, Bud31, fatty acid metabolism

3. ACCOMPLISHMENTS:

What were the major goals of this project? What was accomplished under these goals?

Task 1: To determine if BUD31 interactions with lipid metabolism putative binding partners are necessary for the dependence of Myc-driven breast cancer on BUD31 AND Task 2: To confirm the Myc-induced reliance on FAMs in breast cancer and identify the mechanism of dependence of Myc-dependent breast cancer on fatty acid oxidation

As described in our year 1 progress report, we were able to successfully confirm the myc synthetic lethal phenotype of fatty acid metabolism gene depletion. However, our in vitro breast cancer cell line work and bioinformatics analysis of a large panel of cell lines yielded inconsistent results. As such, we proposed to focus on in vivo experiments/models to test our hypothesis that fatty acid oxidation, as this is ultimate pre-clinical data necessary to support a clinical relevance. No additional data to report for these tasks.

Task 3: To systematically examine BUD31/FAMs requirements for Myc-driven breast cancer in vivo.

We have developed a novel set of technologies that allows us to induce depletion of genes of interest in a human breast cancer cells at the time and physiological setting of our choosing. To test the ability of our system to sort out the most effective fatty acid metabolism MySLs, we compared the effects of depletion in three settings: in vitro, in established primary tumors, and in established metastatic lesions (Figure 1). We reasoned that by pitting each candidate against each other in 3 different relevant settings we could identify the most robust therapeutic intervention points. We chose to directly compare depletion of BUD31 with that of NDUFB10, an electron transport chain gene that facilitates the use of fatty acid oxidation byproducts, since NDUFB10 had 3 independent shRNAs with a strong myc synthetic lethal phenotype in the original screen (Kessler et al. 2012). In an in vitro competition assay, cells with depletion of NDUFB10 phenocopy the effects of BUD31 depletion suggesting that our original screen identified excellent candidates for further study (Figure 1A). However, this effect is not reproduced in primary tumors, where NDUFB10 depletion has little effect on overall presence of NDUFB10 shRNAs expressing LM2 cells, as monitored by qPCR detection of surrogate barcodes (Figure 1B). Interestingly, when these exact cell populations are placed in the microenvironment of the lung and form metastases, NDUFB10 depletion has a much more profound

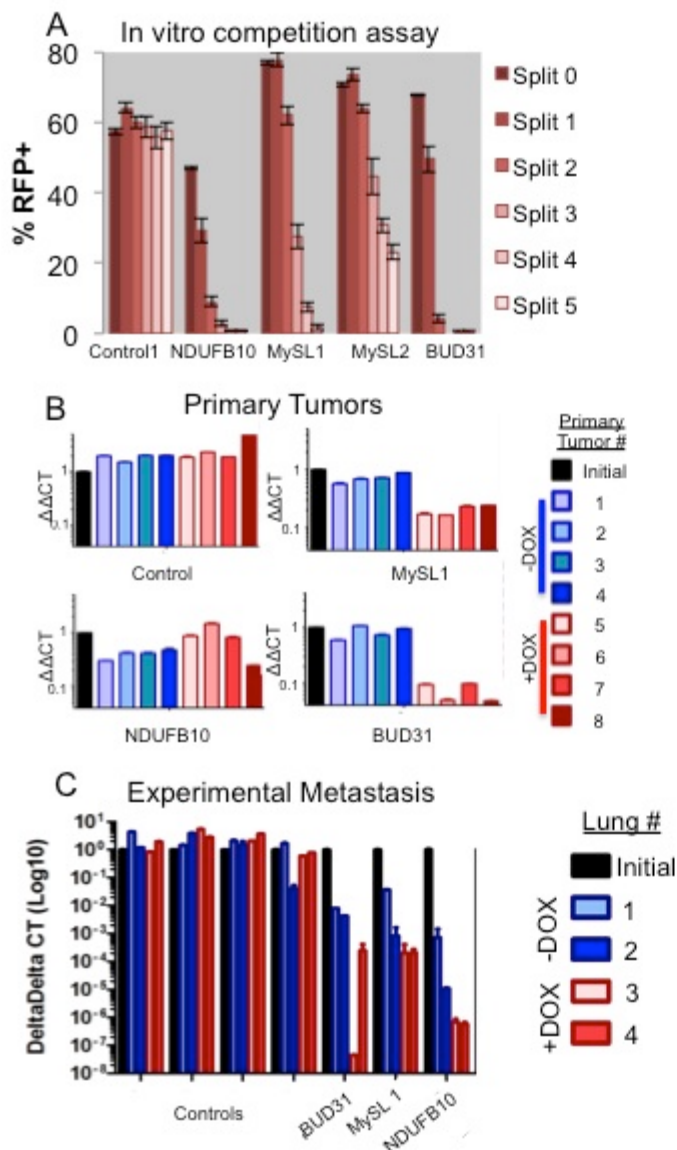


Figure 1. NDUFB10 depletion is preferentially toxic to LM2 human breast cancer cells in metastases

LM2 cells were genetically manipulated to express doxycycline-inducible shRNA vectors targeting the following: negative control sequences; MySL (1 and 2)- not related to FAO; BUD31, and NDUFB10 (an electron chain gene downstream of FAO). A. Manipulated cells were mixed 60:40 with parental lines, passaged in the presence of doxycycline, and then assayed for RFP as a surrogate for manipulated cells. These in vitro competition assays demonstrate strong selection against cells expressing shRNAs targeting BUD31, NDUFB10, and MySL1. B. Manipulated cells were mixed together in vitro, injected subcutaneously into mice and allowed to form tumors. After randomization onto control or doxycycline treatment, tumors were analyzed for detection of barcodes as a surrogate for presence of shRNA expressing cells. Only BUD31 and MySL1 shRNA cells demonstrated consistent loss in the presence of doxycycline. C. Manipulated cells were mixed together in vitro, injected subcutaneously into mice and allowed to form metastases. After randomization onto control or doxycycline treatment, lungs were analyzed for detection of barcodes as a surrogate for presence of shRNA expressing cells. BUD31 and NDUFB10 depletion is highly toxic to cells in the metastatic setting. **Note that while all animal experiments were performed under full IACUC approval, metastasis studies were performed without the use of funds from this CDMRP DOD fellowship.**

effect on cell viability, suggesting that even the same breast cancer cell line will respond differently to targeting metabolism depending on context. These findings support the idea that context matters and the experimental constraints by which we assay our FAM MySLs are highly important. These data support continued study of these players in an in vivo setting, rather than the less physiologically relevant conditions posed by cell culture in vitro (e.g. excess glucose).

During this funding year, we became aware of two groups working on very closely related topics to those proposed herein. Indeed, one study, just recently accepted for publication in Cell Reports by our colleague Dr. Benny Kaiparettu, directly supports our hypothesis that fatty acid oxidation is an excellent therapeutic intervention point for the treatment of breast cancer (Park et al., 2016). Interestingly, they found that pretreatment of MDA-MB-231 cells in vitro and continued treatment in vivo with etomoxir, an inhibitor of the rate-limiting step of FAO, significantly blocks the metastatic colonization of the lung in an experimental metastasis paradigm where cells are injected into the tail vein. Additionally, Dr. Andrei Goga and colleagues at UCSF have presented studies in which they suggest that fatty acid oxidation is myc synthetic lethal and its inhibition is a good strategy for treating breast cancer (SABCS abstract from Dr. Andrei Goga and colleagues).

Spontaneous Metastasis

Experimental Metastasis

PDX	Pulmonary Metastasis H&E*	qPCR
6257	ND	2 of 8
5471	33.3%	2 of 2
4013	21.4%	8 of 10
3807	0%	3 of 4
4913	0%	6 of 6
4272	28.6%	10 of 10
2665	7.1%	10 of 10
3204	28.6%	5 of 5
3611	0%	8 of 9
3107	0%	9 of 9

p53 Null GEMM	Pulmonary Metastasis
T2 Basal	0 of 2
T11 C.L.	0 of 3
T12 C.L.	0 of 4
2151R C.L.	1 of 3
2153L basal	2 of 6
2225L basal	3 of 3
2336R basal	0 of 4
2396R basal	0 of 2

Cells injected	Mets? (qPCR)
1000	0 of 5
5000	2 of 5
10000	3 of 5
100000	4 of 4

A histological image of lung tissue stained with Hematoxylin and Eosin (H&E). The image shows a large area of lung parenchyma on the left, labeled 'Lung'. On the right, there is a distinct area of metastatic tumor growth, labeled 'Metastasis'. The metastatic area shows dense cellular proliferation with hyperchromatic nuclei and disrupted architecture compared to the surrounding lung tissue. Below the image, the text 'H&E (6 weeks)' indicates the staining method and time point.

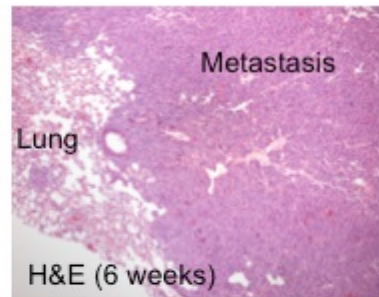


Figure 2. Building Models of TNBC Metastasis

To identify models of breast cancer metastasis, lungs from tumor-bearing mice of the following models were collected: 10 TNBC patient derived xenografts (all basal) and 8 TNBC transplantable genetically engineered mouse models (5 basal and 3 claudin low (CL)). The tables reflect detection of metastasis by tumor cell specific, highly sensitive qRTPCR assays. For the panel of PDXs, detection of metastasis in lungs from tumor bearing mice had previously been assessed using hematoxylin and eosin sections (*Data from Zhang et al. Cancer Research 2013). These data support the use of these models for studying spontaneous pulmonary metastasis. Additionally, many models that do not spontaneously metastasize, will do so via experimental metastasis assays. 6 weeks after tail vein injection of varying numbers of GEMM T11 cells, lungs were collected and qRTPCR assays were used to detect metastases. 100% penetrance of metastases across mice was achieved with injection of 100,000 cells. Representative H&E stained sections of lungs depict macro-metastases. Similar analysis has been performed across many of the GEMM lines. **Note that while all animal experiments were performed under full IACUC approval, this work did not utilize funds from this CDMRP DOD fellowship.**

Our striking findings with NDUFB10 depletion in metastasis coupled with the results presented by Dr. Benny Kaiparettu and colleagues in Cell Reports support the further exploration of these molecular pathways in the context of established metastasis. Additionally, it is well established that myc activation is associated with worse outcome and a propensity to metastasize. Indeed, examples of MYC amplification being detected in metastases but not in matched primary tumors have been reported suggesting an important role in progression to metastatic disease (Singhi et al., 2012). Given that metastasis is the leading cause of mortality for breast cancer patients, a thorough understanding of if and how a proposed therapeutic strategy works across a diverse set of metastatic models seems warranted.

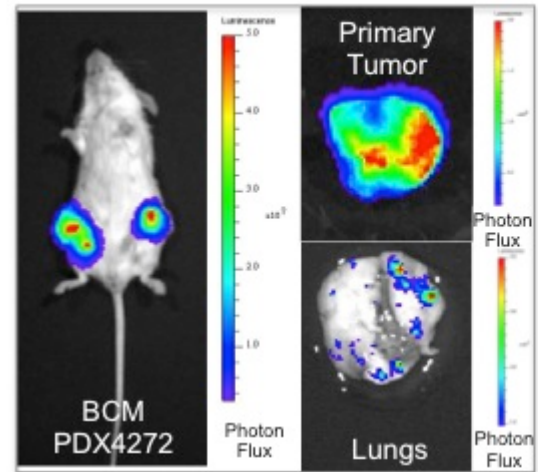


Figure 3. Labeling of PDX Models of Metastasis
Data shown here by whole animal and ex vivo organ bioluminescent imaging represents one example of a patient derived xenograft that has been engineered to express luciferase. Primary tumors were allowed to grow to maximum allowable size and then bioluminescence imaging was performed. Similar labeling has been achieved across a variety of our PDX and GEMM models of TNBC. **Note that while all animal experiments were performed under full IACUC approval, this work did not utilize funds from this CDMRP DOD fellowship.**

Unfortunately, there is currently a dearth of readily available models of breast cancer metastasis, with the field predominantly relying on a few cell lines (i.e. LM2 and 4T1) to model a highly heterogeneous and complex disease. Figure 2 depicts some of our ongoing work to expand the diversity of models to study metastatic TNBC. We have developed and utilized highly sensitive, quantitative qPCR assays to detect the presence of tumor cells in secondary organs. Currently, we have profiled a panel of 10 TNBC patient-derived xenografts (PDXs) and 8 transplantable genetically engineered mouse models (GEMMs) for their spontaneous metastatic capacity (Figure 2). In addition to identifying models that metastasize from the orthotopic site and complete the entire metastatic cascade, we have determined the conditions and kinetics necessary for experimental metastasis assays. Furthermore, to support future pre-clinical studies and testing hypothesis in metastatic disease, we have developed protocols in which we can infect both PDXs and GEMMs with lentiviral bioluminescent reporter constructs. For example, we have successfully labeled PDX models with luciferase to allow for monitoring of these cells in live animals and ex vivo (see Figure 3 for one example). The development of these types of protocols allow for manipulation of PDXs and GEMMs either for labeling and tracking or genetic experiments like those described with cell lines above. By building these tools, this will enable us to systematically test FAO and other pathways as intervention points in MYC-driven metastatic breast cancers. Moreover, these models and methods will be critical for the field in testing other biologies and intervention point.

References:

Kessler et al. Science. 2012 Jan 20;335(6066):348-53. A SUMOylation-dependent transcriptional subprogram is required for Myc-driven tumorigenesis.

Hsu et al. Nature. 2015 Sep 17;525(7569):384-8 The spliceosome is a therapeutic vulnerability in MYC-driven cancer.

Park et al. Cell Reports. 2016. Fatty Acid Oxidation-Driven Src Links Mitochondrial Energy Reprogramming and Oncogenic Properties in Triple-Negative Breast Cancer.

Singhi et al. Mod Pathol. 2012. Mar; 25(3); 378-387. MYC Gene Amplification is Often Acquired in Lethal Distant Breast Cancer Metastases of Unamplified Primary Tumors

What opportunities for training and professional development has the project provided?

Training during Year 2:

As indicated on the training plan for this fellowship and as in last year, I have continued to participate in and attend both Rosen and Westbrook laboratory meeting, journal clubs relevant to breast cancer, breast disease research group seminars, and a variety of other breast cancer-relevant seminars at Baylor College of Medicine and MD Anderson Cancer Center. These events have provided excellent knowledge of the latest research and findings in the field of breast cancer. I have learned new technical skills and developed novel protocols, including working with and genetically manipulating PDX models of breast cancer. As part of necessary training for transitioning out of my postdoctoral position upon completion of year 3 of this fellowship, I have been attending monthly meetings with BCM's Career Center and participating in the BCM career development seminar series to acquire and refine additional skills, such as CV development. As part of honing my grant writing skills necessary for advancement after fellowship completion, I have worked closely with my mentor, Dr. Jeffrey Rosen, to write a Diana Helis Henry Medical Research Foundation Grant. My focus has been on amassing and enhancing both technical and soft skills and will continue to be such in year 3.

Symposia and Conferences attended during Year 2:

- BCM Breast Center Research Meeting- Aug.27-28, 2015, Montgomery, TX
 - I was an invited speaker for this event and was voted 1st place speaker
- BCM Metastatic Breast Cancer Conference- September 18-19, Houston, TX
 - I was the only invited trainee speaker for this outstanding conference of clinicians, scientists, and advocates.
- AACR Advances in Breast Cancer Research: Oct.17-20, 2015, Bellevue, WA
- AACR Tumor Metastasis: Nov. 30-Dec. 3, 2015, Austin, TX
 - My abstract was selected for a short oral presentation.
- Annual Presidential Career Symposium- Feb. 4, 2016, Houston, TX
 - As part of this year's symposium, I served on the planning committee and was instrumental in executing this year's symposium, including bringing in the keynote speaker.

Publications during Year 2:

Hsu TY, Simon LM, Neill NJ, Marcotte R, Sayad A, Bland CS, Echeverria GV, Sun T, **Kurley SJ**, Tyagi S, Karlin KL, Dominguez-Vidaña R, Hartman JD, Renwick A, Scorsone K, Bernardi RJ, Skinner SO, Jain A, Orellana M, Lagisetti C, Golding I, Jung SY, Neilson JR, Zhang XH, Cooper TA, Webb TR, Neel BG, Shaw CA, Westbrook TF. The spliceosome is a therapeutic vulnerability in MYC-driven cancer. **Nature.** 2015 Sep 17;525(7569):384-8. doi: 10.1038/nature14985.

How were the results disseminated to the communities of interest? The findings from the Nature publication referenced above were highly publicized to local and national media and it was widely reviewed/highlighted in subsequent scientific news pieces.

What do you plan to do during the next reporting period to accomplish the goals? The statement of work describes the plan of action for accomplishing year 3 goals. However, as indicated in the accomplishment section and described section 5, we will be exploring how our hypothesis fits more specifically into metastatic TNBC and focusing our attentions on in vivo work.

4.IMPACT: Our work on BUD31 and splicing in myc-driven breast cancer has provided strong pre-clinical evidence to support this therapeutic intervention point for the treatment of breast cancer. We are actively pursuing additional hypothesis stemming from these findings and addressing how this may be translated into clinical studies.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change: As described above in the accomplishments section, two separate groups have been pursuing work very closely related to the work proposed in this fellowship. Since redundancy does not serve the objectives of this funding agency nor promote the career objectives of the fellowship recipient, we will be turning our efforts toward testing how these biological processes described above are at play in metastatic TNBC. We have reported some of the significant advancements made in the development of metastasis models, models that will not only serve this project, but will also be more broadly applicable to understanding metastatic breast cancer. Indeed, we have successfully used these models to test the efficacy of therapies of interest to treat established metastatic lesions. While this experimental paradigm is not routinely utilized, it is no doubt of high clinical relevance to model patients presenting with metastatic lesions (data not shown). During year 3, we will focus on myc-driven breast cancer metastasis and how the pathways described above are at play in this important event in breast cancer.

Actual or anticipated problems or delays and actions or plans to resolve them: Nothing additional to report.

Changes that had a significant impact on expenditures: Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents: All animal studies described above have been approved by Baylor College of Medicine's IACUC board and did not use funding from this fellowship unless indicated and also covered by ACURO approval.

6. PRODUCTS: Nothing to report

7. PARTICIPANTS&OTHER COLLABORATING ORGANIZATIONS:

What individuals have worked on the project?

Name:	Sarah Kurley, PI
Project Role:	PI
Nearest person month worked:	12
Contribution to Project:	Dr. Kurley has executed and guided data described herein. Training tasks listed in this postdoctoral fellowship were also completed by Dr. Kurley.

Has there been a change in active other support? Nothing to report

What other organizations were involved as partners? Nothing to report

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period? Nothing to report

8. SPECIAL REPORTING REQUIREMENTS: Not applicable

9. APPENDICES: No appendices.